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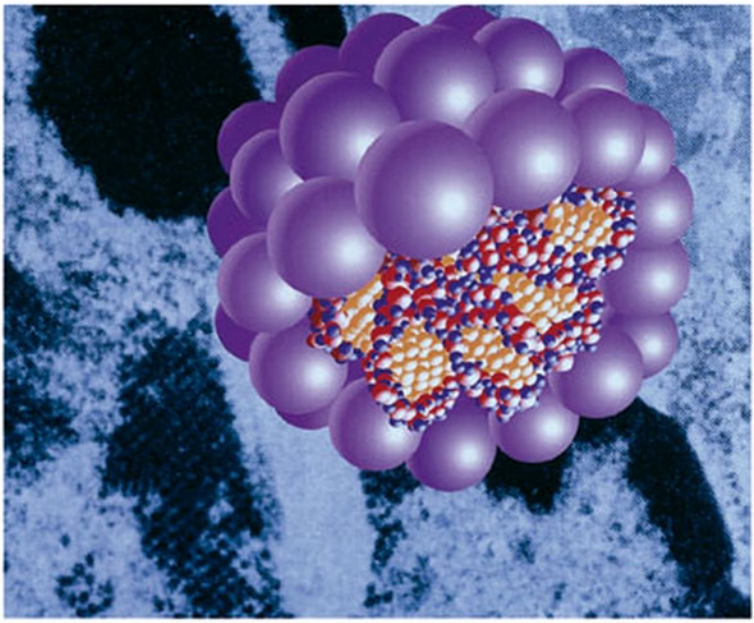
Harald zur Hausen

Infections Causing Human Cancer



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Infections Causing Human Cancer

With a contribution of
James G. Fox, Timothy C. Wang
and Julie Parsonnet

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Preface

For many years I have been tempted to write a comprehensive book on the role of infectious agents in human cancers. Progress has been particularly rapid in this field during the course of the past 25 years, and today we can convincingly report that approximately 20% of the global cancer incidence is initiated or promoted by infectious events. I had admired the task carried out by Ludwik Gross. Since his two-volume publication *Oncogenic Viruses* in 1961, with additional editions in 1970 and in 1983, a number of books have appeared on similar topics, virtually all of them authored by multiple scientists and some of them very heterogeneous in content and structure. For these reasons, I planned to write a book which attempted to develop a more unifying concept and a consistent structure for the individual chapters. Considering the overwhelming magnitude of data, I was sure that I could not undertake this task during my active period as scientific director of the German Cancer Research Center in Heidelberg, and so postponed this for "active retirement". Ultimately, I was pleased that I was able to persuade James Fox from Harvard University to contribute Chapter 10, on *Helicobacter*, as this would have been beyond my personal experience. He immediately consented and jointly with Timothy C. Wang and Julie Parsonnet delivered the chapter in time.

The book is not intended to cover the structure and molecular biology of the agents presented in great detail, but rather aims to concentrate on those aspects that link the respective agents to human oncogenesis. The book should introduce interested colleagues, clinicians, and students to the field, and help to analyze some of the developments that even 20 years ago attracted only minimal attention. Today, this research has culminated in the development of the first – and apparently successful – vaccines for the prevention of specific, common human cancers, cervical carcinomas, and liver cell cancer. Within the book we have tried to provide the readers with an extensive bibliography after each individual chapter, in order to permit further studies on the subject. However, even an attempt to select the most important papers in the field will almost inevitably miss some publications that our colleagues consider as very important. Consequently, I apologize in advance to all of those readers who feel that we did not cover their own or other research areas adequately.

Fortunately, the response on the part of my colleagues was friendly and generous, and they provided helpful suggestions and corrected some of my statements. I am

particularly indebted to George and Eva Klein, Stockholm, for their many extremely helpful comments, to Bernhard Fleckenstein, Erlangen, and Georg Bornkamm, Munich, Vladimir Vonka, Prague, Nikolaus Müller-Lantzsch, Homburg, to Reinhard Kurth, Norbert Banner, and Georg Pauli, all Berlin, and to my Heidelberg colleagues, Frank Rösl, Rainer Schmidt, Lutz Gissmann and Henri-Jacques Delecluse. My secretary, Gudrun Küthe, competently and patiently checked the entire manuscript and corrected initial mistakes. Sherryl Sundell, the Managing Editor of the *International Journal of Cancer*, tried to correct at least some of the "Germanisms" in the language.

A special note of gratitude goes to my wife, Ethel-Michele de Villiers, who not only patiently tolerated two-and-a-half years of evenings and weekends devoted to reading and writing, but also actively contributed by discussing and modifying part of the text. Last, but not least, I would like to mention my granddaughters, Talisa and Johanna, who were to some extent neglected during this period. This is hopefully going to change now.

Harald zur Hausen
Heidelberg, April 2006

1
Historical Review

1.1
The Early Period (1898–1911)

On March 24, 1882 Robert Koch presented his famous lecture at the Physiological Society in Berlin, suggesting that tuberculosis is caused by a bacterium. It was probably this surprising discovery of the infectious etiology of tuberculosis – a disease which until then was not suspected to be caused by an infectious agent – that turned the interest of microbiologists at the end of the nineteenth century towards a possible infectious cause of other chronic conditions, among them cancer. Interestingly, at the turn of the past century, the first positive reports incriminated parasites, liver flukes and *Schistosoma* infections with specific human cancers: in 1900, Askanazy reported a link between *Opisthorchis felineus* infection and liver cancer in the former East Prussia. Only five years later, another report incriminated *Bilharzia* infections (schistosomiasis) in bladder cancer (Goebel, 1905). Goebel mentioned in his paper (without citation) that Griesinger and Bilharz, Zancarol, Kartulis, Harrison, Chaker, Rütimeyer, Scheube, Lortet and Vialleton, Brault and also Albarran had demonstrated previously that chronic schistosomiasis might eventually lead to the development of cancer. Possibly based on these findings, Johannes Fibiger in Denmark reported in the early 1920s the identification of a nematode, *Gangylonema neoplasticum* (initially named *Spiroptera carcinoma*), in rat tumors and drew far reaching conclusions from these investigations also for other types of cancer. For his studies, Fibiger was awarded the Nobel Prize in Medicine in 1926. Unfortunately, his results have not been confirmed by other groups, though an account of the developments leading to his award was published recently (Stolt et al., 2004). Subsequently, at least one other nematode, *Spirocerca lupi*, has been identified as causing esophageal granulomas in dogs, some of them converting into sarcomas (Bailey, 1963).

In those years the evidence for a role of these parasites in human cancer was based exclusively on epidemiological observations and on the findings of clinical studies. Nevertheless – and based on numerous subsequent observations – a panel established in 1994 by the International Agency for Research on Cancer (IARC) in Lyon concluded that there exists sufficient evidence for a role of two parasites, *Schistosoma haematobium* and *Clonorchis viverrini*, in human cancers (IARC, 1994). The same report claimed that there is limited evidence for a carcinogenic function of *Schistosoma japonicum* and for the liver fluke *Clonorchis sinensis*.

The early reports of a link between parasitic infections and human cancers were even preceded by a study of M'Fadyan and Hobday in England. In 1898, these authors published details of the transmission of dog warts by papilloma filtrates known to retain all characterized bacteria identified to that time. Warts, of course, were not considered as "true" tumors, and therefore this publication received little attention. The existence of a "contagium fluidum", which passed filters that would retain all known microorganisms, had been established previously by Iwanowski in St. Petersburg in 1894, and also by Beyerinck in Amsterdam in 1898. Both M'Fadyan and Hobday and Iwanowsky successfully transmitted tobacco mosaic disease by cell-free extracts. In addition, Sanarelli in Montevideo in 1898 had recognized filterable agents as the cause of an acute proliferative disease, myxomatosis in rabbits.

Even in 1907, when Ciuffo in Rome conducted self-inoculation experiments with cell-free extracts from human warts and subsequently developed cutaneous warts at the inoculation site, this result barely created enthusiasm. One year later, in 1908, Ellermann and Bang in Copenhagen reported the cell-free transmission of chicken leukemia. The nature of leukemia and its relationship to malignant diseases were not known in those days. Thus, these experiments were not immediately recognized as the first successful transmission of a natural malignancy. However, this changed in 1911, when Peyton Rous at the Rockefeller Institute in New York demonstrated the cell-free transmission of a solid tumor – a chicken sarcoma – which was, undoubtedly, acknowledged as a malignant neoplasm. This was a first turning point in the public recognition of studies on the infectious origin of cancers.

1.2 Frustration and Successes (1912–1950)

Unfortunately, the following years failed to provide evidence for the applicability of the Rous data to human tumors, and even the cell-free transmission of many animal tumors to the same or to different species attempted during this period commonly resulted in frustration. In fact, more than 20 years passed before any further progress was recognized.

In 1932, Richard E. Shope at the Rockefeller Institute in Princeton noted fibromatous tumors in a wild cotton-tail rabbit. Shope could readily transmit these fibromas by cell-free extracts to either domestic or cotton-tail rabbits. Interestingly, this infection caused a partial cross-immunity against rabbit myxomatosis virus. Both conditions were later identified as being caused by members of the poxvirus family.

Only one year later (in 1933), Shope also discovered a filterable agent in cotton-tail rabbit papillomas; this induced papillomas when inoculated into the scarified skin. Shope partially characterized this virus and noted its remarkable heat stability as it was able to tolerated exposure at 65 C for 30 minutes. In 1934, Rous and Beard discovered that this infection also had malignant potential; this was especially noted in domestic rabbits, where many of the initial papillomas converted into squamous cell carcinomas. This conversion also occurred sporadically in its natural host, the cotton-tail rabbit, albeit at a lower rate (Syverton and Berry, 1935). During the following

years, Rous and his coworkers continued to study this system, and investigated in particular the interaction of this virus infection with chemical carcinogens. Even after systemic application of the virus, they found a remarkable degree of synergism between infection and skin tarring or treatment of the skin with defined chemical carcinogens in carcinoma development (Rous and Kidd, 1938; Rous and Friedewald, 1944). In 1961, Ito and Evans showed that the carcinomas contained infectious papillomavirus DNA. Peyton Rous was conceptually far ahead of his time, his early studies having resulted in defining initiation as an early event in carcinogenesis, despite his not understanding the underlying mechanism. Finally, in 1966 – some 55 years after his seminal discovery in 1911 – Rous was awarded the Nobel Prize.

The 1930s were a relatively fruitful period for tumor virology: in 1936, Bittner described a "milk factor" which was transmissible from lactating mice to their offspring. The milk factor was first visualized in 1948 (Porter and Thompson), and Kinosita et al. (1953) characterized the virus in ultrathin sections by electron microscopy. The virus was later identified as a member of the retrovirus family, and its name was changed from Bittner factor or milk factor to mouse mammary tumorvirus (MMTV).

In 1938, Lucké reported a carcinoma of the kidney in the leopard frog (*Rana pipiens*), which was apparently caused by a transmissible virus. This tumor was known to be more prevalent in frogs during the cold season than in summer (McKinnel and McKinnel, 1968), and subsequently in 1956 Fawcett identified typical herpesvirus particles (now labeled as Lucké herpesvirus) in the tumors of winter frogs.

In 1907, an infectious chicken neurolymphomatosis had been recognized by the Hungarian veterinarian J. Marek, the condition subsequently being designated as Marek's disease. Some 20 years later, in 1926, Pappenheimer et al. recognized the neoplastic nature of this disease, while in 1969 Witter et al. identified the infectious agent as a member of the herpesvirus family. Thus, by the late 1960s two animal herpesviruses were being considered as etiological agents for malignant tumors in frogs and chickens.

Unfortunately, the promising studies of the 1930s were interrupted by the Second World War, and during the postwar period it took about 10 more years before any significant progress in this area re-emerged.

1.3 The Period from 1950 to 1965

Although numerous attempts had been made previously to identify an infectious etiology of at least some human tumors, the results had proved – until 1964 – to be rather disappointing. The involvement of parasites seemed true for some cancers outside of Europe and the United States, and appeared to represent an exotic curiosity. Even up to the early 1980s, most epidemiologists at best marginally considered a possible relationship between infections and cancer. Yet, the foundations for our present understanding of the specific function of tumorviruses were laid between 1950 and 1965.

In 1950 and 1951, Ludwik Gross in New York published the results of his pioneering studies on the transmission of murine leukemias following the inoculation of cell-free extracts into newborn mice. Gross had noticed that the susceptibility to cancer induction by viruses (later identified as members of the retrovirus family) depended largely on infections early in life. His studies in 1953 and 1955 resulted in the discovery of another cancer-inducing agent, later identified and described in more detail by Stewart et al. (1957) and designated polyomavirus.

In 1956 and 1957, Charlotte Friend isolated a virus which caused erythroblastosis in mice, and was able to pass it serially in weanling mice. This virus, which caused rapid enlargement of the spleen and liver and led to progressive anemia, was later identified as a member of the retrovirus family. In contrast to the earlier observations by Gross, Friend was also able to induce this proliferative condition in adult mice.

During the ensuing years, a number of additional retrovirus types were analyzed in other rodents, in chicken, cats, cattle, and even in nonhuman primates. Most frequently these infections were linked to leukemias or lymphomas in their respective hosts, and for these reasons many virologists suspected that human proliferative diseases of the hematopoietic system might also be caused by members of the same virus family.

Two other important observations were made during the early 1960s: (i) the discovery of a transforming and tumor-inducing small DNA virus, initially isolated from rhesus monkey kidney cells; and (ii) the identification of tumor-inducing properties of the widely spread human adenoviruses.

In 1960, Sweet and Hilleman described the simian vacuolating virus, labeled simian virus 40 or SV40, which was isolated from rhesus and cynomolgus monkey kidney cell culture material. One year later, Eddy et al. (1961) noted that the inoculation of rhesus monkey kidney extracts into newborn hamsters resulted after several months in invasively growing tumors. During the following year, this group identified the "oncogenic" substance as simian virus 40 (Eddy et al., 1962).

The tumors induced by SV40 failed to synthesize infectious virus, but did produce a virus-specific antigen, the Tumor- or T-antigen (Black et al., 1963 a). Similar observations were made two years later for polyomavirus-induced tumors (Habel, 1965). Subsequently, T-antigen expression was also found in the early phase of lytic infection (Pope and Rowe, 1964; Rapp et al., 1964). The availability of a virus system which would readily transform a variety of tissue culture cells without virus production, permitted the development of novel experimental approaches aimed at understanding the molecular mechanisms of cancer. The persistence of integrated SV40 DNA in transformed cells added to the interest (Sambrook et al., 1968), and within a short time studies on cell transformation by SV40 became a favorite system of a large number of tumorvirologists. The subsequent isolation of two related DNA viruses directly from humans, namely BK virus (Gardner et al., 1971) and JC virus (Padgett et al., 1971), seemed to underline further the importance of this virus group as potential carcinogens.

The other important result obtained during this period was the identification of the oncogenic properties of virus infections that were widespread among the

human population; these were the adenoviruses, which most frequently caused either respiratory or gastrointestinal symptoms upon infection. In 1962, Trentin and coworkers reported that adenovirus type 12 induced tumors when inoculated into newborn hamsters. These results were soon confirmed and extended by Huebner et al. (1962) for adenovirus type 18, by Girardi et al. (1964) for adenovirus type 7, and by Pereira et al. (1965) for adenovirus type 31. Huebner's group also demonstrated specific complement-fixing antigens in adenovirus-free hamster and rat tumors (Huebner et al., 1963).

Thus, by the mid-1960s several human pathogenic viruses had been identified which possessed oncogenic potential for newborn rodents. Although these viruses were also able to stimulate the permanent growth of tissue culture cells (immortalization), and left their footprints as T-antigens in every tumor cell, none of them transformed human cells or seemed to persist in human cancers. Nevertheless, this period stimulated a number of laboratories to search for viruses, or their footprints, in human tumors.

1.4
A First Human Tumorvirus?

In 1958, Dennis Burkitt, a British surgeon working in equatorial Africa, noted a specific childhood lymphoma that occurred only in specific geographic regions. As these regions coincided with areas of holoendemic malaria, Burkitt speculated that this tumor should have an infectious etiology, most likely vectored by an arthropod, possibly by a mosquito (1962). These initial observations by Burkitt stimulated interest among the scientific community, and consequently Pulvertaft (1964) in Western Nigeria and Epstein and Barr (1964) in Bristol, UK, began to develop tissue culture techniques for these tumors and to establish a number of lymphoblastoid lines. Epstein et al. (1964) noted herpesvirus-like particles in a small fraction of these cells, but in contrast to herpesviruses known at this time, they were unable to transfer the infection to other cell systems, embryonated chicken eggs, or to conventional laboratory animals. These authors concluded at an early stage that they had found a new member of the herpesvirus family which later, was named Epstein–Barr virus (EBV).

The subsequent development of an immunofluorescent test to detect viral antigens in virus-producing cells facilitated further studies (Henle and Henle, 1966). The availability of this test system resulted in the detection of highly elevated antibody titers against viral antigens in patients with Burkitt's lymphoma (Henle and Henle, 1966; Henle et al., 1969), and subsequently also in a second human cancer, in nasopharyngeal carcinomas (Old et al., 1966). In addition, these tests indicated that EBV must be widely spread among all human populations. In 1968, Henle's group identified EBV as the causative agent of infectious mononucleosis (Henle et al., 1968).

The first hints for an oncogenic potential of EBV originated from co-cultivation studies of lethally X-irradiated Burkitt's lymphoma tissue culture cells with umbili-

cal cord lymphocytes (Henle et al., 1967). This resulted in the regular establishment of lymphoblastoid lines of cord blood origin, and was further underlined by the discovery of persisting EBV DNA in "virus-negative" Burkitt's lymphoma cells (zur Hausen and Schulte-Holthausen, 1970), as well as in primary biopsies from Burkitt's lymphomas and nasopharyngeal cancer (zur Hausen et al., 1970). The discovery of a complement-fixing specific nuclear antigen (Pope et al., 1969; Reedman and Klein, 1973), the induction of lymphoproliferative disease after inoculation of EBV into cotton-top marmosets (Shope et al., 1973) or owl monkeys (Epstein et al., 1973), and the identification of EBV persistence in epithelial nasopharyngeal carcinoma cells (Wolf et al., 1973) added to the evidence in this early phase.

Retrospectively, it is surprising that these exciting discoveries received relatively little attention from the scientific community. This was due in part to difficulties in understanding a role for a virus in cancer induction that is persistently present in the vast majority of all human populations. The remarkable geographic restriction of the incriminated human cancers, Burkitt's lymphoma and nasopharyngeal cancer, posed an additional problem. Another reason was that problems which arose during the 1970s created an atmosphere of general disbelief for an infectious etiology of human cancers.

1.5 The Difficult 1970s

The frequent identification of retroviruses from animal leukemias and lymphomas, as well as from some epithelial tumors, resulted in intensified efforts to identify members of this virus family which also corresponded with human malignancies. A number of reports were published during these years, claiming the isolation of C-type viruses from human leukemias or finding components of these viruses in the respective tumor cells. One series of reports characterized a virus isolated from acute myelogenous leukemia (Gallagher and Gallo, 1975; Gallagher et al., 1975; Teich et al., 1975). This virus proved to be closely related to – if not identical with – woolly monkey type C virus. In another set of experiments, DNA from several patients with leukemia was found to hybridize with 70% of RNA from baboon endogenous C RNA virus (BaEV), whereas DNA from normal human tissues hybridized only with 23% of BaEV-RNA (Reitz et al., 1976; Wong-Staal et al., 1976). Based on these data, the authors claimed the horizontal transmission of a BaEV-related virus among humans. In 1977, the same group (Aulakh and Gallo, 1977) reported sequences complementary to Rauscher murine leukemia virus in some patients with leukemia, Hodgkin's disease, and multiple myeloma. These sequences were not detected in non-neoplastic spleen and kidney biopsies from a patient without neoplasia. Thus, at the time these authors suggested that at least three types of type-C viral sequences were present among the human population.

The discovery of a viral RNA-dependent DNA polymerase, reverse transcriptase (which was postulated by Howard Temin in 1964, and demonstrated by Temin and Mizutani and by Baltimore in 1970), seemed to open a new experimental approach

for the search of retroviruses in human tumors. Several reports were made on the detection of a high molecular-weight RNA associated with an RNA-instructed DNA polymerase in various human tumors, such as human leukemias (Gallo and Spiegelman, 1974), lymphomas (Spiegelman, 1975), human melanomas (Hehlmann et al., 1978) and human skin cancers (Balda et al., 1975). Unfortunately, none of these reports was later confirmed by other groups.

A third line of publications covered the possible presence of MMTV-like viruses in human breast cancer and milk. Electron microscopic investigations, as well as biochemical and biophysical analyses, suggested the presence of MMTV-like particles in human milk and malignant breast tumors (Moore 1974; Schlom et al., 1975). Particularly in the milk from women of the Parsee community in India, where there was a high incidence of breast cancer, polymerase and RNA studies provided early evidence for the existence of a MMTV-related virus in humans (Das et al., 1972). Although even controversial today, these reports still require confirmation.

It was the coincidence of these numerous reports during the 1970s (part of them presumably originating from inadvertent contaminations), and the inability of many other groups to confirm these findings that resulted in a widespread distrust and disbelief in a role of viruses in human cancers.

One other aspect added to the problems of tumor virology during the 1970s. In 1976, when Stehelin et al. reported the cellular origin of retroviral oncogenes, this had an immediate effect on tumorvirus research. Although modified cellular protooncogenes were clearly sufficient to stimulate cell growth and mediate cellular transformation, Knudson in 1971 proposed another class of genes – the tumor suppressor genes – based on his studies on retinoblastoma development. The failure of these genes is supposed to activate potential oncogenic functions within the affected cell. The identification and isolation of the retinoblastoma susceptibility gene Rb in 1986 and 1987, the demonstration of its modification in retinoblastomas and osteosarcomas (Friend et al., 1986; Lee et al., 1987), and the identification of a number of additional tumor suppressor genes paved the way for a new interpretation of cancer development. Accordingly, cancer development results from an interruption of the interplay of tumor suppressor genes and protooncogenes. This is usually mediated by mutations or loss of the suppressing alleles, or by activating mutations in oncogenes. This relatively simple and straightforward concept did not require any interaction with foreign, predominantly viral nucleic acids. In fact, it was only disturbing an otherwise clear-cut concept.

It was only consequent that, based on these considerations, a substantial number tumorvirologists turned to cell biology and the characterization of gene/gene interactions in normal and malignant cells.

1.6
The Re-Emergence of a Concept

Ten years later, the situation gradually started to change, due mainly to the contributions of three independent findings: (i) the discovery of a role of hepatitis B virus in

liver cancer; (ii) the identification of a retrovirus in a rare form of human leukemia; and (iii) the characterization of novel types of papillomaviruses causing the second most frequent cancer in females, cancer of the cervix.

The history of the hepatitis B virus and its role in hepatocellular carcinoma (HCC) is not easily unraveled. As early as the 1950s, British and French pathologists in Africa noted the frequent coincidence of hepatitis infections and hepatocellular cancer (for a review, see Szmuness, 1978). In 1956, Payet et al. suggested directly that HCC is the consequence of chronic viral hepatitis. Further, some of the early epidemiological studies stressed a role for chronic hepatitis B infections in HCC development (Prince et al., 1970; Vogel et al., 1970; Denison et al., 1971; Sankalé et al., 1971; Teres et al., 1971; Nishioka et al., 1973; Trichopoulos et al., 1975; Larouzé et al., 1976). Clear-cut evidence of this was presented a few years later when, in a prospective study conducted in government employees in Taiwan, Beasley et al. (1981) noted an increase in relative risk by a factor of 103 for HCC of hepatitis B carriers in comparison to hepatitis B-negative individuals. This clearly emphasized an important role of hepatitis B in the development of liver cancer.

Although it is still unclear by which mechanism hepatitis B virus contributes to the emergence of liver cancer, vaccination studies performed today underline the importance of this infection for hepatocellular carcinomas (this will be discussed in Chapter 5, Section 5.3).

In 1980, the isolation of a human T-lymphotropic retrovirus (HTLV-1) was reported from a cutaneous T-cell lymphoma (Poiesz et al., 1980). The virus could be propagated in T-cell growth factor-stimulated lymphocytes. The factor, interleukin-2, was previously purified and characterized by the same group (Mier and Gallo, 1980). Japanese researchers later identified the same virus (Miyoshi et al., 1981), and linked this infection to adult T-cell leukemia, which is endemic in the coastal regions of Southern Japan (Hinuma et al., 1981). These findings were rapidly reproduced, and firmly established at least one member of the retrovirus family as the causative agent of a rare form of human leukemia. Today, the link is basically proven.

Studies on papillomaviruses have a long history, in part already described for the cotton-tail papillomavirus. Early studies on the cell-free transmission of bovine warts were initiated by Magelhaes in Brazil (1920) while later, in 1951, Olson and Cook showed that the transmission of these viruses to another species, horses, resulted in the induction of sarcoids. These invasively growing but nonmetastasizing tumors are also noted in domestic horses under natural conditions. The Olson group made another striking observation, namely the induction of bladder tumors in cattle by bovine papillomavirus (BPV) infection (Olson et al., 1959). Only four years later, two additional reports by Black et al. (1963 b) and Thomas and colleagues (1963) demonstrated the transforming activity of BPV preparations for bovine fetal and murine cells.

The analysis of human papillomatous lesions and their relationship to virus infections and carcinogenesis had a much slower start. Although the infectious etiology of human warts had been clearly established based on their cell-free transmission, they were mainly regarded as a cosmetic nuisance and not thought to be of any significant medical interest.

The gradual change of this view originated from the description of a syndrome first reported by Lewandowsky and Lutz in Basel, in 1922. These authors described an hereditary condition, characterized by an extensive verrucosis, epidermodysplasia verruciformis. In these patients, at sun-exposed sites such as the forehead, the face, and the backs of the hands and arms, some papillomatous lesions converted into squamous cell carcinomas. Lutz, in 1946, and subsequently Jablonska and Millewski in 1957, proved the viral etiology of these warts in autoinoculation experiments. It was mainly the merit of Stefania Jablonska to point out a potential role of the human papillomavirus (HPV) particles seen in these warts as causal factors for the subsequent development of squamous cell cancers of the skin (Jablonska et al., 1972). Working jointly with Gérard Orth, these groups successfully demonstrated the presence of novel types of papillomaviruses, most frequently HPV 5, within epidermodysplasia verruciformis lesions and within biopsies of squamous cell carcinomas of those patients. (Orth et al., 1978, 1979).

Although HPV 5 represents the first papillomavirus infection regularly detected in cutaneous squamous cell cancers of epidermodysplasia patients, the rarity of the syndrome, the difficulties in obtaining sufficient clinical materials for extensive studies, and the absence of tissue culture lines from these carcinomas were probably the reasons for a limited interest in this condition. Only in recent years has the study of cutaneous papillomavirus infections and their relationship to nonmelanoma skin cancer in immunosuppressed and immunocompetent patients received increasing attention.

Another track of papillomavirus research resulted in the identification of specific HPV types as causative agents for cancer of the cervix, other anogenital cancers, and a subset of oropharyngeal carcinomas. These investigations began with the search for a viral etiology of cancer of the cervix, but by the late 1960s and 1970s the results of serological studies had suggested a role for human herpes simplex virus type 2 (HSV 2) in this cancer (Rawls et al., 1968; Naib et al., 1969). The present author's group was unable to confirm these findings, and sought alternative viral candidates. A number of anecdotal reports on the malignant conversion of genital warts (condylomata acuminata), scattered among the medical literature of the preceding 100 years, attracted attention. Subsequently, a possible causal role of papillomavirus infections for cervical cancer was postulated, and initial attempts were begun to characterize the viral DNA in genital warts (zur Hausen et al., 1974, 1975; zur Hausen 1976, 1977). These and other studies had the early consequence of discovering the heterogeneity of the papillomavirus family (Gissmann and zur Hausen, 1976; Orth et al., 1977; Gissmann et al., 1977), presently counting close to 106 fully sequenced genotypes (de Villiers, 1994; also Personal communication). However, the eventual isolation of HPV DNA from genital warts, labeled as HPV 6 (Gissmann and zur Hausen, 1980), and from laryngeal papillomas (HPV 11) two years later (Gissmann et al., 1982), did not yield reproducibly positive data for these viruses in cervical cancer. Yet, the use of their DNA in hybridization experiments, performed under conditions of reduced stringency, permitted the subsequent cloning of HPV 16 (Dürst et al., 1983) and HPV 18 DNA (Boshart et al., 1984), the two papillomavirus types most frequently found in cervical cancer. This allowed further ex-

periments to be conducted that would prove the role of these papillomaviruses in causing this malignancy (see Chapter 5).

The identification of three viral families with representative types that clearly cause cancers (including common carcinomas such as cancer of the cervix and liver) gradually resulted in an acceptance of infectious agents as important human carcinogens. The subsequent identification of additional infections clearly linked to other cancers further strengthened the role of infectious agents in human cancers. The hepatitis C virus was identified in 1989 (Choo et al., 1989), and initial reports on its relationship to a subset of hepatocellular carcinomas appeared in the same year (Bargiggia et al., 1989; Simonetti et al., 1989). Some earlier reports had been made, however, linking non-A, non-B hepatitis infections to liver cancer (Kiyosawa et al., 1982; Resnick et al., 1983; and others). Human herpesvirus type 8 was discovered in 1994 (Chang et al.) as being the most likely causative agent for Kaposi's sarcoma, while in 1989 (Forman et al., 1991; Nomura et al., 1991; Parsonnet et al., 1991) and 1993 (Wotherspoon et al.), *Helicobacter pylori*, as a bacterial infection, was added to the list of potential human carcinogens. Subsequently, a large number of additional HPV genotypes has been added, the pathogenic significance of which remains to be determined. The same is true for the recently discovered TT viruses; these clearly represent a new virus family, establishing probably life-long persistent infections in a high proportion of the human population.

Thus, today – more than 100 years after the first attempts to link infections to human cancer, and after more than 80 years of mainly frustrating experimentation – infections causing cancer emerge as a major factor in human carcinogenesis. This mode of research leads to new approaches towards cancer diagnosis and treatment, and – most importantly – of cancer prevention.

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2
The Quest for Causality

Whenever human cancers are analyzed for the presence of viruses, depending upon on the method used for detection, a number of different agents may be identified. The sensitivity of the polymerase chain reaction (PCR) in particular permits the discovery even of very small concentrations of DNA or RNA of infectious agents. In the majority of cancers caused by viral infections the viral DNA is present in very small copy numbers, usually less than one DNA copy per 10 tumor cells. For instance, it is possible to detect human cytomegalovirus (CMV), human herpesvirus (HHV) type 6 (HHV 6), Epstein–Barr virus (EBV) and even herpes simplex virus type 1 (HSV-1) DNA in a broad spectrum of different tumors, and even more so a larger number of TT virus genotypes (de Villiers et al., 2002). Similarly, several reports have claimed the presence of polyomavirus types, BK, JC and SV40 virus in human cancer biopsies (for a review, see Gazdar et al., 2002). However, it is unclear whether these agents are causally involved in the development of those cancers.

In several types of human cancer, including nasopharyngeal carcinoma, cancer of the cervix, specific B-cell lymphomas and Kaposi's sarcoma, the discovery of virus-specific DNA was taken as a first clear-cut hint for a viral etiology. In almost all of these systems every individual tumor cell frequently contained multiple copies of viral DNA. In addition, this viral DNA was present in a clonal form, implying its presence at early onset of malignant proliferation. Although in all these systems additional data later on confirmed a close link of these infections to the malignant outcome, it remains unclear as to whether the presence of low copy numbers (e.g., < 1 in 10 tumor cells) excludes causality.

Evidently, there is a need to define causality here against a background of multiple genetic changes, apparently underlying the vast majority of human cancers, and the stepwise progression to malignant proliferation commonly over a period of several decades. During the last decades of the nineteenth century, Robert Koch developed his postulates based on straightforward observations in bacterial infections: he established methods to cultivate bacteria on semisolid media, to purify them from other, contaminating microorganisms, and to use the purified types to induce specific clinical symptoms in laboratory animals. Koch was able to re-cultivate the agent from the sick animal and to re-induce the disease in further experimentation (Koch, 1881). His beautiful documentation of the causal role of specific bacteria in causing *anthrax*, *diphtheria*, *cholera* and *tuberculosis*, respectively, underlined the

validity of his postulates. For decades, these served as baseline to link infectious agents to human and animal diseases. The question remains, however, as to whether they are applicable to establish a causal role of infectious agents in human cancers.

Clearly, the answer to this question is "no". One major problem is the long latency period between the primary infection and the eventual emergence of cancer, frequently covering periods of several decades. In addition, most of these agents are widely spread among the human population, yet, only a fraction of infected individuals develops cancer – a fact which suggests the presence of additional events affecting the infected cell prior to malignant conversion. Tumorviruses frequently integrate their DNA into the host cell genome. The viral genome is commonly modified in the process of integration, often revealing deletions or mutations. Thus, no infectious progeny can be produced, even if the viral DNA were to be excised from the host cell genome. The detection of persisting viral DNA usually relies on hybridization procedures or PCR technology. An isolation of the infectious agent in the sense of Koch's postulates is regularly impossible. In some of these infections, however, viral DNA can be recovered that, upon transfection into suitable host cells or laboratory animals, induces cell transformation or tumors, respectively. To some extent this fulfills the criteria raised by Robert Koch.

Nonetheless, the major difficulty arises from the fact that different tumor-inducing infectious agents perform this function using different mechanisms. Increasing evidence exists that several infections that result eventually in cancer development act indirectly and do not require the persistence of the microbial genome. In all likelihood, this is the case for *Helicobacter pylori* infections which lead, after a long time of persistence, to gastric cancer or to gastric lymphoma. The same is true for parasitic infections such as schistosomiasis or liver flukes. Human immunodeficiency viruses (HIV) provoke a severe immunodeficiency with a drastically enhanced risk for B-cell lymphomas or Kaposi's sarcoma. Herpesvirus infections may lead to an amplification of persisting other DNA viral genomes (polyoma-type and papillomaviruses), and thus contribute indirectly to an enhanced gene expression of the latter. As yet, it remains unclear as to whether hepatitis B or C genome persistence and gene expression is necessary for liver cancer proliferation. Finally, an increasing number of virus infections are being discovered that prevent apoptosis in persistently infected cells, enabling these cells to continue proliferation even under conditions of extensive genome damage.

Evans and Mueller summarized their view in establishing causality neatly in 1990, by concentrating specifically on oncogenic viruses, as follows:

- The long incubation or induction period between initial infection with the putative virus and the cancer(s) with which it is associated.
- The common and ubiquitous nature of most candidate viruses and the rarity of the cancer with which they are associated.
- The initial infection with the candidate virus is often subclinical, so that the time of infection can not be established by clinical features.

- The need for co-factors in most virus-related cancers.
- The causes of cancer may vary in different geographic areas or by age.
- Different virus strains may have different oncogenic potential.
- The human host plays a critical role in susceptibility to cancer, especially the age at the time of infection, the genetic characteristics, and the status of the immune system.
- Cancers result from a complex and multistage process, in which a virus may play a role at different points in pathogenesis in association with alterations in the host's immune system, oncogenes, chromosomal translocations, and a variety of events at the molecular level.
- The inability to reproduce many human cancers in experimental animals with the putative virus.
- The recognition that a virus, toxin, chemical, altered gene, or other causal factor may all be capable of initiating or promoting the processes that result in a cancer with the same histological features.

These considerations clearly demonstrate the difficulties in establishing uniform criteria to define causality in cancer induction by infections. A number of attempts have been made to solve this dilemma. Bradford Hill, for example (1965), attempted to develop criteria to define environmental causes of disease, based on strength of association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experimental evidence, and analogy. As stressed by Vonka (2000), Hill was convinced that none of his "nine viewpoints" would "…bring indisputable evidence for or against the cause and effect hypothesis and none can be required as a *sine qua non*". In 1990, Evans and Mueller based their criteria for an etiological relationship between virus infection and tumor development more on immunological aspects, in part influenced by a previous publication by Rivers (1937). They subdivided their "…guidelines for relating a putative virus to human cancers" into an epidemiological and virological part. For epidemiological evidence, they summarized four points:

- The geographic distribution of infection with the virus should be similar to that of the tumor with which it is associated when adjusted for the age of infection and the presence of co-factors known to be important in tumor development.
- The presence of the viral marker (high antibody titers or antigenemia) should be higher in cases than in matched controls in the same geographic setting, as shown in case-control studies.
- The viral marker should precede the tumor, and a significantly higher incidence of the tumor should follow in persons with the marker than in those without it.
- Prevention of infection with the virus (vaccination) or control of the host's response to it (such as delaying the time of infection) should decrease the incidence of the tumor.

The following three requirements should be provided on the virological side:

- The virus should be able to transform human cells *in vitro* into malignant ones.
- The viral genome or DNA should be demonstrated in tumor cells and not in normal cells.
- The virus should be able to induce the tumor in a susceptible experimental animal and neutralization of the virus prior to injection should prevent development of the tumor.

The considerations by Evans and Mueller were also influenced by a relatively large set of seroepidemiological data obtained up to that period on a role of EBV in human tumors (see Chapter 5, Section 5.11). As it turned out, however, elevated antibody titers against EBV antigens are not limited to EBV-linked cancer patients, but are also found under conditions of T-cell immunosuppression.

Interestingly, successful vaccination against the incriminated agent to prevent the respective tumors – an argument that should strongly support causality – seems not to have been considered before 1990 in various publications. Yet, although prevention of cancer development after vaccination with the suspected agent or its eradication seems to argue strongly for causality, on a second glance it is also highly questionable. For example, holoendemic malaria seems to be a contributing factor to the development of Burkitt's lymphoma in equatorial Africa (Kafuko et al., 1969; Kafuko and Burkitt, 1970). It is possible that malaria eradication in endemic areas for Burkitt's lymphoma will drastically reduce the rate of these cancers. However, it will only prove that malaria is indeed an important contributing factor for this malignancy – the "causation" seems to rely on other infections. The same accounts for the role of HIV infections in B-cell lymphomas and Kaposi's sarcoma. It is likely that successful vaccination or antiviral treatment of HIV infections will result in a drastic reduction of Kaposi's sarcoma and immunoblastoma. Although HIV is a contributing factor, it is not the cause of those sarcomas. It is known today that prolonged immunosuppression paves the way for a tumorigenic function of another herpesvirus infection, HHV type 8 (HHV-8), which seems to be the primary cause of Kaposi's sarcoma or EBV, which causes immunoblastomas.

Clearly, this is an extremely complex issue. Present knowledge of basic mechanisms of viral oncogenesis may, however, provide some encouragement to attempt a new definition. In order to escape from the previously described difficulties, there is a need to develop different criteria for those agents which induce cancer by direct interaction, which require persistence and possibly continued expression of the respective genome, and for those which contribute indirectly to tumorigenesis.

2.1 Infectious Agents as Direct Carcinogens

Infectious direct carcinogens are most readily defined among small DNA-containing viruses. In particular, the transformation of human cells by papilloma- and poly-

oma-type viruses is regularly accompanied by persistence of the whole genome or specific parts of it, by transcriptional activity of derived viral gene products, and by the expression of specific viral "early" proteins (viral oncoproteins). The use of temperature-sensitive mutants in the case of polyoma-type viruses (Butel et al., 1975; Brugge and Butel, 1975) showed that the transformed phenotype depends on the functioning of viral T-antigens. The results of these experiments reveal that the function of viral oncogenes is necessary for maintaining the transformed state of the respective cells. In the case of cervical carcinoma cells, harboring high risk papillomavirus DNA, the malignant phenotype can be reversed by blocking the expression or function of viral E6 and E7 genes (Storey et al., 1991; von Knebel Doeberitz et al., 1992, 1994; Shillitoe and Steele, 1992; Tan and Ting, 1995). Cervical cancer represents the first case where molecular techniques directly prove a necessary role of viral proteins for the maintenance of the malignant phenotype. However, *necessary* does not mean *sufficient* and, as discussed later, viral oncogene expression is not sufficient for cell transformation, as additional modifications of the host cell genome are required in order to develop invasive growth properties. Yet, the expression of viral oncogenes is clearly an essential and determining factor for continued cell proliferation. The availability of epidemiological studies demonstrating that the incriminated virus infection represents a significant risk factor for the respective cancer type strongly underlines causality further.

Based on these considerations, it is not too difficult to define criteria for a causal role in direct carcinogenesis by infectious agents (zur Hausen, 1991):

- the regular presence of the genome or parts of it of the infectious agent in every cancer cell;
- transfection of this nucleic acid into tissue culture cells or suitable laboratory animals should result in cell immortalization or tumor induction, respectively;
- excision of this nucleic acid from transfected cells or inhibition of its function should lead to a reversion of the immortalized or malignant phenotype of cells carrying the respective genome or parts of it;
- epidemiological case/control and prospective studies should identify the agent as a major risk factor for the respective tumor type.

The prevention of such cancers by specific immunization against this agent here would, of course, further underline causality.

If an attempt is made to identify agents which fit this definition, then three groups of viral infections can be labeled as direct carcinogens:

- Viruses expressing specific oncogenes which are necessary for the transformed phenotype (examples are "high-risk" human papillomaviruses, Epstein-Barr virus, human herpesvirus type 8, and human T-lymphotropic retrovirus).
- Viruses with acquired cellular oncogenes ("acute" transforming retroviruses, e.g., Rous sarcoma virus and others).

- Viruses which, when inserted into specific cellular chromosomal sites, activate cellular oncogenes and mediate transformation of the infected cells (e.g., mouse mammary tumor virus).

At this stage only members of the first group have been identified as carcinogens for humans, though clearly only one part of the spectrum of infectious agents engaged in carcinogenesis is covered by this definition. A leukemia of cattle caused in Mediterranean and East African countries by protozoon infections with *Theileria parva* and *Theileria annulata*, requires a special consideration. Here, the production of an epidermal growth factor-like protein by the parasite seems to be sufficient for induction of the disease. Successful treatment of the parasite also cures the leukemia. The mechanism by which these parasites cause leukemia still fits into the definition of direct carcinogenesis. The continuous presence of the protozoon is required for maintaining the malignant phenotype. However, different criteria must be developed to characterize other infectious carcinogens.

2.2 Infectious Agents as Indirect Carcinogens

At present, several possible mechanisms can be defined by which infectious agents may contribute to the development of specific carcinomas or lymphomas in humans. The most obvious one is the induction of immunosuppression. The pronounced immunosuppression caused by HIV infections in a number of patients results in the development of tumors that are commonly rejected in immunocompetent individuals due to their T-cell antigenicity. These tumors are mainly caused by persistent EBV infections (B-cell lymphoblastomas) and by infections with HHV-8. Whereas EBV and HHV-8 act as direct carcinogens under conditions of immunosuppression, HIV contributes indirectly to carcinogenesis, via immunosuppression. Successful treatment or prevention of the HIV infection would also prevent at least the vast majority of these EBV- or HHV-8-induced malignant proliferations, in spite of the fact that the latter are the direct cause and essential factors for the respective carcinogenesis.

Another example for indirect carcinogenesis is the prevention of apoptosis by cutaneous papillomavirus infections, as well as by a number of other viruses (see Chapter 5). Cells which are persistently infected with these viruses and acquire genetic damage due to physical or chemical factors may survive under conditions which otherwise would result in apoptotic cell death. The accumulation of mutational events within these cells may occasionally result in a loss of growth control and eventually lead to cancer development. Squamous cell carcinomas of the skin are suspected to be caused by this mechanism (Jackson et al., 2000, 2002).

There exist several other (most likely indirect) modes by which infectious agents may contribute to carcinogenesis: parasites and bacteria induce cancer by relatively poorly defined mechanisms. Chronic inflammation, increased development of oxygen radicals, the production of carcinogenic metabolites, as well as growth-stimulat-

ing factors excreted by these parasites are suspected as being the prime drivers for the eventual development of cancer in the infected tissues (see Chapters 10 and 11). At this stage, persistence of the inducing agents is no longer required.

A number of additional modes for indirect carcinogenesis by infectious agents can be envisaged, among which the induction of amplification of persisting polyoma- or papillomavirus genomes by herpesvirus infection (Schlehofer et al., 1983; Schmitt et al., 1989) deserves attention. At present, the evidence for this is based only on laboratory observations, while the clinical significance remains undetermined. Mutations and chromosomal rearrangements of host cell DNA induced by various viruses (e.g., vaccinia, adenoviruses, herpesviruses), even under conditions of abortive infections, could occasionally contribute to the development of cancer. In addition, there exists the possibility that some persisting virus infections may introduce long-lasting chromosomal instability into the infected cells that may again, in rare instances, result in invasive growth properties of the affected cell.

Thus, carcinogenesis mediated by infectious agents by an indirect mode is clearly complex. It is extremely difficult to define clear-cut criteria for identifying and characterizing these agents, though in somewhat vague terms the following criteria may act as a baseline:

- clinical observations, experimental and animal studies should point to a role of the respective agents as co-carcinogenic factors;
- epidemiological studies should identify these infections as risk factors for cancer development;
- vaccination against or successful treatment of the respective infection should provide significant protection against cancers suspected to be co-induced by these infections.

Thus, while there remains a very soft definition of indirect carcinogenesis by infectious agents, it seems nonetheless useful to some extent. The previously used term of "hit and run" mechanisms characterizes the existing situation very poorly: neither under conditions of immunosuppression by HIV, nor in cases of chronic bacterial or parasitic infections is cancer development necessarily accompanied by any loss of the contributing agent. Hence, the term "indirect carcinogen" appears to describe the existing situation more appropriately.

2.2.1
Induction of Chromosomal Aberrations

The induction of chromosomal aberrations may represent a specific indirect effect by which infectious agents could contribute to human cancers. Alterations of human chromosomes by virus infections have been noted for DNA and RNA viruses, among them adenovirus, HSV, varicella virus, EBV, human CMV, and polyomaviruses (for reviews, see Nichols, 1970; Stich and Yohn, 1970; Harnden, 1974; Fortunato and Spector, 2003). Most of the observed changes represent random modifications such as chromatid breaks, translocations, chromosome pulverization, coiling deficiencies and persistent overcondensation, though specific chromosomal abnormalities have been noted for three viruses (Table 2.1).

Table 2.1 Viruses inducing specific modifications of human chromosomes.

Virus	Type of change	Localization of modification	Reference(s)
Adenovirus type 12	chromatid break	1p36 1q21 1q42–43 17q21–22	zur Hausen (1967) McDougall (1971)
Herpes simplex virus	chromatid break	1p32 1q32 3p21	Mincheva et al. (1984)
	uncoiling of pericentric regions	1 9 16	Peat and Stanley (1986)
Humanic cytomegalovirus	chromatid break	1q21 1q42	Fortunato et al. (2000)

The first report on specific chromosomal changes induced by a virus infection was made in 1967 (zur Hausen, 1967). Subsequently, specific changes were also reported for HSV infections (Mincheva et al., 1984) and human CMV (Fortunato et al., 2000). The localization of these changes is shown in Table 2.1.

It is striking that adenovirus type 12 and human CMV appear to affect the same region at two loci on chromosome 1. The region of 1q42 harbors a putative tumor suppressor gene (Li et al., 1995), the *ADPRT* gene involved in DNA repair and replication (Baumgartner et al., 1992), and the 5S rRNA locus (Sorensen et al., 1991). The region 1q21 again contains among others a putative tumor suppressor gene (Bieche et al., 1995) and a family of snRNA pseudogenes, apparently not transcribed as stable RNA (Lindgren et al., 1985 a, 1985 b; Lund, 1988). The two additional sites of adenovirus type 12-induced changes seem to code for small structural RNAs and consist of tandemly arranged sequences (Durnam et al., 1988; Lund, 1988). The adenovirus E1B 55 000 protein which blocks p53-dependent and p53-independent apoptosis emerges as the necessary protein to induce specific changes on chromosomes 17q21–22 and 1p36 (Schramayr et al., 1990). In HSV infections the *ICP4* gene seems to be required for induction of the chromosomal modifications (Chenet-Monte et al., 1986; Johnson et al., 1992). According to Fortunato et al. (2000), CMV-induced chromosomal changes do not require cellular DNA synthesis.

It is presently unproven whether the described chromosomal aberrations play a role in human oncogenesis. Adenovirus type 12 is a potent carcinogen when inoculated into newborn hamsters and mice. The induction of chromosomal changes may contribute to viral carcinogenesis by mutating host cell genes that may interfere with viral oncogene expression or function.

No convincing evidence exists for a role of HSV and for human CMV in the etiology of specific human cancers. These viruses, however, have been reported to

transform rodent cells; indeed, CMV has even been claimed to immortalize specific human cells (see Chapter 4, Sections 4.1.1 and 4.2.1, respectively). The modification of host cell chromosomes may be important, especially in rodent cell transformation.

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3
Tumors Linked to Infections: Some General Aspects

3.1
Tumor Types Linked to Infections

The spectrum of tumors linked to infectious events reveals a remarkable diversity. Leukemias and lymphomas were the original prime suspects and, indeed, the relationship of Epstein–Barr virus (EBV) to Burkitt's lymphoma and to immunoblastic lymphomas, and the role of human T-lymphotropic retrovirus to adult T-cell leukemia seemed to confirm the preferential role of infectious events in the etiology of proliferative diseases of the hematopoietic system. Yet, almost simultaneously two important epithelial cancers emerged as being caused by infections, namely hepatocellular carcinoma and cancer of the cervix. The subsequent discovery of a relationship between gastric cancer and *Helicobacter pylori* and, to a more limited degree also with EBV, linked another important epithelial tumor system to infectious causes. The tumor types presently linked to, or associated, with infections are listed in Table 3.1.

Table 3.1 Tumor types presently linked to infections

Tumor type	Infectious cause
B-cell lymphomas in immunocompromised patients (ca. 50%) and in a subset of T-cell lymphomas	EBV
Burkitt's lymphomas	
Nasopharyngeal cancer	
Hodgkin's disease (30–40%)	
Gastric cancer (~10%)	
Cancer of the cervix, anal and perianal cancers	various HPV types
Vulvar, penile and vaginal cancers	
Oropharyngeal cancers (ca. 25%)	
Specific squamous cell carcinomas of the skin	
Hepatocellular carcinomas	HBV and HCV

Table 3.1 (Continued)

Tumor type	Infectious cause
Adult T-cell leukemia (ATL)	HTLV-1
Seminomas?	Endogenous human retroviruses
Kaposi's sarcoma	HHV-8
Gastric cancer, gastric lymphoma	**Helicobacter pylori**
Bladder cancer (Rectal cancer)	**Schistosoma haematobium, (japonicum, mansoni)**
Cholangiocarcinoma	**Opisthorchis viverrini and felineus, Clonorchis sinensis, (Helicobacter bilis?)**

There exist a number of other cancers (these will be discussed subsequently) which may have an infectious etiology. In particular, not only leukemias and lymphomas but also epithelial cancers linked to inflammatory events (e.g., cancer of the prostate) deserve attention. In addition, for seminomas a role of specific endogenous retroviruses has been postulated. The analysis of a potential role of these viruses for various human tumors remains to be intensified.

3.2 Global Contributions of Infections to Human Cancers

An estimation of the global contribution of infections to human cancers depends on the information received from regional cancer registries. A first estimation taking into account contributions of major viral infections linked to human cancers [EBV, human papillomaviruses (HPV), hepatitis B virus] was published in 1986 (zur Hausen). This was based on a previous publication of Parkin et al. (1984), according to whom approximately 15% of human cancers were linked to viral infections. The subsequent identification of the involvement of *H. pylori* in human gastric cancers resulted in an upgrading of this percentage (Parkin et al., 2002). In the calculation by Parkin and his colleagues, the participation of high-risk papillomavirus infections in oropharyngeal cancers was substantially underestimated. The majority of studies conducted came to the conclusion that approximately 25% of these cancers were linked to papillomavirus infections. In addition, Parkin's estimates did not include approximately 10% of gastric cancers which are presently linked to EBV infections. Thus, the estimates in Figures 3.1 and 3.2 are derived from the published data of Parkin et al., but contain some modifications.

By including cancers at all genital sites linked to HPV infections (ca. 90% of perianal and anal cancers, and ca. 50% of penile and vulvar cancers, vaginal cancers), adult T-cell leukemia, Kaposi's sarcoma and cancers caused by parasitic infections,

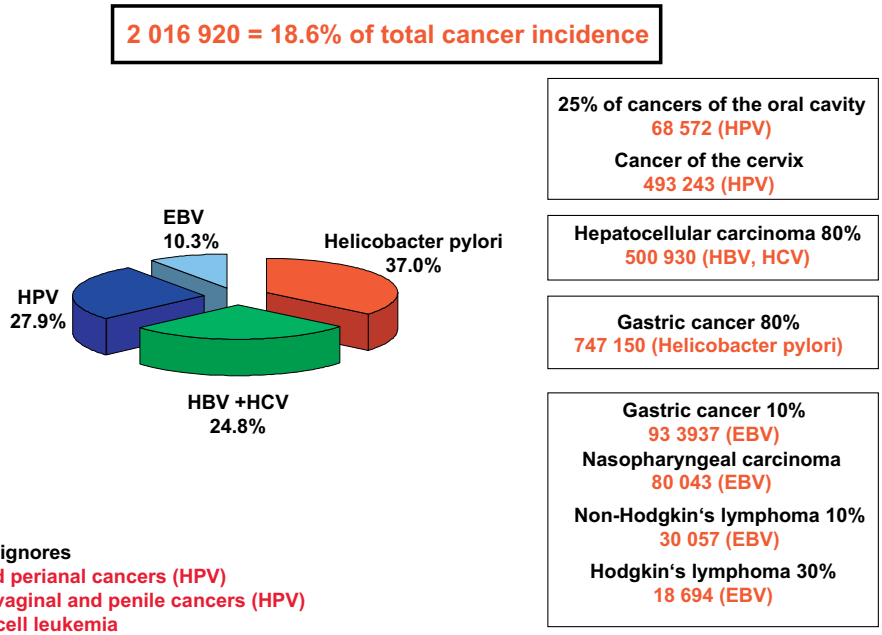

Fig. 3.1 Estimation of the annual contribution of Epstein–Barr virus, human papillomaviruses, hepatitis B and C viruses and *Helicobacter pylori* to global cancer incidence. (Modified from: Parkin et al., Global Cancer Statistics, 2002.)

slightly more than 20% of the global cancer incidence should presently be due to infectious events.

There exist remarkable differences between males and females in the incidence of cancers linked to infections: high-risk HPV infections are the main contributers to cancers in females, due to the high rate of cervical cancers worldwide (Fig. 3.2). In males, the role of this infection is relatively small, but *H. pylori* plays the main role in gastric cancer, which is still one of the most frequent cancers globally.

The geographic distribution of cancers linked to infections differs substantially between developed and developing parts of the world (Fig. 3.3).

In particular, sub-Saharan Africa is most severely affected by these types of cancer. Australia, North America and Central and Western Europe reveal the lowest percentages of infection-linked cancers.

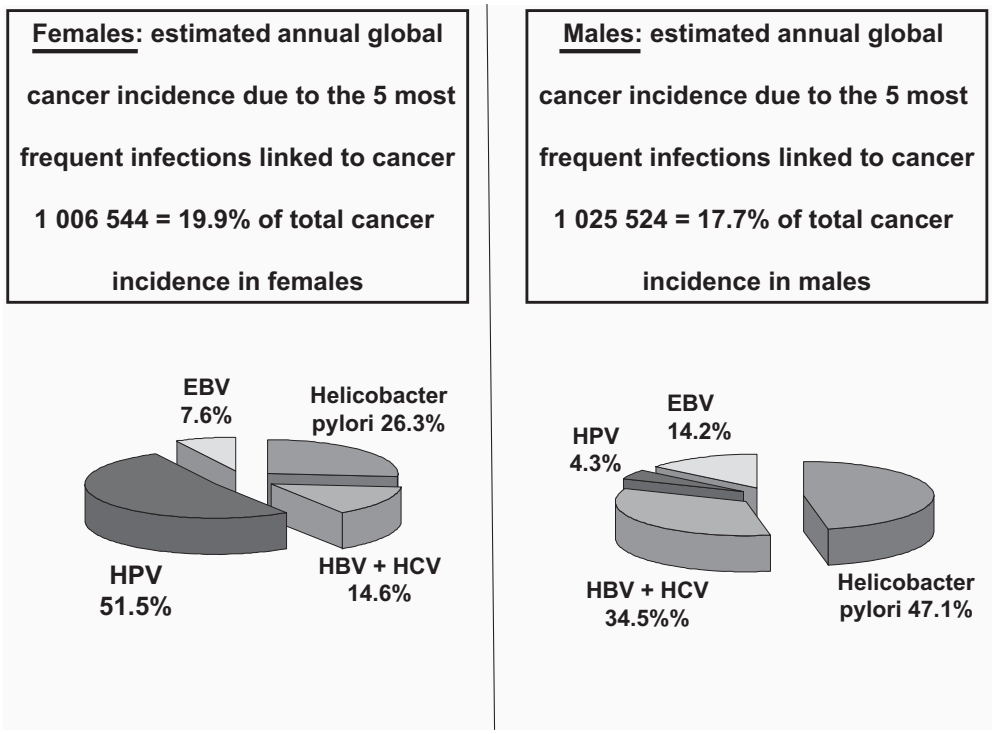

Fig. 3.2 Estimation of the annual contribution of Epstein–Barr virus, human papillomaviruses, hepatitis B and C viruses and *H. pylori* to global cancer incidence in females and males.

3.3
Host Interactions with Potentially Carcinogenic Infections: The CIF Concept

An early postulation of the loss of a cellular control of persisting tumorviruses resulted from: (i) the long latency periods elapsing between primary infection and subsequent tumor emergence; (ii) the observed synergism between physical and chemical mutagens and oncogenic viruses (reviewed by Casto and DiPaolo, 1973); (iii) the obvious stepwise progression to malignant growth; and (iv) the growing knowledge of monoclonal tumor development, even of those cancers associated with viral infections (zur Hausen, 1977). These observations formed the background for suspecting the existence of a cellular interference factor (CIF), the allelic deletion of which could explain most of the previously mentioned observations.

Subsequent developments clearly indicated that a single interference factor could not be responsible for a growing number of observations linked to persistent tumorvirus infections and for the protection against malignant proliferations of persistently infected cells (zur Hausen, 1994). This resulted in an extension of the hypothesis to a cellular interference factor cascade (CIF-cascade). Today, there exists ample evidence for the existence of at least three protective signaling cascades, providing custody to prevent malignant conversion of those virus-infected cells.

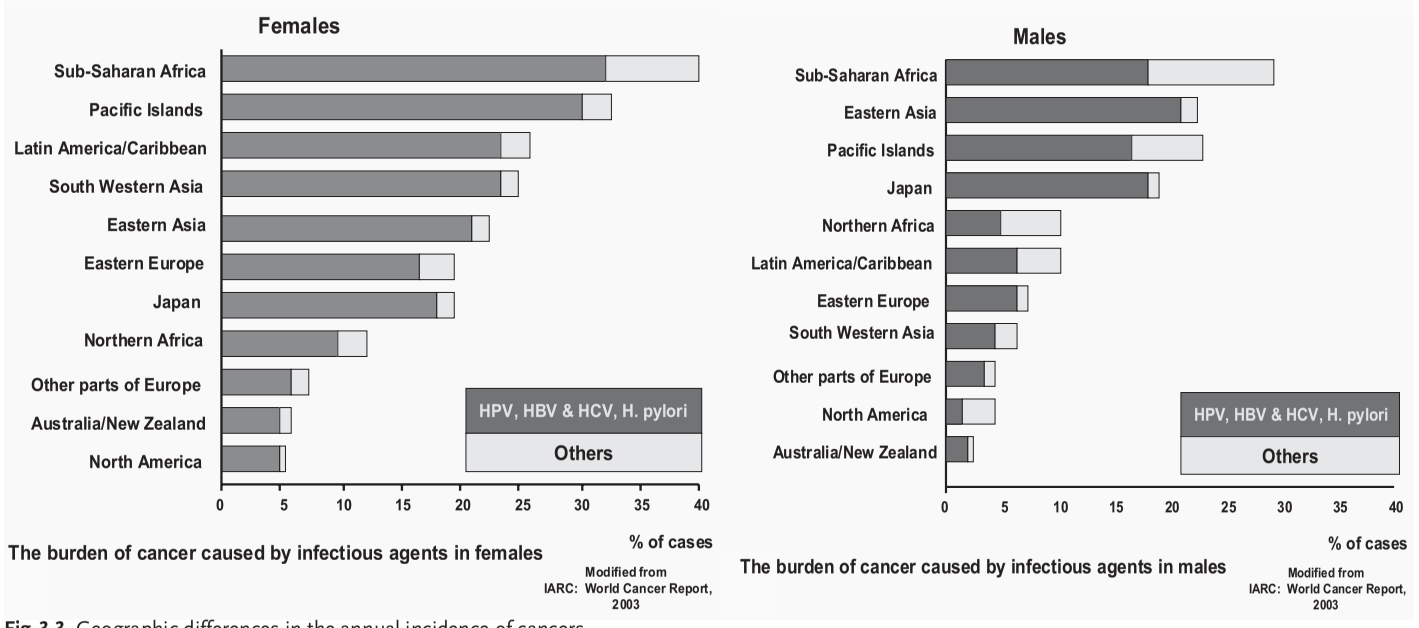

Fig. 3.3 Geographic differences in the annual incidence of cancers linked to infection in males and females. (Modified from World Cancer Report, Stewart, B.W. and Kleihues, P. (Eds.), Lyon, France: IARC Press, 2003.)

3.3.1 The CIF-I Cascade

Immunosuppression substantially increases the risk for malignant tumors linked to infectious events. This accounts in particular for EBV-associated lymphomas, human herpesvirus type 8-linked Kaposi's sarcomas and high-risk human papillomavirus (HPV)-caused intraepithelial neoplasias at cervical, anogenital and oral sites (see Chapters 4.3.1, 4.4.1 and 5.4, respectively). Clearly, in immunocompetent individuals malignant progression of persistently infected tissues is at least in part controlled and prevented by immune functions of the host. Most or all of the viruses mentioned here are able to suppress specific acquired or innate immune functions (in case of herpes group viruses, frequently the HLA class I and II presentation [Walev et al., 1992; Brander et al., 2000; Neumann et al., 2003; reviewed in Schust et al., 1999]. E5 functions of HPV down-regulate MHC class I molecules [Cartin and Alonso, 2003; Ashrafi et al., 2005]. Expression of HPV 16 E5 also perturbs MHC class II antigen maturation [Zhang et al., 2003], and hepatitis B and C viruses block interferon expression [Twu and Schloemer, 1989; Foster et al., 1991; Fernandez et al., 2003; Breiman et al., 2005; Zhang et al., 2005]). Although this suppression may facilitate long-term viral genome persistence, it is not sufficient to result in premalignant or malignant proliferations. Clearly, additional modifications must take place that enable the viral genome-carrying cells to continue growth.

In EBV infections at least three different events may interfere with immune functions and lead to malignant proliferations:

1. A general suppression of cell-mediated immune functions, as occurs in organ allograft recipients treated with immunosuppressive drugs or in HIV-infected patients. Apparently, the mere immunosuppression is still not sufficient for the outgrowth of EBV genome-carrying B cells, as indicated by a prolonged period of immunosuppression prior to the onset of lymphomatous growth and the common monoclonality of the arising lymphomas (Levine et al., 1992; Kimura et al., 2001). It is therefore highly likely that additional genetic or epigenetic modifications must take place before the appearance of continuously proliferating cells.
2. A modification in Xq25 of the SAP-gene in the X-chromosome-linked lymphoproliferative disease, as a hereditary disorder disturbing the immunological control of persistently EBV-infected cells (see Chapter 4).
3. The c-myc translocation, altering the EBV transcriptional regulation (Pajic et al., 2000) and resulting in a defect of c-myc to promote apoptosis due to a failure to induce the BH3-only protein Bim (a member of the B-cell lymphoma 3 [Bcl-2] family) and effectively to inhibit Bcl-2 (Hemann et al., 2005).

In high-risk papillomavirus-infected cells modifications in the antigen-presentation system appear to occur more randomly, yet, more than 95% of HPV-positive cervical cancers (in which the E5 gene is commonly deleted) reveal defects in the HLA class I presentation system (for a review, see Stern, 2005). Related observations, which here mainly affect the innate immune system, have also been reported for hepatitis C (see Chapter 7 and Jinushi et al., 2004).

Thus, either adaptive immunity or its auxiliary systems including innate immune functions need to gain specific defects, probably to ensure a state of long-term persistence. This is shown schematically in Figure 3.4.

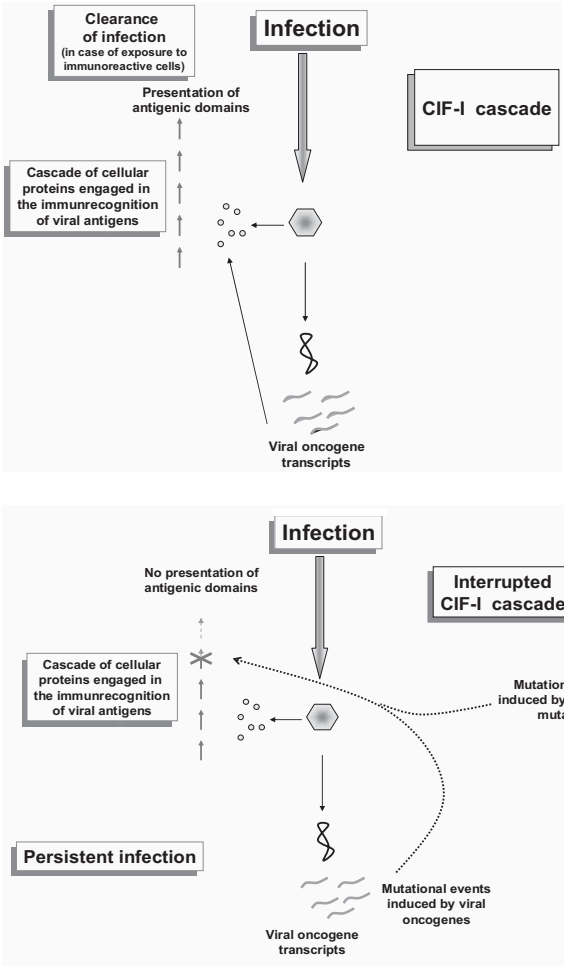

Fig. 3.4 Schematic outlines of the CIF-I cascades whose failure abolish or reduce immune recognition of viral genomes facilitating genome persistence.

3.3.2
The CIF-II Cascade

An intracellular control of viral oncogene functions was postulated during the late 1970 s (zur Hausen, 1977). This was in part based on the observation that a number of human and other primate viruses (BK, JC, several types of human pathogenic adenoviruses, SV40) possess oncogenes which efficiently induce malignant tumors if inoculated into newborn rodents (mice, hamsters, rats), but fail to be tumorigenic in their native hosts. Clearly, this effect is not due to the immune competence of the human host, since until now tumors linked to these infections have neither been detected in immunosuppressed allograft recipients, nor in HIV-infected persons. Since BK and JC polyoma-type viruses can be reactivated under conditions of immunosuppression without subsequent tumor induction, it is apparently not an immune function that negatively controls the oncogenic potential of these viruses. Thus, it is likely that an intracellular control developed during the course of human evolution protects our species against the potentially deleterious effects of these infections.

This concept is nicely supported by somatic cell fusion experiments conducted either with SV40- or HPV 16-immortalized cell lines. Somatic cell hybridization of different clones of such cells with each other frequently results in complementation to senescence with complete loss of their proliferative capacity in spite of ongoing viral oncoprotein synthesis (Pereira-Smith and Smith, 1981; Whitaker et al., 1992; Chen et al., 1993; Seagon and Dürst, 1994). In the absence of immunological interference and of contacts with other cell compartments and in presence of viral oncogene expression, this clearly points to an intracellular control of viral oncogene function. Senescence seems to be the consequence of complementation of different mutated genes in the respective cell fusion partners. This is outlined schematically in Figure 3.5.

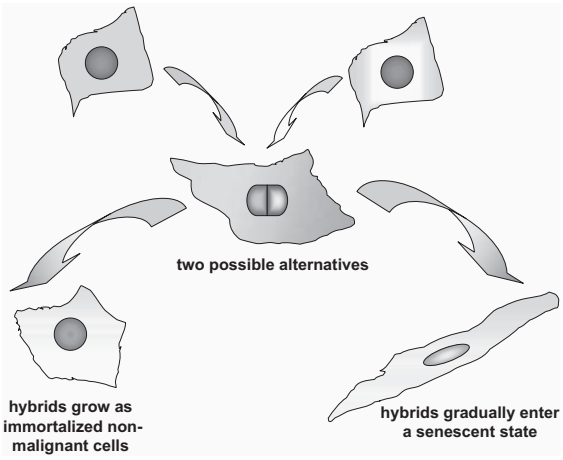

Fig. 3.5 Schematic outline of somatic cell hybridization between two cell clones expressing the same viral oncogene. Some combinations comple-

Thus, apparently several different genes suppress the viral oncogene function, possibly within the same signaling cascade.

Cyclin-dependent kinase inhibitors are proteins relatively clearly identified in suppressing functions of high-risk papillomaviral oncogenes (see Chapter 5). In particular, $p16^{INK4}$ negatively interferes with the E6 function, whereas some evidence points to an important role of $p14^{ARF}$ in blocking E7 functions. Mutagenic events, in part induced by functions of the viral oncogenes, in part also triggered by exogenous mutagens or epigenetic modifications, result in the interruption of the CIF-II cascade. This is outlined schematically in Figure 3.6.

CIF-II functions thus prevent the development of premalignant lesions in latently papillomavirus- and probably also polyomavirus-infected cells. It seems that polyomavirus and adenovirus infections in their respective natural hosts are commonly – but not uniformly (e.g., polyomavirus infection in mice) – controlled by more than one intracellular signaling cascade, probably as an adaptive response of the host during the course of evolution. On the other hand, this type of control may be absent in virus-linked tumors emerging after a relatively short incubation period, such as EBV-linked immunoblastic lymphomas, Kaposi's sarcoma and the endemic form of Burkitt's lymphoma. The highly specialized cells (B cells and endothelial cells) from which these tumors originate seem to have lost this control mechanism, possibly as consequence of their degree of differentiation.

3.3.3 The CIF-III Cascade

Evidence for a third signaling pathway negatively interfering with the transcription of viral oncogenes originated from studies on a paracrine transcriptional control of high-risk HPV infections (see also Chapter 5). Human keratinocytes immortalized by HPV 16 actively transcribe the viral oncogenes E6 and E7 under tissue culture conditions (Bosch et al., 1990; Dürst et al., 1991, 1992; Stoler et al., 1992). Following

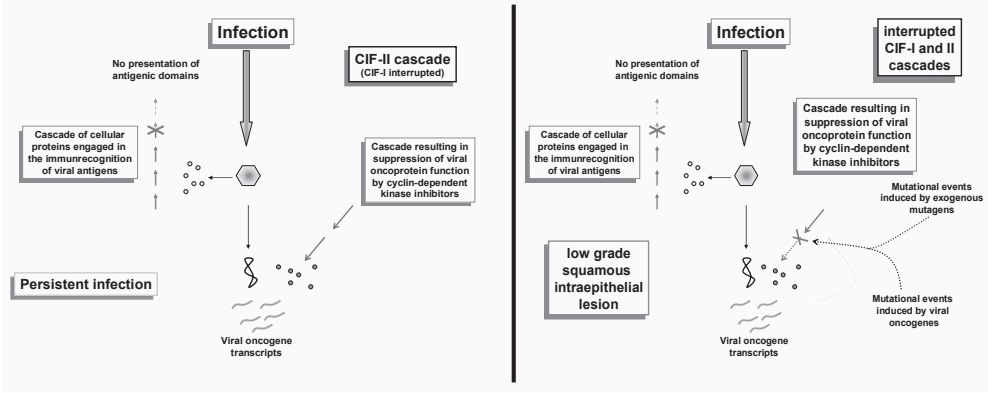

Fig. 3.6 Schematic outlines of the CIF-II cascades which interfere with the function of viral oncogenes.

xenotransplantation of these cells into nude mice, however, cell growth ceases and transcription of viral DNA is drastically reduced. In contrast to HPV-containing malignant cells, HPV-immortalized cells fail to form tumors under these conditions and persist for long periods of time as small nodules. Malignant cells commonly grow as xenotransplant and do not reveal any inhibition of viral oncogene transcription.

The existence of a paracrine transcriptional control exerted by cell compartments surrounding the immortalized cells in xenotransplants was further supported by experiments revealing the inhibition of HPV transcription in non-malignant HeLa-fibroblast hybrids by the addition of activated macrophages (Rösl et al., 1994) and subsequently also by tumor necrosis factor (TNF)-α (Delvenne et al., 1995; Vieira et al., 1996; Soto et al., 1999). The mechanism of this inhibition was partially clarified: TNF-α caused a modification of the transcription factor AP-1 within the HPV promoter, modifying pre-existing jun/jun dimers to jun/Fra-1 heterodimers (Soto et al., 1999, 2000). Malignant HeLa cells, containing HPV 18 DNA, reveal at the same site c-jun/c-fos heterodimers. Overexpression of c-fos in immortalized cells results in malignant conversion of those cells in a single step (Soto et al., 1999; Prusty and Das, 2005). Recent data point to a dysregulation of c-fos expression in malignant cells due to the absence of c-fos repression by the negative regulator Net (van Riggelen et al., 2005). A scheme depicting the interruption of this third regulatory cascade is shown in Figure 3.7.

It is likely that similar regulatory mechanisms exist in other viral systems which may lead to malignant transformation of human cells. Cells immortalized by EBV also fail to form tumors after xenotransplantation into nude mice. Similarly, hybrids between EBV-positive Burkitt's lymphoma cells and lymphoblastoid cells from the same donor are non-tumorigenic, in spite of the tumorigenic properties of the parental Burkitt's lymphoma line (Wolf et al., 1990; Jox et al., 1998). After an initial period of growth they regularly undergo apoptosis (Figs. 3.8 and 3.9). The underlying mechanism is presently not understood, but it is likely that the apoptotic events are triggered by the surrounding cellular environment.

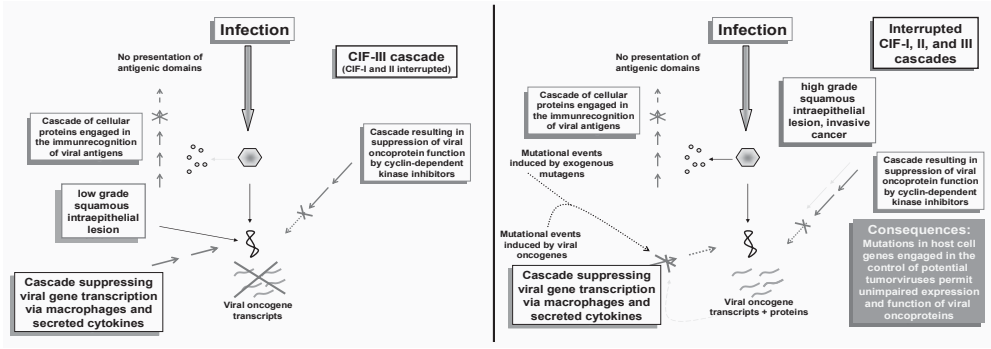

Fig. 3.7 A third regulatory cascade (CIF-III) suppressing the transcription of HPV oncogenes in immortalized cells (left) and its interruption in malignant proliferations (right).

A similar situation seems to exist for HTLV-1 immortalized cells (for reviews, see Ambinder, 1990; Suzuki and Yoshida, 1997), although no detailed mechanistic studies are available.

It should be noted that a large number of studies have shown in the past that immortalization of a variety of cell types by animal viruses (e.g., SV40, polyoma, adenoviruses) does not result in a tumorigenic phenotype (Asselin and Bastin, 1985; Pontén, 1985; Steinberg and Defendi, 1983). Similarly, some human viruses immortalizing animal cells (e.g., BK or JC polyomaviruses, adenoviruses types 12, 18, 31) do not induce a malignant phenotype under these conditions (Casto, 1968; Wright et al., 1976).

In stark contrast to these observations, the inoculation of certain tumor viruses into evolutionarily not too distantly related species (e.g., herpesvirus saimiri, herpesvirus ateles and EBV virus into cotton-top marmosets) may result in the acute development of leukemias and lymphomas. Apparently, adaptive mechanisms interfering with these infections do not exist in those species which are not infected by these viruses under natural conditions. This points to an interesting correlation between the length of the latency period between primary infection and subsequent tumor development and the existence of cellular interfering factors. The absence of

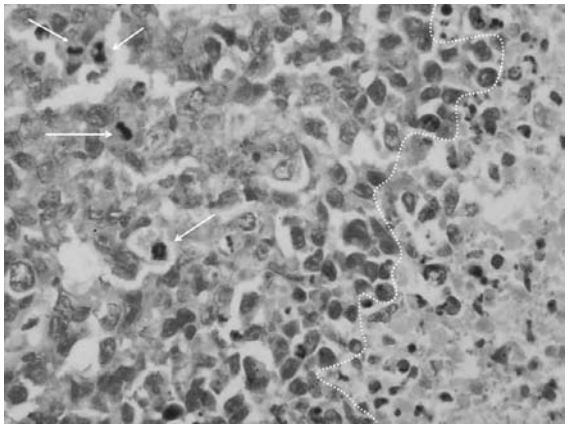

Fig. 3.8 Epstein–Barr virus-immortalized cells may initially grow rapidly after xenotransplantation into nude mice. After several days a central necrosis becomes visible and the tumors regress completely. The figure shows a tumor in regression. The white line reveals a relatively sharp demarcation of still actively proliferating cells (left side, arrows depict cells in mitosis) and progressive apoptosis (right side).

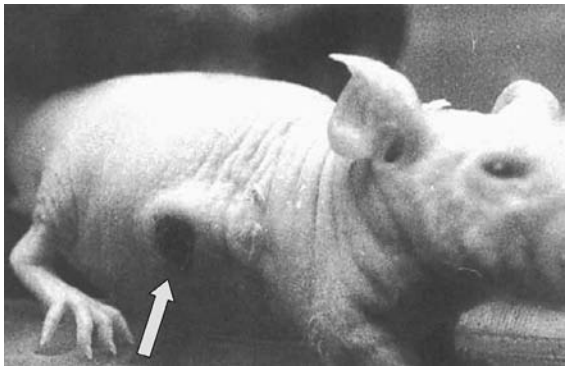

Fig. 3.9 Regressing tumor of Figure 3.8, revealing a typical central necrosis. (Illustration courtesy of Jürgen Wolf, Cologne.)

the latter seems to result in immediate cell proliferation and tumor formation, their partial absence or reduced function abbreviates the latency period. Latency periods of 20 years and more probably underline long periods of co-evolution between the virus and the human host.

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4
Herpesviruses and Oncogenesis

There are presently eight members of the herpesvirus group known as human pathogens: herpes simplex viruses type 1 and 2 (also known as HSV-1 and HSV-2 or HHV-1 and HHV-2); varicella-zoster virus (VZV or HHV-3); Epstein–Barr virus (EBV or HHV-4); human cytomegalovirus (CMV or HHV-5); human herpesvirus 6 (HHV-6); human herpesvirus 7 (HHV-7); and Kaposi sarcoma-associated herpesvirus or human herpesvirus type 8 (KSHV or HHV-8). The virion contains a core with linear double-stranded DNA, varying in size between 120 and 230 kbp, an icosahedral capsid of about 100–110 nm in diameter with 162 capsomeres. The capsid is surrounded by an envelope containing viral glycoproteins on its surface.

Herpesviruses are found in a large number of different species, and almost 100 herpesviruses have at least been partially characterized (Roizman, 1996). Virtually all of these types contain linear DNA with terminal repeat sequences, some of them juxtaposed internally. All of these viruses share the property of persisting in a latent state in specific types of cells. The cell type harboring persisting viruses of this family differs according to the virus type. In the state of latency and prior to viral DNA replication in productive infections, the DNA circularizes.

The current classification subdivides the herpesvirus family into three subfamilies. This subdivision is based on DNA sequence homology, similarities in genomic sequence arrangements and the relatedness of viral proteins:

- Alphaherpesvirinae: These are characterized by a variable host range and the establishment of latent infections in sensory neuronal cells. Human representatives of this group are HSV-1 and -2 and the VZV.
- Betaherspevirinae: These reveal a rather restricted host range, a slow reproductive cycle, and remain latent preferentially in salivary gland cells, cells of the hematopoietic system, kidneys and some other tissues. Human representatives of this group are CMV and HHV types 6 and 7.
- Gammaherpesvirinae: Viruses in this group specifically infect B and T lymphocytes, where they result either in lytic or latent infections. Some are also able to infect epithelial, fibroblastic and endothelial cells. This subfamily contains two genera, the *Lym-*

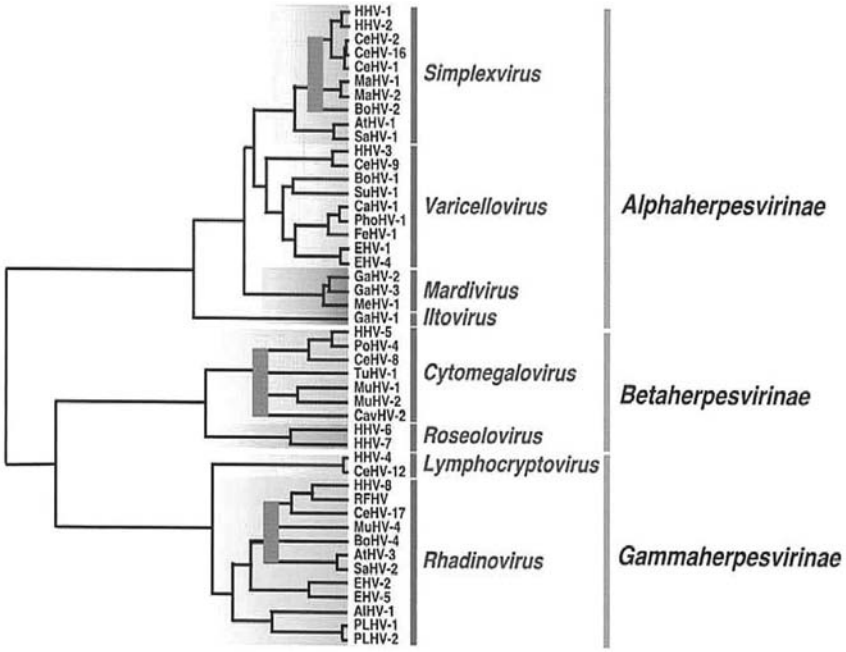

Fig. 4.1 Phylogenetic tree of the herpesvirus family. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The Double Stranded DNA Viruses, Herpesviridae, 211. Copyright 2005, with permission from Elsevier.)

phocryptoviruses (e.g., EBV) and the *Rhadinoviruses* (e.g., HHV type 8).

Whereas EBV and HHV type 8 were linked immediately after their discovery with human malignancies, a role of other members of this family remains uncertain until today. Human herpesviruses induce mutations in host cell DNA (Schlehofer and zur Hausen, 1982). Even under conditions of abortive infections these virus infections effectively amplify persisting genomes of small DNA tumorviruses (e.g., polyoma-type and papillomavirus genomes) (Schlehofer et al., 1983; Matz et al., 1984; Schmitt et al., 1989; Heilbronn and zur Hausen, 1989; Heilbronn et al., 1990). Human CMV even permits the replication of the human JC polyomavirus co-transfected into CMV-infected human fibroblasts (Heilbronn et al., 1993). This may represent an indirect mode by which these infections could contribute to human carcinogenesis, although epidemiological evidence for their contribution under *in-vivo* conditions is still missing.

Besides human pathogenic herpesviruses, a number of animal representatives of this group are known to represent potent carcinogens, either in their natural species of infection or upon inoculation into related species. *Marek virus* of chickens induces

an infectious neurolymphomatosis in chickens, while the *Lucke frog herpesvirus* emerges as the responsible agent for kidney carcinomas in American leopard frogs. It is interesting to note that Marek's herpesvirus may incorporate complete genomes of avian retroviruses, such as the chicken reticuloendotheliosis virus, into its own DNA under natural as well as under experimental conditions (Isfort et al., 1992; Jones et al., 1993; Witter et al., 1997; Davidson and Borenshtain, 2001). *Herpesvirus saimiri* and *herpesvirus ateles*, which persistently infect their natural hosts, are potent leukemogenic agents in related species and induce acute leukemias and lymphomas there. A large number of species have been identified harboring EBV-related herpesviruses.

All these observations stress the importance of members of this virus family as important carcinogens or as potential candidates for human carcinogenicity.

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4.1
Alphaherpesvirinae

4.1.1
Herpes Simplex Viruses Types 1 and 2

Herpes simplex virus infections are very common and affect a high percentage of all human populations. In 1962, Schneweiss identified two different serotypes, subsequently confirmed by Nahmias and Dowdle (1968). Whereas HSV-1 is found predominantly in oropharyngeal infections, HSV-2 has a strong predilection for anogenital sites. Both types of viruses cause a large number of different clinical symptoms. Their molecular biology, as well as clinical aspects of herpetic infections, have been described extensively elsewhere (Roizman, 1996; Whitley, 1996).

In 1968 and 1969, the first reports appeared on increased antibody levels against HSV-2 antigens in women with cervical cancer (Rawls et al., 1968; Naib et al., 1969). Studies confirming these findings were published one year later (Nahmias et al., 1970; Rawls and Kaufman, 1970). These studies initiated a burst of activities which attempted to link herpetic infections to human cancers, particularly to cancer of the cervix. Reports appeared identifying virus-specific antigens as markers for cervical cancer (e.g., Aurelian et al., 1977, 1983; MacNab et al., 1980; Gupta et al., 1981). Fifteen years after the first report by Naib et al., a large-scale prospective study conducted in the former Czechoslovakia failed to provide serological evidence in support of a role of HSV in cervical cancer (Vonka et al., 1984 a,b). At approximately the same time, specific types of human papillomaviruses (HPV) were incriminated as the main cause of cervical cancer (Dürst et al., 1983; Boshart et al., 1984; Schwarz et al., 1985). The combined effect of these publications was most likely the prime reason for a reduced interest in seroepidemiological studies analyzing the relationship between HSV infections and anogenital cancer. Very recently, however, two further large-scale seroepidemiological studies have been published, one linking HSV-2 seropositivity with an increased risk of cervical squamous cell carcinoma (relative risk [RR] 2.19) and of adeno- or adenosquamous cell carcinoma (RR 3.37) after adjustment of potential confounders (Smith et al., 2002). A follow-up of a cohort of 550 000 women in Scandinavia (Lehtinen et al., 2002) arrived at an opposite result: after adjustment for smoking and HPV infections, the relative risks for HSV-2 were 1.0 and 0.7, respectively, and even in a meta-analysis the relative risk for HSV-2 was 0.9. Thus, seroepidemiological evidence for a role of HSV-2 in cervical cancer remains inconclusive.

Additional experimental studies, however, seemed to support a possible role of HSV in human cancers. These originated in part from reports of HSV DNA or RNA persistence in cervical cancer cells: in 1972, Frenkel et al., and in 1973 Roizman and Frenkel, identified a fragment of HSV-2 in one cervical cancer biopsy and also analyzed transcripts of this DNA. Two other groups reported similar findings: initially, HSV RNA was detected in premalignant cervical cells only (McDougall et al., 1980), but subsequently RNA and HSV protein was found in tumors of the uterine cervix (McDougall et al., 1982), the RNA representing limited regions of the viral genome.

Four years later, the same group reported the occasional detection of HSV and HPV DNA and RNA within the same tumor (McDougall et al., 1986).

These data require cautious interpretation: several studies noted regions of homology between HSV and mammalian (including human) DNA (Peden et al., 1982; Puga et al., 1982; Jones et al., 1985; Gomez-Marquez et al., 1985). Although most of the homologies were located within the long and short inverted repeat regions, an additional one was noted near the center of the long unique region. Hybridization occurred even at relatively stringent conditions (Peden et al., 1982). The *in-situ* hybridization labeling not only of cervical carcinoma cells, but also of apparent macrophages by the HSV-specific probe (McDougall et al., 1982), could suggest a homology with cellular RNA expressed preferentially in proliferating cells and activated macrophages. In addition, other reports failed to confirm the presence of HSV-2 DNA in cervical cancer cells (zur Hausen et al., 1974; zur Hausen, 1976).

Another puzzling aspect originates from *in-vitro* transformation studies of rodent cells with partially inactivated HSV DNA or with DNA fragments of these viruses. Duff and Rapp (1971, 1973) reported initially oncogenic transformation of hamster embryo cell after exposure to partially inactivated HSV-2 or HSV-1. A number of additional reports followed which revealed the transformation also of murine and rat cells, in part by fragments of HSV DNA (for a review, see Minson, 1984). In one of these studies persistence of HSV-2 DNA sequences was noted in transformed hamster cells (Galloway et al., 1980), corresponding to a sequence also found in some cervical cancers (McDougall et al., 1984).

Overall, three distinct transforming regions have been identified: in HSV-1 DNA the fragment BglII (map units 0.31–0.42), the HSV-2 fragment BglII N (map units 0.58–0.63) and the HSV-2 BglII C fragment (reviewed in Di Luca et al., 1995). Although the BglII N fragment contains the information of at least five polypeptides (Galloway et al., 1982), subsequent publications by the same group stated that the transforming part of this fragment may not specify a viral polypeptide (Galloway et al., 1984). An insertion sequence-like structure was incriminated as the possible transformant. As a consequence of these observations, a "hit-and-run" mechanism was postulated for HSV oncogenesis (Skinner, 1976; Galloway and McDougall, 1983).

In-vitro transformation studies could represent a strong correlate to seroepidemiology and nucleic acid hybridization studies. It is somewhat unfortunate, however, that human cells proved to be refractory to cell immortalization by HSV DNA fragments or by partially inactivated virus particles. In rodent cells, spontaneous transformation is a relatively frequent event which may be enhanced by nonspecific exposures, such as DNA transfection. The activation of endogenous retroviruses by HSV or its DNA fragments (Hampar et al., 1976; Boyd et al., 1978, 1980) may further complicate the interpretation of transformation studies in rodent cells. The activation of murine type C viruses is not limited to transformed murine cells, but occurs also in nontransformed cells (Hampar et al., 1977). Even in human cells, endogenous HERV-K and HERV-W retrovirus long terminal repeats (LTRs) become activated by HSV infection (Kwun et al., 2002; Lee et al., 2003). HSV infection also results in activation of the LTR of human immunodeficiency virus (HIV) (Gendelman et al., 1986;

Mosca et al., 1987), which seems to result from an interaction between HIV Tat and Rex and the HSV US11 protein (Popik and Pitha, 1994; Schaerer-Uthurralt et al., 1998). Thus, the reported transformation of cells by HSV may have resulted from specific retrovirus insertion after their activation by the HSV infection.

In addition, there exists a remarkable paucity of further transformation studies during the past two decades, when compared to the 1970s and the early 1980s. Thus, the evidence for a role of HSV infections in human cancers remains inconclusive.

Some other experimental approaches still could point to an indirect role of HSV infections in carcinogenesis: HSV infection actively induces chromosomal aberrations in infected host cells, even under conditions of an abortive infection (Hampar and Ellison, 1961; Boiron et al., 1966; Waubke et al., 1968; O'Neill and Miles, 1969). This points to a potential mutagenic activity of these virus infections. Direct proof for a mutagenic effect of HSV infections originated from a number of different experiments: Schlehofer and zur Hausen (1982) and Pilon et al. (1986) showed that partially UV-inactivated HSV or HSV-2 infecting nonpermissive cells induced mutations within host cell DNA. Clarke and Clements (1991) revealed that mutagenesis occurring after infection with HSV types 1 or 2 does not require virus replication. Shillitoe et al. (1986) and Das and colleagues (1994) induced mutations in a bacterial assay with the cloned Bam HI G fragment of HSV type 1, and showed that the UL 26 gene, expressed with a different carboxy terminus early in infection, codes for a mutagenic peptide. Brandt et al. (1987) induced mutations within the HGPRT gene by transfection with fragments of HSV-2 DNA.

Another interesting possibility by which HSV infection may contribute to cell transformation is the ability of these viruses to amplify genomes of persisting small DNA tumorviruses even under conditions of abortive infection. Initially, Sara Lavi (1981) showed the amplification of persisting SV40 DNA in Chinese hamster cells after treatment of these cells with chemical carcinogens. The mutagenic activity of HSV infections for host cell DNA prompted similar experiments after HSV infection of SV40-transformed cells (Schlehofer et al., 1983), resulting also in the amplification of persisting SV40 genomes. It was subsequently shown that the HSV DNA polymerase is an essential component for this amplification (Matz et al., 1984). Amplification is not limited to polyoma-type viruses, but has also been noted in HSV-infected papillomavirus-positive cells (Brandt et al., 1987; Schmitt et al., 1989; Hara et al., 1997).

The induction of chromosomal aberrations, of mutations in host cell DNA, and the amplification of other DNA tumorvirus genomes after infection with HSV could all contribute (as an indirect mode) to human carcinogenesis. Unfortunately, epidemiological support for such a role is still very weak and hardly convincing. Thus, as there exists no evidence that HSV-1 or -2 do act as direct carcinogens, even their role as indirect carcinogenic factors remains to be established.

4.1.2
Varicella-Zoster Virus

Varicella-zoster virus is the etiological agent of chickenpox, an acute infectious disease, resulting in life-long persistence of viral DNA in the dorsal root ganglia. Reactivation of the virus may occur under conditions of long-lasting stress or immunosuppression, resulting in typical localized zoster eruptions. Patients at high risk for zoster eruptions are AIDS patients, organ allograft recipients, patients with malignant hematopoietic disorders, and those receiving chemotherapy (for a review, see Arvin, 1996). The zoster lesions produce infectious virus and may give rise to new chickenpox epidemics among nonexposed children. A detailed account of the molecular biology and clinical features of VZV infections is provided elsewhere (Cohen and Straus, 1996; Arvin, 1996).

Data relating VZV infections to human malignant tumors are scarce, and based on a few studies on the transformation of rodent cells and the biochemical transformation of mammalian cells. In 1980, Gelb et al. reported the oncogenic transformation of primary hamster embryo cells after infection with VZV. Inoculation of these cells into inbred hamsters resulted in aggressively growing fibrosarcomas. The tumor-bearing hamsters developed antibodies to VZV antigens. In a later study, Gelb and Dohner (1984) state that transformation of cells by VZV is a very rare event, and one that may require a recent clinical isolate. Transformed hamster cell lines did not retain VZV DNA.

Transformation to thymidine kinase expression of mouse L-cells lacking the enzyme thymidine kinase (Ltk^-) by VZV infection was reported by Yamanishi et al. (1981). The transformed cells showed almost the same growth rate as the parental Ltk^- cells. The converted clones revealed, however, a nuclear VZV-specific antigen and their tk-activity was neutralized by hyperimmune serum against VZV.

Altogether, the available publications presently provide no evidence for a role of VZV infection in the causation of human malignancies.

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4.2
Betaherpesvirinae

4.2.1
Human Cytomegalovirus

Human CMV infects the majority of individuals in all human populations, results commonly in a latent infection, and is responsible for a large number of different clinical symptoms. Serological and molecular studies have attempted to link this infection to cervical carcinoma, to adenocarcinomas of the prostate and colon, and to Kaposi's sarcoma (reviewed in Doniger et al., 1999; Harkins et al., 2002; Samanta et al., 2003). These studies either yielded conflicting results or were based on individual reports. Detailed descriptions of its molecular biology and of the clinical symptoms caused by this infection have been published elsewhere (Mocarski, 1996; Britt and Alford, 1996).

Human CMV shares at least three properties with HSV infections: it induces chromosomal aberrations (Lüleci et al., 1980; AbuBakar et al., 1988; Deng et al., 1992) and amplifies the persisting DNA of polyoma-type viruses, SV40 (Pari and St. Jeor, 1990a,b) and human JC virus (Heilbronn et al., 1993), permitting replication of JC DNA in otherwise nonpermissive human fibroblasts. The enhancement of bovine papillomavirus (BPV)-induced transformation of NIH 3T3 cells by co-infection with human CMV (Goldstein et al., 1987) seems to follow a different pattern, since amplification of BPV DNA was not noted in these experiments. Induction of specific chromosomal aberrations by human CMV has also been reported (Fortunato et al., 2000). The induction of host cell DNA synthesis by human CMV (St Jeor et al., 1978) seems to result in a subsequent block in cell cycle progression and cell division, mediated by the immediate early protein 86 (Murphy et al., 2000). Although the biological behavior of betaherpesvirinae differs substantially from that of HSV, transforming properties have also been reported for human CMV and HHV-6 infections. As another common property, similar to HSV, CMV and HHV type 6 also act as helper viruses for adeno-associated virus (McPherson et al., 1985).

Based on their data on transformation of hamster embryo cells by ultraviolet (UV)-irradiated HSV, Rapp and his colleagues initiated similar experiments with human CMV. In 1973, Albrecht and Rapp reported malignant transformation of hamster embryo fibroblasts following exposure to UV-irradiated human CMV. Induction of cellular DNA synthesis and increased mitotic activity was enhanced by irradiation with UV-light of the virus prior to infection (Albrecht et al., 1976). Three years later, the same group published the oncogenic transformation of human embryo lung cells by human CMV strain Mj (Geder et al., 1976). Here, the persistent infection of human embryonic lung fibroblasts with a genital isolate of CMV resulted in transformed human cells inducing progressively growing tumors in weanling athymic nude mice. The cells expressed virus-specific antigens, although at that time a potential contamination with transforming anogenital papillomavirus types could not be ruled out. An additional report claimed the transformation of human embryonic lung cells by additional CMV strains, BT1757 and Towne (Huang et al., 1986). A cell line obtained from human prostate cancer reacted positively with CMV-immune sera in indirect immunofluorescence (Geder et al., 1977). There was, however, no direct evidence that this cell line resulted from the exposure to human CMV. One of the transformation studies of human cells by CMV was subsequently clouded by the isolation of *infectious bovine rhinotracheitis virus* (IBRV) from the transformed human embryo lung fibroblasts (Geder et al., 1979). This virus apparently induced permanent growth upon infection of a primary human kidney cell culture. The apparent demonstration of intracellular immunofluorescent antigens specific for CMV resulted in the speculation that CMV might be involved in the development of prostatic neoplasia (Geder and Rapp, 1980). A CMV-induced transformation of human endothelial cells was reported by Smiley et al. (1988); CMV transformed these cells to anchorage-independent growth, persistently producing infectious CMV particles. Dog embryo kidney cells were also transformed by human CMV infection (Yelle et al., 1990). These cells are nonpermissive for this virus and, after infection, showed an unlimited division potential, and were poorly tumorigenic in mice.

Several additional reports stress a potential role of CMV in human cell proliferation: the cellular proto-oncogenes *fos*, *jun* and *myc* are transcriptionally transactivated by CMV infection (Boldogh et al., 1991). The prevention of apoptosis, mediated by viral immediate-early genes, may point to a role in indirect carcinogenesis (Zhu et al., 1995). The immediate-early genes IE 72 and IE 86 cooperate with adenovirus E1A in the transformation of baby rat kidney cells (Shen et al., 1997). Physical interaction of IE 86 with p53 has been noted without affecting p53-mediated cell cycle growth arrest (Bonin and McDougall, 1997). A similar interaction has been observed with pRb (Sommer et al., 1994; Fortunato et al., 1997).

In several studies the characterization of a transforming fragment of the human CMV genome was reported. Nelson et al. (1982) defined a 2.9-kilobase fragment between map units 0.123 and 0.14 on the prototype molecule of the CMV AD169 strain as transforming segment for NIH 3 T3 cells. This fragment was not retained within the transformed cells. The region was designated as "morphological transforming region I" (*mtr* I). A different fragment was identified by Clanton et al. (1983). By

using a different CMV strain (Towne), their *Xba*I-E fragment immortalized primary diploid Syrian hamster cells and transformed established NIH 3T3 cells. A subclone containing the 3.0 kbp *Xba*I-*Bam*HI EM fragment with efficient transforming activity was labeled as *mtr* II. A further characterization of the transforming part of the *Xba*I-E fragment published by *Clanton et al.*, revealed a small, potentially spliced open reading frame (ORF) which possessed some of the signals involved in eukaryotic gene expression (Kouzarides et al., 1983). Jariwalla et al. (1989) reported that the terminal fragments (EJ [designated as *mtr* III] and EM) of the XbaI-E segment of human CMV can independently induce tumorigenic conversion of immortalized Rat-2 cells. Both fragments cooperated in tumorigenic conversion, inducing foci at a tenfold higher frequency than the individual fragments. The transformants commonly retained the *mtr* II sequences, frequently in multiple copies, whereas *mtr* III or *mtr* I did not persist (el-Beik et al., 1986). The genomic organization of human CMV is outlined in Figure 4.2.

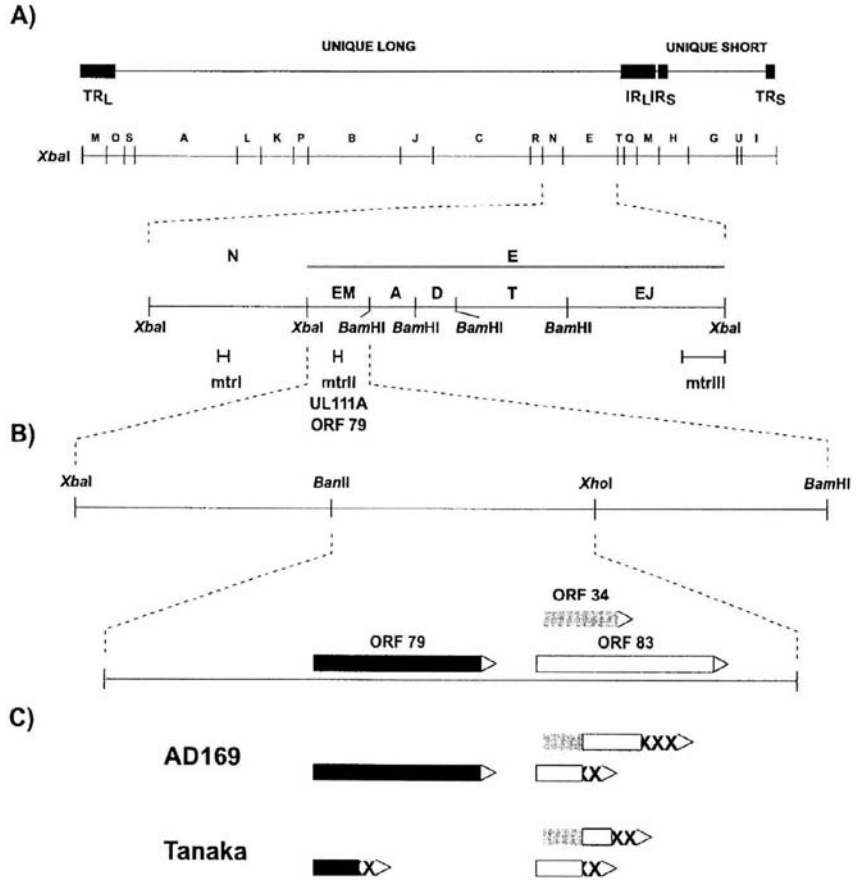

Fig. 4.2 Cytomegalovirus genomic organization. Supposedly transforming regions are indicated in the lower panel in black. (Doniger et al., 1999. With permission.)

The persistence of the *mtr* II region in immortalized and transformed cells promoted additional interest in these sequences. The transforming activity was further localized to a 980-bp subfragment with three ORFs with a coding capacity of 79, 83 and 34 amino acids (Razzaque et al., 1988). In the 5' terminus of the ORFs several regulatory elements and transcription initiation signals were located, in addition to six copies of the heptanucleotide sequence GGTG(A/G)TC. This motif has similarity to the SV40 enhancer core consensus sequence. All three putative proteins were observed 24 h after infection with CMV. Interruption of the *mtr* II ORF resulted in loss of transforming activity (Inamdar et al., 1992), strongly suggesting a direct involvement of these proteins in transformation and virtually excluding promoter insertion as mechanism for the observed transforming activity. Mutational analyses conducted by Thompson et al. (1994) pointed to a specific role of ORF 79 for the transforming activity of *mtr* II. An additional report (Muralidhar et al., 1996) claimed an interaction of mtr II protein with p53. mtr II also inhibited p53-activated transcription in transient transfection assays, as well as in transformed cells. In stably *mtr* II-transformed NIH 3 T3 cells the level of p53 was ten- to twenty-fold higher than in parental cells.

Based on these data, it is extremely difficult to assess a potential role for CMV in human carcinogenesis. Clearly, specific genes of this virus have transforming potential for rodent cells. The data on immortalization of human cells are not yet fully convincing and require further confirmation. The ubiquity of this infection, and its frequent persistence in normal and neoplastic tissues, makes it difficult to analyze any potential tumorigenicity. The situation might be reminiscent of infections with oncogenic human adenovirus types where, in spite of efficient tumor induction in rodents, no evidence for a role in human cancers has been obtained. Clearly, this field requires that further studies be conducted since, at present, there exists no convincing evidence for a direct role of CMV in human cancer, though an indirect contribution cannot be excluded.

4.2.2 Human Herpesvirus Type 6

Human herpesvirus type 6 (HHV-6) was initially named human B-lymphotropic virus (HBLV) and identified by Salahuddin et al. (1986) from patients with lymphoproliferative disorders or from HIV-positive individuals. This virus grows well in phytohemagglutinin-stimulated umbilical cord blood lymphocytes and T-cell lines, and requires interleukin (IL)-2 addition for efficient propagation (Black et al., 1989; Frenkel et al., 1990). HHV-6 is a ubiquitous virus which infects children at a young age, persisting subsequently and being found in at least 90% of all adults (Segondy et al., 1992; Portolani et al., 1993). It causes exanthema subitum and roseola ("sixth disease") in approximately 30% of infected children. Two subgroups of HHV-6 have been identified, namely HHV-6A and HHV-6B; these have an overall nucleotide identity of 90% (Dominguez et al., 1999), with a most divergent region in direct repeats (DR) and the right end of the unique region with 85% and 72% nucleotide sequence identity, respectively. A novel homologue of HHV-6B was isolated from

chimpanzees, with about 95% sequence homology at the nucleotide and amino acid levels (Lacoste et al., 2005). Recent studies have demonstrated a difference in the biological behavior of both subgroups: whereas HHV-6B virus depletes predominantly $CD4^+$ T cells, HHV-6A virus infection results in a depletion of both, $CD4^+$ and $CD8^+$ T cells (Grivel et al., 2003). HHV-6 has been suggested as a cofactor in HIV infections, as both viruses infect CD4 human T cells and accelerate the cytopathic effect (Lusso et al., 1989).

The original isolation of this virus from various malignant lymphatic disorders raised the suspicion of a possible etiologic involvement of this infection in those conditions. Indeed, especially in some lymphoproliferative disorders, increased antibody titers against HHV-6 antigens were demonstrated. Elevated HHV-6 titers were noted in acute myeloid leukemia (AML), Hodgkin's disease and low-grade non-Hodgkin lymphomas (NHL) (Clark et al., 1990), and in myelodysplasia and chronic myeloproliferative diseases (Krueger et al., 1994). For AML this was also reported by Gentile et al. (1999). Another study reported elevated titers in lymphoma, multiple myeloma and leukemia patients, but not in patients with solid tumors (Carricart et al., 2002). In children with acute lymphoblastic and myeloid leukemia, an elevated level of IgA antibodies against HHV-6 was noted (Salonen et al., 2002). In Hodgkin's disease, Alexander et al. (1995) showed a significant difference in geometric mean titers between Reed–Sternberg cell EBV-positive cases (mean titer 11.5) and those with Reed–Sternberg cell EBV-negative patients (mean titer 73.7), compared to a value in healthy individuals of 20.5. In another study, the pre-treatment sera of Hodgkin patients did not reveal significantly different antibody titers when compared to controls; however, the titers increased during the course of follow-up of patients who relapsed, but decreased significantly over time in patients who did not (Levine et al., 1992). In contrast to the demonstration of EBV latent membrane antigens in Reed–Sternberg cells of EBV-positive Hodgkin biopsies, HHV-6 antigens or viral DNA were not demonstrated in Reed–Sternberg cells of HHV-6 positive biopsies. Rather, they were restricted to macrophages and surrounding follicular cells (Maeda et al., 1993; Valente et al., 1996).

A number of studies reported an elevated level of positivity for HHV-6 DNA in patient materials from acute myeloid leukemias, NHL and Hodgkin's disease (Fillet et al., 1995; Valente et al., 1996; Pan et al., 1998; Schmidt et al., 2000; Hermouet et al., 2003). It is noteworthy that in some of these as well as in other studies, reactive lymph nodes were also found frequently to be positive for HHV-6 DNA (Borisch et al., 1991; Sumiyoshi et al., 1993; Valente et al., 1996). There exist additional reports of HHV-6 in lymphoproliferative disorders, particularly in AIDS-related lymphomas, contrasting the rarity of HHV-6 in HHV-8 positive primary effusion lymphomas (Asou et al., 2000). In addition, HHV-6 DNA has been noted in ocular lymphomas (Daibata et al., 2000), in B- and T-cell lymphomas (Ohyashiki et al., 1999), and in T-cell acute lymphoblastic leukemia (Luka et al., 1991). One report claims that HHV-6-positive Hodgkin biopsies are exclusively of the nodular sclerosis subtype of Hodgkin's disease (Collot et al., 2002). With regard to solid tumors, two reports have revealed the presence of HHV-6 DNA in some nasopharyngeal carcinoma tissue (Kositanont et al., 1993; Chen et al., 1999).

In our own studies (E.-M. de Villiers and H. zur Hausen, unpublished results), by using herpes consensus primers covering predominantly the herpes DNA polymerase region, we noted a relatively high percentage of positive bladder cancers (~10%), of gastric cancers (~12%), lung and rectum cancers (~15%), esophageal cancers (~20%), and of cancers of the colon (~28%). Biopsies from several other tumors were negative in this test series. The data obtained are shown in Figure 4.3.

It should be of substantial interest that HHV-6 infection trans-activates high-risk HPV gene expression in HPV-immortalized or malignant HPV-containing cervical carcinoma cell lines (Chen et al., 1994), and also trans-activates the HIV promoter (Horvat et al., 1989). A few reports describe an oncogenic potential of HHV-6. Full-length HHV-6 DNA or subgenomic clones transformed NIH 3 T3 cells to colony formation and tumorigenicity (Razzaque, 1990; Thompson et al., 1994). Neoplastic transformation was also recorded in immortalized human epidermal keratinocyte lines after transfection with subgenomic fragments of HHV-6 (Razzaque et al., 1993). The transforming region was localized by Kashanchi et al. (1997) to ORF-1 (DR-7) in the *Sal*I-*Hin*dIII subfragment of HHV-6 DNA. Western blot analysis confirmed the expression of ORF-1 in transformed NIH 3 T3 cells which produced fibrosarcomas in athymic nude mice. ORF-1 sequences were detected in five of 12 lymph nodes from patients with angioimmunoblastic lymphadenopathy, but rarely in tumor tissue. The protein coded for by ORF-1 (pORF-1) binds p53 (Kashanchi et al., 1997). ORF-1 is able to transactivate the minimal HIV-1 promoter (Kashanchi et al., 1994). In addition to the reported transforming activities, HHV-6A was shown to

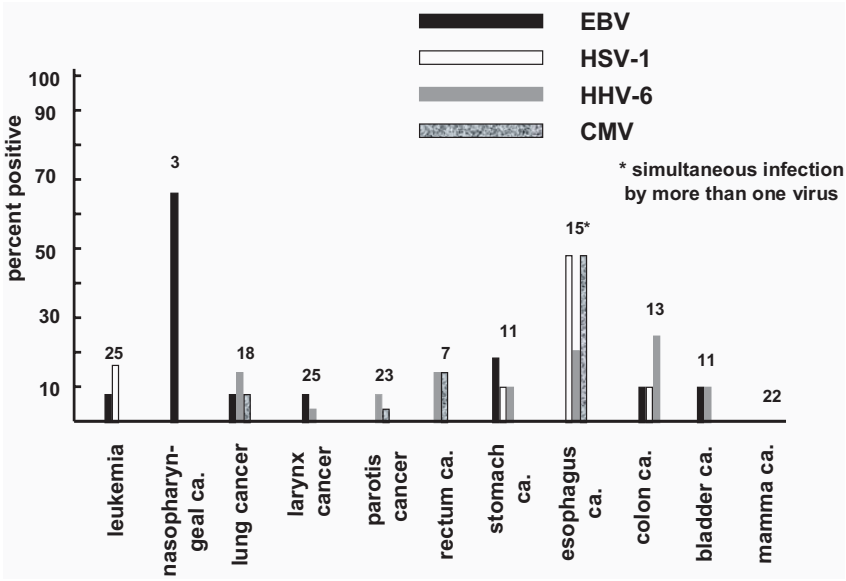

Fig. 4.3 Detection of various human herpes-group viruses in human tumor biopsies. The numbers at the top of the columns indicate the total number of biopsy materials tested from the respective tumor type. (E.-M. de Villiers and H. zur Hausen, unpublished data.)

contain a 1473-bp transformation suppressor gene that inhibits the transformation of NIH 3 T3 cells by H-ras and transcription of H-ras and HIV-1 promoters in transient transfection experiments (Araujo et al., 1997). This gene also down-regulates the BPV p89 and the HPV type 16 p97 promoter. Figure 16 shows The organization of the HHV-6 genome is illustrated in Figure 4.4.

Under certain conditions, HHV-6 appears able to integrate stably into host cell chromosomal DNA. Luppi et al. (1993) and Torelli et al. (1995) described the integration of HHV-6 sequences into the telomeric region of chromosome 17 in peripheral blood cells of individual cases of one Hodgkin lymphoma (among 55 cases investigated), one NHL (among 64 cases), and in one patient with multiple sclerosis (31 cases studied). A detailed analysis revealed chromosome band 17p13.3 as the integration site (Morris et al., 1999). It is interesting to note that HHV-6 DNA has been found integrated into various chromosomal sites, in a case of a woman with HHV-6 infected Burkitt's lymphoma in chromosome 22 q13 (Daibata et al., 1999). An EBV-negative, HHV-6-positive cell line was established from this Burkitt's lymphoma (Daibata et al., 1998 a). The asymptomatic husband of this patient also carried HHV-6 DNA integrated into chromosome 1q44 of peripheral blood mononuclear cells.

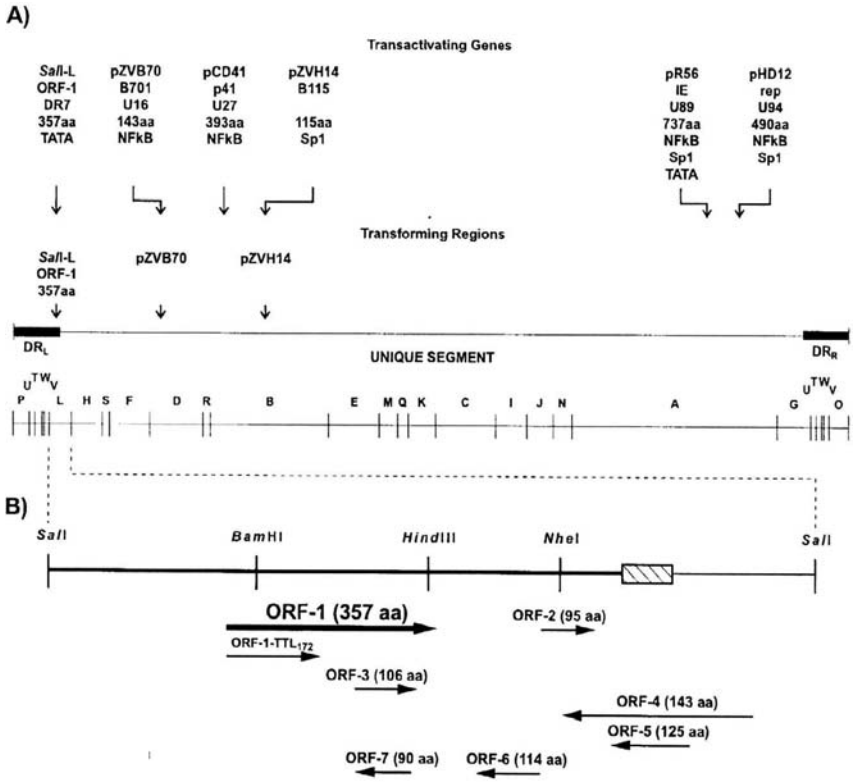

Fig. 4.4 Schematic representation of the HHV-6 genome. (Doniger et al., 1999. With permission.)

Their daughter had HHV-6 DNA in both chromosomes, 22q13 and 1q44. Another B- and T-cell line was established from a patient with acute lymphocytic leukemia after transformation with EBV or *herpesvirus saimiri*, respectively. Both cell lines contained integrated HHV-6 sequences, located in the long arm of chromosome 1q44 (Daibata et al., 1998 b). A summary of these results is provided in Table 4.1.

In 1991, Thomson and colleagues described the presence of the adeno-associated virus (AAV) type 2 *rep* gene within the HHV-6 genome. This gene is required for AAV-2 replication, and trans-regulates homologous and heterologous gene expression. It is also responsible for the AAV-mediated inhibition of cell transformation. The HHV-6 linked *rep* gene complements the replication of *rep*-deficient AAV-2 genomes and activates the LTR of HIV in fibroblast cell lines but not in T cells (Thomson et al., 1994). This contrasts the function of the native AAV-2 *rep* which inhibits the HIV LTR both, HIV LTR activity in fibroblasts and T cells. Thus, the properties of HHV-6 *rep* and AAV-2 *rep* are related, but not identical.

The rep sequence is found at the right end of the HHV-6 genome and is present in the A and B variants of HHV-6. Although 13 of the 17 ORFs at this right end differ in the amino acid composition derived from these ORF by more than 10%, the rep region differs by only 2.4% (Dominguez et al., 1999). This seems to point to an important function of this gene. In another investigation, HHV-6A and HHV-6B isolates differed in the *rep* gene by only 3.5% and 2.5% in nucleotide and amino acid sequence, respectively (Rapp et al., 2000). Seventeen clinical and geographical disparate isolates of HHV-6A differed in this region by between 0.2% and 0.6%, while 13 HHV-6B isolates were identical. Transcripts of this gene were present at low abundance (~10 copies per cell 3 days after infection). Mori et al. (2000) demonstrated three transcripts of the HHV-6 rep gene of 9.0, 5.0, and 2.7 kb, where the 2.7-kb transcript was most abundant. HHV-6 rep binds to the human TATA-binding protein

Table 4.1 Reports on the integration of HHV-6 DANN into host cell chromosomes

Human material	Chromosomal localization	Reference(s)
M. Hodgkin (peripheral blood lymphocytes – PBL)	17 p13.3	Luppi et al. (1993)
Non-Hodgkin lymphoma (PBL)	17 p13.3	Morris et al. (1999)
Multiple sclerosis (PBL)	17 p13.3	
Burkitt's lymphoma[a]	22 q13	Daibata et al. (1999)
Asymptomatic PBL[a]	1 q44	Daibata et al. (1998 a)
Asymptomatic PBL[b]	1 q44 and 22q13	Daibata et al. (1998 a)
Lymphocytic leukemia B- and T-cell line[c]	1 q44	Daibata et al. (1998 b)

[a] Wife and husband.
[b] Daughter of both.
[c] EBV-transformed B-cell line and Herpesvirus saimiri-transformed T-cell line.

through its N-terminal region. In Western blot analysis, an anti-rep monoclonal antibody recognized a 56-kDa polypeptide (Dhepakson et al., 2002). The same authors also demonstrated a binding activity of this protein to single-stranded DNA.

It seems that the role of the *rep* gene (U94) in HHV-6 relates to viral DNA persistence and to maintenance of the latent state. In HHV-6 infected lymphoid cell lines this gene was stably expressed in the absence of viral DNA replication (Rotola et al., 1998).

Overall, published data on cell transformation by HHV-6 are persuasive, but not fully convincing, and further studies are required in this respect. Nevertheless, the number of positive reports of HHV-6 DNA in a certain percentage of myeloid leukemias, NHL, and in Hodgkin's disease may point to a potential role of this virus in these malignancies. It should be noted that HHV-6, similar to HSV infections, is able to up-regulate HIV-1 expression by inducing the LTR activity of the latter virus (Ensoli et al., 1989; Campbell et al., 1991; Garzino-Demo et al., 1996). Under specific conditions of infection (e.g., of dendritic cells) however, HHV-6 may even suppress HIV replication (Carrigan et al., 1990; Asada et al., 1999). Conversely, the tat gene of HIV may enhance replication of HHV-6 (Sieczkowski et al., 1995). Until now, no reports have been published analyzing the potential activation of human endogenous retroviruses by HHV-6. It is interesting to note that HHV-6 replication – particularly in the leukemic HSB cell line – results in HHV-6 particles with the frequent attachment of exosome-like particles (Fig. 4.5). The nature of the latter has not yet been identified.

Although HHV-6 has frequently been noted in reactive lymph nodes, it seems interesting that this has rarely been found and incriminated in specific inflammatory conditions, such as morbus Crohn or polyarthritis. Except for efficiently transforming gamma-herpesviruses, however, it is presently very difficult to assess the role of other herpesvirus subfamilies, including herpes simplex and human CMV and VZV in human cancers.

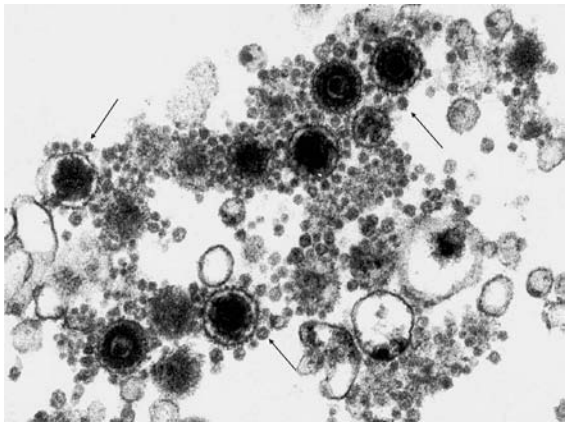

Fig. 4.5 HHV-6 particles with exosome-like particles attached.

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4.3
Gammaherpesvirinae (Lymphocryptoviruses)

4.3.1
Epstein–Barr Virus

The Epstein–Barr virus (EBV) was initially seen by electron microscopy in lymphatic tissue culture cells derived from Burkitt's lymphoma (Epstein et al., 1964). The viral structure is illustrated in Figure 4.6. The virus could not be transmitted to other tissue cultures or common laboratory animals, nor did it infect the chorioallantoic membrane of embryonated chicken eggs. The development of an indirect immunofluorescence test to detect EBV-specific antigens (Henle and Henle, 1966) and the identification of the fluorescent cells as EBV-producing cells (zur Hausen et al., 1967), permitted an early approach to study the seroepidemiology of this infection. Within a short time it became clear that EBV represents a new member of the herpesvirus family, that infections with EBV are ubiquitous (Henle et al., 1969), and that close to 90% of the population became infected during childhood or adolescence. Two types of human cancers were detected initially with high antibody titers against EBV antigens, namely Burkitt's lymphoma (Henle et al., 1969) and nasopharyngeal cancer (Old et al., 1966; de Schryver et al., 1969). Shortly thereafter, EBV was recognized as the causative agent of infectious mononucleosis (Henle et al., 1968). The subsequent development of an immunofluorescence test to detect early EBV-induced antigens (Henle et al., 1970, 1971) showed again the specificity of a highly elevated immune response against EBV in patients with Burkitt's lymphoma or nasopharyngeal carcinoma. Electron micrographs showing EBV particles are shown in Figure 4.6.

Three other developments occurred during the late 1960s and early 1970s: a first biological function of EBV infections was identified in 1967 and 1968 (Henle et al., 1967; Pope et al., 1968), when these groups each revealed immortalizing activity of EBV-producing lethally X-irradiated Burkitt's lymphoma cells for human lymphocyte preparations in co-cultivation experiments or of cell-free filtrates from an EBV-producing leukemic cell line. In addition, zur Hausen and Schulte-Holthausen (1970) demonstrated persistence of EBV DNA in a "virus-free" cell line, Raji, of Burkitt's lymphoma origin. Shortly thereafter, the same group demonstrated EBV DNA in biopsies from Burkitt's lymphoma and from nasopharyngeal carcinomas (zur Hausen et al., 1970). Three years later, it was shown that EBV persists not only in cells of lymphatic origin, but also in epithelial nasopharyngeal carcinoma cells (Wolf et al., 1973). Viral DNA persistence in Burkitt's lymphomas was soon confirmed by Nonoyama and Pagano (1973), and in epithelial cells of nasopharyngeal carcinomas by Klein et al. (1974).

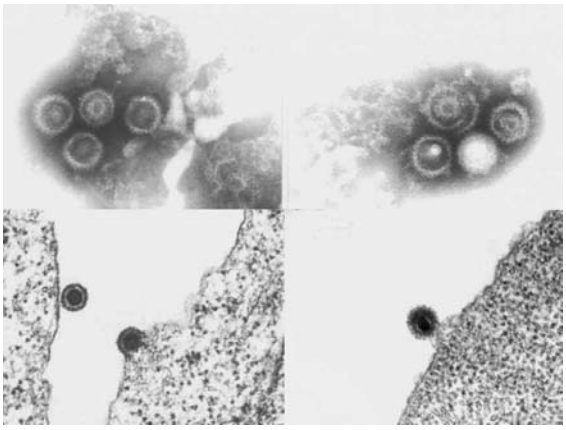

Fig. 4.6 Epstein–Barr virus particles seen in negative contrast staining (upper panel) and budding from the cell surface (lower panel). (Illustration courtesy of Birgit Hub, Heidelberg.)

Two other developments marked this period: the identification of a complement-fixing antigen in latently EBV-infected cells (Pope et al., 1969), and its demonstration by anticomplement immunofluorescence tests (Reedman and Klein, 1973). The other important step was the induction of lymphoproliferative disease resembling malignant lymphoma in cotton-top marmosets (Shope et al., 1973) and in owl monkeys (Epstein et al., 1973). At this time, the evidence was firm that EBV represents a HHV infection with carcinogenic potential, though the etiologic role for Burkitt's lymphomas and nasopharyngeal carcinomas remained questionable.

Today, this virus infection has been linked with several human malignancies, including B-cell lymphomas in immunocompromised patients, the endemic form of Burkitt's lymphomas, nasopharyngeal cancer, a subset of Hodgkin's lymphoma, with nasal natural killer (NK)/T-cell lymphomas, and with approximately 10% of gastric cancers. Data pertinent to EBV oncogenicity will be outlined in the following sections, while for a detailed account of the molecular biology of EBV infections the reader is referred to the reviews of Kieff (1996) and Rickinson and Kieff (1996).

4.3.1.1 **Characterization of the Virus, and its Biological Properties**

The complete nucleotide sequence of the double-stranded genome of EBV was initially determined for one specific EBV isolate (B95–8) with 172 282 base pairs (Baer et al., 1984), and subsequently for another EBV strain from nasopharyngeal carcinoma with 171 656 base pairs (bp) (Zeng et al., 2005). Among different isolates, the nucleotide sequence varies between 168 000 and 184 000 bp, and encodes more than 85 genes. Terminal repeat regions and an internal region of repeats of 3072 bp divide the genome into a short and a long unique region containing most of the genetic information (Cheung and Kieff, 1982). Upon latent infection of cells, the linear EBV genome circularizes with characteristic numbers of terminal repeats preserved from the parental genome.

Two subtypes of EBV have been identified (EBV-1 and EBV-2); these differ in genes coding for nuclear proteins EBNA-LP, -2, -3 A, -3 B, and -3 C, with differences in the predicted amino acid sequence of between 28% and 47% (Dambaugh et al., 1984; Adldinger et al., 1985; Sample et al., 1990). They also differ in their transforming activity; EBV-2 transforms B lymphocytes less efficiently than EBV-1, apparently due to the different amino acid composition of EBNA 2 (Rickinson et al., 1987; Cohen et al., 1989). There exists no clear-cut evidence for differences between the two EBV subtypes in pathogenicity, although about 85% of nasopharyngeal carcinomas from Taiwan contain EBV-1 (Shu et al., 1992).

Although EBV was initially recognized as a B lymphotropic virus, it became clear recently that the virus can also infect epithelial cells, probably depending upon the production of specific glycoproteins, gHgL (Borza and Hutt-Fletcher, 1998; Borza et al., 2004) or gp 110 (Neuhierl et al., 2002). In most of the earlier publications in which the cell tropism of EBV was studied, viral preparations derived from the B95–8 line were used. This line resulted from EBV infection of marmoset leukocytes and produced spontaneously relatively high concentrations of EBV (Miller et al., 1972). EBV derived from these cells barely expresses gp110 (Gong et al., 1987), whereas EBV derived from some other lines, such as MA-BA (originating from a nasopharyngeal carcinoma patient) and Akata (originating from a Japanese Burkitt's lymphoma), produce high quantities of this protein and do infect epithelial cells (Neuhierl et al., 2002).

4.3.1.2 **EBV Gene Products in Latent Infection**

EBV gene products synthesized in latent infection are of particular interest in relation to the oncogenic properties of this virus. A number of gene products have been characterized which are expressed in specific tumors or in nontransformed, latently infected cells. Some of these share structural and functional homologies with cellular gene products, or interfere with the function of the latter. The nuclear EBNA-1 protein is consistently expressed. This is a sequence-specific DNA-binding phosphoprotein that fulfills a central role in the episomal maintenance of the EBV genome and is required for its DNA replication (Middleton and Sugden, 1994). At least in Burkitt's lymphoma cells it is a survival factor and prevents apoptosis (Kennedy et al., 2003). A role is also suggested for this protein in malignant transformation, by directing its expression to B cells of transgenic mice, where this results in B-cell lymphomas (Wilson et al., 1996).

EBNA-1 binds to the origin of replication within the EBV plasmid which contains two distinct EBNA-1 binding sites (Rawlins et al., 1985; Reisman et al., 1985; Jones et al., 1989; Ambinder et al., 1990). The EBNA-1 binding elements are located in a family of repeats and the dyad symmetry binding elements (Wysokenski and Yates, 1989). They contain multiple EBNA-1 binding sites each of 18 bp. The binding of EBNA-1 to the plasmid origin of replication may initiate viral DNA replication, with the additional aid of host cell enzymes (Frappier et al., 1994). The EBV origin of replication contains both, the initiation and termination sites of viral plasmid DNA rep-

lication (Gahn and Schildkraut, 1989). The EBNA-1 protein is divided into amino- and carboxy-terminal domains by a glycine-glycine-alanine repeat sequence, which varies in size for different EBV strains. This repeat domain acts as a cis inhibitor of MHC class I-restricted presentation, and blocks antigen processing in the ubiquitin/proteasome pathway (Levitskaya et al., 1995). EBNA-1 interacts and suppresses functions of the suppressor of metastasis and cell migration, Nm23-H1 (Murakami et al., 2005).

EBV deprived of EBNA-1 is able to induce B-cell proliferation, albeit with a 10 000-fold lower frequency than wild-type virus (Humme et al., 2003). Expression of dominant-negative derivatives of EBNA-1 decreases the survival of EBV virus-positive cells (Kennedy et al., 2003), this inhibition being due to the induction of apoptosis. Thus, EBNA-1 apparently prevents apoptosis in EBV-positive cells. Tumor induction by EBNA-1 in transgenic mice has been reported in these animals (Wilson et al., 1996), and EBNA-1 also appears to cooperate with myc in lymphomagenesis (Drotar et al., 2003). EBNA-1 transgenic mouse lymphocytes induce *BLC-xL* and *RAG* genes and reveal an enhanced response to IL-2 (Tsimbouri et al., 2002). Another study failed to confirm these data (Kang et al., 2005), though this may of course depend on the genetic background of the mouse strains used. Even in the negative study an increased level of pulmonary adenomas was noted in EBNA-1 transgenic lineages. The structure of the EBV genome is depicted in Figure 4.7.

EBNA-2 is a nuclear protein which plays a critical role in lymphatic cell immortalization, and also acts as a transcriptional coactivator. It regulates the expression of a number of viral and cellular genes (Ambinder et al., 1990). Deletion of the EBNA-2 gene covering also the last two exons of EBNA-LP in the transformation-defective

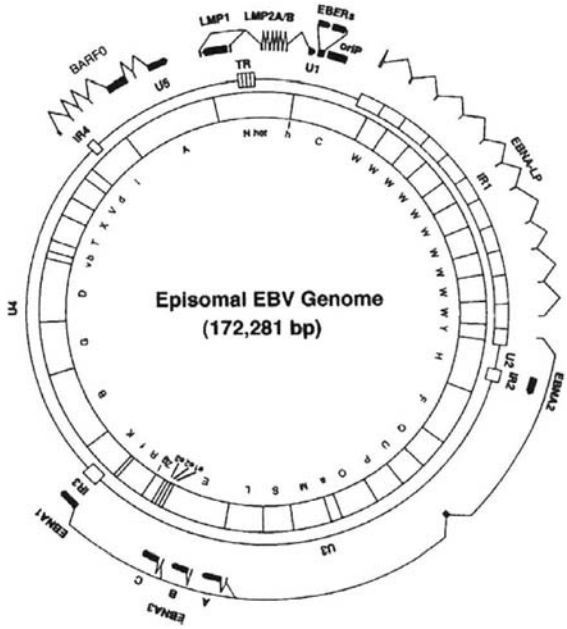

Fig. 4.7 The genome organization of the Epstein-Barr virus. (Reprinted from SEMINARS IN CANCER BIOLOGY, Vol 7, Issue 4, Osato, T. and Imai, S. Epstein-Barr virus and gastric carcinoma, 175–182. Copyright 1996, with permission from Elsevier.)

P3 HR-1 line initially revealed an important role for EBNA-2 in cell immortalization (Rabson et al., 1982; King et al., 1982). The correct location of the deletion was determined in the same year (Bornkamm et al., 1982). The reintroduction of EBNA-2 into this viral DNA resulted in restoration of the transforming properties, and emphasized the role of EBNA-2 in immortalization (Hammerschmidt and Sugden, 1989). EBNA-2 trans-activates a number of cellular genes, among them CD 23, CD 21, and *c-frg* (Wang et al., 1987 a; Cordier et al., 1990; Knutson, 1990). EBNA-2 abolishes the repression mediated by a protein complex including the DNA-binding Jκ-recombination binding protein (RBP-jκ) (Henkel et al., 1994; Grossman et al., 1994; Zimber-Strobl et al., 1994; Hsieh et al., 1995), and functionally replaces the intracellular region of Notch (Sakai et al., 1998). Conversely, Notch 1 IC partially replaces EBNA-2 function in EBNA-2 estrogen-dependent EBV-immortalized B cells (Gordadze et al., 2001). EBNA-2 also regulates the expression of other latency-associated EBV genes, namely LMP-1 and LMP-2 (Wang et al., 1987 b; Abbot et al., 1990; Fahraeus et al., 1990; Wang et al., 1990; Zimber-Strobl et al., 1990).

4.3.1.2.1 **LMP-1**

LMP-1 functions as a constitutively activated member of the tumor necrosis factor (TNF) receptor superfamily. This leads to the activation of several signaling pathways, including the NFκB transcription factor pathway, the MAP kinase cascade, the JAK/STAT, and the PI3 K/Akt pathways (Gires et al., 1999; Dawson et al., 2003; Young and Murray, 2003). This results in the up-regulation of anti-apoptotic proteins (e.g., BCL2 and A20) and the stimulation of cytokine production (Laherty et al., 1992; Eliopoulos et al., 1997, 1999). Although, with the exception of Burkitt's lymphomas, LMP-1 is expressed in most EBV-linked tumors, only 20–60% of nasopharyngeal cancers unequivocally are LMP-1 positive (Fahraeus et al., 1988; Niedobitek et al., 1992). LMP-1 induces several cellular chemokines, as for instance the interferon-γ-inducible protein 10 (IP-10) (Vockerodt et al., 2005) or CCL/RANTES (Uchihara et al., 2005). In addition, the two members of the inhibitor of differentiation (Id) family, Id1 and Id3, were also induced by expression of LMP-1 in C33 A and Rat-1 cells (Everly et al., 2004). A high level of LMP-1 expression can be induced by the ectopic expression of Rta, an immediate-early protein of EBV, initiating the lytic cycle (Chang et al., 2004). The expression of Rta is induced during the course of terminal differentiation of plasma cells (Laichalk and Thorley-Lawson, 2005).

4.3.1.2.2 **LMP-2 A**

LMP-2 A modulates the NFκB pathway (Stewart et al., 2004), and is consistently expressed in nasopharyngeal carcinomas. In tonsillar epithelial cells, LMP-2 A expression causes them to become migratory and invasive, and also enhances integrin-α-6 (ITGα6) expression (Pegtel et al., 2005). LMP-2 A is not essential for transformation of B lymphocytes (Tierney et al., 1994), but it interferes with B-cell receptor signaling (Dykstra et al., 2001). In primary B cells, the initiation of B-cell receptor signaling re-

sults in the activation of *Src* family protein tyrosine kinases and in the binding of *Syk* protein tyrosine kinases to the immunoreceptor tyrosine-based activation motifs (ITAM) present in the B-cell receptor (Kurosaki, 1999; Benschop and Cambier, 1999). LMP-2 A shares some of these properties, but renders B cells unresponsive to stimulation by B-cell receptor and prevents the reactivation of EBV virus from latency following surface immunoglobulin crosslinking (Miller, C.L. et al., 1994). LMP-2 A inhibits the autocrine secretion of IL-6, which seems to result from its property of negatively modulating the expression of NFκB (Stewart et al., 2004). In epithelial cells, the expression of LMP-2 A as well as LMP-2 B is associated with an increased capacity of the cells to spread and migrate on extracellular matrix (Allen et al., 2005).

The cellular transcription factor NF-κB seems to play a central role in all EBV-associated tumors. It fulfills several functions in these conditions, besides inhibiting the lytic replication of gammaherpesviruses (Brown et al., 2003): it is constitutively activated by LMP-1 (Eliopoulos et al., 2003; Thornburg et al., 2003), its activity is required for the deregulation of the rearranged c-myc expression in Burkitt's lymphoma cells by activating the immunoglobulin heavy chain enhancer (Ji et al., 1994; Kanda et al., 2000). Finally, its expression regulates the anti-apoptotic activity mediated by LMP-1 (Devergne et al., 1998; D'Souza et al., 2004).

4.3.1.2.3 **LMP-2 B**

The LMP-2 B and LMP-1 promoters are separated by 200 bp, and act bidirectionally. The function of LMP-2 B has been relatively poorly analyzed. It has been reported that mutations in either LMP-1 or LMP-2 do not affect the immortalizing activity for EBV in human lymphocytes, nor do they modify the growth properties of the immortalized cells in tissue culture (Kim and Yates, 1993; Longnecker et al., 1993). A more recent publication, however, documents that the LMP-2 A gene is important for efficient B-cell immortalization (Brielmeier et al., 1996).

Besides EBNA-1, EBNA-2, LMP-1 and LMP-2 A and -2 B, at least eight additional EBV genes are active in latent infection. Two of these code for small non-polyadenylated RNAs, EBER 1 and EBER 2, while four others code for EBNA 3 A, 3 B, 3 C, and LP. The EBNA 3 A, 3 B and 3 C genes are tandemly placed within the EBV genome. Their RNAs initiate at the CP or Wp EBNA promoters and represent the least abundant EBNA mRNAs. They accumulate in the nuclei and localize in clumps sparing the nucleoli (Hennessy et al., 1983, 1986; Kallin et al., 1986; Petti and Kieff, 1988; Petti et al., 1990). EBNA 3 C can up-regulate CD21 mRNA and LMP-1 expression (Wang et al., 1990; Allday et al., 1993). EBNA 3 A and EBNA-3 C are essential for B-cell immortalization (Tomkinson et al., 1993), and EBNA 3 C cooperates with RAS in rodent fibroblast transformation (Parker et al., 1996). EBNA 3 B has been reported to bind retinoblastoma and p53 proteins (Szekely et al., 1993), and may thus play an important additional role in transformation events.

EBNA LP, besides EBNA 2, represents the first viral protein expressed in B-lymphocyte infection (Alfieri et al., 1991). It is a nuclear protein which is partly spread through the nucleus, and partly concentrated in small nuclear granules

(Wang et al., 1987 a; Petti et al., 1990). It seems to cooperate with EBNA-2 in cell immortalization (Hammerschmidt and Sugden, 1989; Mannick et al., 1991). A recent study shows that the EBNA LP protein binds p14 ARF, a nucleolar protein that regulates the p53 pathway. In addition, it binds the v-fos transformation effector FTE (FTe-1/S3 a) which enhances v-fos-mediated cellular transformation (Kashuba et al., 2005). EBV-induced B-cell transformation leads to the up-regulation of FTe/S3 a.

4.3.1.2.4 **EBER 1 and EBER 2**

Recently, an important role of the commonly abundant EBER transcripts was reported which remain, however, untranslated (Takada and Nanbo, 2001). These authors postulated a key role for those transcripts in maintaining the malignant phenotype of Burkitt's lymphoma cells. According to their results, the EBERs confer clonability in soft agarose, tumorigenicity in nude mice, and resistance to interferon-α-induced apoptosis (Nanbo et al., 2002). EBER also confers resistance to apoptosis mediated through Fas by blocking the PKR pathway (Nanbo et al., 2005). In addition, EBER transcripts induce transcription of IL-10, acting as an autocrine growth factor in Burkitt's lymphomas (Takada and Nanbo, 2001). The putative secondary structure of EBER-1 and Eber-2 was published by Rosa et al. (1981) (see Fig. 4.8).

Other abundantly expressed RNA is transcribed from the *Bam*H1 region of the EBV genome. This family of spliced transcripts is commonly designated as *Bam*H1 A rightward transcripts (BARTs). They are expressed in all EBV latency programs whose TATA-less promoter regions reveal different expression patterns in epithelial and B cells (Chen et al., 2005). They contain ORFs, although no BART-pro-

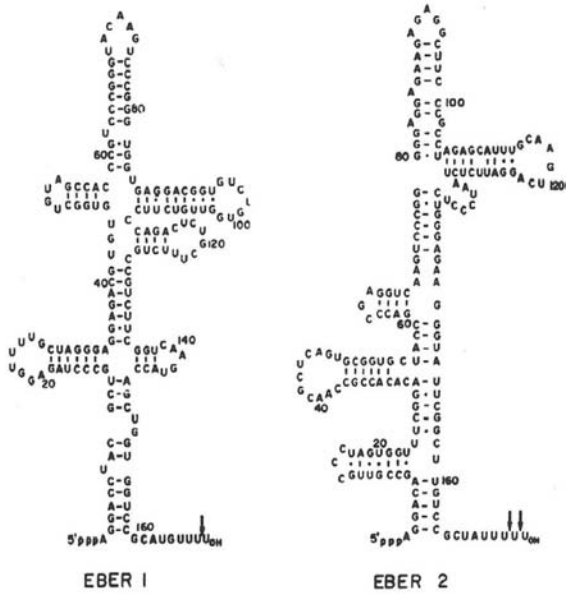

Fig. 4.8 Putative structure of EBER transcripts. (Rosa et al., 1981. With permission.)

tein has yet been identified (Karran et al., 1992; Smith et al., 2000). Recently, *micro RNAs* originating from the intronic regions of the *BART* gene, were detected in all latent stages which seem to target regulators of cell proliferation and apoptosis, B-cell-specific chemokines and cytokines, transcriptional regulators and components of signal transduction pathways (Pfeffer et al., 2004). Three other regions coding for microRNAs were discovered in the BHRF1 (*Bam*H1 fragment H rightward open reading frame 1) gene. These three other regions appear to be preferentially active in lytic infections.

An additional transcript from the *Bam*H1 A region is represented by *BARF-1*, encoding a 31-kDa protein, mainly found in nasopharyngeal and EBV-positive gastric carcinomas (Decaussin et al., 2000; zur Hausen et al., 2000). *BARF-1* shares some homology with human colony-stimulating factor 1 receptor, and is able to transform rodent fibroblasts and simian primary epithelial cells (Sheng et al., 2001). Another transcript from this region, *BARF-0*, codes for a 279-amino acid protein. It interacts with cellular Notch and epithelin and mediates their proteasome-dependent degradation (Thornburg et al., 2004).

Several viral proteins share some homology with cellular genes, and some of these are expressed in lytic infections. Thus, the BCRF-1 protein reveals an 84% homology with human IL-10 (Vieira et al., 1991). The latter is known as an activation factor for B-cell proliferation (Miyazaki et al., 1993). Another EBV protein (BDLF2) which has been detected in oral hairy leukoplakia shares some homology with human cyclin B1 (Hayes et al., 1999). BHRF-1 shows a limited degree of homology with BCL-2 (Henderson et al., 1993). In addition, BARF-1 reveals some homology with intracellular adhesion molecule 1 and with the human colony-stimulating factor 1 receptor (Strockbine et al., 1998). It seems likely that further structural and even more functional homologies will be discovered in the future, permitting the persistent EBV to interfere specifically with host cell signaling pathways.

4.3.1.3 Transforming Properties of EBV and Tumor Induction in Animals

Since the initial studies by Henle and colleagues (1967) on the transformation of human lymphocytes by co-cultivation with X-irradiated EBV-producing Burkitt's lymphoma cells and by cell-free filtrates of EBV-positive cells (Pope et al., 1968), the transforming properties of EBV have been intensively investigated. The virus is extremely effective in immortalizing B lymphocytes. Since most human adults carry latently infected B cells in their peripheral blood, outgrowth of these cells can be readily achieved, particularly by depleting or inactivating accompanying T cells (von Knebel Doeberitz et al., 1983).

Interestingly, a number of different EBV-coded proteins possess immortalizing properties, at least for specific cell types. Immortalized B lymphoblasts express the six EBV nuclear antigens (EBNA 1, 2, 3 A, 3 B, 3 C and LP) and also three latent membrane proteins (LMP-1, -2 A and -2 B). In addition, small, non-polyadenylated RNAs, EBER-1 and EBER-2 are abundantly expressed. A list of EBV latency-associated proteins and their partly putative functions is provided in Table 4.2.

Table 4.2 Gene products of Epstein-Barr virus expressed in viral latency[a]

Gene	Protein or RNA function	Engaged in immortalization
EBNA-1	Transcriptional regulation of other EBNAs maintenance and replication of EBV episomes	Survival factor for EBV-positive BLs, B-cell lymphomas in transgenic mice
EBNA-2	Interaction with RBP-Jκ, activation of LMP-1 and LMP-2A and the cellular gene CD23	Essential component for B-cell immortalization
EBNA-3A EBNA-3C	Disruption of cell cycle checkpoints Cooperation with RAS	Essential for B-cell immortalization. EBNA-3C with Ras transforms rodent cells
EBNA-3B	Interaction with RB2/p130 and p53	Interruption of checkpoint control
EBNA-LP	Interacts with EBNA-2	Mediates efficient transformation of B-cells
LMP-1	Activation of several cellular pathways and of anti-apoptotic proteins and cytokines	Main transforming protein of EBV, a "classic" oncogene
LMP-2A	Drives proliferation and survival of B-cells receptor (BCR)	Non-essential for immortalization
LMP-2B	Similar functions as LMP-2A (?)	Non-essential for immortalization
EBER-1 EBER-2	May inhibit ds-stranded RNA – activated Protein kinase (PKR), induce interleukin-10, inhibit apoptosis	Increase tumorigenicity of BL-cell lines
BART	RNA of unknown function	No evidence
BARF-1	31 kDa protein, shares homology with human colony-stimulating factor 1	Expressed in nasopharyngeal and gastric cancers

[a] Modified from *Young and Rickinson (2004)*.

EBNA-1, EBNA-2, EBNA-3 A, EBNA3 C, and LMP-1 emerge as essential components for the immortalization of B lymphoblasts. The amino-terminal domains of EBNA-3A, 3B, and 3C interact with RBP-Jκ (Robertson et al., 1996). For EBNAs 3A and 3C, it has been shown that these proteins repress RBP-Jκ-EBNA-2-activated transcription by inhibiting the binding of RBP-Jκ to DNA (Waltzer et al., 1996). In addition, EBNA-3C binds Nm23-H1, a known suppressor of cell migration and metastasis, and up-regulates metallomatrix proteinase-9 (MMP-9) (Kuppers et al., 2005). EBNA 3 C also mediates coactivation of the LMP-1 promoter with EBNA-2 (Lin et al., 2002). A number of additional interactions of these EBNAs with cellular proteins have been described (Rosendorff et al., 2004).

Although the immortalization of B lymphoblasts by EBV is remarkably efficient, the cloning of immortalized cells is relatively difficult. It appears that several of the EBV-infected and growth-stimulated lymphoblasts go into crisis (Counter et al.,

1994; Sugimoto et al., 1999). Those which survive stabilize relatively short telomeres and retain telomerase activity in later passages. The genetic background of the lymphocyte donor appears to affect the frequency of immortalizing events (Sugimoto et al., 2004). During the early stages most of the immortalized lines are diploid and nontumorigenic when inoculated into nude mice; chromosomal rearrangements may occur during later passages.

Early experimental proof for a tumorigenic function of EBV was obtained after inoculation of EBV into cotton-top tamarins (*Sanguinus oedipus*) (Shope et al., 1973) and owl monkeys (*Aotus trivirgatus*) (Epstein et al., 1973). These animals develop a lethal lymphoproliferative disorder resembling immunoblastic lymphoma in humans. The tumors are either mono- or oligoclonal (Zhang et al., 1993).The proliferating cells express EBNA-1, EBNA-2, and EBNA-LP, as well as LMP-1 (Young et al., 1989), and EBV-containing cell lines are readily established from diseased animals. Occasionally, even latent EBV infection without lymphoma development was noted in a cotton-top tamarin (Niedobitek et al., 1994). A poorly defined mononucleosis-like syndrome has been reported after EBV infection of the common marmoset (*Callithrix jaccus*) (Wedderburn et al., 1984).

Another animal model which works surprisingly well results from human B- and T- cell-reconstituted severe combined immunodeficient (SCID) mice. If the human lymphocytes originate from EBV-positive donors, the animals frequently develop a B-cell lymphoproliferative disease within 8–16 weeks (Mosier et al., 1988). This model represents a convenient system to analyze therapeutic interferences with these lymphoproliferations.

Virtually all Old World monkeys and nonhuman primates harbor species-specific EBV-like agents as natural persisting infections. They all reveal extensive regions of homology with human EBV. To date, 30 different lymphocryptoviruses have been detected in nonhuman primates by using a panherpesvirus PCR assay (Ehlers et al., 2003). Although no obvious pathogenicity has been observed in these infections, immunosuppression induced by simian immunodeficiency virus (SIV) in cynomolgus monkeys (*Macaca fascicularis*) results in these animals in the development of immunoblastic B-cell lymphomas, driven by a species-specific EBV-related herpesvirus (Feichtinger et al., 1992; Schatzl et al., 1993).

It is interesting to note that EBV infection apparently trans-activates the human endogenous retrovirus HERV-K18 (Sutkowski et al., 2001). The *env* gene of the latter possesses superantigen activity.

4.3.1.4 **Various Stages of Epstein–Barr Viral Latency**

Shortly after the first identification of viral gene products in latent infection, it was recognized that the expression pattern of viral DNA in EBV-immortalized lines is different from that of Burkitt's lymphoma cells and other EBV-positive tumor types (Rowe et al., 1987). Three different latency patterns emerged from this and additional studies.

- Latency pattern I was first analyzed in Burkitt's lymphoma cells (Rooney et al., 1986; Rowe, D. et al., 1986; Rowe, M. et al., 1987; Kerr et al., 1992). This stage is characterized by the expression of EBNA-1, EBER and BARF RNA. While the Cp/Wp promoters are silenced, the downstream promoter FQp is activated, initiating the transcription of EBNA-1 mRNA with a unique splice structure (Schaefer et al., 1991; Sample et al., 1991). The expression of LMP transcripts is abrogated; this gene expression pattern seems to be specific for Burkitt's lymphoma cells. Body cavity lymphomas, carrying HHV type 8 and EBV genomes also express the EBV type I pattern (see Section 4.4.1).
- Latency pattern II was recognized in the following EBV-positive tumor types: nasopharyngeal carcinoma, Hodgkin's disease, gastric cancer and T-cell lymphomas (Contreras-Brodin et al., 1991; Kerr et al., 1992). Here again, the EBER and *Bam*H1 RNAs are expressed, the Cp/Wp promoters are silenced, and FQp is activated as in the previous latency pattern. In contrast to the latter, however, LMP promoters are activated, resulting in the expression of LMP-1 and also in several biopsies of LMP-2 A and/or LMP-2 B (Kerr et al., 1992).
- The expression of viral latency genes in EBV-immortalized lymphoblastoid cells and in immunoblastic lymphomas has been described as latency pattern III. Here, all genes listed in Table 4.3 are expressed.

There seems to exist a fourth latency pattern, here tentatively labeled as latency 0: it has been demonstrated that EBV persists in resting memory B cells in the blood (Babcock et al., 1998; Joseph et al., 2000). It has also been anticipated that the latently EBV-infected cells would produce solely EBNA-1 which would not be recognized by cytotoxic T cells (Levitskaya et al., 1995). Recently, however, evidence has been pres-

Table 4.3 Various stages of Epstein-Barr virus latency[a]

Pattern	Cell type	Mode of expression
I	Burkitt's lymphoma	EBNA-1, EBER-RNA, BART-RNA Cp/Wp promoter silenced, Qp promoter activated
II	NPC, gastric cancer, HD, T-cell lymphomas	EBNA-1, EBER-RNA, BART-RNA Cp/Wp promoter silenced, Qp promoter activated. LMP-1, in several biopsies also LMP-2 A and LMP 2 B
III	Immunoblastic lymphoma	EBNAs 1, 2, 3 A, 3 B, 3 C, EBNA-LP, LMP-1, 2 A, 2 B Cp/Wp promoter active EBER and BART RNA, BARF
0	Resting memory cells (Hochberg et al. 2004)	EBNA-1 only during cell division (Laichalk and Thorley-Lawson, 2005)

[a] Modified from *Young and Rickinson (2004)*.

ented for a virtual null expression of EBV genes in resting memory cells (Hochberg et al., 2004). These cells start to produce EBNA-1 if stimulated for cell division. Since the dividing cells seem to produce solely EBNA-1, this led to the speculation that Burkitt's lymphoma (see below) originates from memory B cells but differs from them because of the constitutive expression of EBNA-1. According to the same research group, cell differentiation into plasma cells also regulates the switch from viral latency to a replicative cycle of the virus (Laichalk and Thorley-Lawson, 2005).

4.3.1.5 **EBV in Infectious Mononucleosis**

Epstein–Barr virus has been identified as the causative agent of infectious mononucleosis (Henle et al., 1968), a self-limiting lymphoproliferative disease, developing as the consequence of hyperproliferation of EBV-containing B cells and a reactive T-cell response. The primary infection frequently occurs during the first years of life (Henle et al., 1969 b; Lang et al., 1977), and is often not noticed by the infected person. Particularly in young adults, previously growing up in a hygienically protected environment, the primary EBV infection more regularly leads to sometimes severe symptoms of infectious mononucleosis. The transmission occurs in most cases orally via the saliva. This resulted in the original name for infectious mononucleosis as *college* or *kissing disease* (Hoagland, 1955). Cell-free virus is detectable in the throat washings and saliva of acute infectious mononucleosis patients, and there exist indications that initial rounds of viral replication occur in epithelial cells lining the tongue, the nasopharynx and the parotid duct (Chang and Golden, 1971; Gerber et al., 1972; Morgan et al., 1979; Sixbey et al., 1984; Wolf et al., 1984). Subsequently, in a still asymptomatic phase, the virus colonizes the B-lymphoid system, before a later dominating reactive T-cell response results in the typical clinical symptoms. The state of infected B lymphoblasts corresponds to EBV latency state III and thus seems to correspond to *in-vitro* EBV-immortalized B cells (Tierney et al., 1994).

The immunological responses to EBV infection in patients with infectious mononucleosis or in symptom-free carriers have been described extensively (Henle and Henle, 1979), and are only briefly recorded here: an early transient IgM-response against viral capsid antigen (VCA) is quickly followed by IgG antibodies to VCA which persist in most cases for lifetime. Similarly neutralizing antibodies persist. Antibodies against early antigens (EA) are frequently noted in the symptom phase and either disappear later or persist at a low level The development of EBNA-2 and in particular of EBNA-1 antibodies follows at a somewhat later stage, and commonly results in their life-long persistence. Cytotoxic T lymphocyte levels are high during the acute phase of infectious mononucleosis and remain at a lower level in the asymptomatic carrier state.

The primary infection with EBV results in a carrier state for the patient's life time and in a low level of continuous virus secretion into the saliva. This explains the remarkable success of this virus in infecting more than 90% of the world population. The high global prevalence of this infection has caused substantial problems in explaining its role in malignant diseases, specifically in view of the wide variation in geographic endemicity of some of the EBV-linked cancers.

4.3.1.6 EBV in X-Chromosome-Linked Lymphoproliferative Disease

The X-linked lymphoproliferative (XLP) syndrome can be characterized as a rare inherited immunodeficiency, initially described by Purtilo et al. (1975) as an inappropriate response to EBV infection. The disease typically affects young boys, and results in a fulminant EBV-caused infectious mononucleosis with usually fatal outcome, malignant B-cell lymphomas, and dysgammaglobulinemia. More than 70% of these patients die before the age of 10 years (Seemayer et al., 1995). In individuals not infected by EBV, other lymphoproliferative disorders, such as malignant lymphomas and lymphoid vasculitis are noted (Morra et al., 2001 a).

In 1998, three groups identified a mutated gene in XLP (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). This gene maps to the X chromosome at Xq25, receiving initially three different designations (*SAP, SH2 D1 A, DSHP*), and is presently labeled as *SAP* (signaling lymphocytic activation molecule [*SLAM*]-associated protein). It contains four exons coding for a protein of 128 amino acids. Its expression is mainly restricted to T and NK cells. Memory B cells also seem to express SAP (Feldhahn et al., 2002). A wide range of different mutations within the *SAP* gene is observed in XLP patients (Morra et al., 2001 a). Some of these cause premature stops, others result in reduced stability of the SAP protein, and still others affect the *SAP* function. Although those mutations are clearly linked to the XLP-phenotype with a family history of more than one case, approximately 30–50% of XLP patients do not reveal detectable *SAP* mutations (Latour and Veilette, 2003). These are commonly patients with no family history of XLP (Sumegi et al., 2000).

SAP contains a single SH2 domain with a 28-amino acid tail (Morra et al., 2001 b) which binds to tyrosine 327 of SLAM after previous phosphorylation (Li et al., 2003). SAP expression emerges as an absolute requirement for SLAM-triggered protein tyrosine phosphorylation events in T cells (Latour et al., 2001). This function reflects the specific capacity of SAP to recruit and activate the src-related protein tyrosine kinase FynT. This interaction is critical for further signaling by SLAM and its modulation of cytokine production. For a detailed description of the events involved, the reader is referred to reviews by Latour and Veilette (2003) or Engel et al. (2003).

The failing function of the SAP/SLAM complex or its absence emerges as a reason for the inability of the immune system to cope with EBV-immortalized cells or cells potentially modified by other as yet putative agents engaged in lymphomagenesis. Thus, XLP represents a beautiful example where molecular biology and immunology clarified at least an important part of the underlying cause of a serious lymphoproliferative condition, triggered in many cases by EBV infections.

4.3.1.7 EBV in Immunoblastic Lymphoma

Non-Hodgkin lymphoma (NHL), besides lung cancers and melanomas, represents the fastest rising malignancies in most parts of the Western world. In the United States, they account for approximately 4% of all cancers (Vose et al., 2002). The overall incidence is about 50% higher for males than for females. The incidence rates in

whites are about 35 % higher than in blacks. In children, NHLs account for 10.9 % of childhood cancers in the United States (Linet et al., 1999).

NHL are particularly frequent under conditions of immunosuppression: in patients receiving immunosuppressive drugs after undergoing organ or bone marrow allografting the relative risk is increased 30- to 50-fold (Young, 1989; Hoover, 1992). In HIV-infected patients the risk is even more than 100-fold higher than that of the general population (Beral et al., 1991). NHL is the most frequent malignancy in persons with Wiskott–Aldrich syndrome, ataxia teleangiectasia, XLP syndrome and combined immunodeficiencies (Filipovich et al., 1992; Vose et al., 2002). With the recent developments of HIV-antiviral therapy and increasing $CD4^+$ T-cell counts, the frequency of immunoblastic lymphomas decreased substantially, whereas the rate of Burkitt and Burkitt-like lymphomas with Ig/myc translocations remained constant (G. Klein, personal communication).

With the exception of Burkitt's lymphomas, spontaneously arising lymphomas in immunocompetent individuals are commonly EBV-negative. In immunosuppressed patients, however, the situation is remarkably different: the majority of arising NHLs is of B-cell origin, contains EBV genomes, and reveals most often the latency III expression pattern of EBV gene expression (Young et al., 1989). In the early phase of immunosuppression, EBV infection or reactivation leads to a polyclonal expansion, while additional genetic changes acquired subsequently (in particular of the BCL-6 locus) result in the emergence of clonal proliferations (Cesarman et al., 1998; Knowles, 1998). Thus, at least in the early phase these tumors seem to represent an *in-vivo* counterpart to EBV-immortalized cells *in vitro*. This renders it likely that in these EBV-positive B-cell lymphomas the failing immunosurveillance permits the proliferation of EBV-transformed B lymphoblasts and implies that EBV is the driving force (direct carcinogen) for the malignant phenotype of these lymphomas. This is further underlined by the observation that reconstitution of the immune system frequently results in lymphoma regression (O'Reilly et al., 1998). Usually, EBV-negative forms of post-transplant lymphomas appear later than EBV-positive tumors, originate mainly from T cells, and grow more aggressively (Dotti et al., 2000; Nelson et al., 2000). Central nervous system lymphomas, which are seen especially in highly immunosuppressed AIDS patients, are almost uniformly EBV-positive (Cesarman and Mesri, 1999; Young and Rickinson, 2004). However, these lesions have virtually disappeared since the introduction of antiviral therapy.

4.3.1.8 **EBV in Burkitt's Lymphoma**

Burkitt's lymphoma (BL) was originally described by Dennis Burkitt (1958, 1962) as an endemic lymphoma in equatorial Africa, frequently affecting the jaw of children aged 5 to 12 years. The peculiar endemic pattern of this tumor led Burkitt to the initial speculation that this tumor may be caused by an arthropod-borne virus (1962). Subsequently, he and others suspected that holoendemic infections with the malaria parasite *Plasmodium falciparum* may contribute to the lymphoma development (summarized in Magrath, 1990). The discovery of EBV particles initially in tissue

culture cells derived from this tumor (Epstein et al., 1964), and subsequently also EBV DNA by molecular hybridization directly in tumor biopsies (zur Hausen et al., 1970), may be considered as the starting point for human tumor virology.

Today, endemic as well as sporadic forms of BL have been described, combining a group of somewhat heterogeneous B-cell lymphomas of germinal center origin, classified as small, non-cleaved-cell lymphomas which respond well to chemotherapeutic interference (Harris et al., 1994; Magrath et al., 1996). One central feature of almost all of these lymphomas is a chromosomal translocation, initially discovered by Manolov and Manolova (1972) that dysregulates the expression of c-myc (Dalla-Favera et al., 1982; Taub et al., 1982). This dysregulation was an early postulate of G. Klein (1979), who inferred this from chromosomal analyses of murine T-cell leukemia cells. Within the same year, Klein and his coworkers demonstrated a reciprocal 8;14 translocation in EBV-negative B-cell acute lymphocytic leukemia with Burkitt-type cells (Mitelman et al., 1979).

The c-myc translocation occurs in the vast majority of all clinical forms of BL: the endemic form, predominantly found in equatorial Africa and also in New Guinea, the relatively rare sporadic form arising in all regions of the world, frequently in adolescents and young adults, and the human immunodeficiency-associated form of Burkitt's lymphoma (Hecht and Aster, 2000). It uniformly involves the c-*myc* locus on chromosome 8q24 and results in most cases in a reciprocal translocation from chromosome 14 at q32. Less frequently, the q11 locus on chromosome 22 and the p12 locus on chromosome 2 are involved. In each of these cases expression of the c-myc gene becomes controlled by elements regulating immunoglobulin genes. Their enhancer elements bind B-cell-specific factors which may activate transcription located up to 500 kb away (Hecht and Aster, 2000). The breakpoints on chromosomes 14, 22, and 2 are within or in the immediate neighborhood of the IgH, Igλ or Igκ genes, respectively. A schematic outline of these recombinations is shown in Figure 4.9.

It is interesting to note – and possibly also of pathognomonic significance – that the breakpoints in endemic and sporadic BL frequently differ. In the endemic form the breakpoints are found in some distance 5' to the first c-myc exon (in some cases more than 100 kb upstream). The breakpoint on chromosome 14 is located in the IgH joining regions (Joos et al., 1992). In sporadic BL, the breakpoints are regularly found within exons 1 and 2 of the c-myc gene on chromosome 8 and within the IgH Sμ switch region on chromosome 14 (Pellicci et al., 1986; Neri et al., 1988; Shiramizu et al., 1991; Gutierrez et al., 1992; Yano et al., 1993). In the variant recombination, the breakpoints occur on chromosomes 2 or 22 of the κ and λ constant regions, respectively. In these cases the breakpoints on chromosome 8 occur at variable distances downstream of c-*myc* (Magrath, 1990; Gerbitz et al., 1999). Although immunoglobulin gene rearrangements are clearly engaged in these recombinations, the mechanism causing c-myc breaks is unknown. Conflicting data exist on the role of activation-induced cytidine deaminase (AID) for the induction of c-myc/IgH translocations (Ramiro et al., 2004; Unniraman et al., 2004). There exist no regions of homology between these breakpoints and sites of the V(D)J or switch recombinase recognition sequences (Hecht and Aster, 2000). A recent speculation

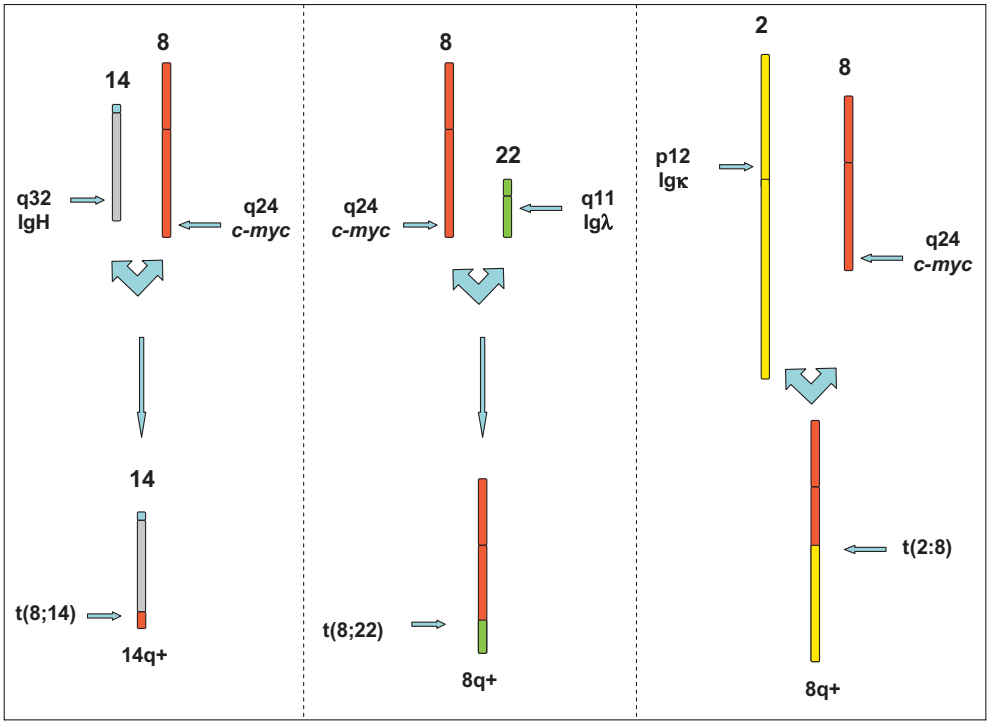

Fig. 4.9 Reciprocal translocation of c-*myc* and immunoglobulin genes.

postulates that specific persistent infections with TT-like viruses may contribute to these recombinations by inducing specific recombinations (zur Hausen and de Villiers, 2005).

C-myc translocations are not limited to BLs. They occur occasionally in large B-cell lymphomas (Sigaux et al., 1984; Thangevelu et al., 1990), lymphoblastic lymphoma (Slavutsky et al., 1996), in a subset of very aggressive follicular lymphomas (Thangevelu et al., 1990; Akasaka et al., 2000), and in late stages of multiple myelomas (Sawyer at al., 1995; Shou et al., 2000). Occasionally, however, lymphoma-associated translocations have also been detected in B cells from normal individuals (Limpens et al., 1995). Since constitutive c-myc activation drives cells into apoptosis, they may however be rescued by EBV infection or by high levels of B-cell stimulatory cytokines, as are found in malaria- or HIV-infected persons (G. Klein, personal communication).

The role of EBV in BL is not easily explained. Viral DNA persists in the EBV-positive tumors commonly at multiple copies per tumor cells (zur Hausen and Schulte-Holthausen, 1970; zur Hausen et al., 1970; Nonoyama and Pagano, 1973). Although most of the genomes persist as circular episomes, some Burkitt-derived cell lines also contain integrated copies (Lawrence et al., 1988; Delecluse et al., 1993; Takakuwa et al., 2004). The expression pattern represents the classical stage latency I with the sole expression of EBNA-1 and EBER and BARF RNA. Although EBNA-1 is

immunologically not silent and its expression results in specific antibody production and specific CD4+ T cells, it is not processed to appropriate HLA class I-associated target peptides, due to the glycine-alanine repeat-dependent processing defect (Lee et al., 2004). Interestingly, myc overexpression imposes a non-immunogenic phenotype on EBV-infected B cells (Staege et al., 2002).

There exist reasons to assume that the virus plays an important role in EBV-positive tumors: the presence of monoclonal viral episomes suggests that EBV infection preceded the proliferation of the precursor B cells (Neri et al., 1991); viral DNA is present in slightly more than 90% of endemic tumors, but in only about 15% of sporadic BLs. In BLs arising under immunosuppression the percentage seems to be slightly higher than in other sporadic cases (Subar et al., 1988). Recent evidence suggests that EBV infection, at least in tumor-derived cell lines investigated thus far, determines the malignant phenotype of the respective cells. Sublines of the Akata line, originating from a Japanese patient with Burkitt's lymphoma, lost their EBV genomes and concomitantly became non-tumorigenic after inoculation into nude mice (Shimizu et al., 1994). Reinfection with EBV restores the malignant growth properties of these cells (Komano et al., 1998). Even the sole transfection of these cells with BARF-1 conferred tumorigenicity to Akata cells again (Sheng et al., 2003). In another set of experiments, Kennedy et al. (2003) demonstrated that EBV provides a survival factor for the EBV-positive cells. Here in particular EBNA-1 was shown as a protective factor against apoptosis. There seems to exist an interesting difference between EBV-positive and EBV-negative BLs concerning reactive oxygen signaling. EBV-positive BLs express high levels of mitogen-activated protein kinase and reactive oxygen species (ROS), whereby ROS directly regulate NF-κB stimulation, whereas EBV-negative tumors do not show elevated levels of ROS (Cerimele et al., 2005).

Additional arguments can be raised from studies which reveal a synergistic interplay between the rearranged c-myc and EBV: human lymphoblasts immortalized *in vitro* by EBV become tumorigenic upon transfection with a rearranged myc gene (Lombardi et al., 1987). The introduction of an activated c-myc gene into an EBV-immortalized line in which EBNA-2 expression was estrogen-dependent, induced continuous proliferation even without expression of EBNA-2 and LMP-1 (Polack et al., 1996). Specific mutants of c-myc retain their ability to stimulate cell proliferation and activate p53, but are defective in promoting apoptosis, as they fail to induce the BH3-only protein Bim and effectively inhibit Bcl2 (Hemann et al., 2005). Thus, mutant myc proteins selectively disable a p53-independent pathway to enable tumor cells to evade p53 functions.

A number of additional genetic changes have been noted in BLs: p53 mutations occur in approximately 30% of these lesions (Gaidano et al., 1991; Ichikawa et al., 1993; Schoch et al., 1995), and it was suggested that this represents a late effect during lymphoma progression (Cherney et al., 1997). Some of the BL lines expressing wt p53 reveal p14ARF deletions or show HDM2 overexpression (Lindstrom et al., 2001; Capoulade et al., 1998). Silencing of the p73 gene has been reported in 30% of BLs (Corn et al., 1999). The retinoblastoma-related gene RB2/p130 is also frequently mutated in endemic BL (Cinti et al., 2000a,b). The Rb pathway is also frequently inactivated in BLs by epigenetic modifications of Rb or $p16^{INK4}$.

Transcriptional silencing in BLs affects the cellular gene coding for the death-associated protein (DAP) kinase (Katzenellenbogen et al., 1999), enabling the generation of death signals by interferon γ, tumor necrosis factor and Fas (Levy-Strumpf et al., 1998; Cohen et al., 1999). The BCL-6 gene is also involved in translocations and mutations in 30–50% of BLs (Capello et al., 1997, 2000). In addition, chromosome 6q abnormalities occur at high frequency in this type of tumor (Parsa et al., 1994).

EBV infection of EBV-negative subclones of the Akata BL cell line (Komano et al., 1998) and two other pairs of initially EBV-positive lines with EBV-negative subclones induces a cellular transcript of the T-cell leukemia I (*TCL-1*) oncogene (Kiss et al., 2003). Although expression of the *TCL-1* gene is normally restricted to very early thymocytes (Teitell et al., 1999; Narducci et al., 2000), biopsies from both endemic and sporadic cases of BL are commonly positive, irrespective of the presence of EBV genomes. It is speculated that other yet undefined events lead to TCL-1 induction in EBV-negative sporadic BL cases (Bell and Rickinson, 2003).

In conclusion, the obvious cooperation between rearranged c-myc and EBV in malignant transformation, the dependence of the malignant phenotype of EBV-negative subclones of previously EBV-positive BL cells on EBV reinfection, and the demonstration of an anti-apoptotic function of EBNA-1, are results that stress the important role of EBV infection in EBV-positive BLs in the endemic regions for this disease. A number of important questions, however, remain completely open:

- What determines the geographic restriction of the endemic form of BL?
- Which cofactors contribute to regional differences in tumor incidence?
- Why do endemic and sporadic cases of BL differ in c-myc breakpoints?
- Is there another viral factor involved in the etiology of EBV-negative BLs?

There are no clear-cut answers to any of these questions. Although the geographic restriction of the endemic form of BL has frequently been attributed to the induction of B-cell proliferation by holoendemic infections with the malaria parasite *P. falciparum* (Rowe et al., 1987), there exist regions in India, in Cambodia, Malaysia, and Indonesia with a comparatively high rate of *P. falciparum* transmission but a low rate of BL (Sharma et al., 2004). Clearly, immunosuppression in organ transplant recipients – and even more so in AIDS patients – represents a risk factor for BL development. It does, however, neither explain the endemic cases nor the sporadic cases arising without recognizable immunosuppression. Attempts have been made to explain the different c-myc breakpoints by different stages of maturation between germinal center pre-B cells in early childhood in endemic regions in comparison to adolescents and young adults most frequently affected by sporadic BL (Hecht and Aster, 2000). Yet, the reasons for modifying specifically the c-myc gene at different sites in endemic and sporadic BLs remain unexplained. It also remains an open question as to whether additional infectious agents contribute in particular to the EBV-negative cases of BL. A recent hypothesis attempted to explain at least several of

these open questions (zur Hausen and de Villiers, 2005). According to this view, latent infections with specific types of TT-like viruses contribute to chromosomal translocations. The geographic prevalence of the endemic form of BL would be due to such virus types with predominance in the equatorial BL tumor belt and a preference for affecting the upstream region of the c-*myc* gene.

Although BL represents the first human malignant tumor, harboring (at least in endemic regions) a latent tumor virus in every tumor cell, it represents a tumor system in which 40 years of intensive research did not provide any clear-cut answers to the quest for causality.

4.3.1.9 **EBV in Nasopharyngeal Carcinoma**

In 1966, Old and colleagues reported immunoprecipitation studies revealing a specific reactivity of sera from patients with nasopharyngeal carcinoma (NPC) against an antigen present in BL cells. Immunofluorescent tests developed to detect EBV antigens in BL cells subsequently confirmed the high reactivity of NPC sera against EBV antigens (Henle and Henle, 1969). One year later, EBV DNA was detected in NPC cancer biopsies by nucleic acid hybridization (zur Hausen et al., 1970). Original assumptions that the viral genomes may reside within the infiltrating lymphocytes were refuted three years later (Wolf et al., 1973; Klein et al., 1974). EBV DNA persisted usually in multiple copies within the epithelial tumor cells. The link between EBV infection and NPC was further strengthened by the demonstration of high IgG and IgA antibody titers not only against VCAs, but also against viral EAs (Henle and Henle, 1976).

The histological classification of NPC depends on the degree of differentiation. According to the WHO classification, three types can be distinguished: (i) a keratinizing squamous cell carcinoma (type 1); (ii) a non-keratinizing carcinoma (type 2); and (iii) the undifferentiated carcinoma (type 3) (Shanmugaratnam, 1978). The latter type is also designated as lymphoepithelioma or Schmincke tumor. Serological studies in particular linked types 2 and 3 to EBV infections, but less so type 1. The presence of EBV DNA in type 1 tumors remains somewhat controversial: one study consistently found EBV DNA in these tumors (Raab-Traub et al., 1987), while another failed to detect EBV DNA within the differentiated form (Niedobitek et al., 1991). By analyzing NPC cases from Europe, our group consistently was unable to detect EBV DNA in a small percentage of NPC biopsies (H. zur Hausen, unpublished results; E.-M. de Villiers and H. zur Hausen; unpublished results). In our view, the situation seems reminiscent of EBV in BLs with some EBV-negative cases, even in areas of high tumor incidence.

4.3.1.9.1 **Epidemiology and Risk Factors**

Nasopharyngeal carcinoma shows a peculiar geographic distribution, reaching particularly high levels among Cantonese males in Southern China with 20–30 cases per 100 000 population (Yu et al., 1986). The incidence is also high in Hong Kong

(21.4), in Singapore (16.3), among Hawaiian Chinese (10.7), in Hanoi, Vietnam (10.4) and in Manila, Philippines (7.2) (Parkin et al., 2002). Outside of Asia a higher incidence is reported for the Northwest Territories of Canada (9.2), for Californian Chinese (7.6), Algeria (2.7) and Uganda (1.8) in Africa, and in Europe for Malta (2.6). In most other parts of the world the incidence is below 1 per 100 000 inhabitants.

Initially, it was proposed that the consumption of Cantonese-style salted fish is a possible risk factor for the increased incidence of NPC in this area (Ho, 1972). In subsequent studies, salted fish specimens were shown to be low in volatile nitrosamines and in bacterial mutagenicity (Tannenbaum et al., 1985), although exposure to nitrite yielded substantial quantities of nitrosamines and mutagenic activity. A few additional preserved foods (fermented fish sauce, salted shrimp paste, moldy bean curd and preserved plum) were reported as risk factors, independent of salted fish exposure (Yu and Henderson, 1987; Yu et al., 1989). Although very suggestive, the accurate role of dietary factors in the etiology of NPC remains unclear, especially in their interaction with persisting EBV infections.

An interesting hypothesis was put forward by Ito and colleagues (1983 a). Based on the observation that tumor promoters of the diterpene ester type efficiently activate the lytic cycle of persisting herpes-type viruses (zur Hausen et al., 1979), these authors investigated the EBV activation by tung oil from the Chinese tung oil tree, *Aleuritis fordii* (Ito et al., 1983 b). One of the main constituents of this oil, 12-*O*-hexadecanoyl-16-hydrophorbol-13-acetate (HHPA), retained its EBV-inducing activity even after treatment for 2 h at 120 °C (Yanase and Ito, 1984). Soil samples collected under tung oil trees which are frequently planted in high-risk areas for NPC, revealed the EBV-inducing activity (Ito et al., 1983 c). Ito and colleagues speculated that the regular activation of EBV after exposure to tung oil might represent one contributing factor to the geographic clustering of NPC in Southern China.

The frequency of NPC incidence in specific Chinese and among Innuit populations suggested, at an early stage, the involvement of genetic factors in the occurrence of this disease. Indeed, it seems that two specific HLA haplotypes HLA-A2 Bw46 and A19 B17 increase the risk for NPC by two- to four-fold (Chan et al., 1983). Two other alleles, HLA-A11 and B13, reveal a protective effect. This seems to indicate that genes closely linked to the HLA locus, but not necessarily identical with HLA, seem to influence susceptibility to NPC (Lu et al., 1990). According to a more recent publication, among Taiwanese Chinese the extended haplotype HLA-A*3303-B*5801/2-DRB1*0301-DQB1*β201/2-DPB1*0401 was associated with a statistically significantly decreased risk for NPC (OR = 0.24, 95 % CI = 1.1 to 6.4) (Hildesheim et al., 2002).

It remains presently very difficult to assess the relevance of these published observations in order to pinpoint modifications of specific genes as factors in NPC development. More direct information may originate from the analysis of individual genes or specific chromosomal changes in cancer tissue from NPC patients. Studies in Tunisia revealed a polymorphism of the stress protein HSP70–2 gene as a susceptibility risk factor for NPC (Jalbout et al., 2003). A polymorphism of a gene *CYP2 E1* (a cytochrome p450), the product of which is involved in the activation of nitrosamines into reactive intermediates, has also been shown to be associated with

an increased risk for NPC (Hildesheim et al., 1997). This led to an investigation of polymorphisms in genes involved in DNA repair (Cho et al., 2003), and the subsequent identification of high-risk polymorphisms for the genes *hOGG1* (codon 326) and *XRCC1* (codon 280) which, in combination with *CYP2 E1*, resulted in a drastically increased OR of 25 (95 % CI = 3.5–177).

Chromosomal aberrations specific for NPC have been described for several chromosomal sites: non-random changes were observed in chromosomes 1, 3p, 3q, 5q, 9p, 11q, 12, 13q, 14q, and X (for a review, see Lo and Huang, 2002). The inactivation of tumor suppressor genes on 3p, 9p, 11q, 13q, 14q, and 16q and alterations in oncogenes on chromosomes 8 and 12 seem to be particularly important for NPC development. The most frequent change involves a deletion in chromosome 3p. A most interesting candidate region seems to locate at 3p21.3. The critical target here appears to be an isoform of the *RASSF1* gene, *RASSF1A* (Lo et al., 2001). Epigenetic inactivation of this gene has also been noted in small-cell lung cancer, non-small-cell lung cancer, breast cancer, and in renal carcinoma (Dammann et al., 2000; Burbee et al., 2001; Dreijerink et al., 2001). Other loci frequently modified in NPC involved the gene coding for the fragile histidine triad protein (FHIT) on chromosome 3p14.2, and the gene coding for the retinoic acid receptor β 2 (for a review, see Lo and Huang, 2002). On chromosome 9p, modifications involve the site 9p21 containing the genes for the cyclin-dependent kinase inhibitors $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ (Lo et al., 1995). Transfer of normal chromosome 11 suppressed the malignant phenotype of NPC cells (Cheng et al., 2002). Here, it is likely that the TSLC1 gene located at 11q23 plays a significant role (see Lo and Huang, 2002). Genes located in other chromosomal regions have thus far not been precisely identified. Inactivation of the genes discussed here is frequently mediated via promoter methylation.

4.3.1.9.2 **EBV Genome Persistence and Gene Activity in NPC Cells**

As in BL cells, the EBV genome circularizes after infection of nasopharyngeal cells and persists in this state in NPC. In some tumors, however, integration of viral DNA occurs (Kripalani-Joshi and Law, 1994). The structure of the viral termini serves as a marker of clonal cellular proliferation (Raab-Traub and Flynn, 1986). Indeed, the identical number of terminal structures within one individual tumor permits the conclusion that EBV-infection preceded the development of malignant outgrowth and suggests a role of the viral infection within this process. *In-situ* hybridization and Northern blots permitted the detection of EBNA-1, LMP-1 and LMP-2, and EBER RNA transcription in tumor biopsy material (Yeung et al., 1993; Raab-Traub et al., 1983; Wu et al., 1991). Approximately 60 % of EBV-positive NPC biopsies were LMP-1-positive (Fahraeus et al., 1988), while other studies found only in part of the tumors evidence for LMP-1 protein expression (Young et al., 1988; Niedobitek et al., 1992), whereas LMP-2 protein has not been discovered within the tumor tissue, although NPC patients reveal increased antibody titers to this protein (Frech et al., 1993). This latter finding, as well as the variable detectability of LMP-1 protein in NPC materials, suggests that very low levels of these proteins are probably expressed in all NPC tumors. LMP-2 A seems to influence the migratory pattern of epithelial

cells and induces an invasive phenotype by activating the integrin-α-6 gene expression (Pegtel et al., 2005).

NPC cells commonly express the EBV latency pattern II. EBNA 1 expression is constantly observed in these cells, a situation reminiscent of Burkitt's lymphomas. EBNA 2 and all EBNA 3s are absent. In both types of tumors the promoter in BAM HI F/Q is used for EBNA 1 transcription, leaving all other EBNAs unexpressed (Sample et al., 1990, 1991; Smith and Griffin, 1992). In spite of the regulation of the LMP-1 promoter by EBNA 2 and EBNA-LP, in epithelial cells LMP-1 is expressed from a larger mRNA (Gilligan et al., 1990 a,b). The initiation starts from a promoter regulated by SP1 and STAT3 which is constitutively active in NPC (Sadler and Raab-Traub, 1995; Chen et al., 2001). LMP-1 induces the cyclooxygenase-2 in nasopharyngeal carcinoma cells, and this seems to result in an increased vascular endothelial growth factor production (Murono et al., 2001). Thus, LMP-1 may play a role in angiogenesis in NPC.

EBER RNA is consistently expressed in NPC cells. It was recently reported that EBER is responsible in nasopharyngeal carcinoma-derived cell lines for the induction of insulin-like growth factor 1, and that this may directly affect the pathogenesis of NPC (Iwakiri et al., 2005). There exists however, a remarkable heterogeneity in EBER-RNA expression within the same NPC biopsies (Fig. 4.10). The EBER-negative cells have not yet been analyzed for the presence of EBV DNA.

Interestingly, another group of abundant and consistent transcripts exists in NPC tumors: three mRNAs can be detected in Northern blots mapping at the 3'-end of the

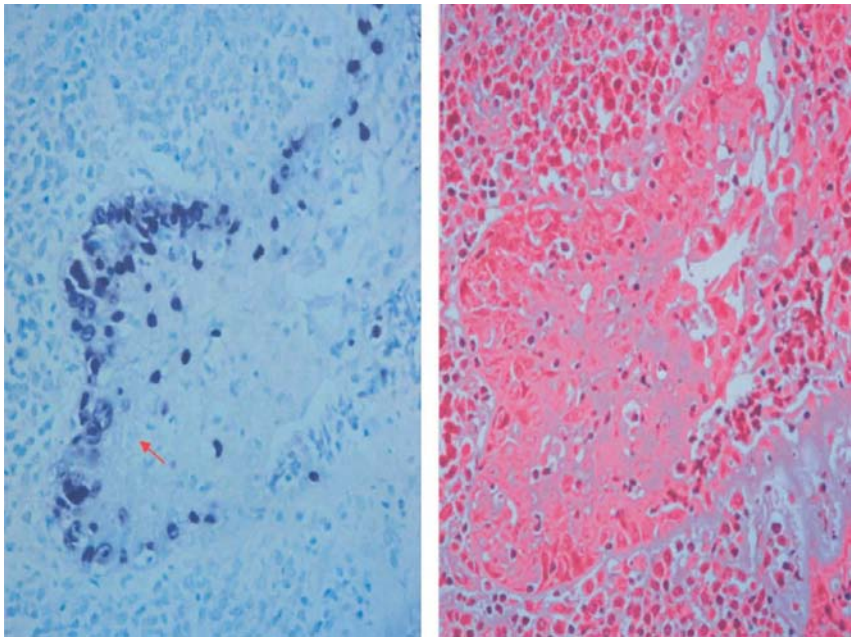

Fig. 4.10 EBER-RNA expression in a nasopharyngeal carcinoma biopsy.
(Illustration courtesy of Kwok Wai Lo, Hong Kong.)

BAM H1 A rightward frame 0 (BARF0). They originate from an alternate splicing of seven exons forming several ORFs (Sadler and Raab-Traub, 1995). Although protein translation of the BARF transcripts has not been demonstrated, some experiments point to a possible interaction of specific BARF transcripts and their *in-vitro*-derived proteins with Notch family proteins, resulting in Notch protein degradation and translocation of unprocessed Notch into the nucleus (Kusano, 2001). In the EBNA2-negative line P3 HR-1, expression of one of the BARF ORFs results in the induction of LMP-1 (Kusano and Raab-Traub, 2001).

4.3.1.9.3 **EBV Strain Variation and NPC**

The peculiar geographic pattern of NPC incidence suggested at an early stage that different EBV strains, prevalent in endemic areas for NPC, may play a significant role. Although two genotypically different EBV strains have been identified (EBV types 1 and 2), no stringent relationship between infection by these types and NPC has been uncovered. The two types are almost identical, except for differences in EBNA-LP, -2, -3A, -3B and -3C proteins, ranging between 16% and 47% (Dambaugh et al., 1984; Adldinger et al., 1985; Sample et al., 1990). A polymorphism was, however, noted within the LMP-1 gene (Hu et al., 1991). LMP-1 gene polymorphisms were found quite consistently in Chinese populations and Alaskan Innuits (Miller et al., 1994; Sung et al., 1998). In addition, differences in an 11-amino acid repeat element of LMP-1 were noted, in some strains with a 5-amino acid insertion (Miller et al., 1994), yet a disease-specific association remains questionable. It is interesting to note that some LMP-1 variants isolated from NPC possessed an increased ability to activate NF-κB (Miller et al., 1998; Johnson et al., 1998).

4.3.1.9.4 **EBV in Premalignant Lesions of NPC**

Few studies have attempted to identify the presence of EBV DNA in premalignant lesions of NPC. This is due mainly to the difficulties in identifying these lesions and in obtaining sufficient material for a detailed analysis. Whereas one study describes the consistent presence of EBV in all precursor cells of nasopharyngeal cancer (Pathmanathan et al., 1995), another study had difficulties in discovering EBV in early lesions, although the virus was present in all late stages studied (Yeung et al., 1993). This leaves the question open, as to whether EBV infection is a relatively late event in possibly genetically premodified epithelial cells of the nasopharynx (as discussed for EBV-linked gastric cancer), or not. It also permits the speculation that EBV might play a key role as a malignizing factor in an already pre-damaged cellular environment.

In conclusion, the consistency of association of EBV, at least with the undifferentiated histological type of NPC, the presence of viral DNA in all tumor cells of a virus-positive nasopharyngeal carcinoma, the specific pattern of virus antigen expression, and the monoclonal pattern of virus persistence, point strongly to a role of EBV in the causation of these tumors. Despite vast geographic differences in NPC incidence, EBV-positive tumors occur everywhere in the world.

Many questions regarding the role of EBV in NPC remain unanswered, however:

- Present observations on local risk factors, genetic predisposition, and EBV strain variation do not fully explain the wide variations in the incidence of NPCs.
- It is not clear at which stage of proliferation (normal cell, early precursor lesion or late precursor lesion) EBV enters the scene. Although it is well established that EBV can infect epithelial cells, there exists the possibility that late-stage precursors become more readily infected than early stages.
- It is unclear which type of host cell genetic modifications interact with EBV during the course of malignant conversion, and to what extent the environment contributes to the development of these modifications.
- The existence of EBV-negative NPCs requires further studies. The lesions may arise from specific host cell modifications in regulatory pathways, but potentially other viruses might also be involved.

These and other questions need to be answered before EBV can be considered as the major contributing factor for the causation of NPC and as a direct carcinogen for nasopharyngeal cancers.

4.3.1.10 **EBV in Hodgkin's Disease**

The suspicion that Hodgkin's disease (HD) is caused by an infection reaches back for more than 70 years (for a review, see Kaplan, 1980) with, initially, environmental factors being implied as risk factors for this condition (Fraumeni and Li, 1969; Dörken, 1975). Dörken suggested a possible relationship of HD with exposure to pets and farm animals, and this resulted in the speculation that HD might represent a zoonosis. Hodgkin-like disease has been occasionally noted in domestic animals, particularly in cats (Maeda et al., 1993; Blomme et al., 1999; Walton and Hendrick, 2001). Several studies have pointed to a higher prevalence of this condition in rural residences, in agricultural occupations, and among woodworkers (Milham and Hesser, 1967; Acheson, 1967; McCunney, 1999). The peculiar age distribution of HD suggested the delayed exposure to a common infectious agent (Gutensohn and Cole, 1980). Males are more frequently affected than females, with the male:female gender ratio ranging between 1.2 and 2.0

Initial studies to detect EBV DNA in biopsy material from patients with HD failed due to the insensitivity of the method used at that time (zur Hausen et al., 1970; Nonoyama et al., 1974). Several serological studies performed at the same time in various laboratories revealed elevated antibody titers of HD patients against Epstein–Barr viral capsid and early antigens (Johannsson et al., 1970; Levine et al., 1971; Henle and Henle, 1973; Henderson et al., 1973; Hesse et al., 1973). The data were partially obscured by reports from some of the same groups, describing elevated antibody titers directed also against other members of the herpesvirus group.

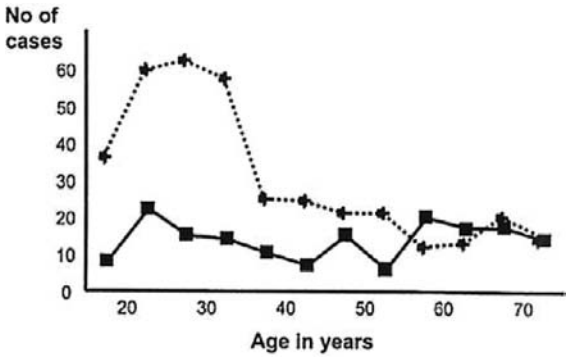

Fig. 4.11 Age distribution of Hodgkin's disease in Scotland and the North of England, 1994–119, by EBV status (solid line) EBV-associated cases (dashed line) non-EBV-associated cases. (Jarrett, 2002. With permission.)

Subsequent epidemiological studies demonstrated a two- to four-fold increased risk for HD within the first three years following infectious mononucleosis (Rosdahl et al., 1974; Munoz et al., 1978). Direct evidence for a role of EBV in at least a proportion of HD patients originated from the detection of monoclonal viral genomes in several biopsies by Southern blotting and terminal repeat analysis and the subsequent demonstration of EBV persistence within the Hodgkin Reed–Sternberg cells by in-situ hybridization (Anagnostopoulos et al., 1989; Weiss et al., 1987, 1989). A number of subsequent studies confirmed these results. Based on the differentiation of Hodgkin types according to the Rye classification (Lukes et al., 1966), there exist substantial differences in EBV positivity for the four histological types of classical HD (Fig. 4.11): the lymphocytes-predominant subtype is rarely linked to EBV infections, the nodular sclerosing type in 10–30% is EBV-positive, whereas lymphocyte-depleted and mixed cellularity types are 80–90% EBV-positive (Pallesen et al., 1993; Jarrett, 2002).

It is of interest to note that in Western Europe and the United States the nodular sclerosis and lymphocyte-predominant types prevail, occurring mainly in late adolescence or in young adults. In contrast, in economically less developed parts of the world early childhood cases are more common, revealing predominantly the mixed cellularity histology (Correa and O'Conor, 1971; Macfarlane et al., 1995; Glaser and Jarrett, 1996). The Rye classification of HD is depicted in Table 4.4.

Table 4.4 The Rye classification of different histologies in Hodgkin's disease

Histological classification	Epstein-Barr virus-positive* [%]
Lymphocyte predominance	Rare
Nodular sclerosis	10–30
Mixed cellularity	80–90
Lymphocyte depletion	80–90

* The EBV-negative types prevail in Europe and in the United States.

In addition to these four forms of classical HD, a nodular lymphocyte-predominant form of Hodgkin's lymphoma has been described with clonal immunoglobulin gene rearrangements and ongoing mutations. This defines a mutating germinal center clone with antigen-selection properties as a possible precursor of lymphocytic and histiocytic cells (Küppers et al., 1994; Braeuninger et al., 1997; Marafioti et al., 1997). In spite of functional immunoglobulin rearrangements, Hodgkin and Reed–Sternberg cells are defective in immunoglobulin transcription (Marafioti et al., 2000). This is in part due to destructive mutations within the rearranged molecules (Kanzler et al., 1996; Marafioti et al., 2000), in part to the failure of transcription factors to bind to the mutated promoter (Jox et al., 1999; Theil et al., 2001), and also to the absence of immunoglobulin-specific transcription factors (Re et al., 2001; Stein et al., 2001; Torlakovic et al., 2001).

4.3.1.10.1 **EBV Genome Persistence and Gene Expression**

Hodgkin's lymphoma is characterized by the appearance of Reed–Sternberg cells, large binucleated or polynucleated cells. They are surrounded by a reactive infiltrate of T cells, histiocytes, eosinophils and plasma cells (reviewed in Thomas et al., 2004). The cellular origin of Reed–Sternberg cells was clarified by micromanipulation of single cells from Hodgkin biopsies (Küppers et al., 1994). The clonal rearrangement of immunoglobulin genes and somatic mutations within these genes characterized them as germinal center or post-germinal center B cells (Kanzler et al., 1996). Occasionally, clonal T-cell receptor rearrangements were noted, pointing to the T-cell origin of some HD cases (Müschen et al., 2000; Seitz et al., 2000).

In EBV-positive Reed–Sternberg cells the pattern of EBV genome expression is restricted and corresponds to the latency pattern II. The cells express EBNA1, LMP-1, -2A and -2B proteins and EBER RNAs (reviewed in Rickinson and Kieff, 1996). Expression of LMP-1 is unusually strong in EBV-positive Reed–Sternberg cells (Herbst et al., 1991; Pallesen et al., 1991). In EBV-positive, as well as in EBV-negative cases, expression of members of the tumor necrosis factor receptor superfamily CD30, CD40, and CD95 is observed in a high proportion (Kim et al., 2003). The expression of LMP-1 seems to mimic a constitutively active CD40 receptor (Eliopoulos and Rickinson, 1998). Both bind tumor necrosis factor-associated factors, resulting in the activation of NF-κB, AP-1 and STAT (Devergne et al., 1996; Izumi and Kieff, 1997; Kieser et al., 1997; Gires et al., 1999). LMP-1 appears to be more potent than CD40 in signaling activation (Brown et al., 2001), and may thus contribute to cell transformation. Germinal center B cells depend for survival on signals provided by B-cell receptor and CD40 molecules (Lam et al., 1997). Without immunoglobulin expression these cells undergo apoptosis. Since Reed–Sternberg cells do not express immunoglobulins at their surface, it is likely that LMP-1 and also LMP-2 A contribute to their survival (Jarrett, 2002). The survival mechanism of EBV-negative Reed–Sternberg cells is presently unknown.

LMP-2A increases the expression of genes engaged in cell cycle induction and prevention of apoptosis, and decreases the expression of B-cell-specific factors and genes associated with immunity (Portis et al., 2003). Thus, the function of EBV-in-

duced membrane proteins in Reed–Sternberg cells facilitates their survival and contributes to their proliferative capacity.

Interestingly, both EBV-positive and EBV-negative Hodgkin cells reveal a constitutive strong activation of NF-κB (Bargou et al., 1996). Activation of this transcription factor appears to play a central role in HD, since the introduction of a dominant-negative mutant of IκBα, binding NF-κB irreversibly in the cytoplasm, results in apoptosis of Hodgkin and Reed–Sternberg cells (Bargou et al., 1997; Hinz et al., 2002). The constitutive activation of NF-κB results partially from mutations in the IκBα gene, from amplification of the NF-κB/REL locus, or from constitutively active CD30, CD40, Notch 1 and RANK pathways (Jundt et al., 2002; Emmerich et al., 1999; Annunziata et al., 2000; Joos et al., 2000; Martin-Subero et al., 2002). An additional group of constitutively active transcription factors in HD is represented by the STAT family (Kube et al., 2001). STAT3 and STAT6 appear to be regularly activated (Skinnider et al., 2002).

4.3.1.10.2 Immunosuppression and Hodgkin's Disease

Whereas the incidence of B-cell lymphomas dramatically increases in organ allograft recipients and AIDS patients under conditions of immunosuppression, there is a more moderate increase in risk for Hodgkin's lymphoma. In AIDS patients, the relative risk for non-Hodgkin lymphomas varies between 225 and 650 (Hessol et al., 1992; Cote et al., 1997). Solely for Burkitt's lymphomas, the risk increases 260-fold (Cote et al., 1997). For HD, the relative risk seems to be in the range of 10 to 20 (Hessol et al., 1992; Grulich et al., 1999; Dal Maso and Franceschi, 2003). The mixed cellularity type of HD is predominant in these patients, pointing to a high prevalence of EBV-associated cases (Levine, 1998).

In conclusion, several data favor a causal role of EBV in the EBV-positive cases of HD:

- Viral DNA persists within the malignant Reed–Sternberg cells and is not a contaminant of the surrounding lymphoid stroma.
- The clonality of viral DNA indicates its uptake prior to the onset of malignant growth.
- Viral antigens expressed (in particular LMP-1 and LMP-2) should promote a proliferative and transformed phenotype.
- EBV-induced infectious mononucleosis in young adults significantly increases the risk for the subsequent development of EBV-positive HD.

The major open questions at this stage concern the EBV-negative cases of HD. It has been speculated that these might result from a "hit-and-run" mechanism where, after an initial modification of the infected host cell due to genetic modifications, viral persistence may be no longer required. An alternative possibility would be the persistence of Epstein–Barr viral subgenomic fragments in those "EBV-negative" cases. There exists no evidence in either direction: ample data support the view that several of the EBV-negative HD patients had not been infected with EBV in their past

(Chapman et al., 2001; Gallagher et al., 2003). Similarly, the search for integrated EBV-fragments has been unsuccessful in EBV-negative HD materials (Staratscheck-Jox et al., 2000). Occasionally, cured Hodgkin's lymphoma patients develop EBV-induced infectious mononucleosis years after their successful treatment (H. zur Hausen, unpublished results). In addition, late first exposure and infectious mononucleosis increase the risk for EBV-positive HD cases (Alexander et al., 2003), but not for the EBV-negative form of the disease. Even the age distribution between EBV-positive and EBV-negative patients seems to differ, with EBV-negative cases commonly occurring at a greater age (Jarrett, 2002).

These considerations lead to the conclusion of a different etiology of the EBV-negative HD cases. It remains at present an open question whether a hitherto unknown infectious agent is involved, or whether merely genetic modifications suffice under certain circumstances to produce the clinical picture of HD. Thus far, the search for infectious agents has been unsuccessful. The use of consensus primers for herpes-, polyoma-, and parvoviruses did not result in the identification of an unknown agent in cell lines derived from Hodgkin biopsies or in primary biopsy material (Gallagher et al., 2002; E.-M. de Villiers and H. zur Hausen, unpublished results). Recently, TT virus-like sequences were discovered in the EBV-negative Hodgkin lymphoma line L1236 (zur Hausen and de Villiers, 2005), though the significance of this observation remains to be established.

4.3.1.11 **EBV in Gastric and Esophageal Carcinomas**

Gastric cancer is commonly considered as a malignancy linked to *Helicobacter pylori* infections. In 1990, Burke et al. demonstrated EBV-DNA in one lymphoepithelioma-like gastric cancer. Shortly thereafter, in 1992, Shibata and Weiss identified the presence of EBV-DNA in 16% (22 out of 138) of gastric cancers. Subsequently, a number of other groups confirmed these findings, revealing on average approximately 10% of gastric cancers as EBV-positive (Tokunaga et al., 1993; Takada, 2000). Taking into consideration the high global rate of gastric cancers of approximately 870 000 new cases every year (Parkin, 2001), an estimated number of 80 000 EBV-associated cases probably points to this cancer as the most frequent EBV-associated malignancy (zur Hausen, 2004). Major geographic differences in EBV-positivity have been reported, with the highest rates in the United States and Germany (Ott et al., 1994; Takada, 2000). It seems that EBV-positive gastric adenocarcinomas represent a distinct clinicopathological entity with a low frequency of lymph node involvement (van Beek et al., 2004).

Esophageal cancer is rarely found to be associated with EBV infection. Moreover, the scarce data available from the literature do not permit an accurate estimation of the presence of EBV genomes in these cancers. Although present in the rare undifferentiated lymphoepithelioma-like tumors of the esophagus (Mori et al., 1994), EBV was also found to be associated with esophageal squamous cell carcinomas (Jenkins et al., 1996; Yanai et al., 2003). One report describes an unusually high number of EBV DNA in squamous cell carcinomas (35%) and in adenocarcinomas

(36%), although the authors did not differentiate between EBV presence within the tumor cells and in infiltrating lymphocytes (Awerkiew et al., 2003). Thus, although rare, EBV might be etiologically involved in a small percentage of esophageal cancers.

In contrast to some other EBV-linked malignant tumors (NPC and BL), which are endemic in south-east Asia and equatorial Africa, EBV-associated gastric cancers are non-endemic throughout the world (Takada, 2000). Histomorphologically, it is difficult to differentiate them from EBV-negative gastric cancers; usually, they are characterized by a high number of infiltrating CD8 cells (Kuzushima et al., 1999). They are commonly found in the proximal part of the stomach (Takada, 2000), and occur more frequently in men than in women (Tokunaga et al., 1993). Partial gastrectomy seems to increase the risk for EBV-associated gastric cancer (Yamamoto et al., 1994).

Since the pattern of terminal repeats within the EBV genome is constant (clonal) for individual gastric cancers, the tumors clearly arose from a single EBV-infected cell (Kida et al., 1993; Imai et al., 1994; Ott et al., 1994). The expression pattern of viral antigens in EBV-positive gastric cancers is somewhat intermediate between EBV latency type I found in BLs and latency pattern II of nasopharyngeal cancer: EBNA-1, BARF-0, BARF-1, and EBER 1/2 are consistently expressed (Sugiura et al., 1996; zur Hausen et al., 2000; Luo et al., 2005), whereas the latent membrane protein LMP-2B seems to be unexpressed. In some cases LMP-1 and LMP-2A have been recorded in several cells (Kida et al., 1993; Sugiura et al., 1996).

Patients with EBV-associated gastric cancers commonly reveal high antibody titers against VCAs and viral EAs. Approximately 60% of the patients also expressed IgA antibodies against VCAs, usually at lower levels than in nasopharyngeal cancer (Imai et al., 1994,). High antibody titers seem to precede the diagnosis of EBV-linked gastric cancer (Levine et al., 1995).

Based on epidemiological studies, *H. pylori* infections have been strongly linked to gastric cancer (Parsonnet et al., 1991). It is interesting to note that there seems to be no difference in *Helicobacter* infections between EBV-positive and EBV-negative cases of gastric cancer (Yanai et al., 1999; Torlakovic et al., 2004). Based on these results, it is presently not possible to postulate an interaction between these bacterial and EBV infections of the gastric mucosa, although at this stage it is also equally impossible to rule this out.

An EBV-positive gastric cancer cell line has been established with EBNA1 and LMP-2A expression, but negative for EBNA2 and LMP-1 (Oh et al., 2004). Another EBV-positive gastric carcinoma cell line has been kept by continuous transplantation in SCID mice (Iwasaki et al., 1998). These cells also retained the same expression pattern of EBV antigens as the original tumor. Two additional lines were established from non-cancerous tissue portions of two patients with gastric cancer (Tajima et al., 1998); these lines express EBNA-2 and LMP-1 and produce infectious EBV spontaneously. This virus is able to transform primary B cells and to induce EA in Raji cells. In an EBV-negative gastric cancer line, EBV infection induced the expression of insulin-like growth factor 1 (IGF-1) (Iwakiri et al., 2003), the induction depending on the EBV-encoded EBER-RNA. The result was an accelerated growth of

the infected cells. However, antibodies to IGF-1 blocked the growth stimulation, suggesting that the EBER-induced IGF-1 acts as an autocrine growth factor.

As observed in other virus-linked cancers, several modifications of host cell genes have been noted in EBV-linked gastric cancers: reduced expression and promoter methylation has been recorded in these carcinomas (Osawa et al., 2002). Promoter hypermethylation and aberrant expression was also reported for the E-cadherin promoter (Sudo et al., 2004). Methylation of the p16 as well as of the E-cadherin promoter was higher in EBV-positive than in EBV-negative cases of gastric carcinomas. Along with the p16 promoter methylation and a p16 protein loss in 90% of EBV-positive cancers, a similar percentage of tumors revealed methylation of the CDKN2A promoter, whereas EBV-negative tumors showed this methylation pattern only in 32% of cases (Vo et al., 2002). Additional observations revealed the amplification and overexpression of the c-met gene in EBV-linked gastric cancers, corresponding however to similar observations in EBV-negative gastric tumors (Kijima et al., 2002). Furthermore, up-regulation of the truncated basic hair keratin 1 (hHb1-DeltaN) has been noted in EBV-positive gastric carcinoma cells (Nishikawa et al., 2003), suggesting an interference of this unstable protein with functions of the keratin cytoskeleton and/or interference with transcriptional regulation. It may also point to an inhibition of differentiation driving the growth of NPC cells.

Distinct chromosomal aberrations were also reported in EBV-carrying gastric carcinomas. Copy number gains were recorded for chromosome 11 (Chan et al., 2001), losses of 11p and 4p were selectively found in EBV-positive tumors, while gains of 13q, 3q, and loss of 1q were solely observed in EBV-negative gastric tumors (zur Hausen et al., 2001).

Based on the published data, it is difficult to assess firmly the role of EBV in gastric cancer. Monoclonality of the infecting virus and the pattern of latent antigen expression, corresponding to other EBV-linked tumor systems, favor an etiological relationship. Several observations suggest that EBV infection occurs in the dysplastic stage and not in the normal mucosa (reviewed by Takada, 2000). According to A. zur Hausen et al. (2004), the absence of EBER 1/2 transcripts in the presumed preneoplastic gastric lesions suggests a very late infection. It is possible that EBV contributes a "malignizing" factor for pretransformed cells. This is shown in EBV-negative Akata BL cells which regain malignant growth properties after reintroduction of the EBV genome (Shimizu et al., 1994). In other EBV-positive BL lines the requirement for EBNA-1 expression for cell survival was demonstrated (Kennedy et al., 2003). The other aspect which requires further study is the possible interaction of EBV infections with pre-existing H. pylori manifestations.

4.3.1.12 **EBV in NK/T-Cell Lymphomas**

EBV is also found in a small percentage of T-cell and NK-cell lymphomas (Jones et al., 1988). Specifically, an extranodal angiocentric T-cell lymphoma, which is relatively common in south-east Asia, turns out to be EBV-positive (Harabuchi et al., 1990; Su et al., 1991; Zhou et al., 1994). The tumors are monoclonal and of CD4+ or

CD8+ T-cell origin. They arise after an acute primary infection or after chronic active EBV infection as a hemophagocytic syndrome, and commonly express the EBV latency pattern II (Kanegane et al., 2002; Young and Rickinson, 2004). Another EBV-linked lymphoma, again relatively common in south-east Asia, is represented by the lethal midline granuloma, which starts as an erosive lesion in the nasal cavity (Kanavaros et al., 1993; van Gorp et al., 1994). It is, however, presently included in the category of NK/T-cell lymphomas. A recent publication points to the growth promotion of T cells by EBV due to the induction of IL-9 by EBV-EBER RNA (Yang et al., 2004). Another observation by Takahara et al. (submitted for publication) that induction of LMP-1 expression in EBV-positive, but LMP-1-negative NK/T lymphoma lines, up-regulates CD25 which represents the IL-2 receptor. This may confer a growth advantage on EBV-carrying cells, as their proliferation depends on IL-2. LMP-1 induction was mediated by IL-4 and IL-10 in an EBNA-2 independent manner (Kis et al., 2006 a,b). There are at present no reports available which could hint at any specific properties of EBV strains found in these malignancies.

4.3.1.13 **EBV and Other Human Cancers**

Epstein–Barr virus has also been found in the rare leiomyosarcomas in AIDS patients, although detailed studies on its role in this malignancy are still not available (McClain et al., 1995; van Gelder et al., 1995; Timmons et al., 1995). Reports on a role for EBV in human breast and liver cancer (Labrecque et al., 1995; Bonnet et al., 1999; Sugawara et al., 1999) remain presently unconfirmed, and are also controversial (Herrmann and Nietobitek, 2003; Speck et al., 2003). Occasional replicating foci of EBV even in normal breast epithelium have been recently demonstrated (Huang et al., 2003), but do not lend support to a role for EBV in this malignancy.

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4.4 Rhadinoviruses

4.4.1 Human Herpesvirus Type 8 (HHV-8, Kaposi's sarcoma-associated herpesvirus)

4.4.1.1 Historical Background

In 1872, a Hungarian dermatologist, Moriz Kaposi, working at that time in Vienna, described a new tumor type of the skin that he characterized as an "idiopathic multiple pigmented sarcoma of the skin". He observed these rare tumors on the legs and arms of elderly men, particularly among inhabitants of the Mediterranean region, of Eastern Europe, and in males of Jewish descent. Subsequently this tumor, which was designated as Kaposi's sarcoma (KS), remained rare in Europe for more than 100 years. Besides this sporadic and "classic" form of KS, an endemic form of the tumor was detected in equatorial Africa (reviewed in Hutt, 1983). In addition, in the years between 1969 and 1973 it was noted that organ transplant recipients were at increased risk of developing this tumor (Siegel et al., 1969; Penn and Starzl, 1970; Fahey, 1971; Haim et al., 1972; Birkeland and Kemp, 1973) (Fig. 4.12).

In 1981, highly aggressive forms of KS were identified in homosexual men who had previously acquired the newly identified AIDS infection (Borkovic and Schwartz, 1981; Gottlieb et al., 1981; Hymes et al., 1981). Subsequently, it was shown that close to 30% of HIV-1-positive homosexual men developed AIDS-associated KS (Beral, 1991). This and another study suggested that KS should have an infectious

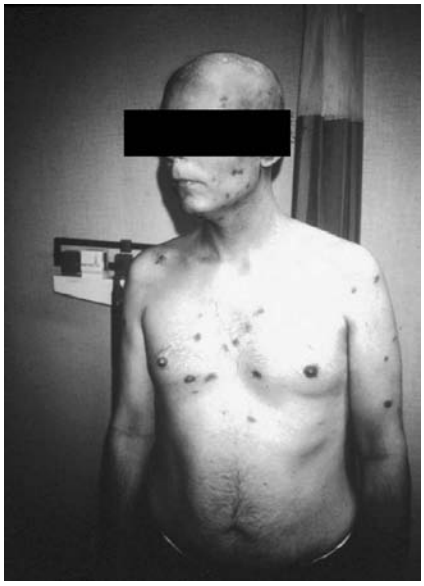

Fig. 4.12 Kaposi's sarcoma. (Source: Centers of Disease Control and Prevention (CDC), Atlanta, and courtesy of D.P. Drotman and H. Haverkos.)

etiology activated under immunosuppression (Weiss and Biggar, 1986). A DNA fragment of the putative responsible agent was identified in 1994 by Chang and Moore and their colleagues, and this resulted in the identification of a new human herpesvirus, human herpesvirus type 8 (HHV-8) or Kaposi sarcoma-associated human herpesvirus (KSHV) (Russo et al., 1996; Neipel et al., 1997). The availability of viral DNA sequences permitted the detection of HHV-8 in two additional lymphoproliferative conditions, primary effusion lymphoma (PEL) (Cesarman et al., 1995, 1996), and in a variable percentage in multicentric Castleman disease (MCD) (Soulier et al., 1995; Corbellino et al., 1996). During the past 10 years the epidemiology and the molecular biology of this interesting virus infection have been intensively studied.

4.4.1.2 **Epidemiology and Mode of Transmission**

The epidemiology of KS has been described in detail in a recent review (Dourmishev et al., 2003), wherein differentiation was made between the classic or sporadic form, the African endemic, the AIDS-epidemic, and the iatrogenic (immunosuppression of allograft recipients) forms of the lesion. Although all of these forms seem to differ in their histology, aggressiveness, morbidity and mortality, they are all linked with HHV-8 infections. This infection emerges as a prerequisite for the development of KS.

The classic form has been more often observed in elderly men in Mediterranean regions in Italy, Greece, Turkey, and Israel (Schwartz, 1996; Iscovich et al., 2000). In Italy, the seroprevalence of antibodies against HHV-8 differs substantially from the

north to the south. In northern Italy, 7.3% of blood donors are HHV-8-positive, in central Italy 9.5%, and in the south 24.6% (Whitby et al., 1998). Kaposi's sarcoma develops in the Mediterranean in only 0.03% of HHV-8-infected men above the age of 50 years (Vitale et al., 2001), and this suggests the involvement of additional cofactors in tumor development. It has been proposed that a combination of mild immunosuppression (lower lymphocyte and CD4 cell numbers) and of immune activation (increased serum neopterin and β_2-microglobulin levels) may account for the sporadic KS cases (Touloumi et al., 1999).

Since family members and heterosexual partners of sporadic KS patients reveal increased HHV-8 seroprevalence (Angeloni et al., 1998; Brambilla et al., 2000; Plancoulaine et al., 2004), transmission of the virus probably occurs by sexual as well as by nonsexual contacts. Tonsillar swabs from KS patients are frequently HHV-8-positive, suggesting that saliva contact, similar to EBV transmission (Cattani et al., 1999), and possibly also breast milk (Dedicoat et al., 2004), are the most important routes for HHV-8 transmission in sporadic KS cases. In endemic populations from Central Africa, the most likely route of HHV-8 transmission occurs from mother to child and between siblings (Plancoulaine et al., 2004). Thus far, no evidence has been reported for vertical transmission of HHV-8 during pregnancy (Sarmati et al., 2004), although vertical transmission has been suggested for African HHV-8 infections (Dupin and Calvez, 2000).

Today, the African endemic form of KS is difficult to study in view of the high prevalence of HIV infections in virtually all of those countries in which KS was previously endemic. These were mainly the north-eastern regions of the Congo (Zaire), western Uganda and Tanzania, although KS covered a broad band of equatorial Africa (Dourmishev et al., 2003). In the pre-AIDS period from 1954 to 1960, KS amounted to 6.2–6.6% of male cancer patients in Uganda without female cases; about 30 years later, the prevalence in males rose to 48.6%, and in females to 17.9% (Wabinga et al., 1993). Today, two age groups are mainly affected by KS: adults at an average age of 35 years, and children with an average age of 3 years. In Cameroon, approximately 4 % of all childhood cancers are due to KS (Kasolo et al., 1997). Besides oral and sexual modes of virus transmission, in certain African regions ritual skin piercing and tattooing may also contribute to transmission of the virus (Enbom et al., 2002).

HHV-8 infects a number of target cells (B cells, macrophages, endothelial cells and keratinocytes) *in vivo* via cell surface heparan sulfate, and uses $\alpha_3\beta_1$ integrin as one of its entry receptors (Akula et al., 2002; Naranatt et al., 2003). The infection of endothelial cells results in a semipermissive response: the cells are converted into spindle shape, part of them produce virus, resulting in cell death, while surviving cells enter the stage of viral latency (Gao et al., 2003). In addition, HHV-8, similar to other members of the herpesvirus group, induces chromosomal instability in primary human endothelial cells (Pan et al., 2004), and is also able to transform these cells (Flore et al., 1998). Early in the course of infection HHV-8 induces acetylation of microtubules and the activation of *RhoA* and *Rac* GTPases (Naranatt et al., 2005). The activation of Rho GTPases significantly enhances the nuclear delivery of viral DNA.

AIDS-associated KS is strikingly linked to the epidemiology of HIV infections. Dual infections by HHV-8 and HIV-1 are high in the AIDS epicenters, such as California and New York (Dourmishev et al., 2003). During the early 1980s, the incidence of AIDS-associated KS in homosexual men in San Francisco was about 40% (Beral et al., 1990), but by the mid-1900 – due to the introduction of effective anti-AIDS therapy – it had declined to 15–25% (Whitby et al., 1995; Moore et al., 1996a; Gao et al., 1996; Kedes et al., 1996). In Thailand, where there is a low incidence of KS but a high rate of HIV infections, only 2–12% of homosexual men were reported HHV-8-positive in 2002 (Ayuthaya et al., 2002). It is interesting to note that the introduction of highly active antiretroviral therapy (HAART) resulted, in the United States, to an 8.8% annual decline in KS incidence (Jones et al., 2000). Clearly, the immune restoration occurring due to this therapy plays the most important role in this respect; typically, a long period of treatment (> 24 months) results in undetectable HHV-8 viremia (Bourboulia et al., 2004). Since HIV-specific protease inhibitors (e.g., indinavir and sequenivir) are also anti-angiogenic (Sgadari et al., 2002), this may, in addition, contribute to the effect of HAART therapy in preventing KS.

Sexual transmission is clearly the most prominent route of HHV-8 infection in HIV-infected populations (Martin et al., 1998). The risk for KS development is calculated to be more than 10 000-fold higher in HIV-infected homosexuals than in the general population (Goedert, 2000). Correspondingly, hemophiliacs and injection drug addicts have a markedly reduced HHV-8 infection rate and a low KS risk (Beral et al., 1990; Atkinson et al., 2004). Orogenital sex seems to represent a particularly high-risk factor (Dukers et al., 2000).

Immunosuppressive therapy in organ allograft recipients in HHV-8-positive patients may lead to the iatrogenic form of KS. This occurs in about 0.4% of transplant patients in the United States and Western Europe (Farge et al., 1993; Penn, 1997), in contrast to 4.0–5.3% of renal transplant patients in Saudi-Arabia (Qunibi et al., 1988; Penn, 1993). The type of immunosuppressive therapy plays an important role, in particular cyclosporine treatment results more frequently in KS development (Penn, 1993), possibly due to the reactivation of latent HHV-8 (Hudnall et al., 1999). Although the vast majority of these patients is HHV-8-seropositive before organ transplantation, seronegative patients may also become infected from the donated organ (Parravicini et al., 1997; Barozzi et al., 2003).

4.4.1.3 Pathogenesis: Other Diseases Associated with HHV-8 Infections

Besides Kaposi's sarcoma, primary effusion lymphoma (PEL) is a rare HHV-8-associated cancer. This lesion constitutes only 0.13% of all AIDS-associated lymphomas, but AIDS patients who previously acquired KS are at increased risk for this lymphoma (Mbulaiteye et al., 2002).

Another partially HHV-8 associated rare angiolymphoproliferative disorder is that of multicentric Castleman disease (MCD) (Soulier et al., 1995; Corbellino et al., 1996). Among AIDS patients with MCD, more than 90% are positive for HHV-8, whereas among HIV-seronegative MCD patients only 40% are HHV-8-infected

(Grandadam et al., 1997). Lytic infection is more frequently detectable in MCD lesions, which suggests that the viral gene expression program differs here from that of KS and PEL (Teruya-Feldstein et al., 1998; Katano et al., 2000; Oksenhendler et al., 2000; Parravicini et al., 2000). A viral involvement in the HHV-8-negative MCD proliferations has not yet been identified.

Several other diseases were suspected of being linked to HHV-8 infection (for a review, see Dourmishev et al., 2003), but as yet no reproducible results have been recorded.

4.4.1.4 **Viral Genes Expressed in Viral Latency**

Detailed reports of viral genes and genome functions have been provided in three excellent recent reviews by Dormishev et al. (2003), Moore and Chang (2003), and Verma and Robertson (2003). Consequently, this chapter will only detail those genes and their functions that are engaged in viral latency and tumorigenesis, and which permit escape of the virus from host cell immune functions.

One gene that is highly expressed in all HHV-8-associated malignancies and all infected cells is the latency-associated nuclear protein *LANA* (Kedes et al., 1997; Dupin et al., 1999). This gene is located in ORF 73 and is expressed as a polycistronic mRNA jointly with viral cyclin, originating from ORF 72, and viral Fas-associated death domain IL-1B converting enzyme inhibitory protein encoded by ORF 71 (Sarid et al., 1999). LANA represents a 222 to 232-kDa nuclear protein, and is important for viral episome maintenance during latent infection. It tethers the viral episomes to host cell chromosomes, and is also bound to the terminal repeat elements of the viral genome (Ballestas et al., 1999; Cotter and Robertson, 1999). The binding site at the terminal repeat region covers the carboxy-terminal 200 amino acids of LANA (Ballestas and Kaye, 2001; Cotter et al., 2001), whereas the N-terminus of this protein plays an important role in binding to chromatin (Barbera et al., 2004; Wong et al., 2004). Binding to this region as oligomer (Komatsu et al., 2004) is sufficient to permit replication of terminal-repeat-containing plasmids (Fejer et al., 2003; Grundhoff and Ganem, 2003). Disruption of LANA leads to abortive episome persistence (Ye et al., 2004; Barbera et al., 2004). LANA also binds to histone H1 (Cotter and Robertson, 1999) and to the SUV39H1 histone methyltransferase (Sakakibara et al., 2004).

A large number of functions have recently been ascribed to LANA. This protein has a transcriptionally regulatory role and affects gene expression both positively and negatively. LANA regulates its own promoter positively via its C-terminal domain, and binds to a defined site within the core promoter (Jeong et al., 2004). LANA interacts with p53 and down-regulates p53-mediated activation of p53-responsive promoters, thus protecting against apoptosis (Friborg et al., 1999). The 223 amino acids of the LANA C-terminus are sufficient to inhibit p53-mediated activation of the human BAX promoter (Wong et al., 2004). LANA also associates with hypophosphorylated ("active") pRB *in vitro* and under conditions of transient transfections, thus transactivating E2F-responsive promoters. In combination with *H-ras* it

transforms primary rat cells and renders them tumorigenic in the nude mouse system (Radkov et al., 2000). It also prolongs the life span of human primary umbilical vein endothelial cells (Watanabe et al., 2003). A further interesting feature of LANA is the activation of the activator protein 1 (AP-1) response element and the induction of the binding of a *c-Jun-Fos* heterodimer, which in turn results in IL-6 expression (An et al., 2004). In another system, cervical cancer, *c-Jun-Fos* heterodimer formation seems to be one of the characteristics of the malignant phenotype (Soto et al., 2000). LANA also induces the inhibitor of the basic helix-loop-helix transcription factor Id-1 (Tang et al., 2003). The multifunctional activities of LANA are further underlined by the activation of the human telomerase transcriptase promoter (Knight et al., 2001) by directly interacting with the transcription factor SP1 (Verma et al., 2004). LANA interferes with the Wnt signaling pathway by interacting with the glycogen synthase kinase 3 (GSK-3). This overcomes the GSK-3 mediated inhibition of β-catenin and results in increased levels of β-catenin (Fujimuro and Hayward, 2003). Another cellular transcriptional repressor, KLIP1, repressing herpes simplex thymidine kinase promoter activity, interacts with LANA which alleviates its activity (Pan et al., 2003). It seems that LANA also plays a critical role in maintaining viral latency, as it suppresses the replication and transcription activator (*Rta*) of HHV-8, thus, controlling the switch between viral latency and lytic replication (Lan et al., 2004).

The multiple functions of LANA are summarized in Table 4.5. It is somewhat surprising that this protein combines various functions of other viral oncogenes, including the EBNAs, papilloma-, polyoma- and adenovirus oncogenes.

A second important gene product in latently infected cells is the *viral cyclin D* protein, coded for by ORF 72, revealing a 32% identity and 54% similarity to cellular cyclin D2 (Chang et al., 1996; Godden-Kent et al., 1997; Li et al., 1997a; Swanton et al., 1997). This protein forms functional complexes with the cellular cdk-6, phosphory-

Table 4.5 Interaction of the LANA protein with cellular proteins, and resulting consequences

Interacting gene product	Activation/repression	Consequence
p53	Inactivation	Inactivation fo p53-responsive promoters (e.g., BAX)
pRB	Inactivation	Activation of E2F and cdk2
AP-1	Co-activation	Formation of c-jun/Fos heterodimers
Id-1 (helix-loop-helix transcription factor)	Activation	
SP-1	Activation	Activation of telomerase reverse transcriptase
Glycogen kinase 3 GSK-3	Inactivation and altered distribution	β-catenin overexpression
HHV-8 replication and transcription activator Rta	Inactivation	Latency maintenance

lates RB1 and histone H1, and stimulates the G_1 to S transition. The complex v-cyclin/cdk-6 is resistant to the inhibitory functions of the cyclin-dependent kinase inhibitors p16, p21, and p27 and phosphorylates and down-regulates p27 (Swanton et al., 1997; Ellis et al., 1999; Mann et al., 1999; Card et al., 2000). Phosphorylation of Bcl-2 by this complex may lead to apoptosis. In latent infection this is probably counteracted by the inactivation of p53 by interaction with LANA (see above). Similar to LANA, v-cyclin is invariably expressed in biopsies from Kaposi's sarcomas and primary effusion lymphomas (Dittmer, 2003; Jarviluoma et al., 2004). v-Cyclin overrides growth-suppressive signals of the signal transducer and activator 3 (STAT3) by suppressing its activity. This prevents the growth-suppressive effect by the IL-6 type cytokine, oncostatin M (Lundquist et al., 2003). The targeted expression of v-cyclin to B- and T-lymphocyte compartments results in lymphoma development by nine months of age (Verschuren et al., 2004), and all lymphomas had lost p53 expression. This suggests a role for p53 in interacting with v-cyclin.

A third protein originating from ORF 74 clearly represents a viral oncoprotein and possesses homology to the cellular G-protein-coupled receptor (GPCR) (Swanton et al., 1997). It is relatively closely related to the IL-8 receptors CXCR1 and CXCR2, and activates the phosphoinositide pathway. Although the protein appears to be difficult to detect in latently infected tumor cells, the respective RNA is constitutively expressed. Upon transfection, vGPCR transforms rat fibroblasts (Arvanitakis et al., 1997) and NIH 3 T3 cells (Bais et al., 1998). It also has been shown to immortalize human endothelial cells by activating the VEGF receptor-2/KDR (Bais et al., 2003) and to induce angioproliferative tumors in transgenic mice that strikingly resemble human KS (Montaner et al., 2003). These data point to a central role for vGPCR as one of the major pathogenic determinants of KS. cGPCR has been considered as a gene expressed in lytic infection, yet, it has been proposed that its dysregulated expression by HIV-1 Tat, inflammation, or aborted cell cycle progression may trigger its expression and the up-regulation of oncogenic signaling pathways (Sodhi et al., 2004). The demonstration of lytic replication-defective HHV-8 genomes in Kaposi's tumors and PEL cell lines may lend some support for this speculation (Deng et al., 2004). The mode by which vGPCR may contribute to the neoplastic growth was analyzed by Pati et al. (2003) and Montaner et al. (2004). According to these and other authors, vGPCR up-regulates the expression and secretion of several cytokines by stimulating NF-κB, AP-1, and nuclear factor of activated T cells (NF-AT) by the activation of the small G protein Rac1 and its effector, the p21-activated kinase 1 (Pak1) (Dadke et al., 2003). Inhibition of the latter blocked vGPCR-induced transcription and the secretion of cytokines, including IL-6 and IL-8 and the growth-regulated oncogene α (GROα). The constitutive activation of NK-κB and the induction of proinflammatory and angiogenetic factors are consistent with the inflammatory hyperproliferative nature of Kaposi lesions (Pati et al., 2003). NF-AT activation depended on signaling through the phosphatidylinositol 3-kinase-Akt-glycogen synthetase kinase 3 (P13-K/Akt/GSK-3) pathway. Interestingly, NF-AT and NF-κB activation by vGPCR was greatly increased by the HIV-1 Tat protein, whereas Tat alone had little effect. Thus, there seems to exist a collaborative stimulation by vGPCR and Tat.

There exists another latency-expressed protein originating from an adjacent genomic region, ORF 71 (also labeled K13), the v-FLICE-inhibitory protein, v-FLIP. This possesses two death effector domains, and functions as a dominant-negative inhibitor of Fas-receptor-mediated apoptosis by binding to Fas-associated death domain protein and caspase 8 (FLICE) (Belanger et al., 2001). v-FLIP is constitutively expressed. Upon transduction it enhances tumorigenicity of mouse B lymphoma cells in immunocompetent mice (Djerbi et al., 1999) and transforms Rat-1 and Balb/3 T3 fibroblast cells to soft agar growth and tumor formation in nude mice (Sun et al., 2003). v-FLIP activates NF-κB by activating the IκB kinase γ (Chaudhary et al., 1999; Kataoka et al., 2000; Liu et al., 2002; Field et al., 2003). Specifically, the upregulation of p100/NF-κB2 expression and its subsequent procession into the p52 subunit are effected by v-Flip (Matta and Chaudhary, 2004). Silencing of ORF 71 expression by siRNA inhibits the processing of p100 and blocks cell proliferation. v-FLIP is largely responsible for NF-κB activation in latently infected PEL cells, and its elimination results in decreased NF-κB activity, induction of apoptosis, and increased sensitivity to external apoptotic stimuli (Guasparri et al., 2004). It has also been documented that v-FLIP activates the JNK/AP-1 pathway in a TRAF-dependent fashion (An et al., 2003). This results in cellular IL-6 expression, the promoter of which becomes active after NF-κB and AP-1 activation.

Kaposin (K12) represents a small hydrophobic protein of 60 amino acids. It has also been reported to represent a transforming protein after transfection of its gene into Rat-3 tissue culture cells, which then form tumors after inoculation into nude mice (Kliche et al., 2001; Muralidhar et al., 1998, 2000). The K12 locus is complex, it encodes many potential ORFs, the relative importance of which is unclear (Li et al., 2002). Its complex transcripts are abundantly present in KS cells and in PEL cell lines (Zhong et al., 1996; Staskus et al., 1997; Sturzl et al., 1997). Kaposin reorganizes cellular actin (Kliche et al., 2001) and associates with cytohesin-1, a guanine nucleotide exchange factor, regulating integrin activity. Three different Kaposins – A, B, and C – have been identified in PEL cell lines. Induction of the lytic cycle by phorbol ester TPA treatment of HHV-8-positive cells increases the transcription substantially (Li et al., 2002).

The HHV-8 genome contains four tandemly arranged genes coding for viral interferon regulatory factors (vIRF1–4). During the lytic cycle vIRFs 1, 2, and 4 are induced, whereas vIRF-3 is not inducible and constitutively present at low abundancy in PEL cells (Fakhari and Dittmer, 2002; Cunningham et al., 2003; Dittmer, 2003). The protein is also designated as LANA-2. It inhibits p53-mediated transactivation and PKR-triggered apoptosis, and abrogates activation of caspase 3 (Esteban et al., 2003). It seems to be latently expressed exclusively in B cells (Rivas et al., 2001). vIRF3 stimulates the transcriptional activity of cellular IRF-3 and -7 by directly interacting with IRF-3 and -7 through its C-terminal region (Lubyova et al., 2004). It also binds to the transcriptional co-activator CBP/p300. It is recruited to the interferon α-promoters by IRF-3 and -7 and activates genes controlled by these proteins.

Besides vIRF-3, which is not inducible after TPA treatment, the inducible vIRF-1 is transcribed at a low rate during viral latency (Cunningham et al., 2003). The function of vIRF-1 differs from that of vIRF-3, as it blocks the interaction between IRF-3

and p300 and inhibits histone acetylation (Lubyova et al., 2004). Similar to the other two vIRFs (vIRF-2 and -4), there is at this stage no recognizable direct role of vIRF-1 for the viral state of latency, or for events linked to cell transformation.

The last latency-associated protein which apparently plays an important role in cell transformation originates from a family of alternatively sliced transcripts of approximately 7.5 kb, translated with hypervariable protein epitopes (Stebbing et al., 2003). The coding gene (K1) is located between ORF 75 and the terminal repeats at the right end of the HHV-8 genome (Glenn et al., 1999). The gene product represents a latent membrane protein (LAMP). The K1 gene consists of eight alternatively spliced exons and is supposed to encode a 45-kDa transmembrane protein with 12 predicted transmembrane regions (Brinkmann et al., 2003). The C-terminal cytoplasmic domain contains binding sites for SH2 and SH3 domains, and binds in addition tumor necrosis factor receptor-associated proteins. In its organization it shows a remarkable similarity to EBV LMP-1 and LMP-1A. It activates the Ras/mitogen-activated protein kinase (MAPK) and NF-κB pathways. This requires phosphorylation of tyrosine residue 481 within one of the SH2-binding sites which is mediated by tyrosine kinases *Src*, *Lck*, *Yes*, *Hck*, and *Fyn* (Brinkmann et al., 2003). Additional experimental data point to a central role of K1-mediated constitutive *Lyn kinase* activation for the production of VEGF and NK-κB activation (Prakash et al., 2005). Smaller K1 isoforms of the K1 protein activate these pathways to a much lesser degree.

The transforming potential of the K1 gene was first reported by Lee et al. (1998). The expression of K1 in rodent fibroblasts resulted in morphologic changes and focus formation. In case of replacement of a gene encoding the herpesvirus saimiri-transforming protein (which shows no structural relationship to K1) by K1, this recombinant herpesvirus immortalizes primary T lymphocytes to IL-2-independent growth and induces lymphoma in cottontop marmosets. In B lymphocytes, K1 targets the phosphatidylinositol kinase pathway, activates the *Akt* kinase, and inhibits the phosphatase PTEN (Tomlinson and Damania, 2004). Activation of *Akt* results in phosphorylation and inhibition of members of the forkhead transcription factor family which regulate cell cycle progression and apoptosis. Thus, K1 promotes cell survival pathways and prevents cells from undergoing premature apoptosis. K1 probably represents the most important transforming protein of HHV type 8, although the pathogenic spectrum of HHV-8-induced tumors in all likelihood originates from the cooperation of various viral oncogenes.

Our current knowledge of genes expressed under conditions of viral latency is summarized in Table 4.6.

4.4.1.5 **Cellular Genes Regulating Viral Latency**

Activation of the replication and transcription factor (RTA) of γ-herpesviruses initiates the lytic cycle of HHV-8. This transactivator is preserved among all known γ-2-herpesviruses (Damiana et al., 2004). Its transcriptional activation is suppressed by cellular poly(ADP-ribose) polymerase 1 (PARP-1) and the Ste20-like kinase hKFC

Table 4.6 Transforming genes of HHV-8 an genes expressed in latent infection

Gene	Function	Transformation of
LANA	Episome maintenance, transcript. regulator	Transforms primary rat cells jointly with H-ras
v-cyclin D	Binds to cdk-6, phosphorylates Rb1 and histone H1	Lymphomas in transgenic mice after loss of p53 expression
v-FLIP (K13)	Blocks Fas-mediated apoptosis and caspase 8, activates NF-κB	Promotes tumor establishment due to evasion form NK cell killing
LAMP (K15)	Membrane protein, resembles EBV LMP1 and LMP 2 A. Activates Ras and NF-κB pathways	Transforms rodent fibroblasts; immortalizes T-lymphocytes; induces lymphomas in cotton-top marmosets
Kaposin (K12)	Reorganizes cellular actin, regulates integrin activity	Malignant transformation of rat-3 cells
v-IRF-3 (K10.5, LANA-2)	Inhibition of p53-mediated transactiv. stimulates activity of cell. IRF-3 + IRF-7	Constitutively expressed only in hematopoietic cells
G-protein-coupled receptor (GPCR)	Activates VEGF-R, NF- êB, AP-1, and NF-AT	Transforms rat fibroblasts and NIH-3 T3 cells

which interact with the serine/threonine region of γ-herpesvirus RTA (Gwack et al., 2003). The genetic ablation of PARP-1 and hKFC substantially enhances the replication of these viruses. Suppression of the RTA-promoter is also mediated in *in-vitro*-infected endothelial cells by the joint action of interferon-γ, tumor necrosis factor-α, IL-1β, and IL-6 (Milligan et al., 2004), whereas interferon-γ alone effectively induces HHV-8 in PEL cells (Blackbourn et al., 2000; Chang et al., 2000; Mercader et al., 2000).

The expression of the cellular protein *Raf* significantly enhances HHV-8 infection of target cells (Akula et al., 2004). The Raf-induced vascular endothelial growth factor (VEGF) is apparently responsible for this effect since the treatment of cells with a VEGF receptor inhibitor significantly reduced the infection (Ford et al., 2004). Thus, *Raf* seems to modulate the pathogenesis of Kaposi's sarcoma.

4.4.1.6 Interaction Between HIV and HHV-8

The frequency of HHV-8-associated KS development in patients infected by human immunodeficiency viruses (HIV) could suggest that, besides the induction of immunosuppression, HIV infection may specifically activate latent HHV-8 genomes. Indeed, several observations point in this direction. Conditioned media from HIV-1-infected T cells induce the lytic reactivation of HHV-8 in PEL cells (Mercader et al., 2000). Replication of HIV in PEL cells has the same effect (Varthakavi et al., 1999).

Specific HIV proteins (*Tat* and *Vpr*) can induce HHV-8 gene expression; conversely, the *Rta* protein of HHV-8 synergizes with *Tat* in activating the HIV long terminal repeat (Huang et al., 2001). Overexpression of Tat in transgenic mice induces KS-like lesions (Vogel et al., 1988); extracellular Tat is also able to support the growth and survival of KS cells (Ensoli et al., 1990; Barillari et al., 1993; Albini et al., 1995). Tat mediates an anti-apoptotic gene expression program, in part by direct interactions with VEGF receptor 2 and IGF receptor I (Deregibus et al., 2002). In addition, Tat enhances the infectivity of HHV-8. The full-length HIV Tat and a 13-amino acid peptide corresponding to the basic region of Tat enhance the entry of HHV-8 into endothelial and other cells (Aoki and Tosato, 2004). Correspondingly, the median HHV-8 viral load is substantially higher in Kaposi's sarcomas of HIV-1-positive patients than in HIV-negative KS patients (Chandra et al., 2003).

All of these data underline the existence of a remarkable synergism between two virus infections, belonging to two very different virus families.

4.4.1.7 Viral Homologues to Host Cell Genes and Evasion from the Host's Immune Mechanisms

HHV-8 is particularly effective in immune evasion strategies (Moore and Chang, 2003). These strategies involve the MIR proteins, v-FLIP, and viral chemokines. Established strategies and related proteins from other viruses are listed in Table 4.7.

Table 4.7 Genes of HHV-8 with immune modulatory functions. (Reproduced from Moore, 1996 b. With permission)

KSHV protein	KSHV gene	Features and functions	Related proteins from other viruses
vKCP	*ORF4*	Homologue of cellular complement control regulators; inhibits C3 deposition on cell surface	HVS-ORF4, HSV1-gC, Vaccinia-vCP/C21 L, Cowpox-IMP, Vaccinia-SPICE
KIS	*ORF K1*	Constitutively active membrane protein containing ITAM motif; interacts with mu chains of B-cell receptor complex to block surface transport; transforms rodent fibroblasts and primary T cells; activates Syk signaling pathway; variable ectodomain used for KSHV strain analysis	HVS-STP
MIR1/ MIR2	*ORF K3/K5*	Enhanced endocytosis of surface MHCI via ubiquitination; MIR2 also ubiquitinates and down-regulates ICAM-1 and B7.2	MHV 68-K3, CMV-US2
vCCL-2 (vMIP-II)	*ORF K4*	Binds to CCR3 and induces angiogenesis *in ovo*; induces eosinophil and Th2 chemotaxis; CCR8 and vGPCR antagonist	Molluscum-MC148

Table 4.7 (Continued)

KSHV protein	KSHV gene	Features and functions	Related proteins from other viruses
vCCL-3 (vMIP-III)	*ORF K4.1*	Agonist for CCR4 and induces Th2 chemotaxis; induces angiogenesis *in ovo*; protein expressed in KS lesions (by Western blot)	
vCCL-1 (vMIP-I)	*ORF K6*	Bids CCR5 and induces angiogenesis *in ovo*; CCR8 agonist; induces Th2 chemotaxis	Molluscum-MC148
vIRF-1	*ORF K9*	Inhibits IFN signaling; binds CBP/p300; transforms rodent fibroblasts; protein expressed in MCD and PEL cell lines, not detectable in KS or PEL	EBV-EBNA2, Adeno-E1 A, EBV-EBNA2, Adeno-E1 A, SV40-LT, HTLV-Tax
vFLIP	*ORF K13*	Homologue of cellular apoptosis inhibitor (FLIP); transcribed on major latency transcripts (LT)1 and LT2; post-transcriptionals regulation of protein expression *in vivo*	HSV-ORF71
ORF45	*ORF45*	Binds to and inhibits IRF7 nuclear translocation	Vaccinia-E3 L

The two transmembrane proteins MIR1 and MIR2 efficiently inhibit MHC 1 surface expression (Ishido et al., 2000; Stevenson et al., 2000). MHC I is removed from the plasma membrane by endocytosis, lysosomal targeting, and subsequent degradation of the MHC molecules (Coscoy and Ganem, 2000). In addition to MHC I, MIR2 also ubiquinates and down-regulates the accessory immune receptors ICAM-1 and B7.2 (Ishido et al., 2000; Coscoy and Ganem, 2001). MHC I-depleted HHV-8-infected cells escape from the resulting risk of being attacked by NK killer cells (Natarajan et al., 2002) by presenting virus-encoded MHC-like molecules at the cell surface (Tortorella et al., 2000). This is mediated by the expression of v-FLICE-inhibitory protein (v-FLIP) (Thome et al., 1997), which possesses two death effector domains and acts as a dominant-negative inhibitor of *Fas*-induced apoptosis and of caspase 8 (FLICE) (Belanger et al., 2001). Under these conditions, caspase 8 can no longer be recruited into the death-signaling process (Krueger et al., 2001). v-FLIP constitutively activates the NF-κB pathway by directly interacting with the IκB kinase (Liu et al., 2002).

HHV-8 effectively inhibits cell-mediated immune responses through the secretion of virus-encoded chemoattractant proteins (chemokines). The virus codes for three secreted chemokines, v-CCL1 (ORF K6), v-CCL2 (ORF K4), and v-CCL3 (ORF K4.1). All of these differ in their receptor specificities and possess broad antagonistic activities for chemokine receptors and block the chemotaxis for Th1 and NK lymphocytes (Boshoff et al., 1997; Chen et al., 1998; Dairaghi et al., 1999; Endres et al., 1999). v-CCL1 and v-CCL2 are only expressed in lytic infections, while v-CCL3 is also found in KS, stimulates angiogenesis, and may thus play a role in the pathogenesis

(Stine et al., 2000). v-CCL2 signals play a pivotal role in directing antiviral effector cells toward virus-infected organs, and potently inhibit type 1 T-cell-mediated inflammation (Lindow et al., 2003).

A number of additional HHV-8 proteins permit the evasion from innate immunity: the mechanisms involve complement binding, down-regulation of the B-cell receptor, and inhibition of interferon initiation and signaling (Moore and Chang, 2003). A complement control protein (KCP) has homology to human complement regulators (Russo et al., 1996). This blocks human complement-mediated lysis of erythrocytes and serves as a cofactor for factor I-mediated inactivation of complement proteins C3 b and C4 b (Mullick et al., 2003). This protein originates from ORF4 and occurs in three isoforms (Spiller et al., 2003).

The B-cell receptor (BCR) interacts with the complement-binding proteins CD19 and CD21 and regulates the development and functions of B cells. ORF K1 of HHV-8 encodes a small transmembrane glycoprotein, *KIS* (K ITAM-signaling), which possesses immunoglobulin-like properties. Its overexpression causes the transformation of rat fibroblasts, in the context of KIS substitution for the STP oncogene in recombinant herpesvirus saimiri it immortalizes T lymphocytes and induces lymphomas in marmosets (Lee et al., 1998). By interacting with the μ-chain, KIS blocks the intracellular transport of BCR complexes to the cell surface (Lee et al., 2000).

A number of viral proteins act as antagonists of cellular interferons. The secretion of v-interleukin 6 (v-IL-6) results in the activation of STAT1 and STAT3 phosphorylation, the MAP kinase and other serine/threonine kinases (Osborne et al., 1999). N-linked glycosylation at N78 and N89 is required for optimal function of v-IL-6, but not for cellular IL-6 (Dela Cruz et al., 2004). Similar to human IL-6, v-IL-6 maintains the B-cell proliferation in IL-6-dependent murine and human cell lines (Moore et al., 1996 b; Burger et al., 1998). The growth of lymphoma cells, carrying HHV-8 DNA, depends on the autocrine stimulation by v-IL-6 (Chatterjee et al., 2002). v-IL-6 is directly activated by interferon-α, but blocks intracellular interferon signaling. Whereas interferon-α down-regulates the IL-6 receptor, gp 80, v-IL-6 bypasses gp80 and binds directly to the gp130 transducer molecule. v-IL-6 has developed a unique molecular strategy to interact with gp130 with an almost entirely divergent structure of its receptor binding sites (Boulanger et al., 2004). Thus, v-IL-6 prevents the induction of an antiviral state by interferon and sustains the proliferation of HHV-8-positive B-lymphoma cells.

Another HHV-8-encoded protein, v-interferon-responsive factor 1 (v-IRF1), prevents the recruitment of p300 and the CREB-binding protein (CBP) histone acetyltransferase coactivators into the interferon transcriptional complex by cellular IRF3 (Weaver et al., 1998; Li et al., 2000). v-IRF1 acts as transactivator for v-IL-6, but its effect on histone acetylation represses a number of other genes (Li et al., 1997 b; Roan et al., 1999; Li et al., 2000). Expression of v-IRF1 in NIH 3 T3 cells causes full transformation of these cells (Gao et al., 1997).

Besides the inhibition of p53-mediated apoptosis by LANA-1 and LANA-2 (v-IRF-3), v-IRF1 also blocks this p53 effect (Nakamura et al., 2001; Seo et al., 2001). HHV-8 has developed an additional mechanism to interfere with cellular apoptotic signals: it codes for a viral BCL-2-like protein, v-BCL2, which escapes the normally

operating caspase-mediated conversion of its cellular homologue BCL-2 to pro-apoptotic proteins (Bellows et al., 2000). It blocks apoptosis which would be effected by the overexpression of latent v-cyclin/CDK6 complexes. The latter complex inactivates cellular BCL-2 by phosphorylation (Ojala et al., 2000). A further anti-apoptotic factor which acts at the mitochondrial membrane is v-IAP; this is structurally similar to cellular survivin (Feng et al., 2002; Wang et al., 2002). v-IAP stabilizes mitochondrial Ca^{2+} flux under conditions of cellular stress, and blocks apoptosis induced by a variety of different agents (Feng et al., 2002).

HHV-8 emerges as an extremely well-adapted virus in the human host. A larger number of HHV-8-encoded proteins reveal a remarkable molecular mimicry with host cell proteins, commonly interfering with the function of the latter. This permits an efficient evasion from host immune surveillance, as well as from intracellular control mechanisms.

4.4.1.8 **HHV-8-Related Herpesviruses in Nonhuman Primates**

Rhadinoviruses are found in Old and New World monkeys. A new rhadinovirus was reported in 1997 in Old World rhesus monkeys (Desrosiers et al., 1997). Cynomolgus and pig-tailed macaques contain closely related, but species-specific viral sequences (Strand et al., 2000). In addition, two distinct γ-2 herpesviruses were found in African green monkeys (Greensill et al., 2000). B lymphocytes emerge as the major site of viral persistence (Bergquam et al., 1999). In simian immunodeficiency virus (SIV)-infected monkeys only specific strains of rhesus rhadinovirus seem to induce multicentric lymphoproliferative disorders (Wong et al., 1999), whereas others apparently do not contribute to lymphoma development (Ruff et al., 2003).

A novel HHV-8 homologue (PapRV2) was reported in captive baboon species (*Papio anubis* and others) (Whitby et al., 2003). The viral DNA has substantial sequence identity to two HHV-8 genes, viral polymerase, and thymidilate synthase. A colony of almost 200 animals revealed cross-reacting antibodies recognizing HHV-8 or macaque rhadinovirus antigens.

Replication of HHV-8 after infection of rhesus macaques has been reported (Renne et al., 2004), though even rhesus monkeys positive for SIV failed to develop Kaposi-like sarcomas or lymphoproliferative disease.

Among New World monkeys two members of the rhadinovirus family – herpesvirus saimiri and herpesvirus ateles – rapidly produce leukemias and lymphomas in some non-natural hosts (Melendez et al., 1972). Leukemias arise in cotton-top marmosets almost like an acute infection upon inoculation of one of these two virus types. Although an initial report claimed propagation of herpesvirus saimiri in human cells (Ablashi et al., 1971), subsequent studies indicated that in spite of synthesis of early genes most of these infections are abortive (Daniel et al., 1975; Dahlberg et al., 1988). Herpesvirus saimiri may, however, persist in various human hematopoietic and epithelial cell lines (Grassmann and Fleckenstein, 1989; Simmer et al., 1991), and strains of the herpesvirus saimiri subgroup C are even able to stably transform human T lymphocytes (Biesinger et al., 1992). The same strains also immortalize Old World monkey T lymphocytes (Akari et al., 1996).

4.4.2
Marek's Disease of Chickens

Marek's disease was first described by Marek (1907) as a polyneuritis of chicken. Subsequently, it was characterized as a neurolymphomatosis and fowl paralysis, but the causative agent, a herpesvirus, was not discovered until 1967 (Churchill and Biggs, 1967). Viral DNA codes for the RNA subunit of telomerase (Fragnet et al., 2003). The occupational infection of humans with this agent has been suspected to occur, based on the presence of antibodies to Marek's virus in human sera (Choudat et al., 1996; Laurent et al., 2001); however, Marek's disease DNA has been detected only in chicken, and not in human plasma (Hennig et al., 2003). It remains to be seen whether the reported serological reactivity may originate from a related, presently unknown herpesvirus infection. Marek's virus, like several other herpesviruses, has also been reported to transactivate the terminal repeat promoter of the Rous sarcoma retrovirus (Tieber et al., 1990)

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5
Papillomavirus Infections: A Major Cause of Human Cancers

5.1
Introduction

This was the heading of a review which appeared during the mid-1990s (zur Hausen, 1996) and, indeed, papillomaviruses have emerged today as one of the most important group of infectious carcinogens.

As outlined in Chapter 1, the infectious nature of warts became evident around the turn of the twentieth century. The first link of papillomavirus infection to cancer originated from studies on cancer induction by the cottontail rabbit papillomavirus in domestic rabbits and the subsequent elegant studies performed by Peyton Rous and his associates on synergistic effects between these infectious carcinogens and chemical factors (Rous and Beard, 1934; Rous and Kidd, 1938; Rous and Friedewald, 1944). The molecular analysis of papillomaviruses had a slow start. The viruses were first visualized electronmicroscopically in 1949 (Strauss et al.), and their circular double-stranded DNA genome was demonstrated in 1963 (Crawford and Crawford, 1963). Yet, the unavailability of a tissue culture system for viral replication hampered the progress for several additional years.

First evidence for biological functions of a member of this virus group originated from studies with bovine papillomaviruses (BPV). This virus was able to induce urinary bladder tumors in cattle (Olson et al., 1959), was found to be tumorigenic after inoculation into newborn hamsters (Friedmann et al., 1963; Boiron et al., 1964), and transformed calf and murine cells in tissue culture (Black et al., 1963; Thomas et al., 1963). In humans, a detailed analysis of potentially oncogenic papillomaviruses began during the early 1970s, with a publication by Stefania Jablonska in Warsaw (Jablonska et al., 1972), who considered a rare hereditary papillomatosis, *epidermodysplasia verruciformis*, "…as model in studies of papillomaviruses in oncogenesis".

Shortly thereafter it was postulated that papillomaviruses may play a major role in the induction of carcinomas of the cervix (zur Hausen et al., 1974a,b, 1975; zur Hausen, 1975, 1976, 1977). At about the same time, the heterogeneity of human papillomaviruses (HPV) became evident (Gissmann and zur Hausen, 1976; Gissmann et al., 1977; Orth et al., 1977), and a specific HPV type was frequently discovered in skin cancers of epidermodysplasia patients (Orth et al., 1978, 1979). The isolation and identification of specific HPV types in genital warts and laryngeal

papillomas (Gissmann and zur Hausen, 1980; de Villiers et al., 1981; Gissmann et al., 1982) and directly from cervical cancer (HPV 16 and HPV 18) (Dürst et al., 1983; Boshart et al., 1984) and from premalignant genital lesions (Ikenberg et al., 1983) resulted subsequently in an explosion of studies on the role of HPV in anogenital cancers.

Although a number of subsequent experimental studies supported a causal role of "high risk" HPV infections (zur Hausen, 1986 a) in cervical cancer, it took almost a decade, before epidemiologic studies came to the same conclusion (Muñoz et al., 1992; Schiffman et al., 1993; Bosch et al., 1995; Matsukura and Sugase, 1995).

Today, mechanistic aspects of HPV-induced carcinogenesis have been partially unraveled and specific types of HPV have emerged as the most common "human carcinoma viruses" (zur Hausen, 1989 a). There exist remarkable differences in HPV-caused cancers between females and males (Fig. 5.1).

The practical application of our present knowledge in diagnosing patients at risk, in the treatment of early and late lesions, and in particular in preventing these infections by vaccination dominate the research issues in this field.

5.1.1 Structure of the Viral Particle, Transcriptional Regulation, and Taxonomy

Papillomavirus particles have a diameter of approximately 55 nm and contain a double-stranded circular DNA genome. The viral DNA is associated with histone-like proteins (Favre et al., 1977; Pfister and zur Hausen, 1978) and encapsidated by 72 capsomeres (Klug and Finch, 1965). The major capsid protein is encoded by the L1 open reading frame (ORF) which contains type-specific antigenic domains. The L2 ORF codes for a minor L2-component of the viral capsid. This protein possesses group-specific antigenic domains. Virus-like particles can be obtained by expressing either L1 only or L1 and L2 proteins in recombinant systems (Zhou et al., 1991, 1992; Kirnbauer et al., 1992). The L1 protein is highly conserved among different papillomavirus types, whereas the L2 protein is less conserved. The structure of the papillomavirus particle without a lipid-containing envelope renders these viruses relatively resistant to heating and organic solvents (Bonnez et al., 1994).

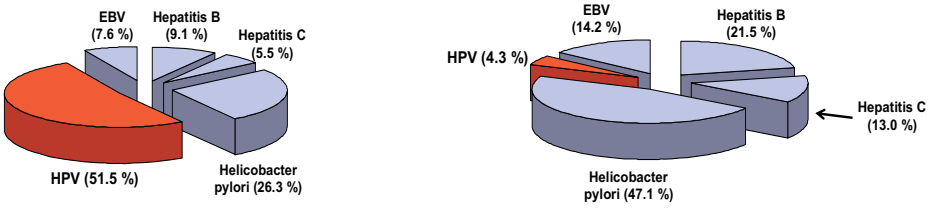

Fig. 5.1 Global percentage of human papillomavirus (HPV)-linked cancers in females and males in relation to other cancers linked to infections.

Papillomavirus genomes contain between 7200 and 8000 base pairs (bp), with up to 10 ORFs. Only one strand of the viral genome is transcriptionally active, transcription occurs only in one direction, and the ORFs of different HPV types reveal a high degree of topic correspondence (Chen et al., 1982; Danos et al., 1982). A long-control region (LCR) or upstream regulatory region (URR) covers about 12% of the genome. As its main functions, the URR regulates the epithelial-specific transcription, the differential expression of viral genes during the course of cell differentiation, receives feed-back signals to control viral gene expression, and responds to host factors on viral gene expression.

The regulation of viral gene expression is complex and controlled by viral and cellular transcription factors. Most of these regulations occur within the URR region, which varies substantially in nucleotide composition between individual HPV types. Additional regulatory mechanisms involve the use of different promoters, differential splicing, particularly affecting the E1 gene, differential transcription termination signals, and variations in the stability of different viral mRNAs. The URRs of anogenital HPVs range in size between 88 and 900 bp, but in other HPV genera – particularly in those found in genus β – they are somewhat shorter. Within the URR, cis-active elements regulate transcription of the E6 and E7 genes, which represent the most important transforming genes for immortalization and the maintenance of the malignant phenotype of HPV-positive cervical cancer cells (Schwarz et al., 1985; Yee et al., 1985; Münger et al., 1989; Hawley-Nelson et al., 1989; von Knebel Doeberitz et al., 1992).

Analysis of the URR regions of different papillomavirus genotypes resulted in the identification of several binding sites shared among most HPV types, as well as a few unique ones. The common sequences include TFIID binding to TATA boxes located approximately 30 bp upstream from early start sites (Longworth and Laimins, 2004a). Sp-1 and AP-1 binding sites, upstream of these sequences, have been identified in all HPV types studied (del Mar Pena and Laimins, 2001). Many different HPV types share binding sites for additional factors, including those for NF-1, TEF-1, TEF-2, Oct-1, AP-2, KRF-1, and YY-1 (Mack and Laimins, 1991; Bauknecht et al., 1992; Ishiji et al., 1992; Butz and Hoppe-Seyler, 1993; O'Connor and Bernard, 1995). The keratinocyte-specific enhancers seem to regulate the tropism of papillomaviruses for epithelial cells. Glucocorticoid-responsive elements emerge as particularly important for anogenital papillomavirus types (Gloss et al., 1987).

In genital HPV types the transcription of late genes is activated in differentiated cells from start sites within the E7 ORF. This seems to be due to a rearrangement of chromatin around the late promoter region during the course of epithelial differentiation (del Mar Pena and Laimins, 2001).

At present, 106 human and 22 animal papillomavirus genotypes have been fully described, yet it is very likely that the total number of human – and certainly also of animal genotypes – will be much higher (de Villiers et al., 2004a; E.-M. de Villiers, personal communication). All known human papillomavirus types, isolation sites and appropriate references are listed in Table 5.1.

The heterogeneity of the HPV family is not restricted to the human members, and the large number of human prototypes reflects the intensity of investigations in our

Table 5.1 All presently known human papillomavirus (HPV) types and sites of their isolation (courtesy of E.-M. de Villiers).

HPV-type	Original isolation from
1	plantar warts
2	common warts
3	flat warts
4	common warts
5	benign and malignant epidermodysplasia verruciformis (EV) lesions
6	genital warts, laryngeal papillomatosis
7	"butcher's warts", oral papillomas of HIV patients
8	benign and malignant EV lesions
9	EV lesions
10	flat warts
11	laryngeal papillomas, genital warts
12	EV lesions
13	oral focal epithelial hyperplasia
14	EV lesions
15	EV lesions
16	CIN III and anogenital and oral cancers
17	EV lesions
18	CIN III and anogenital cancers
19	EV lesions
20	EV lesions and cutaneous squamous cell carcinomas
21	EV lesions
22	EV lesions
23	EV lesions
24	EV lesions
25	EV lesions
26	common warts under immunosuppression
27	common warts
28	flat warts
29	common warts
30	anogenital and oral intraepithelial lesions
31	CIN III and anogenital cancers
32	oral focal epithelial hyperplasia, oral florid papillomatosis
33	CIN III and anogenital and oral cancers
34	low-grade CINs
35	usually low-grade CIN
36	actinic keratoses, EV lesions
37	keratoacanthoma
38	papillomas under immunosuppression and skin cancers
39	CIN lesions and anogenital cancers
40	anogenital intraepithelial neoplasias
41	cutaneous squamous cell carcinomas
42	low-grade CIN
43	low-grade CIN
44	low-grade CIN, condylomata acuminata
45	anogenital intraepithelial neoplasias
46	now designated as HPV 20b
47	EV lesions
48	cutaneous lesions

Table 5.1 (Continued)

HPV-type	Original isolation from
49	flat warts under immunosuppression
50	EV lesion
51	CIN and anogenital cancers
52	CIN III and anogenital cancers
53	anogenital intraepithelial neoplasias
54	anogenital intraepithelial neoplasias
55	anogenital intraepithelial neoplasias
56	anogenital intraepithelial neoplasias and cancers
57	oral and inverted maxillary sinus papillomas
58	CIN III and anogenital cancers
59	anogenital intraepithelial neoplasias
60	epidemoid cysts
61	anogenital intraepithelial neoplasias
62	anogenital intraepithelial neoplasias
63	myrmecia warts
64	anogenital intraepithelial neoplasias
65	pigmented warts
66	CIN and anogenital cancer
67	anogenital intraepithelial neoplasia
68	anogenital intraepithelial neoplasia
69	CIN and anogenital cancers
70	vulvar papillomas
71	anogenital intraepithelial neoplasias
72	oral papillomas in HIV patients
73	oral papillomas in HIV patients
74	anogenital intraepithelial neoplasia
75	common warts in organ allograft recipients
76	common warts in organ allograft recipients
77	common warts in organ allograft recipients
78	skin wart
79	erroneous designation, no longer existing
80	normal skin and ear canal
81	vaginal intraepithelial neoplasia I
82	CIN II and CIN III
83	anal condyloma
84	normal cervical smear
85	cervical scraping and wart
86	CIN I
87	koilocytotic atypia
88	skin wart
89	normal cervical smear
90	normal cervical smear
91	normal cervical smear
92	basal cell carcinoma
93	actinic keratosis
94	squamous cell carcinoma skin
95	skin wart
96	perilesional skin of squamous cell carcinoma
97	genital isolate

Table 5.1 (Continued)

HPV-type	Original isolation from
98	normal skin
99	normal skin
100	premalignant lesion and squamous cell carcinoma skin
101	normal skin
102	genital isolate
103	genital isolate
104	skin wart
105	premalignant skin lesion
106	genital isolate

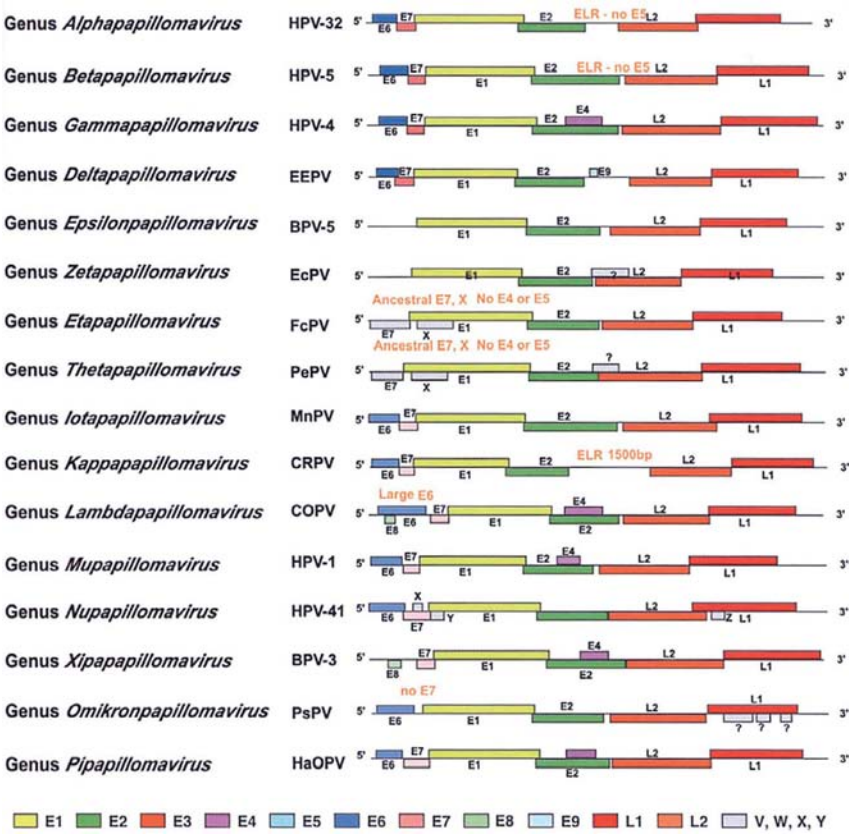

Fig. 5.2 Open reading frames of various HPV types. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The Double Stranded DNA Viruses, Papillomaviridae, 241. Copyright 2005, with permission from Elsevier.)

species. The 22 animal papillomavirus types that have been fully characterized originate mainly from nonhuman primates and from cattle. The genomic organization of the various papillomavirus genera is depicted in Figure 5.2.

It warrants attention that a number of animal papillomavirus types are more closely related to individual members of the human subgroups than are the latter among each other. A papillomavirus, isolated from a penile carcinoma of a rhesus monkey, is very closely related to HPV 52 (Ostrow et al., 1991). Likewise, a pygmy chimpanzee papillomavirus is most closely related to HPV 13 (van Ranst et al., 1992), while the cottontail rabbit papillomavirus and the canine oral papillomavirus, a finch papillomavirus and a porcupine papillomavirus reveal some relatedness to genus μ, containing HPV types 1 and 63 (Giri et al., 1985; Delius et al., 1994; Rector et al., 2005). These observations stress the assumption that, in developmental terms, the papillomavirus genera split off in prehistoric times, probably even before the development of nonhuman primates.

Although originally combined with Polyomaviruses into the family of Papovaviridae, the differences in genetic organization and functional properties resulted in their recognition as a new family of Papillomaviridae. The family is subdivided into 16 genera, labeled by letters of the Greek alphabet form α (alpha) to π (pi). The relative relatedness of individual types is indicated in the phylogenetic tree (Fig. 5.3).

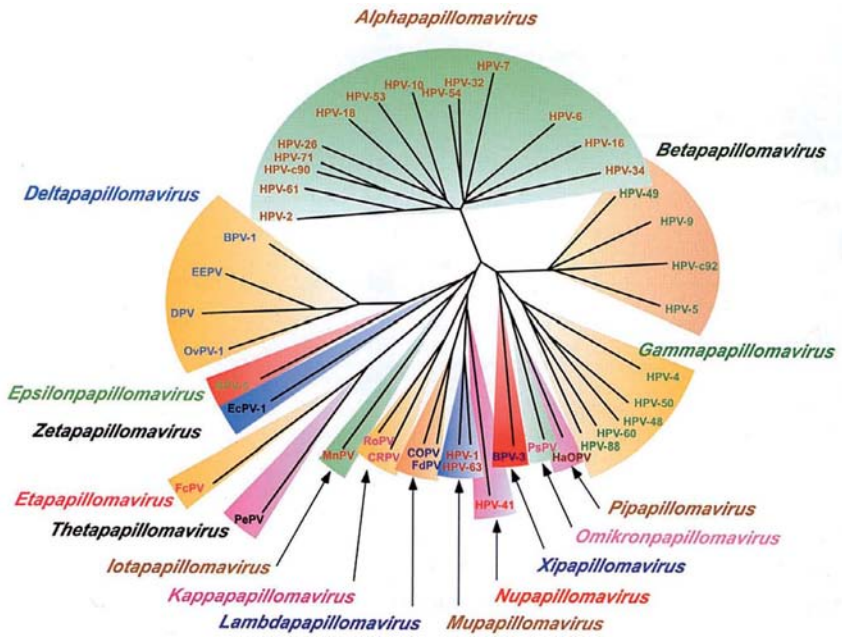

Fig. 5.3 The phylogenetic tree and nomenclature of papillomaviruses. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The Double Stranded DNA Viruses, Papillomaviridae, 252. Copyright 2005, with permission from Elsevier.)

Genotypes are defined by a difference of more than 10% within the most conserved region (L1 ORF), while differences of less than 10% characterize subtypes (de Villiers, 2001). It is interesting to note that this arbitrary separation also seems to define HPV serotypes.

There exist some structural differences between individual genera, as exemplified by the absence of an E5 ORF in genera β, γ, ε, ϕ and ι. The E4 ORF is missing in genera ε and ϕ, no typical E6 is present in genera ε and ϕ, and some genera contain an additional ORF (E8) within the E6 region (genera κ and ζ).

5.1.2 Transmission and Natural History of Papillomavirus Infections

The transmission of HPVs is facilitated by microlesions in the skin and mucosa, and/or by abraded or macerated epithelial surfaces (Oriel, 1971). Anogenital HPV infections are mainly transmitted by sexual contacts, but are rarely detected in sexually inexperienced young women (Fairley et al., 1992; Andersson-Ellström et al., 1994; Gutman et al., 1994; Rylander et al., 1994). It was noted at an early stage that there exists a correlation between the number of sexual partners and the prevalence of HPV infection (Rosenfeld et al., 1989; Moscicki et al., 1990; Bauer et al., 1993; Critchlow and Koutsky, 1995). Occasionally, anogenital HPV infections are also transmitted digitally from one epithelial site to the other (Moy et al., 1989; Euvrard et al., 1993). In addition, they may be transmitted by fomites, by medical instruments, and also by laser plumes (Garden et al., 1988; Ferenczy et al., 1990).

Oral–genital contact may lead to infections at oral sites by anogenital papillomaviruses (Kashima et al., 1992 a). Salivary transmission probably accounts for additional infections in the oral region.

Skin infections by papillomaviruses originate from close skin contact, from contaminated materials, by walking barefoot on abrasive surfaces (Rasmussen et al., 1958; Koutsky et al., 1988), or from accidental wounding with contaminated equipment (Melchers et al., 1993).

The life cycle of HPVs is tightly linked to the differentiation program of human keratinocytes. The production of viral particles occurs exclusively in suprabasal differentiated layers. Recently, however, a replication- and differentiation-independent system for the production of infectious HPV particles has been developed (Pyeon et al., 2005). Infection seems to require the availability of a cell which is still in the proliferation program (for a review, see zur Hausen, 1996). This most commonly appears to occur in microlesions, where basal layer cells are exposed to the surface. It may also happen at the periphery of junctions between different types of epithelial cells. At the distal periphery of the cervical transformation zone, particularly in very young women, proliferating cells touch the surface. This is the most vulnerable region for high-risk anogenital HPV infections.

5.1.3 Functions of Viral Proteins

5.1.3.1 E6

The following sections will mainly deal with functions of high-risk HPV proteins (e.g., HPV 16, 18, and 31); the differing properties of corresponding proteins in low-risk viruses will be detailed separately.

The E6 protein of HPV 16 contains 151 nucleic acids and reveals four Cys-X-X-Cys motifs that mediate zinc binding and which should result in the formation of two zinc finger structures (Barbosa et al., 1989; Grossman and Laimins, 1989; Kanda et al., 1991). E6 proteins are found in the nucleus as well as in the cytoplasm, and bind a larger number of cellular proteins. Expression of the *E6* gene alone results in immortalization of various types of human cells, though at low efficiency and commonly accompanied by the loss of $p16^{INK4}$ expression (Band et al., 1990; Wazer et al., 1995; Reznikoff et al., 1996; Kiyono et al., 1997; Liu et al., 1999). E6, in addition, cooperates with the *ras* oncogene in the immortalization of primary rodent cells (Storey and Banks, 1993). E6 cooperates with the E7 protein in the efficient immortalization of human cells (Münger et al., 1989). It induces anchorage-independent growth of NIH 3 T3 cells and transactivates the adenovirus E2 promoter (Sedman et al., 1991).

The binding and degradation of p53 represents one of the most intensively studied functions of the E6 protein (Werness et al., 1990; Scheffner et al., 1990). p53 has been characterized as a tumor suppressor gene, regulating the expression of genes involved in cell cycle control. Among these, the cyclin-dependent kinase inhibitor p21 plays an important role. DNA damage results in activation of p53 and in the induction of high levels of p21, leading to cell cycle arrest and apoptosis (Ko and Prives, 1996). The activation of apoptotic pathways represents a cellular defense mechanism against the spread of virus infections, as well as a protective function against an undesired accumulation of mutational modifications. A number of viruses – in particular members of the herpesvirus group (see Chapter 4) – have developed mechanisms to interfere with apoptotic pathways. E6 binds to p53 in a ternary complex with E6-AP (Huibregtse et al., 1991). The formation of this complex results in ubiquitination of p53 and subsequent degradation of the latter by the 26 S proteasome (Hubbert et al., 1992; Huibregtse et al., 1993, 1995). E6 also binds to p300/CBP, which is a coactivator of p53 (Lechner and Laimins, 1994; Patel et al., 1999; Zimmermann et al., 1999), and thus interferes also indirectly with p53. High-risk HPV E6 binds and functionally inactivates the cyclin-dependent kinase inhibitors p21 (Funk et al., 1997; Jones et al., 1997), a transcriptional target of p53 (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), and p27 (Zerfass-Thome et al., 1996).

p53 also acts as a transcriptional activator by binding to specific DNA sequences (Kern et al., 1991). It is required for growth arrest following cellular DNA damage (Kuerbitz et al., 1992; Lin et al., 1992). Failure of this function results in continued DNA replication after DNA damage and in chromosomal instability (Livingstone et

al., 1992; Yin et al., 1992). The transcriptional activation function of p53 is also inhibited by high-risk HPV E6 (Gu et al., 1994). Interference with the p53/PUMA/Bax cascade was recently shown to be responsible for the anti-apoptotic function of the E6 protein in cervical cancer cells (Vogt et al., 2006).

Binding of E6 to E6-AP mediates also a self-ubiquitination of the E6-AP protein (Kao et al., 2000). Therefore, it is likely that the reduction of E6-AP concentrations in E6-positive cells will negatively affect the proteolysis of other E6-AP-binding cellular proteins, among them members of the *src*-family of tyrosine kinases (Oda et al., 1999; Frame, 2002). It becomes increasingly apparent that binding of E6 to E6-AP or to other ubiquitin ligases is one of the central events in E6 functions, and influences all other E6-mediated effects described below (Kelley et al., 2005).

Another functionally important interaction results from the carboxy-terminal PDZ (PSD-95, Dlg, and ZO-1 proteins) domain protein-binding motive X-(S/T)-X-(V/I/L)-COOH which can bind a number of cellular PDZ domain-containing proteins. Clearly important here are *hDlg* (human homologue of *Drosophila melanogaster* tumor suppressor protein discs large), *MUPP1* (multi-PDZ domain protein), *hScrib* (human homologue of *Drosophila melanogaster* tumor suppressor *scribble*) (Kiyono et al., 1997; Lee et al., 1997, 2000; Nakagawa and Huibregtse, 2000), and *MAGI-1* (Glaunsinger et al., 2000). The PDZ domains seem to act as molecular scaffold to mediate signal transduction (Gomperts, 1996; Craven and Bredt, 1998). Binding of E6 to these proteins results in their degradation. This binding and degradation process is clearly of substantial importance for cell immortalization by E6, since E6 transgenic mice devoid of PDZ protein expression but expressing a functional p53 do not develop epidermal hyperplasias, regularly observed in wild-type transgenics (Nguyen et al., 2003). Yet, even deletion of the hDlg binding motif of HPV16 E6 permitted the bypass of senescence of human mammary epithelial cells (Kiyono et al., 1998). In this system the activation of telomerase appears to be the major determinant of cellular immortalization. At least in the cervical carcinoma cell line HeLa, containing multiple copies of HPV18 DNA, continued expression of telomerase is not sufficient for the maintenance of the malignant phenotype. Repression of the *E6* gene in these cells, stably expressing an exogenous *hTERT* gene which encodes the catalytic subunit of telomerase, elevated telomerase activity and elongated telomeres did not prevent senescence and apoptosis (Horner et al., 2004). On the other hand, overexpression of the catalytic subunit of telomerase, murine TERT, in basal keratinocytes of transgenic mice results in increased epidermal tumors (Gonzáles-Suárez et al., 2001). This may underline the existing differences in transformability of human and murine cells. Human keratinocytes expressing *hTERT* are able to bypass $p16^{INK4}$-mediated senescence and become immortal, yet they retain normal growth and differentiation characteristics (Dickson et al., 2000).

One report describes that hDlg and MAGIs are degraded by E6 complexing in an E6-AP-independent pathway (Grm and Banks, 2004). It is likely that other E6-associated ubiquitin ligases than E6-AP are important for the degradation of certain E6 targets. One PDZ protein which is degraded by E6/E6-AP is TIP-2/GIPC (Favre-Bonvin et al., 2005); this protein has been found to be involved in transforming growth factor-β (TGF-β) signaling and enhances the expression of TGF-β type III re-

ceptor at the cell membrane. Its degradation diminishes the antiproliferative effect of TGF-β in high-risk HPV E6-expressing cells.

The degradation of PDZ domain-carrying proteins by E6 or E6/E6-AP complexes may also affect the *Notch* signaling pathway. DELTA1 and DELTA4, both Notch ligands, interact with Dlg1 (Six et al., 2004) which is directly affected by the E6/E6-AP complex. JAGGED1, a cell-bound ligand for Notch receptors, contains a PDZ domain, although mutation of the PDZ ligand did not affect the ability of JAGGED1 to initiate Notch signaling (Ascano et al., 2003). Another PDZ domain-containing member of the E3 ubiquitin ligase family, *LNX*, can enhance Notch signaling (Nie et al., 2002). It has been reported that JAGGED1 and Notch ligand are up-regulated in high-grade cervical lesions and in invasive cervical cancer, whereas a negative regulator of JAGGED1-Notch signaling, *Manic Fringe*, is down-regulated (Veeraraghavalu et al., 2004).

E6 has been shown to induce suprabasal DNA synthesis and is alone sufficient to induce carcinomas in transgenic animals (Song et al., 1999). This activity is p53-independent, and correlates with the ability of E6 to bind PDZ domain proteins (Nguyen et al., 2003).

The activation of the catalytic subunit hTERT by E6 emerges as an important factor in cell immortalization (Klingelhutz et al., 1996; Meyerson et al., 1997; Nakamura et al., 1997). Clues of the mechanism leading to hTERT activation by high-risk HPVs originated from experiments conducted by Veldman et al. (2003). A complex of E6/*Myc*/*Max* binds to the hTERT promoter and results in its activation. This effect is specific for high-risk HPV E6. Gewin et al. (2004) proposed that complexes of E6/E6-AP, forming an E3 ubiquitin ligase, target an hTERT repressor, NFX1–91, which becomes highly ubiquitinated and destabilized. E6-independent knock-down of NFX1–91 also results in derepression of the endogenous hTERT promoter and elevated levels of telomerase activity. E6 mutants that fail to bind E6-AP are also defective for increasing telomerase activity and transactivating the hTERT promoter (Liu, X., et al., 2005). E6 functions in solely E6-expressing $p16^{INK4}$-positive or -negative cells are summarized in Figures 5.4 and 5.5.

The induction of chromosomal instability by *E6* oncogene expression is probably one of the very important aspects in the long-term progression of latently high-risk HPV-infected cells. One major reason is the degradation of p53 and the resulting loss of G_1/S checkpoint control and the disruption of p53-dependent G_1 arrest after DNA damage. Kessis and colleagues (1996) showed that either E6 or E7 proteins of high-risk, but not of low-risk HPV, increase the frequency of foreign DNA integration into the host genome. This could be related to the observed HPV DNA integration at late stages of tumor progression – for example, in cervical intraepithelial neoplasia (CIN) III lesions (Klaes et al., 1999) which commonly reveal a similarly high level of viral oncoprotein expression as cervical cancer cells (Dürst et al., 1991; Stoler et al., 1992).

Suppression of both, caspase 3 and 8, by E6 results in the suppression of apoptosis induced by TNF-α via the Fas pathway (Filippova et al., 2004). This seems to be mediated by binding of E6 to cellular FADD and subsequent degradation of the latter. Repression of the E6 gene on the other hand initiates p53-dependent, telomerase-inde-

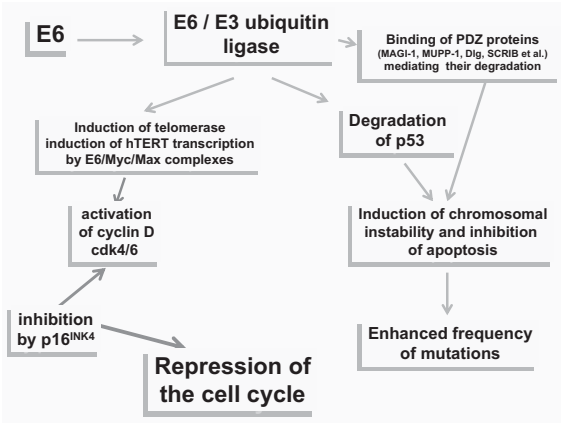

Fig. 5.4 Consequences of E6 expression in $p16^{INK4}$-expressing cells. The activation of the cell cycle is counteracted by $p16^{INK4}$ which interferes by blocking cyclin D/cdk4/6 complexes.

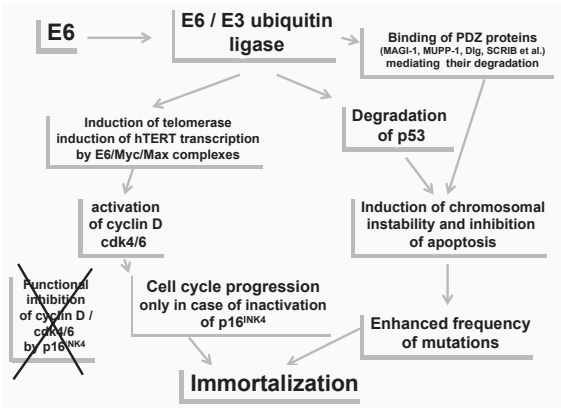

Fig. 5.5 In cells where $p16^{INK4}$ is inactivated (either due to DNA methylation, mutation or deletion), pE6 expression may lead directly to immortalization.

pendent senescence and apoptosis in HeLa cervical carcinoma cells (Horner et al., 2004). The E6 protein also interacts with other pre-apoptotic proteins, most notably with Bak (Thomas and Banks, 1999; Jackson et al., 2000). Restoration of p53 expression sensitizes HPV 16-immortalized human keratinocytes to CD95-mediated apoptosis (Aguilar-Lemarroy et al., 2002).

Several other protein interactions have been reported for E6: among others, with the adhesion and cellular polarity determining factor paxillin, the interferon regulatory factor IRF-3, and the calcium-binding protein E6-BP (Ronco et al., 1998; Tong and Howley, 1997; Chen et al., 1998; Elston et al., 1998). By binding to tuberin, a negative regulator for insulin-induced phosphorylation of S6 kinase and eIF4 E-binding protein 1, E6 also interferes with the insulin signaling pathway (Lu et al., 2004). In addition, E6 seems to be able to promote phosphorylation of the retinoblastoma protein (Malanchi et al., 2004). A compendium of E6 interacting factors has been published by Mantovani and Banks (2001). At present, the significance of the data in this section in terms of cell immortalization and transformation is difficult to assess.

5.1.3.2 **E6***

A series of polypeptides expressed by high-risk HPV types through alternative splicing of E6 and labeled as E6* may point to a viral pathway regulating E6 activity (Schneider-Gädicke et al., 1988). One of these, E6*I, interacts with full-length E6 as well as with E6-AP and blocks the degradation of p53 (Pim et al., 1997). Mantovani and Banks (2001) argue that a possible E6* function could ensure the presence of a limited amount of p53 at viral replication sites. This seems to be supported by the observation that HPV recruits DNA polymerase α for its DNA replication and that p53 may enhance the replicative fidelity of this enzyme (Albrechtsen et al., 1999). It is interesting to note that p53 is also found in replication centers of herpes simplex, cytomegalovirus, and adenoviruses (Wilcock and Lane, 1991; Fortunato and Spector, 1998; König et al., 1999). In addition, an interaction between the HPV ori-complex binding and p53 has been noted (Massimi et al., 1999). A recent study suggests that E6* expression may result in a higher resistance to UV B radiation, and that this is related to a high glutathione peroxidase activity (Mouret et al., 2005). In general, functions of E6* polypeptides have as yet been poorly studied.

5.1.3.3 **E7**

The HPV16 E7 protein represents a zinc finger-binding phosphoprotein with two Cys-X-X-Cys domains composed of 98 amino acids. The zinc finger-binding domain and the two Cys-X-X-Cys motifs show similarity to the E6 protein, suggesting an evolutionary relationship between the two proteins. The amino-terminal part of the E7 protein contains two domains corresponding partially to the conserved region 1 (CR-1) and completely to the conserved region 2 (CR-2) of adenovirus E1 A proteins and to an analogous region in SV40 large T antigen (Phelps et al., 1989). Both of the E1 A regions are involved in cell transformation (Moran and Mathews, 1987). Both corresponding domains in E7 (cd-1 and cd-2) contribute to the immortalizing potential of E7 (Phelps et al., 1992). The E7 protein is able to undergo pH-dependent conformational transitions, exposing hydrophobic surfaces to the solvent (Alonso et al., 2004). Under these conditions it self-assembles into defined spherical oligomeres.

The phosphorylation of E7 is mediated by casein kinase II (CKII). Two S100 family calcium-binding proteins, macrophage inhibitor-related factor 8 (MRP-8) and MRP-14, form a protein complex that inactivates CKII (Tugizov et al., 2005). This complex consequently also inhibits CKII-mediated phosphorylation of E7. Treatment of HPV-immortalized cells with exogenous MRP-8/14 resulted in E7 hypophosphorylation and growth inhibition.

Expression of E7 is able to transform immortalized NIH 3T3 cells and, at very low frequency, also human keratinocytes (Münger et al., 1989; Wazer et al., 1995; Zerfass et al., 1995). This frequency is enhanced by overexpression of hTERT to induce telomerase (Kiyono et al., 1998).

One of the key features of E7 function is its complex formation with the retinoblastoma protein pRb and the related pocket proteins p107 and p130 (Dyson et al.,

1989; Berezutskaya et al., 1997; Classon and Dyson, 2001). The three proteins are differently expressed throughout the cell cycle: Whereas Rb is constitutively expressed, p107 is predominantly expressed during S-phase and p130 prevails in G_0 (Classon and Dyson, 2001; Longworth and Laimins, 2004a). While the unphosphorylated forms of pocket proteins form complexes with E2F/DP1 transcription factors, upon progression from G_1 into S-phase, Rb becomes phosphorylated by cyclin-kinase complexes, releasing E2 F transcription factors. Binding of E7 has the same effect, resulting in the constitutive activation of E2F proteins that activate promoters of genes involved in S-phase progression and apoptosis (Edmonds and Vousden, 1989; Weintraub et al., 1995). This involves genes that are required for DNA synthesis, such as DNA polymerase α and thymidine kinase (La Thangue, 1994; Slansky and Farnham, 1996). There exists a good correlation between the inactivation of pocket proteins by E7 and its transforming potential (Heck et al., 1992; Phelps et al., 1992; Kiyono et al., 1998). Complex formation between E7 and Rb is not restricted to high-risk viruses; indeed, HPV 6 and 11 E7 also bind Rb, though at lower affinity (Piboonniyom et al., 2003). HPV 1 E7 binds Rb with high affinity, yet it is unable to activate E2F-inducible genes or to degrade Rb (Ciccolini et al., 1994; Schmitt et al., 1994). Recently, an association of E7 with the 600-kDa retinoblastoma protein-associated factor, p600, has been reported (Huh et al., 2005). This association is independent of the pocket proteins and is mediated through the N-terminal domain which contributes to cellular transformation independently of pRb binding.

E7 also mediates degradation of the pocket proteins through the ubiquitin proteasome pathway (Edmonds and Vousden, 1989; Jewers et al., 1992; Berezutskaya et al., 1997; Wang et al., 2001). Since Rb also regulates cell cycle exit during differentiation, the abrogation of its function by E7 also permits suprabasal cells to enter DNA replication (Chellappan et al., 1992). Binding of E7 to Rb seems also to be important for maintenance of the episomal state of HPV DNA, though the mechanism is poorly understood (Longworth and Laimins, 2004b). Rb-bound E7 of high-risk HPV types also trans-activates the promoter of p73 (Brooks et al., 2002). In normal cervical epithelium, p73 expression is confined to the basal and suprabasal layers. In neoplastic lesions, however, expression is detected throughout the epithelium and increases with the grade of neoplasia. In addition, a deregulation of expression of the N-terminal splice variant p73Δ2 was observed in a significant proportion of cancers, but not in normal epithelium.

B-myb, a growth-promoting gene regulated by the release of E2 F, is also transcriptionally activated by HPV 16 E7 (Lam et al., 1994). It is inappropriately transcribed during G_1 and constitutively overexpressed in cycling cells containing E7. Regulation of the *B-myb* promoter is apparently mediated by p107-containing complexes binding to the E2 F binding site.

Several other interactions of high-risk HPV E7 with cellular proteins appear to be of substantial importance for its growth-promoting properties: the oncoprotein inhibits the function of the cyclin-dependent inhibitors p21 and p27 (Zerfass-Thome et al., 1996; Funk et al., 1997; Jones et al., 1997). High-risk E7 proteins bind directly to cyclin A/cdk-2 complexes and, through p107, indirectly to cyclin E/cdk-2 complexes by retaining their cdk2-associated kinase activity (Dyson et al., 1992; Arroyo et

al., 1993; Tommasino et al., 1993; McIntyre et al., 1996). In contrast to low-risk E7 proteins, they even increase the levels of cyclin A and E proteins (Martin et al., 1998). The blocking of the cdk inhibitors p21 and p27 by E7 may contribute to the latter effect.

The promyelocytic leukemia (PML) protein induces senescence when overexpressed in primary human fibroblasts. E7 circumvents PML-induced senescence (Bischof et al., 2005). In this case, Rb-related and Rb-independent mechanisms of E7 are necessary to overcome the PML-induced senescence. A senescence inhibitor, DEK, represents an up-regulated target for high-risk HPV E7, but not for low-risk HPVs (Wise-Draper et al., 2005). This up-regulation emerges as a common event in high-risk HPV-driven carcinogenesis.

Another important group of cellular proteins bound to high-risk HPV E7 are the histone deacetylases (HDACs). They also repress E2 F-inducible promoters by binding to Rb proteins (Weintraub et al., 1995; Brehm et al., 1998). Binding of E7 to HDACs occurs independently of E7/Rb interactions (Longworth and Laimins, 2004 b). HDACs remove acetyl groups from the lysine-rich amino-terminal tails of histone proteins and functionally inactivate directly E2F proteins by deacetylation (Marks et al., 2001). Mutation in the HDAC binding domain of E7 results in an inability to stably maintain viral episomes and to extend the lifespan of transfected cells (Longworth and Laimins, 2004 a). Thus, similar to Rb-binding, the association of E7 with HDACs has profound consequences for the E7-expressing cell. The E7 transactivation of the cdc25A tyrosine phosphatase promoter depends on the binding of Rb and HDACs (Nguyen et al., 2002). E7, via HDAC recruitment, also silences the interferon regulatory factor 1 (IRF-1) gene that is important for interferon signaling and immune surveillance of persisting HPV infections (Park et al., 2000). This seems to affect in particular interferon α-inducible genes, but has no effect on interferon γ-inducible genes (Barnard and McMillan, 1999). On the other hand, E7-transfected mouse lymphoma cells were greatly sensitized to interferon α-induced apoptosis (Thyrell et al., 2005).

Besides interactions with the histone deacetylases, one report also describes binding of E7 with the acetyltransferase domain of pCAF which is supposed to function as a co-activator for a variety of transcription factors including p53 (Avvakumov et al., 2003). This interaction reduces the acetyltransferase activity *in vitro*.

Several additional cellular proteins are affected by E7 interactions: these include the S4 subunit of the 26S proteasome (Berezutskaya and Bagchi, 1997), Mi2β, as a component of the NURD histone deacetylase complex (Brehm et al., 1998), the transcription factor AP-1 (Antinore et al., 1996), the fork-head domain transcription factor MPP2 (Lüscher-Firzlaff et al., 1999), insulin-like growth factor (IGF) binding protein 3 (Mannhardt et al., 2000), the TATA box binding protein TBP (Massimi et al., 1996, 1997; Phillips and Vousden, 1997), and a human DnaJ protein, hTid-1 (Schilling et al., 1998).

By blocking Rb and p21, high-risk HPV E7 effectively overcomes cell cycle arrest (Helt et al., 2002) (Fig. 5.6). This seems to represent one reason for the genetic instability of E7-transfected cells, also evidenced by the increased integration of foreign DNA in such cells (Kessis et al., 1996).

Although E6 and E7 can both independently induce chromosomal instability (White et al., 1994), they cooperate in generating mitotic defects and aneuploidy (Münger et al., 2004). The latter appears to be primarily the result of centrosome abnormalities. E6 and E7 become apparent during episomal HPV 16 persistence (Duensing et al., 2001), and levels increase in cells containing integrated HPV DNA (Pett et al., 2004). HPV 16 E7 expression induces primary centrosome and centriole duplication errors in normal diploid cells (Duensing et al., 2001). The mechanism appears to be independent of Rb interactions, since expression of E7 in mouse embryo fibroblasts that lack pRb, p107 and p130 expression also results in increased centrosome abnormalities (Duensing and Münger, 2003). Further data indicate that disruption of the $p16^{INK4a}$/pRb checkpoint of epithelial cell immortalization does not necessarily lead to centrosome-associated genomic instability (Piboonniyom et al., 2003).

In addition to centrosome abnormalities, anaphase bridges and lagging chromosomes, indicating errors in mitotic spindle attachment, and double-stranded DNA breaks are commonly observed in these cells (Duensing and Münger, 2002). Dysregulation of mitotic pathways in cervical cancer and high-risk HPV-expressing cells lines have also been observed by genomic analyses (Patel et al., 2004; Thierry et al., 2004)

Some of the pathways influenced by E7 expression are shown in Figure 5.6.

A newly arising (and doubtless important) aspect of the regulation of E7 expression originates from observations pointing to the importance of the ARF locus ($p19^{ARF}$ for mouse and $p14^{ARF}$ for human) for E7 regulation. The *ARF* gene codes for a tumor suppressor protein with potent cell cycle inhibitory function (Quelle et al., 1995). It acts upstream of p21 by stabilizing and activating p53 (reviewed by Sherr, 1998). This activation occurs by binding of ARF to MDM2, which is an inhibitor of p53. In addition, however, $p14^{ARF}$ seems to act via a p53-independent mechanism in the regulation of cell cycle arrest and apoptosis, which may depend entirely on p21 (Hemmati et al., 2005). ARF also associates with certain members of the E2F family and induces their degradation through the ubiquitin-proteasome pathway (Martelli et al., 2001; Eymin et al., 2002).

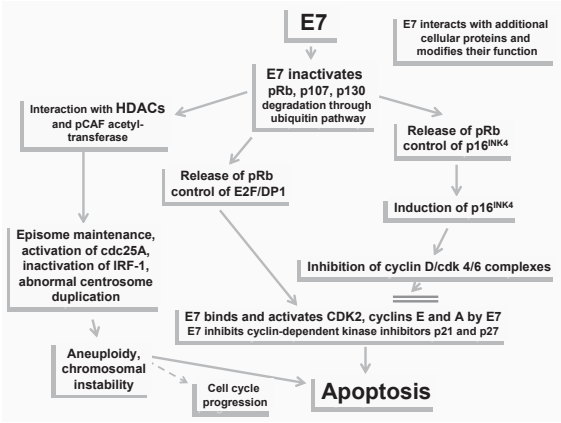

Fig. 5.6 Schematic outline of some of the important pathways influenced by high-risk HPV E7 oncoproteins. The regular consequence of the sole expression of E7 is apoptosis, presumably mediated by the release of E2 F following pRb inactivation.

In 2003, Pan and colleagues demonstrated an effective inhibition of E7 functions by exogenously expressed murine $p19^{ARF}$. ARF caused relocalization of E7 from the nucleoplasm to the nucleolus, and blocked the proteolysis of Rb induced by E7. Although this finding requires confirmation, it could suggest that human $p14^{ARF}$ may provide a natural protective mechanism against E7 expression. It is interesting to note that immunohistochemical data, at the first glance, appear to contradict this expectation: Sano et al. (2002) and Wang, J.L., et al. (2004) reported an increased expression of $p16^{INK4}$ and $p14^{ARF}$ in cervical premalignant and malignant lesions. This was supported by polymerase chain reaction (PCR) analysis revealing an increased transcription of $p14^{ARF}$ (Kanao et al., 2004). On the other hand, this situation is somewhat reminiscent of $p16^{INK4}$ which is commonly inactivated in solely E6-immortalized cells (Reznikoff et al., 1996), but overexpressed in E6/E7-transformed lesions and cell lines. There, the overexpression is due to the inactivation of Rb by E7 and the resulting transcriptional activation of p16 by E2F. Since $p16^{INK4}$ blocks cyclin D/cdk 4/6 complexes, its growth-inhibitory effect is circumvented by E7 by directly stimulating cyclins E and A. The overexpression of $p14^{ARF}$ in advanced cervical lesions may point to a similar effect, where its blocking function on E7, however – for as yet unknown reasons – is no longer functioning. In spite of a high $p14^{ARF}$ expression in cervical cancer cells, this protein does not seem to interfere with the E7 function. Under conditions of hyperproliferation, $p14^{ARF}$ stabilizes p53 by binding HDM2 and inhibiting its HDM2-mediated ubiquitination and degradation (reviewed in Bothner et al., 2001). Upon binding, the conformation of $p14^{ARF}$ and HDM2 changes substantially to extended structures comprised of β-strands. It might be suspected that the preferential binding of E6 to p53 could result in $p14^{ARF}$/HDM2 complexes (Tao and Levine, 1999) which may block the $p14^{ARF}$ inhibitory function for E7 and, at the same time, the functions of HDM2. It has been shown previously that HDM2 negatively regulates the hTERT promoter (Zhao et al., 2005). In HPV-positive cancer cells, however, the HDM2 pathway is completely inactive (Hengstermann et al., 2001), which suggests therefore that binding of p53 by E6 of high-risk HPV may inactivate HDM2 by forming complexes with $p14^{ARF}$ activity. For these reasons, an analysis of $p14^{ARF}$ expression in solely E7-immortalized cells should be of particular interest.

The suspected regulatory networks triggered by E7 and E6 are shown schematically in Figures 5.7 and 5.8.

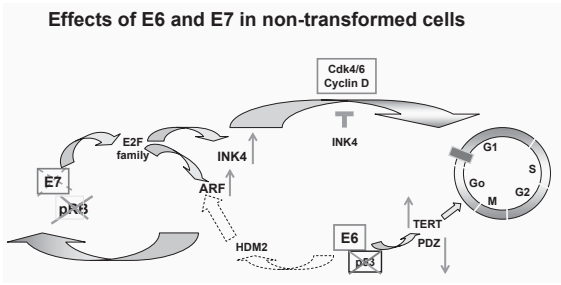

Fig. 5.7 E6 and E7 functions in nontransformed epithelial cells. The initial expression of E7 results in the release of pRb-mediated suppression of E2 F, which activates $p16^{INK4}$ and $p14^{ARF}$. $P16^{INK4}$ expression blocks cell cycle progression at the G_0/G_1 boundary, and $p14^{ARF}$ in turn down-regulates E7.

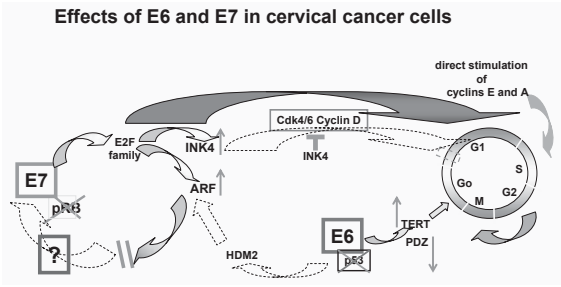

Fig. 5.8 E6 and E7 functions in cervical carcinoma cells. The high expression of E7 results in the release of pRb-mediated suppression of E2 F, which activates $p16^{INK4}$ and $p14^{ARF}$. The inhibition of cell cycle progression by $p16^{INK4}$ expression is circumvented by the direct E7 activation of cyclins E and A. It is presently not clear whether a possible modification of $p14^{ARF}$ is the reason for its failing inhibition of E7, in spite of high levels of $p14^{ARF}$.

Figures 5.7 and 5.8 depict the individual as well as joint functions of E6 and E7 proteins, and speculate on an important role for $p14^{ARF}$. In particular, the direct activation of cyclins E and A by E7 circumvents the block exerted by $p16^{INK4}$ on cyclinD/cdk4/6 complexes. The inactivation of p53 by E6, on the other hand, blocks the E2F-mediated apoptosis as the common consequence of sole E7 expression, and is likely to result in an inhibition of $p14^{ARF}$.

5.1.3.4 **E5**

The E5 protein is the major transforming protein in BPVs (Schiller et al., 1986; DiMaio et al., 1986; Rabson et al., 1986). In HPV infections tested thus far, E5 has only weak transforming activity (Leptak et al., 1991; Leechanachai et al., 1992; Pim et al., 1992). The high-risk HPV *E5* genes code for a protein of approximately 80 amino acids which is highly hydrophobic and mainly localized in the membranes of endosomes, the Golgi apparatus and, to a lesser extent, in the cytoplasmic membranes (Bubb et al., 1988; Halbert and Galloway, 1988; Conrad et al., 1993). In contrast to BPV 1, HPV 16 E5 forms complexes with EGF receptors (Hwang et al., 1995), but does not activate growth factor receptors (Suprynowicz et al., 2005). The E5 proteins associate with the membrane-bound protease-ATPase which is part of the gap-junction complex (Conrad et al., 1993; Finbow and Pitts, 1993). These proteins have the ability to inhibit endosomal acidification, yet they do not uniformly alkalinize intracellular compartments (Disbrow et al., 2005).

High- and low-risk E5 proteins modulate EGF-mediated ERK1/2 MAP kinase activation and down-regulate MHC class I molecules (Cartin and Alonso, 2003; Ashrafi et al., 2005). Expression of HPV 16 E5 also perturbs MHC class II antigen maturation (Zhang et al., 2003). Thus, the interference with cell-mediated immune functions seems to represent an important step in early interactions between virus-in-

fected cells and the host. The inhibition of apoptosis in human foreskin keratinocytes and of TRAIL and FasL-mediated apoptosis in human keratinocyte raft cultures also seem to represent an important contribution to early virus – host cell interactions (Zhang et al., 2002; Kabsch et al., 2004).

The *E5* gene of not only high-risk, but also of some low-risk HPV (HPV 6), cooperates with the *E7* gene to stimulate proliferation of primary cells and of murine 3T3 cells (Bouvard et al., 1994 b; Valle and Banks, 1995). This cooperation may lead to transformation of the murine cells. HPV 16 E5 enhances the immortalization of primary human keratinocytes after transfection with E6/E7 genes (Stoppler et al., 1996). Transformation of rodent fibroblast cell lines by HPV 11 and 16 has been reported (Straight et al., 1993; Suprynowicz et al., 2005). Thus, high-risk HPV *E5* genes seem to play an important role in early events following infection. The frequent loss of this gene in cervical cancers does not support its role in later events of virus-induced oncogenesis.

5.1.3.5 **E1**

E1 shares several properties with SV40 large T antigen (Sun et al., 1990; Seo et al., 1993). The ORF is transcribed into a polycistronic RNA. The protein fulfills an important function in HPV DNA replication, and possesses site-specific DNA-binding functions (Ustav et al., 1991; Ustav and Stenlund, 1991), in that it binds and hydrolyzes ATP and has ATP-dependent helicase activity (Yang et al., 1993). E1 interacts with cellular DNA polymerase α (Bonne-Andrea et al., 1995). The E1 protein binds to the proximal region of the LCR, where the binding site also represents the origin of viral replication (Holt et al., 1994). Bidirectional unwinding of this region represents a prerequisite for viral DNA replication (Li et al., 1993). E1 as a nonstructural protein and the major structural protein L1 are the two most highly conserved regions among different HPV genotypes.

E1 acts as a helicase and is required for efficient viral DNA replication. In HPV 11, a plasmid with the E1 coding region alone supports transient replication of an ori-carrying plasmid, whereas a plasmid containing both the E1 and E2 regions and the viral origin of replication resulted in a self-contained replicon (Deng et al., 2003). Sumoylation of the papillomavirus origin-binding E1 protein emerges as a critical factor for its function (Malcles et al., 2002; Rosas-Acosta et al., 2005). This seems to be mediated by the SUMO E3 ligases of the PIAS protein family, as determined for BPV and HPV 11 E1. HPV replication also requires E1 phosphorylation by cyclin/cdk, which also regulates its nucleocytoplasmic localization (Deng et al., 2004). Another important factor seems to be the recruitment of the major human single-stranded DNA-binding protein, replication protein A (RPA) (Loo and Melendy, 2004).

The crystal structure of E1 has been analyzed (Enemark et al., 2000, 2002; Auster and Joshua-Tor, 2004), the studies having shown that the E1 DNA-binding domain orchestrates assembly of the hexameric helicase on the ori. The results also suggest a mechanism for the transition between double- and single-stranded DNA-binding

required for a functional helicase. The DNA-binding domain of HPV 18 does not share the same nucleotide and amino acid requirements for specific DNA recognition as BPV 1 and HPV 11 E1s. Rather, as discussed in the following section, E1 and E2 form a complex which regulates the efficient replication of papillomavirus DNA.

5.1.3.6 **E2**

The properties of papillomavirus E2 proteins have been reviewed extensively (Hegde, 2002). The *E2* gene codes for at least two proteins which act as transcription factors and regulators of viral DNA replication (Androphy et al., 1987; Ustav and Stenlund, 1991; Chiang et al., 1992; Bouvard et al., 1994a; Demeret et al., 1997). They represent major intragenomic regulators of viral gene expression. The E2 protein is composed of a C-terminal DNA-binding domain and an N-terminal *trans*-activation domain, and forms dimers at specific binding sites (Dostatni et al., 1988; Doorbar et al., 1990). In human cervical keratinocytes the HPV 16 and 18 E2 proteins function as transcriptional activators (Cripe et al., 1987; Phelps and Howley, 1987; Bouvard et al., 1994a). Four positions for E2 binding are highly conserved in the LCR of high-risk HPVs. Three of these emerge as essential for the viral life cycle; their specific arrangement within the URR appears to be of importance (Stubenrauch et al., 1998). If E2 binds to the promoter-proximal binding site 1, it interferes with the recognition of the neighboring TATA box by TATA-box-binding protein (Dostatni et al., 1991). Conversely, human TATA binding protein inhibits HPV 11 DNA replication by antagonizing E1/E2 complex formation on the viral origin of replication (Hartley and Alexander, 2002). This suggests a role for this transcription factor in regulating also viral DNA replication. In addition, binding of E2 to binding sites 2 and 3 may contribute to promoter repression. This is probably due to competition with cellular transcription factors such as Sp1 (Demeret et al., 1997). The HPV 18 E2 protein binds with greatest affinity to binding site 4, and with reduced affinities to binding sites 1 and 2 (Sanders and Maitland, 1994; Demeret et al., 1997). This results in an activation of the E6/E7 promoter at low concentrations of E2, and in repression at higher concentrations, when E2 occupies the binding sites 1 and 2. The recognition of sequences by E2 in the DNA appears to effect sequence-specific conformational changes, depending on the sites occupied by E2 (Bedrosian and Bastia, 1990). The nucleic acid composition of the preferred binding site is 5'AACCGN(4)CGGTT3', where the E2 proteins bind preferentially to sites containing an A:T-rich central spacer (Dell et al., 2003). The fidelity of HPV16 E1/E2-mediated DNA replication seems to depend mainly on the cellular environment; for example, cells which are deficient in XP30RO (deficient in the bypass polymerase ε) reveal a high rate of mutations which can be restored by expressing the enzyme again (Taylor et al., 2003). A cellular protein TopBP1 (topoisomerase II β-binding protein) has been described as a transcriptional co-activator of E2, enhancing the E2 ability to activate transcription and replication (Boner et al., 2002). Chaperone proteins Hsp70 and Hsp 40 abrogate an inhibition of the E1 replicative helicase by the E2 protein (Lin et al., 2002).

Overexpression of E2 acts anti-proliferatively, and results in cell cycle arrest and apoptosis. The latter effect is induced through the extrinsic pathway, involving caspase 8. E2 itself is cleaved by caspases, whereby the cleaved E2 protein exhibits an enhanced apoptotic activity (Blachon and Demeret, 2003).

The crystal structure of the E2 DNA-binding domain has been analyzed for BPV type 1 (Hegde et al., 1992), for high-risk HPV types (Bussiere et al., 1998; Harris and Botchan, 1999), and for the low-risk HPV 11 (Wang, Y., et al., 2004). In addition, the X-ray structure of the papillomavirus E1 helicase in complex with E2 has been unraveled (Abbate et al., 2004). The E2 binding domains are structurally similar among the different papillomavirus types.

Deletion of the E2 ORF occurs relatively frequently in cervical cancer cells as a consequence of viral DNA integration into the host cell genome (Schwarz et al., 1985). This commonly seems to lead to a deregulated expression of viral *E6* and *E7* genes, facilitating further progression to more advanced stages of carcinogenesis. Mutations in the E2 ORF, and specifically also in the E2 DNA binding sites within the viral URR, result in enhanced immortalizing properties of HPV 16 DNA (Romanczuk and Howley, 1992). In cervical carcinogenesis, disruption of the E2 ORF due to integration of the viral DNA is commonly a late event, usually not occurring before the development of CIN III lesions (Matsukura et al., 1989; Dürst et al., 1992; Daniel et al., 1995; Klaes et al., 1999). E2 interacts with E1 in stimulating viral DNA replication (Chiang et al., 1992; Sverdrup and Khan, 1994; Chow and Broker, 1994), and also seems to facilitate the binding of E1 to the origin of replication (Seo et al., 1993).

5.1.3.7 **E4**

E4 originates from a viral transcript formed by a single splice between the beginning of the E1 ORF and the E4 ORF. Its mRNA is the major transcript in HPV-induced lesions (Chow et al., 1987 a,b). E4 is not required for transformation or episomal persistence of viral DNA (Neary et al., 1987); rather, the protein is exclusively localized within the differentiating layer of the epithelium (Doorbar et al., 1986; Breitburd et al., 1987). It probably has been incorrectly assigned as an early gene product, as it seems to play a role in productive infection, apparently by disrupting normal differentiation and by establishing favorable conditions for viral maturation.

The association of E4 proteins with the keratin cytoskeleton has been demonstrated by Doorbar et al. (1991) and by Roberts et al. (1993). E4 tonofilament-like structures which cause a collapse of the cytokeratin network can be visualized electron- microscopically. In HPV 16-infected cells, collapse of the cytokeratin intermediate filament structures is directly mediated by E4, and results from a strong interaction with cytokeratin 18 (Wang, Q., et al., 2004). In addition, E4 sequesters Cdk1/cyclin B1 onto the cytokeratin network (Davy et al., 2005). This prevents an accumulation of active Cdk1/cyclin B1 complexes in the nucleus, and explains the previously observed G_2 arrest of cells expressing E4 (Nakahara et al., 2002; Raj et al., 2004). The G_2 arrest of the infected cells seems to play a significant role in the pro-

motion of HPV genome amplification and S-phase genome maintenance during differentiation (Knight et al., 2004; Davy et al., 2005; Wilson et al., 2005). HPV 16 E4 also associates with a member of the DEAD box protein family of RNA helicases (Doorbar et al., 2000), and binds to mitochondria (Raj et al., 2004). Due to the progressive cleaving of N-terminal sequences, a series of E4 polypeptides is produced which cooperates to negatively influence keratinocyte proliferation (Knight et al., 2004).

Thus, E4 emerges as an important HPV protein, creating optimal conditions for viral DNA replication in suprabasal keratinocytes and the concomitant production of late viral proteins.

5.2 The Concept of Cellular Interfering Cascades: Immunological, Intracellular and Paracrine Host Factors Influencing Viral Oncogene Expression or Function

A cellular interfering factor (CIF) was initially postulated to explain the restriction of tumorvirus gene expression in proliferating cells, and the long latency period between primary infection and the eventual emergence of invasive cancer (zur Hausen, 1986 b, see chapter 3.3.1). Originally, it was proposed that an intracellular function suppresses either transcription of viral oncogenes or functions of viral oncoproteins. When applied to high-risk HPV infections, the suppression of viral transcription in proliferating basal layer cells of low-grade CIN lesions (Dürst et al., 1991, 1992; Stoler et al., 1992) seemed to point initially to a regulatory interference at the level of transcription. The interruption of this cellular function by mutational events or by epigenetic modifications, affecting both coding alleles of the respective gene, was thought to be necessary prior to malignant conversion.

During the following years it became apparent that cellular interference is much more complex: It is based in part not only on innate immunity and cell-mediated immune functions, on intracellular functional inhibition of viral oncoproteins, but also on the paracrine suppression of viral transcription. These findings led in turn to a modification of the original concept which foresees gene silencing in at least three cellular signaling cascades, as shown schematically in Figure 5.9.

The pathogenesis of high-risk HPV-induced cancers, which commonly span a period of more than 20 years, can also be visualized, as outlined in Figure 5.10. The stepwise progression from persistent infection to early lesions, high-grade dysplasias and invasive cancer, which usually covers two to three decades, is interpreted as the consequence of increasing mutations or epigenetic modifications within these three signaling cascades, affecting an allelic set of genes within each of the cascades, respectively. Thus, the number of cellular genes which could be modified in this process should roughly correspond to the number of genes required for the function of these signaling cascades and is, in all likelihood, substantial.

Evidence for the existence of these gene cascades is briefly summarized in the following sections.

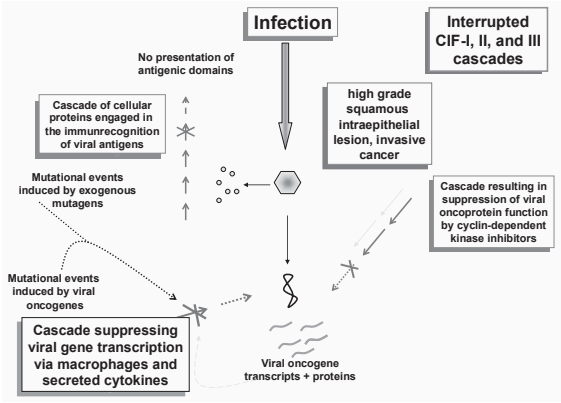

Fig. 5.9 Interruption of at least three signaling cascades by silencing of cellular genes engaged in the control of persistent HPV infection, permitting malignant conversion of the infected cell.

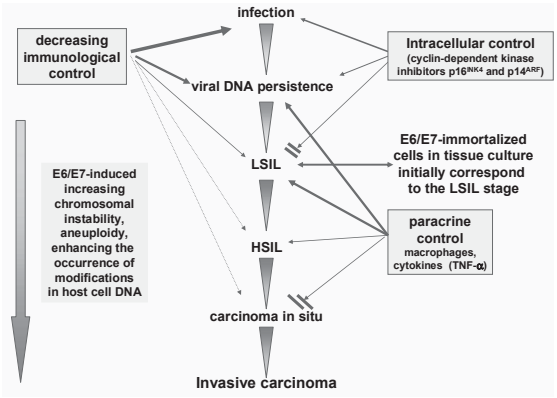

Fig. 5.10 Individual steps required in the progression of a high-risk HPV infection to invasive carcinoma. The time period elapsing between primary infection and invasive growth is commonly in the order of 20 to 25 years.

5.2.1 Immunological Control

The immune response of infected humans to papillomavirus antigens commonly occurs only late after infection, and usually leads only to a low reactivity. Some infected persons remain seronegative, this being most likely due to localization of the virus infection at the surfaces of skin or mucosa, and hence to a low concentration of viral antigens reaching the immunoreactive cells (Ho et al., 2004). Seropositivity is not necessarily protective against reinfections by the same virus type (Viscidi et al., 2004). The immune control of anogenital HPV infections has been recently reviewed (Stern, 2005). It has been suggested that an innate immune response by dendritic cells and Langerhans cells in the skin continuously senses the environment and coordinates with innate immune effectors (macrophages, polymorphic leucocytes and natural killer [NK] cells). As a result, immunemodulatory molecules are synthesized including various interferons, transforming growth factor-β (TGF-β), tumor necrosis factor α (TNF-α) and several interleukins. The Langerhans cells present a combination of MHC peptides, co-stimulatory molecules (CD80, CD86, and CD40), and interleukins

(IL-12 or IL-10) and activate naïve T cells (Niedergang et al., 2004). The subsequent T-cell response shapes the T-cell immunity into a T helper (Th) type 1 or type 2 response (Kalinski et al., 1999; Wang, J.L., et al., 2004). Whereas a Th1 response favors the production of cytotoxic T-cell effectors, Th2 facilitates the stimulation of B cells and their isotype switching to provide neutralizing antibodies.

5.2.2 CIF-I: Recognition System and its Disturbance

The development of immunity and T-cell recognition of HPV target antigens emerge as the prime mechanisms to clear a persisting HPV infection by destruction of the virus-positive cells. E7-specific activity at low systemic levels can be detected in patients with HPV-linked lesions and cancers. This has been revealed by the demonstration of Th cells (Kadish et al., 1997; De Gruijl et al., 1998), cytotoxic T lymphocytes (Bontkes et al., 2000), γ-interferon production (van der Burg et al., 2001), and HLA tetramer assays (Youde et al., 2000).

Clearly, escape from immunological surveillance emerges as one of the prime factors for persistent infection and the subsequent development of lesions. In anogenital neoplasias, a high frequency of HLA class I down-regulation has been noted (Keating et al., 1995), and was found to represent an early change, linked to progressing lesions (Bontkes et al., 1998; Abdel-Hady et al., 2001). Multiple genetic changes may cause the HLA dysregulation (Brady et al., 2000) which is found in 90% of cervical cancers (Koopman et al., 2000). Such changes could involve the transportation activating pathway, the interferon-γ responsiveness of HLA expression, or auxiliary functions required for HLA interactions. The reported hereditary susceptibility and increased risk for cervical cancer of specific HLA haplotypes fits into this consideration (Little and Stern, 1999; Hildesheim and Wang, 2002).

The important role of the immune system in the control of HPV infections is further underlined by the increased incidence of HPV infections of the anogenital tract (Tindle and Frazer, 1994) and the skin (Shamanin et al., 1994a, 1996; Stark et al., 1994; Bouwes Bavinck and Berkhout, 1997) under conditions of immunosuppression. Since 1995, an increasing number of reports have been made analyzing the role of HIV infections on anogenital HPV-linked lesions (Sun et al., 1995; Palefsky et al., 1999; Moscicki et al., 2000; Strickler et al., 2005). Interestingly, the cited studies – and most of many others – identified an increased risk for squamous intraepithelial lesions in HIV-infected women, but failed to demonstrate any significant increase in cervical cancer rates. This may be due to the long latency periods required for malignant conversion of premalignant clones and the high mortality rate of HIV-infected individuals.

Although E5 proteins of high- and low-risk HPVs down-regulate MHC class I molecules (Ashrafi et al., 2005), and the expression of HPV 16 E5 also perturbs MHC class II antigen maturation (Zhang et al., 2003), this seems to facilitate a delayed early recognition by immune functions, though it is clearly not sufficient for prolonged viral DNA persistence. Thus, the interference with cell-mediated immune functions seems to represent an important step in early interactions between

virus-infected cells and the host. The breakdown of immunological control of latently HPV-infected host cells emerges as a critical factor for viral long-term latency, and favors further progression of the affected cells towards invasive growth. It also has negative consequences for attempts to attack later stages of HPV-modified cells by immunotherapeutic approaches. The modifications of cellular genes involved in the presentation of viral antigens may affect different cellular pathways, but merge in one consequence: the inability of the immune system to present or recognize specific antigenic domains of viral oncoproteins. This seems to justify the conclusion of including the control of one major interference factor (the immune system and its failure in tumor progression) as one important regulatory principle. It is here considered as cellular interference factor (CIF) cascade I.

5.2.3
CIF-II: Intracellular Control of Viral Oncoprotein Functions

Transforming properties of sole transfection with the *E6* oncogene of HPV 16 or 18 were initially reported by Band et al. (1990, 1991) for human mammary epithelial cells. Later, Reznikoff et al. (1996) provided indirect evidence for the existence of intracellular mechanisms suppressing E6 viral oncoprotein functions. These authors observed inactivation of the $p16^{INK4}$ gene in all cells immortalized by E6. Four of five clones showed hemizygous deletion of the 9p21 region. In a subsequent study, Foster et al. (1998) showed that the reduction in p16 expression is mainly due to methylation of CpG islands in the p16 promoter. A number of subsequent reports described the absence of $p16^{INK4}$ expression in solely E6 immortalized cells (Kiyono et al., 1998; Jarrard et al., 1999; Tsutsui et al., 2002; Yamamoto et al., 2003). $p16^{INK4}$ causes disruption of the cyclin D/cdk4/6 complexes and replaces them by cdk4/6-$p16^{INK4}$ complexes (Xiong et al., 1996). This disrupts progression of the cell cycle, as shown schematically in Figure 5.11. This disruption, however, is circumvented by the direct activation of cyclins E and A by the high-risk HPV oncoprotein E7, stressing again the cooperative effect of both viral oncoproteins.

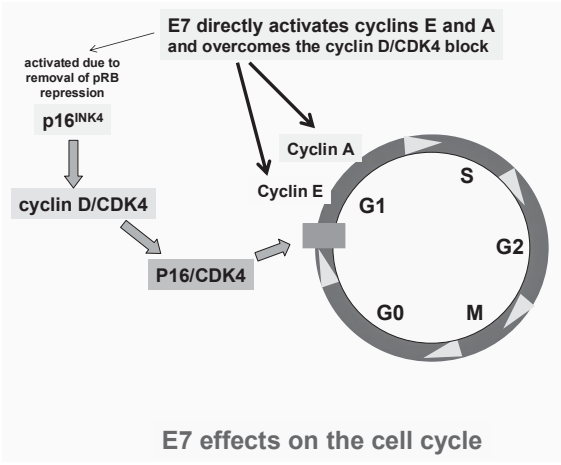

Fig. 5.11 Inactivation of $p16^{INK4}$ permits the unimpaired stimulation of the cell cycle by high-risk HPV E6.

These observations demonstrate the existence of an intracellular control for high-risk HPV E6 proteins at the functional level exerted by $p16^{INK4}$. Clearly, this functional control does not affect cells immortalized or transformed by E6 and E7, since under those conditions $p16^{INK4}$ is overexpressed.

It is therefore a clear question of whether a similar control may exist for E7 protein. This control should become inactivated in solely E7-immortalized cells. A clue to the existence of such a control seems to originate from data published by Pan et al. (2003). These authors demonstrate that the expression of $p19^{ARF}$ (the murine homologue of human $p14^{ARF}$) causes relocalization of E7 from the nucleoplasm to the nucleolus. Two distinct regions in $p19^{ARF}$ overlapping with MDM2 binding sites are necessary for this relocalization of E7. Under these conditions, proteolysis of Rb induced by E7 is blocked. Although these results still need to be confirmed, they do suggest that $p19^{ARF}$ is an effective inhibitor of E7.

Similar to $p16^{INK4}$, $p14^{ARF}$ is overexpressed in HPV-positive cervical cancers (Sano et al., 2002; Kanao et al., 2004). For $p16^{INK4}$, this overexpression apparently finds its explanation in the release of its pRb control by E7-mediated pRb degradation. The direct stimulation of cyclins E and A by Hr-HPV E7 circumvents the potential block of cyclin D/cdk4 complexes by this abundant cyclin-dependent kinase inhibitor. For $p14^{ARF}$, the dysregulation is less well understood, but it seems that *E6* gene expression overcomes a potential E7 inhibition of $p14^{ARF}$ (Stott et al., 1998). In mouse embryo fibroblasts lacking $p19^{ARF}$, E7 stabilizes p53 (Seavey et al., 1999). This effect is probably due to the binding of E7 to MDM2, an inhibitor of p53 in an autoregulatory loop. The cells do not accumulate p53, apparently due to the inactivation of MDM2 functions by E7 binding (Alunni-Fabbroni et al., 2000). As outlined previously, the preferential binding of E6 to p53 may result in $p14^{ARF}$/HDM2 complexes (Tao and Levine, 1999) which may block the $p14^{ARF}$ inhibitory function for E7 and, at the same time, the functions of HDM2. It has been shown previously that HDM2 negatively regulates the hTERT promoter (Zhao et al., 2005). In HPV-positive cancer cells, however, the HDM2 pathway is completely inactive (Hengstermann et al., 2001), which suggests that the binding of p53 by E6 of high-risk HPV may inactivate HDM2 by forming complexes with $p14^{ARF}$ activity.

Clearly, the specific inhibition of E7 by $p14^{ARF}$ requires further investigation. One would predict suppression of $p14^{ARF}$ expression or of its function in solely E7-immortalized cells, but such studies are not presently available. It is tempting to speculate that the presence of E6 is the reason for a functionally inactive overexpression of $p14^{ARF}$ in cervical cancer cells.

To summarize the points of this section, evidence exists for an intracellular control of *E6* and *E7* oncogene functions, and the disruption of this control probably plays a role in the progression of preneoplastic lesions to invasive cancer. Yet, it is difficult to exclude at this stage that the disruption of another signaling cascade (paracrine regulation, see below) may result in the transcriptional activation of both oncogenes which paralyze the described functional control in a joint and coordinated function.

5.2.4 CIF-III: Paracrine Control

The important role of paracrine factors in controlling the potentially deleterious effects of high-risk HPV infections can be easily deduced from studies on the heteroimplantation of HPV-immortalized or malignantly transformed lines into immunosuppressed mice. Immortalized cells are quickly growth-inhibited and do not form tumors, whereas cervical carcinoma-derived cell lines are commonly tumorigenic. The first evidence that a paracrine control acts at the level of HPV transcription originated from studies analyzing the HPV transcription in heterotransplanted nonmalignant HeLa cell – human fibroblast hybrids in comparison to parental HeLa cells (Bosch et al., 1990). In these studies, HPV transcription in the nonmalignant hybrid cells was rapidly suppressed, whereas in the tumorigenic parental cells a high rate of HPV transcription continued. Similar observations were recorded for heterografted HPV-immortalized keratinocytes, when compared to a malignant line derived from these cells (Dürst et al., 1991). These results corresponded to later studies demonstrating suppression of HPV transcription in basal and suprabasal cells of early HPV 16 containing clinical lesions (CIN I/II) in comparison to late stages (CIN III) and invasive cervical cancer which regularly revealed intensive transcription of *E6*/*E7* genes (Dürst et al., 1992; Stoler et al., 1992).

Preceding experiments had demonstrated a cytostatic and/or cytolytic activity of activated murine macrophages against HPV 16-transformed, but not against HPV 18-transformed, murine NIH 3T3 and A31 3T3 cells (Denis et al., 1989). Since these cells, as well as their nontransformed counterparts, were resistant to recombinant TNF-α, these authors claimed that the cell killing was independent of TNF-α. Rösl and colleagues (1994) revealed a selective down-regulation of HPV 18 transcription in nonmalignant HeLa – fibroblast hybrids by activated macrophages, and showed that this effect could be reproduced by the addition of low concentrations of TNF-α. Under these conditions the monocyte chemoattractant protein MCP-1 was highly up-regulated. HPV 16 E6 expression in human or murine cells sensitized these to lysis by macrophages, but not by NK cells (Routes et al., 2005), the lysis being shown to depend on the production of TNF-α or nitric oxide.

A number of additional studies confirmed the selective growth inhibition of nonmalignant HPV-immortalized cells or of low-grade cervical intraepithelial lesions, but not of malignant cells, by TNF-α (Malejczyk et al., 1992; Villa et al., 1992; Kyo et al., 1994; Delvenne et al., 1995; Vieira et al., 1996; Soto et al., 1999). Some of these studies indicate that HPV 18-immortalized cells are less sensitive to TNF-α than those immortalized by HPV 16 (Boccardo et al., 2004), although HPV 18-positive nonmalignant HeLa – fibroblast hybrids proved to be highly sensitive. In contrast to these reports, one group reported stimulation of growth of HPV-immortalized cervical keratinocytes and cervical carcinoma-derived cell lines by TNF-α in epidermal growth factor- and serum-depleted media (Woodworth et al., 1995; Gaiotti et al., 2000).

The mechanism of transcriptional regulation of nonmalignant high-risk HPV-immortalized cells was analyzed in a number of additional studies. Induction of the

MCP-1 gene in nonmalignant HPV-containing cells and low-grade cervical intraepithelial neoplasias (Rösl et al., 1994; Kleine et al., 1995; Kleine-Lowinski et al., 1999), but not in high-grade lesions and invasive carcinomas, by TNF-α emerges as a defense mechanism attracting more macrophages to the respective lesion. On the other hand, *MCP-1* expression is suppressed by a higher level of either *E6* or *E7* expression (Kleine-Lowinski et al., 2003). This suppression seems to be selective, since other chemokines were not affected.

Clues for the molecular basis of TNF-α-mediated transcriptional effects originated from studies analyzing the composition of the transcription factor AP-1, which seems to control the proliferation of HPV-positive cells in clinical lesions (Soto et al., 1999, 2000). AP-1 consists of jun family members (c-jun/junD/junB) either homodimerized or frequently heterodimerized with Fra-1, a member of the c-fos family. In nonmalignant HPV-containing cells, the AP-1 binding site contains to some extent c-jun/Fra-1 heterodimers, and the Fra-1 involvement is substantially increased after TNF-α treatment. Cervical carcinoma cell lines either express low amounts of Fra-1 or are negative for Fra-1 expression, but commonly express high quantities of c-fos. Ectopic expression of c-fos under a heterologous promoter in nonmalignant HeLa – fibroblast hybrids induces tumorigenicity and a change in the jun/Fra-1 ratio towards jun/c-fos heterodimers (Soto et al., 1999; Prusty and Das, 2005). These data suggest that the composition of AP-1 in the HPV promoter plays a crucial role in determining the growth properties of the respective cells, and that this composition is steered by paracrine regulatory factors, prominent among them TNF-α. In most cervical carcinoma cells the TNF-α-mediated pathway is interrupted and functionally inactive. This is further supported by observations showing that the endogenous TNF-α-induced interferon-β synthesis does not function in cervical carcinoma cell lines, in contrast to nonmalignant cells (Bachmann et al., 2002). Apparently, TNF-α-mediated activation of IRF-1 and p48 as key regulatory molecules in the differential interferon-β response fails to function in these malignant cells.

The constitutively increased c-fos transcription in cervical cancer cells seems to be based on a disturbance in c-fos regulation. In malignant cells, c-fos is constitutively expressed even after serum starvation (van Riggelen et al., 2005). c-fos expression is mainly controlled by the serum response element (SRE) motif in the c-fos promoter. Constitutive c-fos activity results from the inefficient expression of the ternary complex factor Net, which negatively regulates endogenous c-fos synthesis. Stable ectopic expression of Net results in a disappearance of c-fos protein from the AP-1 transcription complex. These data seem to place loss of Net and constitutive c-fos expression at the center of the transformation process of cervical carcinoma cells, although the induction of Net by TNF-α requires further study.

One target for the disturbance of TNF-α-mediated signaling could originate from polymorphisms in the TNF-α promoter. Indeed, a number of reports describe such polymorphisms as a risk factor for cervical cancer. In one study, CIN patients had a significantly higher frequency of TNF-α-308 low secretor genotypes compared to controls (Kirkpatrick et al., 2004). New significant associations between several TNF-α single-nucleotide polymorphisms and susceptibility to cervical cancer were reported very recently (Deshpande et al., 2005). In combination with the HLA DQ6

(DQA1*0102-DBQ 1*0602) haplotype, the TNF-α-11 haplotype increased the risk for cervical cancer significantly (Ghaderi et al., 2000, 2001). These data, which revealed a specific modification in the TNF-α promoter, may explain the higher risk for cervical cancer in specific populations, and underline studies demonstrating a hereditary factor in the development of cervical cancer (Magnusson and Gyllensten, 2000). However, they do not provide a reasonable explanation for those cervical cancers in which signaling pathways are blocked after exogenous addition of TNF-α. Possible polymorphisms of the TNF-α receptor which could explain this failure are described as a putative factor in autoimmune and inflammatory pathomechanisms (Csarzar and Abel, 2001); however, for cervical cancers they remain presently unexplored. Alterations in other genes which regulate the TNF-α-mediated signaling cascade may also contribute to the observed disturbances in cervical cancers.

These observations seem to underline a central role of TNF-α in the paracrine regulation of HPV transcriptional activity. They do not exclude, however, the possibility of an involvement of other cytokines or chemokines as regulatory factors. Indeed, TGF-β, IL-1, IL-6, IL-10, amphiregulin and others have been implicated in other studies (Braun et al., 1990; Woodworth et al., 1990, 1995; Gaiotti et al., 2000; Azar et al., 2004). Thus, nonresponsiveness to paracrine signals emerges as one hallmark of cervical cancer. Modifications to this signaling cascade emerge as one *conditio sine qua non* for malignant transformation of human cells containing HPV.

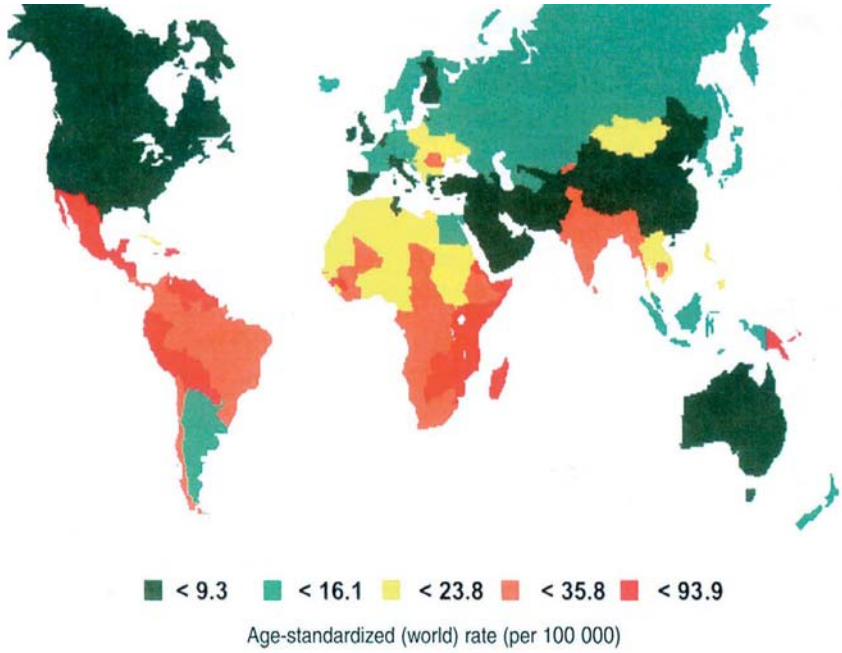

Fig. 5.12 The global age-standardized rate of cancer of the cervix. (Globocan 2002, Cancer Incidence, Mortality and Prevalence Worldwide, IARC CancerBase No. 5, Ferlay, J., Bray, F., Pisani, P., Parkin, D.M. (editors), version 2.0, IARCPress, Lyon, 2004.)

5.3 Cancers Linked to HPV Infections

5.3.1 Cancer of the Cervix

The global distribution of cancer of the cervix differs substantially between the developed and developing regions of the world (Fig. 5.12).

Today, cancer of the cervix probably represents the best-documented case of a human cancer caused by specific viral infections. The evidence has been documented in previous reviews (zur Hausen, 1996; IARC, 2006), and will not be repeated here in all details. The earliest data originated from the demonstration of HPV 16 and 18 DNA in the majority of cervical cancer biopsies (Dürst et al., 1983; Boshart et al., 1984), and the subsequent demonstration of the specific DNA integration pattern and the selective transcription of *E6*/*E7* genes (Schwarz et al., 1985; Yee et al., 1985). The identification of the same high-risk HPV types in precursor lesions of anogenital cancers lent further support for their role in this malignancy (Ikenberg et al., 1983; Crum et al., 1984). Subsequently, it was shown that the DNA of these viruses possesses transforming properties for murine cells (Watts et al., 1984; Yasumoto et al., 1986) and immortalizes human epithelial cells (Dürst et al., 1987; Pirisi et al., 1987, Kaur and McDougall, 1988). The *E6*/*E7* genes were sufficient for these transforming and immortalizing events (Bedell et al., 1987; Schlegel et al., 1988; Münger et al., 1989). Mice transgenic for the high-risk HPV oncoproteins developed carcinomas at sites determined by the selected promoters (Tinsley et al., 1992; Arbeit et al., 1993, 1996; Lambert et al., 1993; Greenhalgh et al., 1994; Sasagawa et al., 1994).

During the following years, the malignant phenotype of cervical cancer cells was shown to depend on continued expression of the two viral oncogenes (von Knebel Doeberitz et al., 1992, 1994; Lappalainen et al., 1994; Rorke, 1997, Alvarez-Salas et al., 1999). Even sole suppression of the E6 oncoprotein by specific peptide aptamers (Butz et al., 2000) or by selective expression and inhibition of HPV 16 E6 in HeLa cells by bovine papillomavirus type 1 E2 protein (Horner et al., 2004), resulted in apoptosis or senescence. Based on these experimental data, it was evident that the HPV infection must play an essential role in cervical cancer development and in the maintenance of malignant growth.

Support for a role of high-risk HPV in cervical cancer was obtained at a relatively late stage, from epidemiologic studies. Although the wide variation in methodological approaches during the late 1980s prohibited useful comparison of the available data (zur Hausen, 1989 b), such comparisons were made and resulted in the statement that "…the available data, although suggestive, do not allow further inferences on causality." (Bosch and Muñoz, 1989). A few years later, large case-control and prospective studies provided full support of the experimental data (Muñoz et al., 1992; Bosch et al., 1992, 1995). Today, numerous additional epidemiologic studies have confirmed these results (for a review, see IARC, 2006), leaving no doubt that cervical cancer is caused by high-risk HPVs. Recently, the definition of papil-

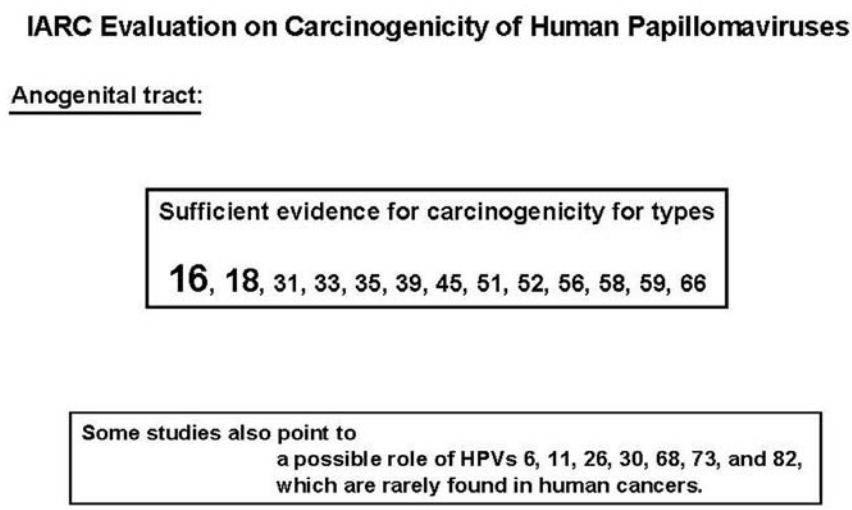

Fig. 5.13 Evidence for a role of specific papillomavirus types in causing human cancers. (Modified from IARC, Volume 90.)

lomavirus types linked to cervical cancer was attempted by an expert group at the IARC (IARC, 2006). Current knowledge on the carcinogenicity of HPV is summarized in Figure 5.13.

As shown in Figure 5.13, there exist a number of HPV types which have been rarely found in malignant tumors, and thus their carcinogenic potential remains to be proven.

The data on high-risk HPV infections in carcinomas of the anogenital tract, as well as of non-anogenital cancers, are summarized in Table 5.2.

The data in Table 5.2 reveal the high prevalence of HPV 16 infections in all of these cancers. Besides HPV 18, all other papillomavirus infections are relatively rare in anogenital and oral cancers; their relative distribution in cervical cancer is shown graphically in Figure 5.14. These data underline the dominating role of HPV 16 and, to a lesser degree, of HPV 18.

The typical precursor lesions of cancer of the cervix are CIN III. Most of these lesions again contain HPV 16 and, to a limited degree, other high-risk viruses. Low-grade CINs frequently contain also other HPV types, such as 6, 11, 34, 35, 40, 42,43, 44, 53, 54, 55, 61, 62, 70, 71, and 74.

One of the early hallmarks of high-risk HPV infection is the development of aneuploidy, most likely due to the uneven amplification of centrosomes (Duensing and Münger, 2003). This event precedes integration of viral DNA in high-grade lesions (Melsheimer et al., 2004). A number of chromosomal aberrations have been noted during the course of progression of the lesions. These involve various chromosomal sites, gains in chromosomes 3 q and deletions in 2 q33-q37 (Rao et al., 2004), loss of heterozygosity in 3p and 6 q (Acevedo et al., 2002), but also 9pter-p13 (Manolaraki et al., 2002), and deletions in 3p (Wistuba et al., 1997). It seems that one of the most consistent changes affects the fragile histidine triad (FHIT) locus on the short arm

Table 5.2 HPV-positivity in anogenital and non-anogenital human cancer

Type of cancer	Papillomavirus types involved	Percent HPV-positive
Cervical cancer	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 (26, 68, 73, 82)	> 95%
Vulva carcinoma		
basaloid	16, 18	> 50%
"warty"	16, 18	> 50%
keratinizing	16	< 10%
Penile carcinoma		
basaloid	16, 18	> 50%
"warty"	16, 18	> 50%
keratinizing	16	< 10%
Vaginal carcinoma	16, 18	> 50%
Anal cancer	16, 18	> 70%
Oral cavity and tonsils	16, 18, 33	~25%
Nail bed	16	~70%

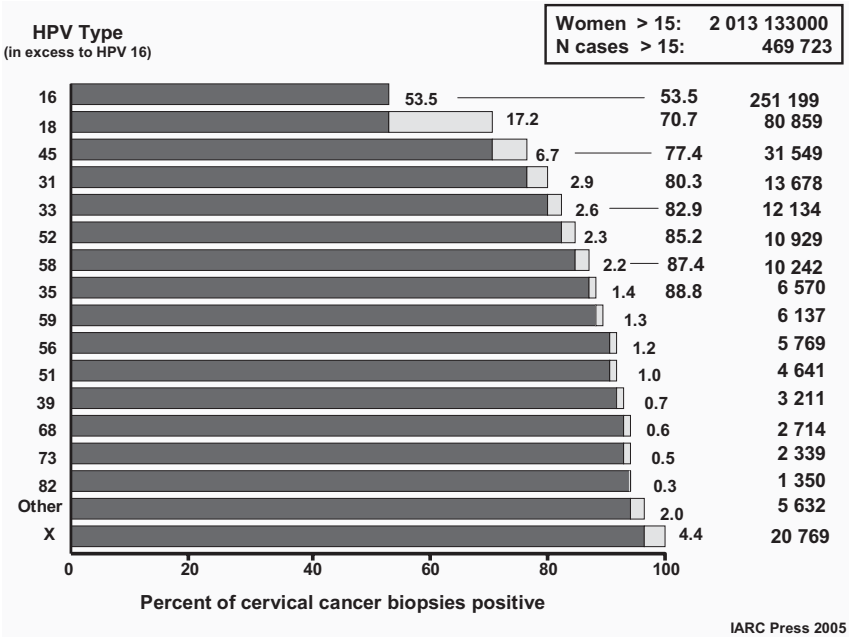

Fig. 5.14 Relative percentages of HPV types found in cervical cancer.

of chromosome 3 (Greenspan et al., 1997; Krivak et al., 2001). In addition to these changes, methylation of the HPV long control region and the flanking L1 gene were noted, low in dysplasia but increasing substantially in carcinomas (Kalantari et al., 2004). Interestingly, microcell-mediated transfer of chromosome 4 into HeLa cells suppresses telomerase activity and results in cellular senescence (Baksch et al., 2001). A mortality gene has been identified in this cell line, mapping to a 130 kb region of human chromosome 4q22-q23 (Bryce et al., 2002). At present, it is unclear as to what extent these chromosomal modifications affect the signaling cascades engaged in the control of HPV infections.

5.3.2
Penile Cancer

The relative positivity of penile cancers for persistent high-risk papillomavirus DNA is indicated in Table 5.2 (for a review, see IARC, 2006). Penile cancer represents a relatively rare malignancy in most parts of the world, although there exist interesting geographical correlations between incidences of cervical and penile cancer (Nandakumar et al., 2005). The histology of this tumor has been extensively reviewed; for relatively recent reviews, see Cubilla et al. (2000) and Bezerra et al. (2001).

A number of clinical observations have documented the malignant transition of genital warts (condylomata acuminata) into penile carcinomas. Some of the older observations were summarized in zur Hausen (1977). Although the majority of virus-positive penile cancers contain HPV 16 DNA, several tumors appeared to be only positive for HPV 6 or 11 DNA (Noel et al., 1992; Pilotti et al., 1993; Dianzani et al., 1998). This correlates with the observation that the invasively growing Buschke–Löwenstein tumors regularly contain DNA of these low-risk viruses (Boshart and zur Hausen, 1986).

5.3.3
Vulvar Cancer

In vulvar cancer the situation is very reminiscent of penile cancers. Typical squamous cell carcinomas of basaloid or warty histology occurring at younger age are commonly HPV-positive, whereas those occurring preferentially at higher age with lichen sclerosus-like lesions are mainly devoid of detectable HPV DNA (Neill et al., 1990; Hørding et al., 1991). To date, the relative rarity of these tumors has prevented a large-scale analysis of the latter for the possible persistence of cutaneous HPV types.

The detection of $p16^{INK4}$ expression seems to represent a useful marker to differentiate even premalignant vulvar and penile lesions for infection with high-risk HPV (Riethdorf et al., 2004; Ruhul Quddus et al., 2005; Rufforny et al., 2005).

The development of a specific T-cell response to HPV E7 antigenic epitopes occasionally results in the clearance of vulvar intraepithelial neoplasias III (Bourgault Villada et al., 2004); however, in the majority of cases it remains ineffective at this

stage of progression (Todd et al., 2004). Measurable antibody titers in premalignant lesions against HPV 16 VLPs were noted in 43 to 82% of cases, even in lichen sclerosus hyperplasia (Heim et al., 2005).

Comparative genomic hybridization of vulvar carcinomas revealed a pattern which corresponded to similar changes in cervical cancer (Huang et al., 2005). Gains were frequently found in 1q, 3q, 5p, and 8q. A high level of amplification was found in some of these tumors. Losses in 2q, 3p, 4p, and 11p were less consistent. Occasionally, integration of viral DNA is seen already in VIN II and III lesions (for a review, see Wentzensen et al., 2004).

5.3.4 Vaginal Cancer

Vaginal cancer accounts for approximately 2% of all female genital malignancies, and has a worse prognosis than cervical cancer (Goodman, 1998). Early tests of vaginal cancer revealed a high degree of HPV positivity (Kiyabu et al., 1989; Ikenberg et al., 1990). Approximately 30% of all cases had been treated for prior occurrence of other anogenital tumors, most often of the cervix (Daling et al., 2002). A prospective seroepidemiological study revealed that seropositivity for HPV 16 was associated with an increased risk of developing vulvar or vaginal cancers (Bjorge et al., 1997). Patients undergoing radiotherapy for advanced cervical or endometrial cancer bear a considerable risk of developing vaginal preneoplastic lesions. High-grade lesions are then commonly positive for HPV 16 DNA (Barzon et al., 2002).

In most other characteristics, vaginal HPV-associated cancers correspond to those described for vulvar cancer (see Section 5.3.3).

5.3.5 Perianal and Anal Cancer

Perianal and anal cancers represent about 4% of all anogenital tumors (Clark et al., 2004), and are highly linked to anogenital high-risk HPV infections, particularly to HPV 16. The rate of positive biopsies usually exceeds 70% (Palefsky et al., 1991; Zaki et al., 1992). The incidence of these cancers, as well as the incidence of the precursor lesions, is high in men who have sex with men (Chin-Hong et al., 2004), and is higher in HIV-positive patients (Haga et al., 2001). Increasing with the histological grade, genetic changes accumulate and a copy gain was mapped to chromosome 3q, a modification also frequently observed in cervical and vulvar cancer (Huang et al., 2005).

Besides cervical and about 25% of oropharyngeal cancers, perianal and anal cancers represent the third most important malignancy consistently linked to high-risk HPV infections.

5.3.6 Cancer of the Head and Neck

The role of papillomavirus infections in head and neck cancer was recently reviewed by Gillison (2004). Early suggestions for a role of HPV in oral squamous cell carcinomas were based on cytological features (presence of koilocytotic atypia) in precursor lesions and tumors (Syrjänen et al., 1983). The first demonstration of HPV 16 DNA in carcinomas and their precursor lesions was reported in 1985 (Löning et al., 1985). A number of additional publications subsequently confirmed the presence of HPV DNA in a subset of oral cancers, in particular in tumors arising in the Waldeyer's ring (Niedobitek et al., 1990; Snijders et al., 1992; Franceschi et al., 1996; Schwartz et al., 1998). Cancer of the oropharynx is causing substantial morbidity and mortality on a global scale, with an annual incidence rate of approximately 275 000 cases (Parkin et al., 2005). Tonsillar carcinomas reveal a particularly high rate of HPV positivity (Niedobitek et al., 1990; Snijders et al., 1992). The prevalence of these tumors increased between 1973 and 1995, from 1.9% to 2.7% per year in Caucasian and African-American males, whereas the prevalence of cancers at other oral sites, except for carcinomas of the tongue, remained unchanged (Frisch et al., 2000a; Gillison, 2004; Shiboski et al., 2005). HPV infections appear to be more common in carcinomas of the base of tongue than in mobile parts of the tongue (Dahlgren et al., 2004).

High rates of HPV positivity were reported from India (31–71%), where betel nut chewing emerges as a major contributing factor (Balaram et al., 1995; D'Costa et al., 1998; Nagpal et al., 2002; Koppikar et al., 2005). Figures from 20 publications of data mainly obtained from Northern America and Europe provide an average rate of HPV positivity of 23.5% (reviewed in Gillison, 2004). By analyzing 60 studies, Kreimer et al. (2005) reported HPV prevalence in 5046 head and neck cancers of 25.9 %, with a higher rate in oropharyngeal cancers (35.6% of 969) than oral squamous cell carcinomas (23.5% of 2646) and a surprisingly high rate of HPV-positive laryngeal squamous cell carcinomas (24% of 1.435). Based on these reviews and a large set of individual publications, a positivity rate of ~25% for oropharyngeal cancers appears to be a realistic assumption.

The majority of oropharyngeal cancers, positive for papillomavirus DNA, contain HPV 16 (~90%); in addition, HPV 18, 33 and 31 have also been detected. HPV 6 has been detected in rare cases (Kahn et al., 1994). Transcription of viral *E6* and *E7* genes has been consistently demonstrated within the positive cancers (Snijders et al., 1992; Wilczynski et al., 1998; Ke et al., 1999; Wiest et al., 2002; Balz et al., 2003), and the integration of viral DNA and overexpression of $p16^{INK4}$ revealed a similar pattern as in cervical carcinomas (Steenbergen et al., 1995; Hafkamp et al., 2003). Loss of the viral E2 region has also been reported (Mellin et al., 2002; Koskinen et al., 2003). These observations underline a similar role of HPV 16 infection in oropharyngeal cancers as established for cervical cancers.

The HPV-positive cancers of the oropharyngeal region reveal molecular, pathohistological and prognostic differences when compared to HPV-negative carcinomas at these sites. In the positive tumors, p53 is targeted by E6 (Balz et al., 2003), although

some tumors revealed the coexistence of HPV DNA and mutated p53 (Snijders et al., 1994; Sisk et al., 2002). Similarly, due to the targeting of pRb by E7, the expression of the former is decreased in HPV-positive oral tumors (Wilczynski et al., 1998; Mork et al., 2001; Wiest et al., 2002).

HPV-positive oropharyngeal cancers arise preferentially from the lingual and palatine tonsils, and reveal distinct basaloid pathology (Brandsma and Abramson, 1989; Fouret et al., 1997; Paz et al., 1997; Gillison et al., 2000). Some of the tumors are also poorly differentiated (van Houten et al., 2001; Klussmann et al., 2003; El-Mofty et al., 2003; Poetsch et al., 2003). The prognosis of HPV-positive cancers is significantly better than that of negative tumors. After adjustment for possible confounding factors, the risk of dying with the former cancer is 59% (Gillison et al., 2000) to 83% (Schwartz et al., 2001) lower than that for HPV-negative cancers of this region. Some studies also report a more favorable response to radiotherapy (Mellin et al., 2000; Lindel et al., 2001; Strome et al., 2002).

An elevated risk for acquiring HPV-positive oropharyngeal cancers is associated with increasing numbers of sexual partners, with a history of practicing oral sex, and also with a history of condylomata acuminata (Schwartz et al., 1998; Herrero et al., 2003; Rajkumar et al., 2003; Smith, E.M., et al., 2004). Seroepidemiologic case-control and prospective studies also revealed an increased risk of exposure to HPV 16 and the development of oral or oropharyngeal cancers (Schwartz et al., 1998; Mork et al., 2001; Herrero et al., 2003), even after adjustment for tobacco and alcohol use (Smith et al., 1998). A history of HPV-linked malignancy represents a risk factor for tonsillar cancer. Women older than 50 years with a history of cervical cancer, and husbands of women with in-situ or invasive cervical cancer also increase the risk for this malignancy (Hemminki et al., 2001). Fanconi's anemia represents a specific risk factor; the risk for head and neck cancers in this population is approximately 500-fold higher than in the general population (Kutler et al., 2003 a) with a high rate of HPV positivity (Kutler et al., 2003 b).

In-vitro immortalization of oral keratinocytes with HPV 16 has been achieved (Kang and Park, 2001). Although nonmalignant for immunocompromised mice, exposure of these cells to tobacco carcinogens enhanced expression of E6 and E7 and resulted in tumor formation in nude mice (Li et al., 1992; Kim et al., 1993). Yet, the relationship of HPV 16 infections to premalignant oropharyngeal lesions is presently less clear. Dysplasias adjacent to HPV-containing cancers in Fanconi lesions have been reported to be devoid of HPV DNA (Kutler et al., 2003 b). There exists a wide variation in HPV positivity of leukoplakias and oral dysplastic lesions in the literature (reviewed in Gillison, 2004). The situation may be analogous to that described for Epstein–Barr virus (EBV) in gastric and nasopharyneal cancers (see chapters 4.3.1.9 and 4.3.1.11), where virus infection seems to occur in dysplastic cells and apparently contributes to late steps in malignant conversion. Clearly, this point requires further investigation.

5.3.7 Other Cancers

Cancers of several additional sites have been described as containing papillomavirus DNA. These involve cancers of the breast, prostate, lung, colon and rectum, ovary, bladder, nasal, sinonasal and conjunctival cancers, larynx, and esophagus. The relevant evidence is discussed briefly in the following sections.

5.3.7.1 Breast Cancer

Several reports have documented negative findings for HPVs 6, 11, 16, 18, 31, 33, and 35 (Ostrow et al., 1987; Wrede et al., 1992; Bratthauer et al., 1992; Czerwenka et al., 1996) in breast cancer. In contrast, a number of laboratories have claimed to find HPV 16 (Di Leonardo et al., 1992), HPV 33 (Yu et al., 2000), HPV 11, 16 and 18 (Liu et al., 2001), and HPV 16 and 18 (Damin et al., 2004), in several of the investigated breast cancer biopsies. Recently, de Villiers et al. (2005) cloned several HPV types from ductal breast carcinomas and from corresponding samples of the mamilla of the same patients. The most prevalent types were HPV 11 and 6. At present, the significance of these findings is presently difficult to assess, but further studies should clarify the existing situation.

5.3.7.2 Prostate Cancer

Similar to cancer of the breast, the data on HPV presence in prostate cancer are conflicting. A few case reports have claimed HPV DNA in these tumors (Serth et al., 1999; Carozzi et al., 2004) or elevated odds ratios for an association between HPV 16 antibodies and prostate cancer (Hisada et al., 2000; Dillner et al., 1998), whereas this was not confirmed in other studies (Dennis and Dawson, 2002; Rosenblatt et al., 2003). It is interesting, however, to note that prostate cancer and sexually transmitted diseases reveal a clear-cut positive association, although responsible factors have not yet been identified (Key, 1985; Hayes at al., 2000; Strickler and Goedert, 2001; Rosenblatt et al., 2001; Taylor et al., 2005; Fernández et al., 2005).

Based on the available data, a relationship between HPV infection and prostate cancer is unlikely. The positive serologic findings may result from a generally increased risk for prostate cancer in individuals exposed to sexually transmitted diseases.

5.3.7.3 Lung Cancer

Although an early study revealed HPV 16 DNA in an anaplastic carcinoma of the lung (Stremlau et al., 1985), it seems that high-risk HPVs are only rarely found in lung cancers (Stoler et al., 1991; Shamanin et al., 1994 b; Szabo et al., 1994; Brouchet

et al., 2005). Yet, occasionally HPV 16- and HPV 18-positive tumors are found. An exceptionally high rate of HPV 16- and 18-positive lung cancers has been reported among non-smoking women from Taiwan (Cheng et al., 2001).

In rare lung cancers developing in patients with recurrent laryngeal and bronchial papillomatosis, however, several reports describe the presence of mainly HPV 11, but also of HPV 6 DNA, in part integrated and transcribed in the malignant lesions (Byrne et al., 1987; Bejui-Thivolet et al., 1990; Guillou et al., 1991; DiLorenzo et al., 1992; Rady et al., 1998; Cook et al., 2000; Lele et al., 2002; Xu et al., 2004). Thus, for this type of cancer there exists good evidence for a role of the low-risk anogenital types 11 and 6. HPV 11 represents the prevailing type in laryngeal papillomatosis.

5.3.7.4 **Colon and Rectum Cancers**

In contrast to anal and perianal cancers, for cancers of the rectum and colon there exists no convincing evidence for an involvement of papillomavirus infections. Except for three reports finding HPV 16, 18 or 45 DNA in a substantial percentage of colon cancers at low copy numbers and in none of 10 control tissues (Sayhan et al., 2001; Buyru et al., 2003; Bodaghi et al., 2005), a number of other publications reported negative results (reviewed in IARC, 2006). Thus, the evidence for HPV involvement in these tumors is far from conclusive.

5.3.7.5 **Ovarian Cancer**

The majority of attempts to demonstrate HPV DNA in primary ovarian cancers have been unsuccessful (reviewed in IARC, 2006). It is of interest, however, that occasionally HPV 16-positive CIN III lesions may spread to the endometrium, fallopian tubes and ovaries (Mai et al., 1996; Pins et al., 1997; Manolitsas et al., 1998), while HPV 6 and 11 may even colonize regions of squamous metaplasia of the endometrium and cause adenoacanthomas (Sherwood et al., 1997; O'Leary et al., 1998).

5.3.7.6 **Bladder Cancer**

There exists no clear-cut evidence for a role of papillomavirus infections in transitional cell carcinomas of the bladder. Most studies analyzing these tumors produced negative results (reviewed in IARC, 2006). In several other PCR-based studies the rate of positivity was about 3%.

Squamous cell carcinomas of the bladder, mainly occurring in *Schistosoma*-endemic countries, were more frequently reported to be HPV-positive. Up to 8% were reported to be positive for HPV 6, 11 or 16 (Kerley et al., 1991; Anwar et al., 1992; Wilczynski et al., 1993; Maloney et al., 1994; Westenend et al., 2001).

In carcinomas of the urethra, HPV 6 and HPV 16 DNA has been discovered repeatedly (Grussendorf-Conen et al., 1987; Mevorach et al., 1990; Wiener et al., 1992; Wiener and Walther, 1994).

5.3.7.7 Nasal, Sinonasal and Conjunctival Cancers

The rare inverted papillomas of the nasal cavity and paranasal sinuses frequently start to grow invasively and convert into malignant tumors in up to 13% of cases (Bernauer et al., 1997). Several of these tumors contain HPV 11 or 6 (Syrjänen et al., 1987; Respler et al., 1987; Kashima et al., 1992 b; Harris et al., 1998) or HPV 57 DNA (de Villiers et al., 1989; Wu et al., 1993; Ogura et al., 1996). Occasionally, also HPV 16 and 18 DNA has been noted in malignant tumors of these sites (Furuta et al., 1992; Buchwald et al., 1997, 2001).

Thus, at these sites HPV 11, 57 and 6 (all low-risk viruses) seem to pose a higher risk for invasive proliferations in comparison to infections by these viruses at other locations. It seems that HPV 6 and 11 play a role in a small percentage of these tumors, and that HPV 16 is also found in a small number of additional biopsies of these cancers.

Several reports have described the presence of HPV 16 and 18 DNA in conjunctival dysplasias and invasive cancers (McDonnell et al., 1989; Saegusa et al., 1995; Scott et al., 2002; Moubayed et al., 2004). One group failed to confirm these findings, but reported HPV 6 and 11 DNA in a substantial percentage of conjunctival papillomas (Eng et al., 2002). One recent publication claimed a very high rate (86%) of squamous cell carcinomas of the conjunctiva positive for epidermodysplasia verruciformis-related papillomavirus types (Ateenyi-Agaba et al., 2004). Related viruses (HPV 5, 20, and 23) were also found in an ocular syringoma (Assdoullina et al., 2000). The available results point to the need to expand the studies on ocular carcinomas for papillomavirus DNA. Most likely as the consequence of the ongoing AIDS epidemic, dysplastic lesions and epithelial tumors of the conjunctive account for approximately 2% of all tumors in Tanzania (Moubayed et al., 2004). Although suggestive, further investigations are required before the involvement of HPV infections may be implied in the etiology of these tumors.

5.3.7.8 Cancer of the Larynx

Laryngeal papillomatosis is caused by HPV 11 and to a lesser degree also by HPV 6. It affects mainly children, but extends in some cases into adult age. Recurrent laryngeal papillomatosis may represent a life-threatening condition. Occasionally – though rarely – the lesions may descend into the bronchial tree, reveal dysplastic histology and convert into squamous cell carcinomas (reviewed in zur Hausen, 1977). Thus, carcinomas of the larynx, occurring predominantly at a higher age, emerged as a possible candidate for a papillomavirus etiology, although other factors such as smoking are also involved.

The first reports on the presence of papillomavirus in laryngeal cancers appeared in 1985 and 1986 (Abramson et al., 1985; Brandsma et al., 1986; Kahn et al., 1986; Scheurlen et al., 1986). Whereas three of these reports found HPV 16 DNA in the cancer biopsies, Kahn et al. described a novel HPV type (HPV 30) in a laryngeal carcinoma. In subsequent years a large number of positive reports were published, although some others failed to find HPV DNA in these tumors (Lindeberg and Krogdahl, 1999; for reviews, see Franceschi et al., 1996; Hobbs and Birchall, 2004). From published data it appears that the figure of 24% of laryngeal carcinomas positive for HPV (Franceschi et al., 1996) is too high, although substantial variations are reported in the literature. A figure of 10% or less is probably more realistic.

Although some of the HPV-positive laryngeal cancers contain HPV 11 or 6 DNA (Brandsma and Abramson, 1989; Zarod et al., 1988; Lie et al., 1996; Lin et al., 1997; Rady et al., 1998), the majority of positive tumors contained HPV 16 DNA.

Laryngeal carcinoma emerges as an additional type of cancer, where a subset of these tumors is linked mainly to high-risk papillomavirus infections, although a fraction appears to be due to the low-risk types HPV 11 and 6.

5.3.7.9 **Cancer of the Esophagus**

Cancer of the esophagus shows wide variations in its geographic distribution, and has been linked to a number of environmental risk factors, including alcohol consumption, smoking, consumption of very hot beverages, fermented fish, and other dietary sources of nitrosamines and micronutrients (Rogers et al., 1995; Castellsague et al., 1999, 2000; Onuk et al., 2002; Ke et al., 2002). The first reports which attempted to link papillomavirus infections to these malignancies were stimulated by the publication of Jarrett et al. (1978), which claimed an interaction of a specific papillomavirus infection with an environmental carcinogen in esophageal cancer of cattle. In 1982, Syrjänen et al. postulated a role for HPV in human esophageal cancers based on the histological detection of koilocytotic cells and immunoperoxidase staining of cells from esophageal papillomas for viral group-specific antigens.

During the following years a large number of remarkably controversial publications appeared, some claiming a high incidence of positive tumors, particularly in high-risk regions of China (Chang et al., 1990; Li et al., 2001; de Villiers et al., 2004 b), whereas other groups reported basically negative results (Loke et al., 1990; Polyak et al., 1998; Peixoto Guimaraes et al., 2001). The data were even controversial in different studies by the same group by analyzing samples from different geographic regions (Chang et al., 1991, 1992). Since in some additional studies novel HPV types were isolated from esophageal cancers and characterized (Togawa and Rustgi, 1995; West et al., 1996; de Villiers et al., 1999; Lavergne and de Villiers, 1999), the likelihood of inadvertent contaminations due to handling procedures should be extremely small.

In esophageal cancer of cattle linked to BPV 4 infections it appears that persistence of viral DNA is not required for the progression and maintenance of the

malignant state (Campo et al., 1985), although C127 mouse cells transformed by BPV 4 DNA maintain amplified and rearranged copies of the BPV 4 genome (Smith and Campo, 1989).

In view of the large number of additional controversial manuscripts that have been published during the past 20 years, two conclusions can be drawn. First, clearly, papillomavirus infections occur within the human esophagus and we may not have identified all types affecting these tissues. Second, there exists an urgent need for broad-based experimental and epidemiologic studies to clarify the role of these infections in the etiology of esophageal cancers and their possible interaction with other environmental carcinogens.

5.3.8
Cutaneous Papillomavirus Infections and Skin Cancer

The first hints for a role of papillomavirus infections in cancers of the skin originated from studies in patients with the rare hereditary disease epidermodysplasia verruciformis (EV) (reviewed in Jablonska and Majewski, 1994). Though rare, this condition occurs worldwide, starting frequently in the age group between 5 and 8 years. The lesions are frequently barely recognizable as papillomas, and usually form reddish plaques. Within the following 20 to 30 years, about one-half of these patients reveal progression of the lesions, initially as actinic keratoses and Bowenoid changes, but subsequently they may convert to squamous cell carcinomas. The tumors are commonly localized at light-exposed sites, such as the forehead, the dorsal hand or on the arms of these patients.

The HPVs found in these lesions belong into the genus β of HPV, and comprise more than 20 different genotypes. The same patient frequently contains multiples of these virus types. Most of the squamous cell carcinomas contain HPV 5, but some harbor a closely related genotype HPV 8. Several other types (e.g., HPV 14, 17, 20, 47) have also been isolated from such tumors (reviewed in Orth, 2005). EV-type HPVs are widely spread in the general population and occur here without induction of apparent lesions (Antonsson et al., 2003).

Carcinomas in EV patients commonly contain high copy numbers of episomal HPV 5 DNA, which is also present in metastases derived from these tumors. Interestingly, most of the persisting viral genomes revealed some rearrangements or deletions (Ostrow et al., 1982; Yabe et al., 1989). Morphological transformation of murine C127 cells has been achieved with HPV-5. The transformed cells retained episomal copies of viral DNA (Watts et al., 1984). The *E6* gene of HPV 5, 8, and 47 also induced morphological changes in rat 3Y1 cells (Hiraiwa et al., 1993). Joint transfection of constructs containing the SV40 promoter/enhancer and HPV 8 *E7* or *E6* and *E7* genes and the activated *Ha-ras* gene resulted in tumorigenic transformation of primary rat embryo fibroblasts (Nishikawa, 1994). In addition, mice transgenic for the complete early region of HPV 8 under the control of the keratin-14 promoter developed in 91% single or multifocal papillomas and in 6% squamous cell carcinomas (Schaper et al., 2005). The cancer cells expressed *E2, E7* and *E6* genes. In benign lesions, HPV 5 DNA transcription is differentiation-dependent (Haller et al., 1995).

All of these data suggest that HPV 5 and 8 – and probably also the types 14, 17, 20, and 47 – possess oncogenic potential, specifically expressed in the rare hereditary condition of EV. It is interesting to note that the susceptibility to papillomavirus infections of EV patients seems to affect selectively members of the genus β, but not other HPV genera. Two genetic modifications have been described in EV patients mapping to the chromosome regions 2p21-p24 and 17q25 (Ramoz et al., 1999, 2002). Nonsense mutations were identified in two adjacent genes, *EVER1* and *EVER2*, that are associated with the disease. Their gene products seem to represent integral membrane proteins and are localized in the endoplasmic reticulum (Ramoz et al., 2002).

EV-like lesions have also been observed in immunosuppressed patients following organ transplantation (Lutzner et al., 1980; Gassenmeier et al., 1986; Rüdlinger et al., 1986). These patients, however, also develop warts caused by other types of HPV (Ingelfinger et al., 1977; Schneider et al., 1983). A different spectrum of HPV types in cutaneous and mucosal lesions emerged in HIV-infected and immunosuppressed patients (Greenspan et al., 1988; Milburn et al., 1988). Oral warts were preferentially found to contain HPV 7, also known as "butcher's wart" virus.

Early studies on non-melanoma skin cancers were performed with specific probes of anogenital HPV types. By using consensus primers which cover a broad spectrum of HPV types the results changed substantially. A large number of different, in part novel, HPV types was found in benign and malignant lesions, initially in immunosuppressed patients (Shamanin et al., 1994a, 1996; Berkhout et al., 1995, 2000; de Villiers et al., 1997). The use of different primer combinations and different sensitivities and specificities of methodological approaches seems to account for some reported discrepancies between individual studies. In squamous cell carcinomas of renal allograft recipients, the detection rate was particularly high, reaching up to 90% of all samples tested (de Villiers et al., 1997). In immunocompetent patients suffering from squamous cell carcinomas of the skin, most reports found a positive association with HPV infections (Boxman et al., 2000). The rate of positivity varied between 27% and 65 % (Shamanin et al., 1996; Harwood et al., 2000; Forslund et al., 2003; Iftner et al., 2003; Meyer et al., 2003). It should be noted, however, that even unaffected skin of immunocompetent individuals revealed a relatively high percentage of HPV-positive samples, particularly when plucked hair or cutaneous tape-stripped biopsies were analyzed (Boxman et al., 1997, 2000; Astori et al., 1998; Forslund et al., 2004), although they were commonly less positive than squamous cell carcinomas. Most studies analyzing basal cell cancer biopsies found viral DNA in the range of normal skin biopsies.

Even serological results seem to point to a specific role for cutaneous HPV infections in squamous cell cancer of the skin, since seroreactivity to five EV HPV types (5, 8, 15, 20, and 24) was significantly increased in patients with this malignancy (Feltkamp et al., 2003).

Although persuasive, the role of cutaneous HPV types in squamous cell carcinomas is by no means settled. One obvious dilemma originates from quantitative evaluations of the viral copy number within positive cancers. In virtually all analyses it is far beyond one DNA copy per tumor cell. In addition, HPV positivity in swab

samples from the top of these tumors was higher than in biopsied material (Forslund et al., 2004). Although HPV types 20, 23, 38, and two newly identified types were more prevalent in one series (de Villiers et al., 1997), this preponderance was less pronounced in other studies. Thus, the role of these viruses clearly does not seem to correspond to that of high-risk anogenital HPV, which commonly persist in higher copy numbers in each tumor cell.

A potential mechanistic explanation for these observations could originate from studies demonstrating an effect of specific cutaneous papillomavirus types in preventing apoptosis in damaged cells. In 1999, Purdie et al. reported that the promoter of a cutaneous HPV type (HPV 77) is stimulated by UV-irradiation and that this responsiveness is mediated through a consensus p53 binding site. Two publications during the year 2000 described the inhibition of UV-induced apoptosis by the E6 protein of several cutaneous papillomavirus types, and of HPV 18 (Jackson et al., 2000; Jackson and Storey, 2000). This appears to be mediated by an E6 deregulation of the p53-dependent transactivation of pro-apoptotic proteins upon UV-B irradiation (Giampieri et al., 2004). A postulated role of this effect in the development of squamous cell carcinomas of the skin finds some further support in the observation that reduced levels of apoptotic activity in squamous cell carcinomas of the skin, but not in basal cell carcinomas, correlate with the detection of cutaneous HPV (Jackson et al., 2002). In addition, the repair of UV-induced thymine dimers is compromised in cells expressing the E6 protein of HPV types 5 and 18 (Giampieri and Storey, 2004).

A tentative scheme of the indirect function by which cutaneous HPVs may contribute to the development of squamous cell carcinomas of the skin is illustrated in Figure 5.15. This scheme emphasizes a joint and synergistic function between persisting infections by these viruses of the skin and UV-exposure at sun-exposed sites. Sunburn episodes in the past, especially at the age of 13 to 20 years, were reported to be associated with an enhanced risk of EV-HPV DNA persistence (Termorshuizen et al., 2004).

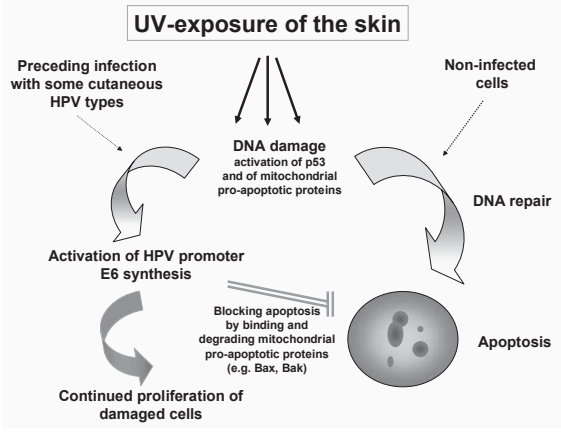

Fig. 5.15 Tentative scheme of the contribution of cutaneous papillomavirus infections to the development of squamous cell carcinomas. In non-infected cells, DNA damage is either repaired or leads to apoptosis. Specific HPV types prevent apoptosis due to interactions with proapoptotic proteins and permit continued proliferation of cells carrying UV-induced DNA modifications.

Interestingly, similar to high-risk HPV-induced high-grade and malignant lesions, both squamous cell carcinomas of the skin and also basal cell carcinomas frequently reveal mutations and functional inactivation of $p16^{INK4}$ and $p14^{ARF}$ (Soufir et al., 1999; Saridaki et al., 2000; Brown et al., 2004).

At least one more aspect of cutaneous papillomavirus infections deserves attention: HPV 38, a type relatively often found in squamous cell carcinomas of the skin, displays transforming properties. Its E7 protein inactivates the tumor suppressor pRb and induces loss of the pRb-mediated G_1/S transition control of the mitotic cycle. HPV 38 induces long-lasting proliferation of primary human keratinocytes and thus, seems to resemble to a certain degree high-risk anogenital HPV infections (Caldeira et al., 2003).

In summary, although a number of observations suggest that cutaneous papillomavirus infections may play a (probably indirect) role in squamous cell cancer of the skin, numerous questions remain unanswered, and the role of these viruses in tumorigenesis is clearly not resolved.

5.4 The Role of Cofactors

5.4.1 Non-Infectious Cofactors

5.4.1.1 Smoking

During the past two decades it has been suspected that, besides HPV, other factors might also interact in the etiology of anogenital cancers. Indeed, the first observations on a role of smoking in cervical cancer date back to the 1960s and 1970s (Naguib et al., 1966; Winkelstein, 1977, 1990). During the past 15 years, many studies have shown a significant association between smoking and cervical cancer or its precursor lesions (reviewed in Plummer et al., 2003). These studies were conducted by either adjusting for HPV infection or by excluding HPV-negative cases (reviewed by Haverkos et al., 2003). The majority of case-control and prospective studies revealed an excess risk for ever smoking of 2.17, for current smokers of 2.30, and for ex-smokers of 1.80 (Plummer et al., 2003). Smoke constituents and mutagenic factors in the vaginal mucus of smokers were detected as early as the 1980s (Sasson et al., 1985; Holly et al., 1986; Schiffman et al., 1987), and increased levels of DNA adducts were subsequently found in the cervix of smoking women (Ali et al., 1994). Prospective studies also revealed a prolonged duration of HPV infections and a lower rate of clearing these infections in present or former smokers, resulting in a significant increase in persisting HPV infections (Giuliano et al., 2002; Minkoff et al., 2004). These experimental and epidemiologic data support the concept that exogenous mutagens contribute and moderately elevate the risk for cervical cancer and its precursors in addition to HPV infection.

5.4.1.2 Hormones and Hormonal Contraceptive Use

In 1987, Gloss and colleagues described a sequence element in the promoter of HPV 16 which confers strong inducibility of the p97 promoter by dexamethasone. The same element was active in HeLa cells. Subsequently, other groups demonstrated the oncogenic transformation of primary cells with a combination of HPV 16 DNA and the activated form of the human *H-ras* oncogene or v-fos by the glucocorticoid hormone dexamethasone (Pater et al., 1988; Crook et al., 1988; Dürst et al., 1989). Progesterone and glucocorticoid response elements were found in high- (HPV 16 and 18) and low-risk (HPV 11) human papillomaviruses (Chan et al., 1989). The induction of the HPV 31 promoter is differentiation-dependent since monolayer cultures are insensitive to this treatment, whereas growth of infected cells in semisolid media results in promoter activation (Bromberg-White et al., 2003)

The potential carcinogenic effects of estrogens have been suspected and demonstrated experimentally for more than half a century (Emge et al., 1949; Kirkman and Bacon, 1950). 16-Alpha-hydroxyestrone has been recognized as one of the metabolic products of estrogen, forming covalent linkages with amino groups on proteins and nucleotides (Bucala et al., 1982; Bradlow et al., 1986). The most estrogen-sensitive cells at the transformation zone are at greatest risk for HPV-related cancers. Explants from the transformation zone are able to promote 16-α-hydroxylation of estradiol (Auborn et al., 1991). Since immortalization of cervical and foreskin epithelial cells by HPV 16 is greatly enhanced by 16-α-hydroxyestrone, Auborn and colleagues proposed a cooperative effect for cell transformation. Approximately 90% of cervical cancers arise at the periphery of the transformation zone. It should be noted that proliferating nondifferentiated cells almost reach the surface of the epithelial layer at these sites, and are therefore most easily infected. Thus, it appears that hormonal effects and the specific localization of infectable cells close to the surface probably contribute jointly to the high rate of cervical cancer in comparison to the low rate of penile cancer in males. Available data on the 16-α-hydroxylation of estrogens and the possible relationship to cervical neoplasias have been reviewed recently (de Villiers, 2003).

In HPV 18 transgenic mice, carrying the bacterial β-galactosidase gene under the control of the URR, estradiol and progesterone activated the viral URR (Michelin et al., 1997). A similar model of K14 HPV 16 transgenic mice demonstrated preferential neoplastic progression of the transformation zone by a combination of low-dose estrogen and low level of HPV oncogene expression (Elson et al., 2000). In these transgenic mice exposure to 17α-estradiol increased the proliferation of cervical and vaginal epithelial cells and carcinogenesis, accompanied by an up-regulation of E6/E7 oncogene expression (Arbeit et al., 1996). In HPV 16 transgenic mice, estrogen contributes to the onset, persistence, and malignant progression of cervical cancer (Brake and Lambert, 2005).

These experimental data are less clearly supported by epidemiologic studies (reviewed in de Villiers, 2003; Green et al., 2003; Smith et al., 2003). The latter have been conducted as retrospective case-control studies or as prospective studies in long-term contraceptive use in women. In women using oral contraceptives for

more than 5–10 years, a slightly increased risk for cervical cancer was noted (Ylitalo et al., 1999; Hildesheim et al. 2001); this was confirmed in nine case-control studies from the International Agency for research of Cancer in Lyon (Franceschi, 2005). Other studies did not find any association with pre-neoplastic or malignant disease in current and long-term oral contraceptive users (Krüger-Kjaer et al., 1998; Lacey et al., 1999; Shapiro et al., 2003; Giuliano et al., 2004). Among prospective studies, one investigation reported a significantly increased risk for women who used oral contraceptives for more than 8 years (Deacon et al., 2000). In two other studies, data from current contraceptive users were analyzed and no increased risk was found for the incidence of cervical intraepithelial lesions (Moscicki et al., 2001; Castle et al., 2002).

Combined with the experimental data, these results point to a role of long-term hormonal contraceptive use in stimulating gene activity of persisting papillomavirus infections, and probably also by enhancing the duration of HPV persistence. This in turn seems to be responsible for the slightly elevated risk for pre-neoplastic lesions and their eventual malignant progression.

5.4.1.3 **Parity**

A number of reports have described an elevated risk for cervical cancer in multiparous women; however, based on the few studies which analyzed only HPV-positive women the association remains inconsistent. Although some retrospective studies reported a significant association between multiparity and ASCUS (atypical squamous cells of unknown significance), squamous intraepithelial lesions and cancer (Kjaer et al., 1996; Hildesheim et al., 2001; Muñoz et al., 2002), other studies failed to confirm this (Krüger-Kjaer et al., 1998; Giuliano et al., 2004).

In view of the described effects of estrogens and progesterone, however, it is likely that multiparity will lead to an increased risk for pre-neoplastic and malignant lesions of the cervix in high-risk HPV-positive women.

5.4.1.4 **Nutrients**

The role of nutrition in cervical cancer development or cervical cancer prevention has been studied repeatedly. Some retrospective studies yielded controversial results. An inverse relationship between the consumption of vegetables and fruits and the risk for cervical cancer was reported from India (Rajkumar et al., 2003). A study analyzing a possible link between vitamins A, C, E, β-carotene, folate, and zinc and the risk for low- and high-grade intraepithelial lesions in Portland, USA, failed to reach significance (Wideroff et al., 1998). Higher levels of serum retinol seemed to show some protective effect for cervical intraepithelial neoplasias in one study (French et al., 2000), but a second investigation failed to demonstrate this effect (Ho et al., 1998). In the latter study, only vitamin C was associated with a significant reduced risk of disease. An elevated risk without statistical significance for cervical

cancer has been reported for women with low serum and red blood cell levels of folic acid (Weinstein et al., 2001).

Among prospective studies no link between serum retinol and serum α-tocopherol and cervical cancer was noted in one investigation (Lehtinen et al., 1999), whereas a second study reported significant links between serum β-carotene and α-tocopherol with transient HPV infections in contrast to persistently HPV-infected women (Giuliano et al., 1997). The same group were unable to detect any association with serum folate, vitamin B_{12} or homocysteine (Sedjo et al., 2003). A protective effect of cis-lycopene, high vegetable consumption and lutein intake was also reported (Sedjo et al., 2002 a,b).

Two Phase II clinical trials have been completed to study the effect of folic acid in cervical cancer prevention (Butterworth et al., 1992; Childers et al., 1995), but both failed to reveal any protective effect. Similarly, five Phase II/III trials on supplemental β-carotene failed to reveal any significant effect (de Vet et al., 1991; Fairley et al., 1996; Romney et al., 1997; Mackerras et al., 1999; Keefe et al., 2001). One of three trials using all-*trans* retinoic acid reported a significant effect of three administrations of the compound on regression of CIN II lesions (Meyskens et al., 1994). In contrast, two other studies failed to demonstrate any effect (Follen et al., 2001; Alvarez et al., 2003).

As a baseline of these data, clear-cut evidence for a role of nutritional constituents in preventing premalignant or malignant cervical lesions is, at present, still missing.

5.4.2 Infectious Cofactors

5.4.2.1 Herpes viruses

Cytological and histological changes typical of herpes simplex virus (HSV) infections, when frequently observed in women with cervical lesions, provided an early hint for the possible involvement of HSV type 2 (HSV-2) in cervical cancers (Naib et al., 1969). These initial suggestions were supported by data from early seroepidemiological studies conducted by three different groups (Rawls et al., 1969; Nahmias et al., 1970; Royston and Aurelian, 1970). These data seemed to be supported by the demonstration of an HSV fragment in only one cervical carcinoma biopsy (Frenkel et al., 1972), and studies which revealed the transforming properties of partially UV-inactivated HSV-2 for hamster cells (Duff and Rapp, 1971).

These positive findings were, however, not confirmed by other groups: zur Hausen et al. (1974 a,b) and Pagano (1975) were unable to detect HSV-2 DNA in cervical cancer biopsies. An early large seroepidemiologic prospective study also failed to confirm the previously reported positive data (Vonka et al. 1984 a,b).

The discovery of a mutagenic activity of HSV infections for host cell DNA and the ability of the virus to induce selective amplification of persisting polyoma- or papillomavirus DNA (Schlehofer and zur Hausen, 1982; Schlehofer et al., 1983; Schmitt et al., 1989; Heilbronn and zur Hausen, 1989; Clarke and Clements, 1991) permitted

the interpretation of an indirect contribution of HSV infections to human carcinogenesis.

Even today, epidemiologic data on a possible role of HSV-2 in cervical cancer remain inconsistent and difficult to interpret. Indeed, two recently conducted large studies arrived at conflicting results. Lehtinen et al. (2002) pooled data and specimens from three population-based Nordic cohorts, containing a total population of more than 500 000 women. They found no difference in HSV-2 seroprevalence of cervical cancer cases and controls, even after adjustment for HPV 16/18/33 VLP antibodies and for cigarette smoking. A study conducted by Smith et al. (2002), which included data from seven case-control studies, reported a significant association of HSV-2 seroantibodies with squamous cell carcinoma of the cervix (odds ratio 2.2). However, by combining data on seroreactivity against HSV-2 in cases and controls obtained during the past 20 years, the majority of these studies failed to detect any significant association. Although some studies reported a high prevalence of HSV DNA in neoplastic cervical material by PCR technology (Koffa et al., 1995; Han et al., 1997), others failed to confirm these findings (Vecchione et al., 1994; Tran-Thanh et al., 2003; Yang et al., 2004).

Thus, although plausible due to a set of experimental data, a role for HSV infection in cervical neoplasias remains open and is, in all probability, at best marginal.

5.4.2.2 **Chlamydia trachomatis**

Chlamydia trachomatis represents a very common bacterial, sexually transmitted infection. These infections frequently remain asymptomatic and may persist for months or even years (Stamm, 1999; Peipert, 2003; Stephens, 2003). Chronic infection may result in pelvic inflammatory disease and squamous metaplasia.

Early suggestions for a possible role of chlamydial infections in cervical cancer date back to 1971 and the following decade (Alexander, 1973; Schachter et al., 1975; Kalimo et al., 1981). The present evidence is mainly based on seroepidemiologic and epidemiologic observations. Most of these studies reported a significant association of *Chlamydia* seropositivity and squamous cell carcinomas of the cervix. The two large epidemiologic studies analyzing also the role of HSV-2 in cervical cancer reported a significant increase of seropositivity in cervical carcinoma patients (Koskela et al., 2000; Smith, J.S., et al., 2004). Since 1991, more than 10 additional studies have been published supporting the seroepidemiologic evidence for an involvement of chlamydial infections in cervical cancer, though most also found high-risk HPV reactivity.

In addition to the serologic findings, *Chlamydia trachomatis* DNA in pre-diagnostic Pap smears was also associated with a subsequent risk for cervical cancer or low-grade squamous intraepithelial lesions (Wallin et al., 2002; Golijow et al., 2005). A mechanism by which chlamydial infections may contribute to cervical carcinogenesis has recently been suggested by Sillins et al. (2005). In their study, the most significant risk factor for the persistent presence of HPV DNA was a self-reported history of previous *Chlamydia trachomatis* infection. This raises the possibility that

chlamydial infections facilitate HPV persistence and may thus contribute indirectly to cervical carcinogenesis.

5.4.2.3 Human Immunodeficiency Virus

Human immunodeficiency viruses (HIV) types 1 and 2 can be considered as the classical indirect carcinogens. Immunosuppression induced by these infections results in an increased incidence of tumors caused by other viruses [see Chapter 4, Sections 4.3.1 (EBV) and Section 4.4.1 (HHV-8)]. The majority of many epidemiological studies also indicate an increased incidence of precursor lesions of anogenital neoplasias in these patients (e.g., Sun et al., 1995; Palefsky, 1995, 1999; Jamieson et al., 2002; Strickler et al., 2005). This is supported by a number of reports on an increased incidence of cervical cancer in these patients (e.g., Goedert et al., 1998; Frisch et al., 2000 b; Dorucci et al., 2001; Mbulaiteye et al., 2003).

It is possible that the effect of HIV in cervical neoplasias is not solely mediated by the virus-induced immunosuppression, but may also be exerted by a co-activation of the HPV promoter by HIV *tat* (Vernon et al., 1993; Buonaguro et al., 1994). HIV infections emerge, however, as one of the important cofactors for cervical neoplasias.

5.4.2.4 Other Infections

A number of other viral and nonviral agents have been suspected of playing a role as cofactors in cervical carcinogenesis. Among the herpesvirus family, human cytomegalovirus, EBV, and human herpesvirus types 6 and 7 have been detected in cervical premalignant and malignant specimens (Young and Sixbey, 1988; Han et al., 1997; Chan et al., 2001). Neither these findings, nor reports on elevated antibody titers against the respective viruses, were confirmed in subsequent studies – which leaves the question open as to whether they play any role in cervical cancer. Cytomegalovirus has been shown in the past to effectively mediate the amplification of the human polyoma-type virus JC (Heilbronn et al., 1993), which points to similar properties of this virus as previously described for herpes simplex viruses. Studies on the role of cytomegalovirus infections on the amplification of HPV DNA are presently not available.

A few studies have reported an association of *Trichomonas vaginalis* infections with cervical neoplasia (Gram et al., 1992; Zhang and Begg, 1994; Viikki et al., 2000). Here, the reported detection of N-nitrosamines in the vaginal vault of women with trichmoniasis could provide some supportive evidence (Nunn et al., 1974; O'Farrell, 1989). The association was, however, not confirmed in other studies (Becker et al., 1994; Schiff et al., 2000; Watts et al., 2005). Similarly, bacterial vaginosis, *Neisseria gonorhoeae*, *Treponema pallidum* and *Candida albicans* infections have been considered as cofactors in cervical carcinogenesis (reviewed in Rotkin, 1973). Recent studies did not provide supportive evidence, however.

Thus, to summarize this section, there exists conclusive evidence for the role of HIV infections as cofactors for HPV-mediated oncogenesis, and for chlamydial infections the role is suggestive but not proven. For HSV-2 infections, a possible role is at best marginal, but for all other anogenital infections the evidence remains inconclusive.

5.5 Preventive Vaccination

Initial attempts to vaccinate against papillomaviruses were made in animal models by using bovine papillomaviruses (Campo et al., 1993), canine oral papillomavirus (Suzich et al., 1995) or cotton-tail rabbit papillomavirus vaccines (Breitburd et al., 1995), and all of these proved to be highly successful. These and subsequent vaccines were based on an observation made by Salunke et al. (1986). This group demonstrated that purified polyoma virus structural proteins are able to self-assemble to virus-like particles (VLPs). The same was shown subsequently by Zhou et al. (1991, 1992) and Kirnbauer et al. (1992) for papillomavirus structural proteins L1 and L2 produced in vaccinia and yeast vector systems. Clinical trials were soon initiated with VLPs of HPV types 6, 11, 16, and 18, which indicated that VLP preparations were well tolerated and induced high titers of neutralizing antibodies, as well as T-cell responses (Zhang et al., 2000; Harro et al., 2001; Brown et al., 2001; Evans et al., 2001; Emeny et al., 2002). The titers usually exceeded those reached in natural infections by more than 40-fold.

A first double-blind study determined whether HPV 16 VLP vaccination prevented the respective infection and also premalignant lesions (Koutsky et al., 2002). Two groups, each including more than 750 HPV 16-negative women, aged 16 to 23 years, received three VLP vaccinations or placebo vaccinations over 6 months, respectively, and were analyzed initially after 17 months. During this period, 41 persistent HPV infections occurred in the placebo group, and none in the vaccinated group. Nine HPV 16-positive squamous intraepithelial lesions (SILs) occurred only in the placebo group, although in each of the two groups 22 lesions of non-HPV 16-associated SIL were observed. Six women of the vaccinated group and 27 among the placebo group acquired a transient HPV 16 infection, without developing SIL.

Two other studies have been published recently confirming and extending these results (Harper et al., 2004; Villa et al., 2005). The study by Harper and colleagues used a bivalent vaccine containing HPV 16 and 18 VLPs. Villa et al. (2005) used a quadrivalent vaccine containing in addition HPV 6 and HPV 11 VLPs. In these studies incident infections were prevented by more than 90%, and persistent infections by 100%.

These data are extremely encouraging and raise the hope that cervical cancer can be successfully prevented in the future. It will of course require long-term observations to demonstrate the preventive effect for cervical cancer, but the prevention of premalignant lesions caused by these high-risk HPVs strongly points into this direction.

At present, a number of additional attempts are being made to produce a cheap and efficient HPV vaccine. One of the most promising candidates is a capsomere vaccine produced in bacteria. L1 proteins, expressed in vesicular stomatitis virus vectors, have provided promising results in preclinical models (Roberts et al., 2004). Bacterially expressed L1 proteins assemble into pentameric structures, corresponding to capsomeres with neutralizing epitopes (Li et al., 1997). In canine oral papillomatosis this vaccine reveal an efficacy similar to that of VLP vaccines (Yuan et al., 2001). Moreover, it can also be applied intranasally, avoiding the use of syringes. The repeated use of syringes is difficult to avoid under conditions of poor hygiene, and bears the risk of transmitting other viral infections such as hepatitis viruses and HIV.

L1 and L2 proteins possess antigenic domains that crossreact with additional HPV types. Antibodies to these regions do not seem to react with VLPs, nor do they neutralize infection (Jenson et al., 1980; Christensen et al., 1990; Hines et al., 1994). Some studies indicate, however, that L2 antibodies neutralize homologous and heterologous HPV types (Kawana et al., 1999, 2003; Roden et al., 2000). Consequently, there is hope that vaccines may be developed which cover a broad spectrum of papillomavirus infections.

Additional attempts are being made to express viral structural proteins in plants, in Salmonella, in vaccinia virus, or to vaccinate with naked viral DNA or viral DNA vectored in various viral vector systems (adeno-associated virus, adenovirus, lentivirus), and these have shown some promising results (Dale et al., 2002; Liu et al., 2005; Berg et al., 2005). A chimeric hepatitis B/HPV16 E7 vaccine encoded by an adenovirus vector may even have a therapeutic application (Báez-Astúa et al., 2005). However, all of these products are currently only in the preclinical phase of testing.

5.6 Therapeutic Vaccination

Early attempts to develop vaccines which might also be effective therapeutically were made by linking a segment of the *E7* gene to the carboxy-terminus of L1 or L2 (Müller et al., 1997; Greenstone et al., 1998). Although preclinical studies demonstrated the induction of neutralizing antibodies to VLPs and T-cell responses to L1 and *E7*, a first clinical trial with these chimeric vaccines failed to reveal a convincing result (Kaufmann et al., 2001).

Since cervical carcinoma cells and high-grade intraepithelial lesions mainly express *E6* and *E7* genes, most studies have focused on eliciting cytotoxic T lymphocytes (CTLs) directed against these oncoproteins. Although preclinical trials in murine systems have been performed successfully (Da Silva et al., 2001; Preville et al., 2005), a number of clinical trials have not yet yielded very convincing results. HLA class I-restricted epitopes have been mapped for E6 and E7 (Kast et al., 1993; Beverley et al., 1994), and several clinical trials using these epitopes have been conducted. The inoculation of such vaccines into 15 HPV 16-positive, A-0201-positive cancer patients resulted in neither any CTL detection nor any measurable clinical ef-

fect (Ressing et al., 2000). Another trial was also unsuccessful (van Driel et al., 1999). However, a third report, in which the vaccine was applied to 18 women with high-grade intraepithelial lesions, recorded 10 CTL responses to the E7 peptide, with three of the women revealing a complete clinical regression (Muderspach et al., 2000). A prolongation of the epitope peptide seems to elicit a more effective CTL response than minimal epitopes, which can be further enhanced by admixture of dendritic cells, including an activating adjuvant (Zwaveling et al., 2002). The linkage of such a peptide to palmitic acid resulted in a CTL response in three out of 12 cervical cancer patients, and in a complete clinical response in one of these (Steller et al., 1998; Steller, 2002). These sets of data suggest that therapeutic vaccines might have some value in patients with premalignant lesions, but they are less likely to be effective in cancer patients. This is in line with previously discussed data, revealing the loss of antigen presentation in cervical carcinoma cells.

Recombinant vaccinia virus expressing mutated HPV 16 and 18 *E7* genes was applied to patients with advanced cervical cancer (Boursnell et al., 1996; Borysiewicz et al., 1996). Only one of the patients developed CTLs with a clinical remission. Among 29 patients with stage Ib or Ia, four developed CTLs and eight revealed serologic responses to the HPV proteins (Kaufmann et al., 2002). In more recent studies, the treatment of patients with intraepithelial neoplasias was found to be somewhat more successful: Baldwin et al. (2003) found some improvement in 83% of 12 treated patients, while Davidson et al. (2003) reported a reduction of high-grade vulvar intraepithelial lesions in diameter by at least 50% in eight out of 18 vaccinated women. Other viral vector systems (E7 or poly-epitope proteins) used in therapeutic approaches were recombinant adenoviruses (Tobery et al., 2003), adeno-associated virus (Liu et al., 2000), RNA-based poliovirus (van Kuppeveld et al., 2002), and alphavirus (Velders et al., 2001). Several of these systems are currently undergoing clinical trials.

In order to increase the immunogenicity of the E7 protein, this oncogene has been fused to the heat shock gene (coding for hsp 70) of *Mycobacterium tuberculosis* (Chen et al., 2000) or to hsp 65 of Bacillus Calmette-Guerin (Goldstone et al., 2002). This should stimulate immunogenicity via the Toll-like receptor 4. Among 14 patients with anogenital warts, three had a complete resolution and an additional 10 had a reduction in wart size. Such DNA vaccines were stimulated in their immunogenic potency by coadministration of a DNA-encoding serine protease inhibitor-6 (Kim et al., 2004). Inoculation of fusion protein of a mutated HPV 16 *E7* gene to the first 108 amino acids of *Haemophilus influenzae* protein D in patients with CIN III and CIN I elicited systemic specific immune responses (Hallez et al., 2004).

Peptide or protein pulsed dendritic cells are thought to be more effective in inducing anti-tumor CTLs than peptides alone (Schoell et al., 1999). In murine cells, E7-loaded dendritic cells transfected with BAK/BAX siRNA, to down-regulate these pro-apoptotic genes, resulted in a strong therapeutic effect (Peng et al., 2005). Monocytes from cancer patients differentiate into dendritic cells following admixture of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF). Several smaller clinical studies have used this approach, though without any significant therapeutic effects (Steller et al., 1998; Santin et al., 1999, 2002; Ferrara et al., 2003; Adams et al., 2003).

Overall, the available data on immunotherapeutic interferences with specific viral antigens or epitopes seems promising for HPV-positive premalignant lesions, but less so for malignant tumors. In the latter cases, immunotherapeutic approaches may be more promising by targeting the modified expression of specific cellular proteins, as for instance $p16^{INK4}$.

5.7 Therapy

This section briefly summarizes some of the most commonly used treatments for HPV-linked mucocutaneous lesions. Surgical treatments include cryotherapy, electrocoagulation, laser surgery, and surgical excision. Cryotherapy usually provides aesthetically satisfying results and is only traumatic to a limited degree. In genital warts, it frequently requires several sessions. Freezing of the tissue results in the destruction of warts (Kouronis et al., 1999; Scala et al., 2002).

Laser surgery permits high-precision surgery which seals small blood vessels immediately. The results are usually excellent, with a very low postoperative morbidity. Similarly, photodynamic therapy by topical application of amino-levulinic acid with subsequent irradiation with light of different wavelengths, can be used for superficial lesions (Roberts and Cairnduff, 1995). The surgical removal of lesions is indicated in case of a limited number of lesions. Extensive intra-anal and vulvovaginal condylomata should be removed under general anesthesia (von Krogh et al., 2001). The surgical removal of malignant lesions will not be detailed at this point.

One special problem is posed by recurrent respiratory papillomatosis, which represents the most common benign neoplasm in the larynx of children (Kimberlin, 2004). It frequently leads to obstruction of the airways and respiratory distress, and even surgical removal cannot protect against multiple recurrences. At present, there is no truly effective therapy against this condition, although indole-3-carbinole, diindolylmethane, interferon and photodynamic therapies are used as additional modalities (Armbruster, 2002; Auborn, 2002).

A number of pharmacological therapies have been used to treat mucosal and cutaneous HPV-linked lesions. One such compound is cidofovir, an acyclic nucleoside phosphonate which specifically inhibits viral DNA polymerases. The reported activity against HPV lesions is surprising in view of the absence of virus-coded DNA-polymerases (Stragier et al., 2002). Close to 50% of lesions seem to be cleared by cidofovir treatment (Snoeck et al., 2001). Cidofovir is presently also used for the treatment of laryngeal papillomatosis, but the observed nephrotoxicity of the drug limits its systemic application (de Clerq, 2003).

Notably in the case of anogenital warts, cytodestruction has been achieved by the application of podophyllin resin, podophyllotoxin, salicylic acid, trichloroacetic acid, and cytostatic agents such as bleomycin and 5-fluorouracil. Podophyllin resin and its derivative podophyllotoxin induce tissue necrosis by blocking cell division and inhibiting microtubule assembly (Manso-Martinez, 1982). Gels of salicylic acid are commonly used for the treatment of non-genital warts (Rivera and Tyring, 2004).

Trichloroacetic acid has been used as an alternative to podophyllin. It induces tissue coagulation and seems to be effective in cervical condylomata acuminata (Menendez Velazquez et al., 1993). Bleomycin and 5-fluorouracil, both of which are mainly applied topically, have also been reported to result in excellent clearance rates (Munn et al., 1996; Syed et al., 2000; Swineheart et al., 1997).

Substantial hope is presently placed on immunomodulatory treatments, specifically on imidazoquinolines. Imiquimod and its homologues act through the activation of Toll-like receptors, and stimulates macrophages and other cells to secrete proinflammatory cytokines (interferon-α, TNF-α, IL-12). This induces a local Th-1 cell-mediated immune response and the production of cytotoxic effectors (Stanley, 2002). The topical use of imiquimod for up to 16 weeks was found to be effective and safe, with a low recurrence rate (Edwards et al., 1998; Cox et al., 2004).

All interferons possess anti-HPV activity. Topical, intralesional or systemic interferon application in some instances leads to partial or complete remission of respiratory papillomatosis, of cutaneous or anogenital warts, in condylomata acuminata in part also in combination with podophyllin (Weck et al., 1986). Interferon-α has been approved by the US Federal Drug Administration for the treatment of genital warts, but has dose-limiting side effects (Wiley et al., 2002).

Some hope is placed on RNA interference for treating cancers caused by viral infection, and here specifically on cervical cancer (Milner, 2003). Interfering RNA (RNAi), triggered by double-stranded RNA, permits gene silencing by targeting specific mRNA transcripts for degradation. Hutvagner et al. (2001) showed that short RNA duplexes, containing 19–22 nucleotides initiated RNAi in mammalian cells. In HPV-carrying cervical carcinoma cells the silencing of *E6* RNA had little effect, whereas the silencing of *E6* and *E7* and of *E7* alone resulted in apoptosis of the transfected cells (Jiang and Miller, 2002). One problem here for clinical use is the mode of application, though at present topical application promises the best results.

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6
Hepadnaviruses

6.1
Hepatitis B

6.1.1
Historical Aspects

Hepatitis B virus (HBV) causes acute and chronic infections of the liver, resulting in part in fulminant liver failure, cirrhosis, and also in hepatocellular carcinoma (HCC). According to data provided by the International Agency for Research on Cancer (IARC, 1994), over 300 million people are estimated to be chronically infected worldwide, and between 250 000 and 1 000 000 die annually from HBV-associated disease.

The detection of the responsible virus has an interesting history. The recognition of hepatitis B as an infection primarily transmitted by parenteral inoculation of human serum causing an acute infection with jaundice dates back to the 1930s (Findlay and MacCallum, 1937). A widespread epidemic of hepatitis among US military troops in 1942 resulted in the acceptance of a high risk of the use of pooled and dried human plasma or after blood transfusion (Morgan and Williamson, 1943; Beeson, 1944). In 1947, the designations "hepatitis B" for "serum hepatitis", and "hepatitis A" for acute infectious hepatitis were coined (MacCallum, 1947). During the 1950s and 1960s, the seroepidemiologic relationship between hepatitis A and hepatitis B was investigated (Murray et al., 1954; Murray, 1955; Krugman et al., 1967), and later, in 1965, Blumberg et al. described a "new" antigen in the blood of an Australia aborigine ("Australia antigen") which formed a precipitin line with a serum obtained from a multiply transfused hemophiliac. Shortly thereafter, the relationship of this antigen to serum hepatitis was recognized (Blumberg et al., 1967, 1969; Prince, 1968). In 1970, 42-nm particles were described by Dane et al., which were suspected to represent hepatitis B particles. In 1973, an endogenous DNA-dependent DNA polymerase was found within the core of these particles (Kaplan et al., 1973). The genome was identified as a small, circular, partially double-stranded DNA (Robinson and Greenman, 1974; Robinson, 1977).

Early reports on a possible role of HBV infections in hepatocellular cancer appeared as early as the 1950s (Payet et al., 1956). Additional early epidemiological studies further reported a role of chronic hepatitis B infections in HCC development

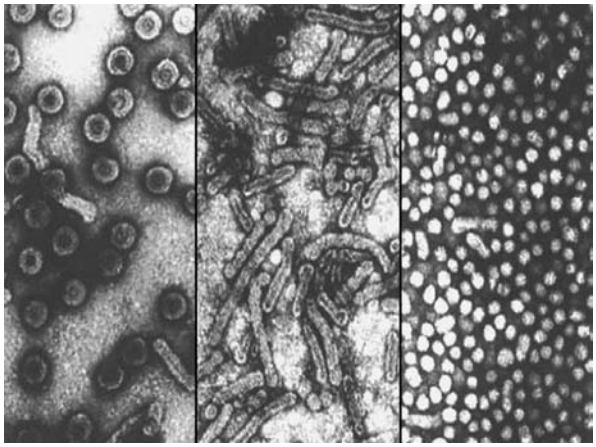

Fig. 6.1 Hepatitis B virus. Left: typical 42-nm particles containing an outer HB surface antigen (HBs) envelope, Center: filamentous forms of 20 nm diameter particles. Right: 20 nm particles. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., et al. (Eds.), Part II – The DNA and RNA Reverse Transcribing Viruses, Hepadnaviridae, 373. Copyright 2005, with permission from Elsevier and courtesy of W. Gerlich.)

(Prince et al., 1970; Vogel et al., 1970; Denison et al., 1971; Sankalé et al., 1971; Teres et al., 1971; Nishioka et al., 1973; Trichopoulos et al., 1975; Larouzé et al., 1976). In 1981, Beasley et al. presented clear-cut epidemiologic evidence for a role of hepatitis B in HCC. In a prospective study conducted in government employees in Taiwan, these authors noted an increase in relative risk for HCC by a factor of 103 of hepatitis B carriers in comparison to negative individuals.

Typical hepatitis B particles are illustrated in Figure 6.1.

6.1.2 Epidemiology and Clinical Symptoms

In Western societies the majority of HBV cases are observed in age groups between 20 and 40 years, whereas in developing countries, infection occurs frequently during the perinatal period. There exist remarkable differences between males and females in liver cancers presently linked to HBV or hepatitis C virus (HCV) infections (Parkin et al., 2005) (Fig. 6.2), the percentage of males suffering from these cancers being approximately two-fold that of females.

There exists also a wide variation in the geographic prevalence of HBV infections, largely corresponding to that of HCCs (Fig. 6.3). The Asia-Pacific region and Africa tend to have the highest prevalence of hepatitis B infection worldwide (Lin, X., et al., 2005).

The mode of infection transmission depends on age: the infection of neonates – particularly those born to persistently infected mothers – reveals in 85% of cases a

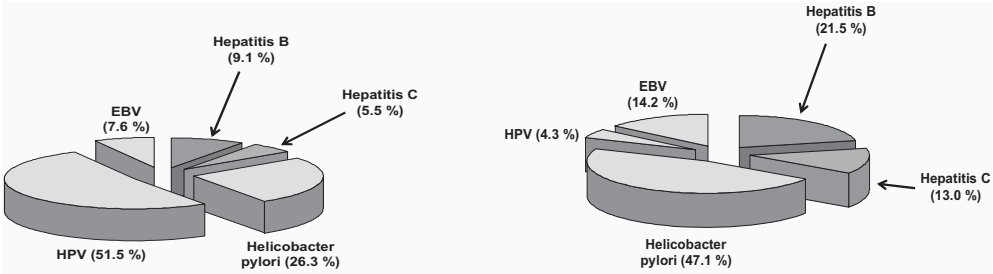

Fig. 6.2 Percentages of hepatitis B and C virus infections on the incidence of liver cancer in relation to other virus-linked human cancers in male (left) and female (right) patients. EBV: Epstein–Barr virus; HPV: human papillomavirus.

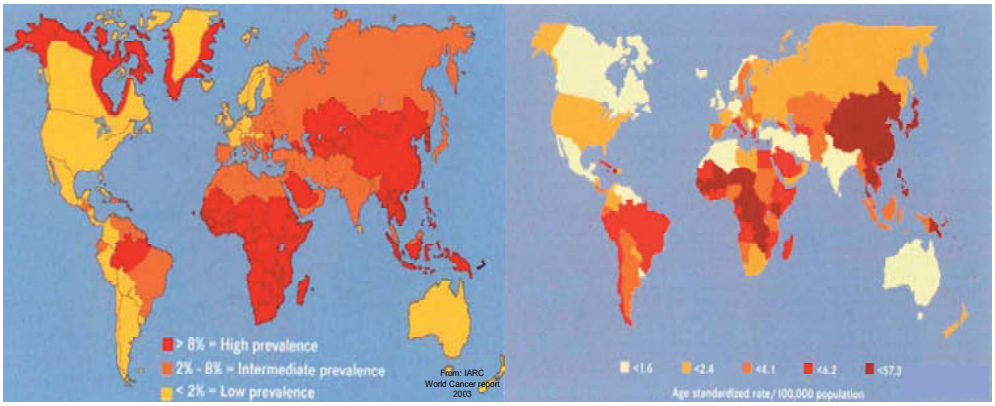

Fig. 6.3 Left: Geographic pattern of the prevalence of hepatitis B virus infections. Right: Geographic pattern of the prevalence of hepatocellular carcinoma. (World Cancer Report, Stewart, B.W. and Kleihues, P. (editors), Lyon, France: IARC Press, 2003 and Vaccines, Immunization and Biologicals, WHO 2000.)

chronic carrier state (Mitsuda et al., 1989). The risk for infection during later childhood increases among children whose siblings have already acquired the infection (Whittle et al., 1990). Contaminated needles, tattooing, syringes and acupuncture equipment have been clearly identified as important sources of infection (Kent et al., 1988). In adulthood, sexual transmission (Szmuness, 1975; reviewed in Atkins and Nolan, 2005) and parenteral infections appear to play the major role. Among homosexual men the rate of infection is commonly high and depends on the number of sexual partners (Alter et al., 1989; Rosenblum et al., 1992; Osmond et al., 1993). Intravenous drug users are at an especially high risk (Alter et al., 1990; Polakoff, 1990; Struve, 1992). Infection in developed as well as in developing countries is frequently facilitated by a low socioeconomic status (Szmuness et al. 1978 a; Toukan, 1987).

For chronic infections the age of infection appears to play the prevailing role. This accounts in particular for neonates, where up to 100% of these children develop a chronic carrier state (Beasley et al., 1977; Wong et al., 1984). Among immunocompetent adults this occurs only in 0.5 to 3% of subjects (Pol, 2005).

The source of the infectious virus is commonly blood, but other secretions and excretions also contribute to infections; among these are included semen, saliva (Ward et al., 1972; Heathcote et al., 1974; Villlarejos et al., 1974; Karayiannis et al., 1985; Davison et al., 1987), vaginal secretions, and menstrual blood (Mosley, 1975). Tears and breast milk have also been recorded as containing infectious HBV. In addition, all other biological fluids from infected patients may be considered as potentially infectious. There exist indirect modes of transmission in renal dialysis units, through common use of fomates such as towels, razors and toothbrushes, as well as by acupuncture needles and medical instruments.

Hepatitis B infections occur throughout the world. Particularly high rates of infection are recorded in developing countries and under conditions of poor hygiene. The highest rates of chronic HBV carriers have been reported from China, Africa, Oceania and the South Pacific, the Middle East, and certain areas in South America (Sobeslavsky, 1975; Szmuness, 1975; Gust et al., 1978; Szmuness et al., 1978 b).

There is no seasonal spreading of the infection and no typical epidemics, except under circumstances of transfusions with contaminated blood or by needle sharing of drug abusers (Maynard et al., 1976). In endemic areas the pattern of transmission is different in comparison to the Western world: in the former, most infections occur as maternal – neonatal transmissions, whereas in the latter group mainly adolescent children or young adults become infected (Szmuness et al., 1978 a).

Asymptomatic infection with hepatitis B occurs in slightly more than 50% of cases, where no medical intervention is required. Between 80% and 85% of infected newborns, however, become chronic carriers if infection occurred during their first 2–3 months of life (Okada et al., 1976; Beasley et al., 1977; Shiraki et al., 1977; Lee et al., 1978). Generally, symptomatic cases occur after a latency period of 3–4 months. The clinical course and diagnosis of acute and fulminant hepatitis B have been described in detail previously (e.g., Zuckerman and Zuckerman, 2000), and will not be described here. In Western societies, chronic infections develop in up to 5% of infected people (IARC, 1994). Infection may result in not only a symptomatic but also an asymptomatic course. The incidence of chronic infections is higher in men than in women. Hepatitis B leads more frequently to asymptomatic infections of children than in adults, and affects particularly immunosuppressed patients (McMahon et al., 1985; Taylor et al., 1988; Edmunds et al., 1993). Viral persistence is accompanied by inflammatory reactions in the liver, necrosis of liver cells and frequently with integration of the hepatitis B genome into liver cell DNA (De Franchis et al., 1993). Continued replication of HBV results in serious problems for the infected patient: primarily benign lobular hepatitis, severe chronic active hepatitis and liver cirrhosis (Fattovich et al., 1990). The presence of HBV DNA in the serum represents a particular risk factor for the development of cirrhosis (Fattovich et al., 1991).

6.1.3 Taxonomy and Viral Genome Structure

Human hepatitis B virus belongs to the family of Hepadnaviridae, which comprises a growing number of mammalian and avian members. Mammalian viruses related to HBV have been isolated from woodchucks (Summers et al., 1978) and Beechey ground squirrels (Marion et al., 1980). In particular, the woodchuck virus was found to be an effective carcinogen in its native host. Additional isolates have been obtained from chimpanzees, gorillas, orangutans and gibbons (Starkman et al., 2003). It is interesting to note that the HBV variants found in orangutans were interspersed with variants from southerly distributed gibbon species (*Hylobates agilis* and *Hylobates moloch*), which occupy overlapping habitats. Gibbons from mainland Asia are phylogenetically distinct. Also in chimpanzees there seems to exist a geographical rather than a subspecies distribution of HBV variants.

Avian hepadnaviruses have been isolated from ducks (Mason et al., 1980), herons (Sprengel et al., 1988), snow geese (Chang et al., 1999), various types of additional exotic ducks and geese (Guo et al., 2005), storks (Pult et al., 2001), and cranes (Prassolov et al., 2003). There exists evidence for an interspecies spread of heron hepatitis B viruses among different species and subspecies of herons (Lin, L., et al., 2005). Phenotypic mixing has been observed of rodent, but not avian, hepadnavirus surface proteins into human HBV particles (Gerhardt and Bruss, 1995). Clearly, the family of hepadnaviruses is widely spread among mammalian and avian species. It is likely that additional virus types belonging to this family will emerge in the future. A phylogenetic tree of human and animal hepadnaviruses is illustrated in Figure 6.4.

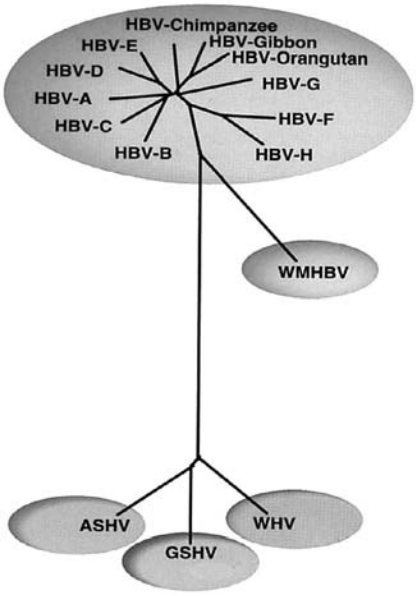

Fig. 6.4 Phylogenetic tree of human and animal hepadnaviruses. ASHV: Arctic ground squirrel hepatitis virus; GSHV: Ground squirrel hepatitis virus; WHV: woodchuck hepatitis virus; WMHBV: Woolly monkey hepatitis B virus. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The DNA and RNA Reverse Transcribing Viruses, Hepadnaviridae, 382. Copyright 2005, with permission from Elsevier.)

Eight different genotypes of human hepatitis B viruses (A–H) have been identified (Kao et al., 2000 b; Kidd-Ljunggren et al., 2002; Shibayama et al., 2005). Their differentiation is mainly based on comparative and phylogenetic analyses of the S region. A sequence divergence of more than 8% of the entire genome, consisting of approximately 3200 base pairs, is commonly used to differentiate between the individual HBV genotypes (Okamoto et al., 1988). The genotypes differ in geographic prevalence: genotype A has been preferentially found in Northwest Europe, sub-Saharan Africa, India and the United States (reviewed in Akuta and Kumada, 2005). Genotypes B and C are more often found in south-east Asia, Japan and Oceania, and genotype D mainly in Mediterranean countries. Genotype E seems to be restricted to Africa, and F to Central and South America. The geographic prevalences of genotypes G and H have not yet been determined. Although not confirmed in all other studies (Sumi et al., 2003; Yuen et al., 2003), case-control studies suggested that genotype C is associated with more severe liver disease and a more rapid progression to cirrhosis and liver cancer (Kao and Chen, 2000; Sumi et al., 2003; Chan et al., 2004). Since cirrhosis is more frequently induced by genotype C infections, the latter suggestion gains some probability. This is further supported by a prospective longitudinal cohort study of chronic hepatitis B patients in Hong Kong (Chan et al., 2004).

The structure of hepatitis B virus DNA is shown in Figure 6.5. The 42-nm particle is surrounded by a phospholipid bilayer envelope which harbors the surface antigens and encloses the nucleocapsid. Depending on the initiation site of transcription, three different sizes of surface proteins of 24, 32, and 39 kDa are produced; the small surface protein (S-antigen) is most abundantly formed. The largest surface antigen form represents the binding site for host cell attachment. The infected cells form large quantities of 22-nm particles consisting of surface proteins (mainly the

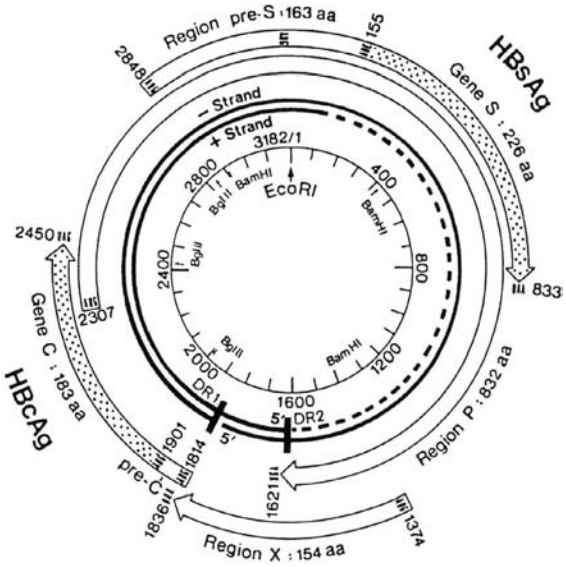

Fig. 6.5 Structure of the hepatitis B DNA and transcripts originating from the genome. (Reproduced from Kao, J.-H. and Chen, D.-C. Overview of hepatitis B and C viruses. In: Goedert, J.J. (Ed.), *Infectious Causes of Cancer* . Humana Press, pp. 313–330, 2000 a. With permission.)

small form of S-antigen) and lipids which immunologically correspond to antigens expressed at the surface of infectious 42-nm particles. The core open reading frame (ORF) (nucleotides 1814 to 2450) contains a precore and a core region. The truncated precore protein is designated as e-antigen and can be demonstrated at the surface of infected cells and in the peripheral blood (Schlicht and Schaller, 1989; Hilleman, 2003). An ORF for 832 amino acids encodes the viral polymerase. The X-protein consisting of 154 amino acids is essential for viral infectivity and represents a transcriptional activator (Ganem and Schneider, 2001).

The genome of HBV consists of partially double-stranded DNA. For replication, the viral nucleocapsid is transported to the nucleus where the relaxed plus-strand is completed to a covalently bound closed circle and subsequently forms a supercoil. RNA transcription follows within the nucleus. The RNA is transported into the cytoplasm and translated into viral proteins. The assembled nucleocapsid takes up one strand of viral RNA where it is packaged. The viral polymerase reverse transcribes the minus-strand DNA, followed by an incomplete synthesis of the positive strand. Budding of the particle occurs from the endoplasmic reticulum where it acquires the lipid envelope with the embedded surface antigens. The reverse transcription of viral RNA without proofreading has a high probability for mutational events. Mutants in the core promoter may lead to enhanced viral replication and within the core and surface antigens to altered antigenicity of the virus (reviewed in Baumert et al., 2005). Mutations in the polymerase gene may confer resistance to antivirals.

6.1.4 Viral Gene Products and Functions

6.1.4.1 Core Antigen

The reading frame for the core antigen contains a pre-core and the core region. The pre-core protein represents a truncated form of the core protein, and is released as a soluble protein on the surface of infected host cells and into the blood stream (Schlicht and Schaller, 1989). It is designated as e-antigen. The regions of the pre-core and core protein are divided in a segment of redundant nucleotide sequences that imparts the common antigenic specificity to the e-antigen and core antigens (Milich et al., 1990; Hilleman, 2003). The HBV core protein contains 183 to 185 amino acids, depending on the subtype. Phosphorylation and dephosphorylation events seem to contribute to intracellular and extracellular forms of the core protein (Pugh and Summers, 1989; Mabit and Schaller, 2000). Phosphorylation precedes the HBS RNA encapsidation (Gazina et al., 2000).

6.1.4.2 Polymerase

The viral polymerase functions as a DNA polymerase and as reverse transcriptase. At about 90 kDa, it represents the largest protein produced by the HBV genome. There exists a structural similarity of the HBV polymerase to the corresponding

domain of the of HIV reverse transcriptase (Das et al., 2001). Upon infection of susceptible cells, within the nucleus the relaxed positive strand is completed and forms a covalently bonded closed-circle supercoil. Subsequently, full-length strands of RNA are transcribed, which are packaged into the assembled capsid, where the RNA is reversely transcribed by the viral polymerase to form the viral minus-strand. The incomplete positive strand is synthesized within the nucleocapsid. It is possible to separate the polymerase domains responsible for initiation of DNA replication and for reverse transcriptase activity (Beck and Nassal, 2001).

6.1.4.3 **HB X Antigen**

A typical ORF coding for the X-protein is only present in mammalian hepadnaviruses, and is either missing or present in a highly divergent form in avian hepadnaviruses (Chang et al., 2001). Since the latter commonly do not induce liver tumors, this led to the suspicion that HBx might play a role in carcinoma induction by animal hepadnaviruses. A comprehensive review on the present knowledge of features and functions of hepatitis B X gene products has been published by Bouchard and Schneider (2004).

The *HBx* gene codes for a protein of 154 amino acids with a molecular mass of about 17.5 kDa. Its amino- and carboxy-terminal regions contain presumptive helical domains and a potential coiled-coil motif (Kodoma et al., 1985; Colgrove et al., 1989). The 50 amino-terminal amino acids seem to contain a negative regulatory element, since their deletion activates HBx transcriptional functions (Murakami et al., 1994). HBx is mainly found in the cytoplasm, but also reaches the nucleus (Doria et al., 1995; Sirma et al., 1998; Hoare et al., 2001).

In woodchucks, a gene corresponding to HBx is essential for the replication of woodchuck hepatitis virus (WHV) (Chen et al., 1993; Zoulim et al., 1994). In human HBV infections, the role of HBx is less clear, although it augments HBV infection and viral persistence (Melegari et al., 1998; Bouchard et al., 2001; Xu et al., 2002). The X protein stimulates transcription of various cellular transcription elements, usually containing binding sites for NF-κB, AP-1 and -2, c-EBP, ATF-CREB or calcium-activated factor NF-AT (Faktor and Shaul, 1990; Maguire et al., 1991; Lucito and Schneider, 1992; Williams and Andrisani, 1995; Lara-Pezzi et al., 1999). HBx also stimulates RNA polymerase I-, II- and III-dependent promoters (Aufiero and Schneider, 1990; Wang et al., 1998). It acts, however, also as a suppressor for other genes. This has been suggested for $p21^{WAF}$ (Ahn et al., 2001). Its transcriptional activity seems to be repressed by HBx-mediated down-regulation of Sp1, which regulates the activity of $p21^{WAF}$.

HBx does not bind DNA directly, but binds to some components essential for basal transcription. Its interaction with the ZIP-region of CREB seems to be responsible for the HBx-mediated activation of CREB-dependent transcription (Maguire et al., 1991; Williams and Andrisani, 1995).

The cytoplasmic HBx activates the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases/NH_2-terminal-Jun kinases, and the p38 kinase

(Benn et al., 1996; Tarn et al., 2001, 2002). HBx also activates indirectly the Ras-pathway by activating nonreceptor tyrosine kinases of the Src-family (Klein and Schneider, 1997; Lee and Yun, 1998; Tarn et al., 2002). This results in modifications of cellular adherens functions (Lara-Pezzi et al., 2001). The activation of Src kinases stimulates the HBV replication in established liver cell lines by 5- to 20-fold (Klein et al., 1999; Bouchard et al., 2001). Some functions of the HBV X protein are summarized in Figure 6.6.

Conflicting reports describe the potential interaction of HBx with p53. Although a direct binding has been reported (Feitelson et al., 1993; Wang et al., 1994; Truant et al., 1995), these results have not been confirmed in other studies (Puisieux et al., 1995; Su et al., 2000). Thus, this field still requires further analyses. The binding to the transcription factor CREB and other b-ZIP transcription factors, however, is firmly established (Maguire et al., 1991; Barnabas et al., 1997; Andrisani and Barnabas, 1999; Choi et al., 1999).

One study showed that HBx inhibits nucleotide excision repair (Jia et al., 1999). The interaction of HBx with the DNA repair proteins DDB1 and DDB2, as revealed by in-vitro and in-vivo studies, seems to be of particular importance (Becker et al., 1998; Sitterlin et al., 2000; Bergametti et al., 2002), although the immediate functional consequences are not entirely clear. Transgenic mice expressing HBx do not reveal an increased rate of spontaneous mutations (Madden et al., 2000). The hepatocytes of the same animals, however, are more sensitive to low levels of chemical mutagens (Madden et al., 2001; Zhu et al., 2004). Although not a primary mutagen, HBx seems to contribute to mutagenic functions of other carcinogens.

Induction of apoptosis by HBx has been controversial. In HBx-transgenic mice, two reports noted increased apoptotic rates in hepatocytes (Terradillos et al., 1997, 2002), whereas another report failed to confirm this observation (Madden et al., 2000). Similarly, several authors reported that HBx blocks apoptosis induced by tumor necrosis factor α (TNF-α), Fas, p53, or transforming growth factor-B (TGF-β) (Elmore et al., 1997; Shih et al., 2000; Pan et al., 2001). On the other hand, HBx expression independent of HBV replication was described as promoting apoptosis in various cell types (Chirillo et al., 1997; Su et al., 2001; Kim and Seong, 2003; Shirakata and Koike, 2003). It is very difficult to reconcile these diverging studies at

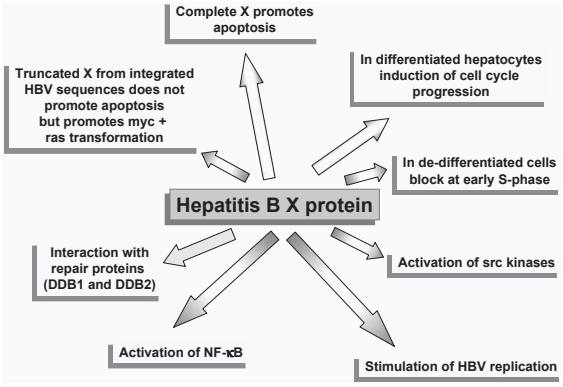

Fig. 6.6 Some functions of the hepatitis B X protein.

this stage. It seems to be important, however, that HBx expressed from replicating HBV genomes in cultured cells results in hypersensitivity to cell killing by TNF-α. This hypersensitivity requires a step involving HBx activation of c-Jun terminal kinase and Myc (Su et al., 2001; Kim and Seong, 2003; Bouchard and Schneider, 2004). Primary mouse hepatocytes are sensitized by HBx to ethanol and TNF-α-induced apoptosis by a caspase-3-dependent mechanism (Kim et al., 2005). In this respect, it is of interest that the differentiation stage of cells plays a role in determining HBx-dependent cell cycle progression (Lee et al., 2002). HBx-expressing differentiated hepatocytes proceed through G_1, S and G_2/S, concomitant with the induction of cyclins D1, A, and B and Cdc2. In HBx-positive dedifferentiated cells, the cells enter G_1 and S-phase, but do not proceed further.

In summary, HBx clearly represents a pleiotropic protein, the individual functions of which still remain at best enigmatic.

6.1.5
Pathogenesis and Immune Interactions

The pathogenesis of HBV infections is complex and involves innate and adaptive immune responses. The virus, in addition, has evolved mechanisms to induce immunotolerance, immune exhaustion and suppression, and immune escape through mutation, modification of the antigenic phenotype, molecular mimicry and others (Tai et al., 2002; Favoreel et al., 2003; Alcami, 2003; Vanlandschoot and Leroux-Roels, 2003).

Usually HBV DNA becomes detectable in the blood circulation within one month following primary infection, but remains at relatively low level of 10^2–10^4 genome equivalents per mL for up to 6 weeks (Rehermann and Nascimbeni, 2005). Subsequently, viral DNA, HbeAg, and HbsAg reach peak titers before specific antibodies arise (Hollinger, 1987). An elevated level of serum alanine amino-transferase which starts to rise approximately 10–15 weeks after primary infection is an indication for T-cell-mediated liver injury. The primary events contributing to liver injury and clearance of HBV infection are mediated by cytotoxic T lymphocytes (CTLs) (Bertoletti and Maini, 2000; Rehermann, 2000). Infected cells are destroyed by MHC class I-restricted CD8 + CTLs (Chisari and Ferrari, 1995). The aggressiveness of this response determines the outcome of the infection. A weak cell-mediated immune response commonly results in a persistent viral carrier state, initially clinically unapparent, but ending frequently in chronic active liver disease (Hilleman, 2003). Pro-inflammatory cytokines, such as interferon-α, interleukin (IL)-12, and IL-18, contribute as innate immune response to the outcome of the infection (Ferrari et al., 1987; Bertoletti et al., 1997). In mice transgenic for the entire HBV genome, transfer of CTLs or of their cytokines (interferon-γ, TNF-α, IL-2), abolishes virus replication and gene expression (Guidotti et al., 1996, 1999; Guidotti and Chisari, 1999; Thimme et al., 2003).

Clinical recovery results in life-long protective immunity, sometimes even in spite of traceable amounts of persisting virus in the blood. Under chemotherapy and severe immunosuppression, reactivation of the infection may even occur (Kawatani,

2001). Allografts from previously HBV-positive patients may lead to HBV transmission in immunosuppressed transplant recipients (Chazouilleres et al., 1994).

Tolerance against HBV antigens may arise as the consequence of in-utero infections (Milich et al., 1990, 1998; Wang and Zhu, 2000). The resultant absence of CTLs prevents clearance of the infected host cells and results in persistent HBV infection. The still immature immune system in perinatal and prenatal infections emerges as the main reason for the high percentage of HBV persistence in infections acquired during this period. Non-responsiveness to viral antigens may also arise from mutations within viral genes: in one investigation, 34 mutations were recorded in the pre-core/core gene, 12 in the polymerase sequence, and nine in the pre-S/S region (Brunetto et al., 1999). These represented in part genetic transitions, insertions, transversions, and deletions. It is likely that a substantial diversity of viral genotypes exists within individual infected liver cells and within the total liver. Interestingly, in this study the X gene was not mutated.

Hepatitis B virus infection also interacts directly with innate immune responses. The HBS core antigen inhibits the transcription of human interferon-β gene by interacting with regulatory DNA sequences 5' to the coding sequence of the interferon-β gene (Twu and Schloemer, 1989; Whitten et al., 1991). The viral polymerase gene, specifically its terminal domain, has also been described as inhibiting the responses to interferons α and ã (Foster et al., 1991). Subsequent studies showed that the HBV capsid protein of defective particles associated with chronic hepatitis B selectively inhibited induction of the MxA protein by suppressing and interacting directly with the MxA promoter (Rosmorduc et al., 1999). The MxA protein represents an interferon-inducible GTPase with antiviral activity against several viruses. HBV-encoded pre-core/core proteins blocked the MxA expression by affecting the interferon-stimulated response elements 2 and 3, upstream of the putative start codon of the MxA promoter (Fernandez et al., 2003).

6.1.6 Role in Hepatocellular Carcinoma

6.1.6.1 HBx Transgenic Mice and HCCs

Although an early report found no evidence for a role of HBx in transgenic mice (Lee et al., 1990), a number of subsequent studies reported a positive correlation of HBx expression and liver cancer in these animals. A construct including the HBx coding region as well as the HBV enhancer I region, the X gene promoter, and the HBV polyadenylation signal induced liver tumors directly linked to HBx expression (Kim et al., 1991; Yu et al., 1999). Particularly high levels of HBx expression resulted in a high rate of liver cancer in male mice (Koike et al., 1994).

Several authors have described a higher susceptibility of HBx-transgenic mice for chemical carcinogens (Slagle et al., 1996; Madden et al., 2001; Zhu et al., 2004). These groups suggest that HBx expression is not sufficient for carcinogenesis, but the *HBx* gene may rather act like a tumor promoter. On a similar line, expression of c-myc driven by WHV virus regulatory sequences in HBx-transgenic mice short-

ened the average tumor latency by 2–3 months (Terradillos et al., 1997; Lakhtakia et al., 2003). Again, these data seem to support a tumor promoter function of HBx. Knock-in experiments resulting in integration of the *HBx* gene into the $p21^{WAF}$ locus resulted within 18 months in HCCs (Wang et al., 2004). One interesting observation made in the latter study was a high up-regulation of the estrogen receptor-β selectively in tumor tissues of male p21-HbsAg mice, providing suggestive genetic evidence that HbsAg might represent the factor explaining the higher prevalence of HCCs in males when compared to females.

Mutations within the X gene region involving nucleotides 1762(T)/1764(A), which are considered as markers for liver cancer development, have been described in HBV genomes derived from the plasma of patients with HCCs (Kuang et al., 2004). Within tumors from patients of Qidong in the Peoples Republic of China, 74.3% carried a double mutation at nucleotides 1762 (T) and 1764 (A). Four of six plasma samples from such patients were also positive. This mutation appears to have also some predictive value, since more than 50% of patients with liver cancer revealed this mutation several years prior to the detection of HCCs.

C-terminal truncation of HBx results in enhanced transformability of murine cells upon co-transfection with ras or myc constructs (Paterlini-Brechot et al., 2000, 2003), and reveals the biological impact of natural C-terminal deletions of hepatitis B virus X protein in HCC tissues (Tu et al., 2001).

6.1.6.2 **HBS Transgenic Mice and HCCs**

A first report appeared in 1990, describing hepatocarcinogenesis due to chronic liver cell injury in hepatitis B S antigen (HbsAg) transgenic mice (Dunsford et al., 1990). Within 4 months these animals developed chronic hepatitis, followed sequentially by the development of regenerative nodules and oval hyperplasia. Liver adenomas developed at 8 months of age, and carcinomas at 12 months. By 20 months, 100% of the animals had developed hepatocellular cancer. Within the same year another report described transgenic mice containing a single copy of HbsAg which revealed an elevated susceptibility to carcinogen-induced hepatocarcinogenesis (Dragani et al., 1990). Interestingly, in carcinogen-induced liver tumors expression of the HbsAg was inhibited, apparently due to de novo methylation of the S region (Farza et al., 1994). The synergistic effect between HbsAg-transgenic mice and chemical carcinogens was soon confirmed for aflatoxin and diethylnitrosamine (Sell et al., 1991). At least in these experimental mouse models, p53 and pRb mutations have not been found, possibly indicating an early stage of hepatocarcinogenesis (Pasquinelli et al., 1992). The development of liver cancer in HbsAg-transgenic mice depends on the level of expression of the transgene (Huang and Chisari, 1995).

TGF-α drastically accelerates hepatocarcinogenesis when overexpressed in TGF-α transgenic mice (Jhappan et al., 1990). Overexpression of this factor in hepatocytes in transgenic mice is sufficient to induce enhanced hepatocyte proliferation and HCCs (Jakubczak et al., 1997). Mice which were bitransgenic for TGF-α and HbsAg revealed a dramatically accelerated appearance of HCCs (Jakubczak et al., 1997).

HCCs in HBV-positive patients also show elevated expression levels of TGF-α (Hsia et al., 1994). Hepatitis B preS1, as part of large HbsAg, activates the transcription of TGF-α and may thus contribute to liver carcinogenesis (Ono et al., 1998). Mutants of the preS1 or preS2-regions cause oxidative stress and DNA damage (Hsieh et al., 2004). Since such mutants are found in late and non-replicative stages of HBV infection, this seems to indicate that preS1/S2 mutants induce oxidative stress and mutations in hepatocytes in late stages of HBV infection. This is shown schematically in Figure 6.7.

Oxidative stress in turn negatively regulates hepatitis B virus gene expression and viral replication (Zheng and Yen, 1994).

In acute HBV infections the small HbsAg is the major form that constitutes the envelope of the virion. In the chronic phase, the large form becomes dominant. Several truncated forms with a partially deleted pre-S region have been identified. Two of these contribute to two distinct histological patterns labeled as "ground glass hepatocyte" types I and II (Fan et al., 2000, 2001). Type I reveals an inclusion-like pattern of HbsAg which is deleted in the pre-S1 promoter region (Hsieh et al., 2004), whereas type II is deleted of nucleotides 4–57 in the pre-S2 region and contains a point mutation at the start codon. This results in a substantial decrease in the synthesis of small and middle surface antigens. In both types, HbsAg accumulates in the endoplasmic reticulum. In particular, type II is highly correlated with cirrhotic

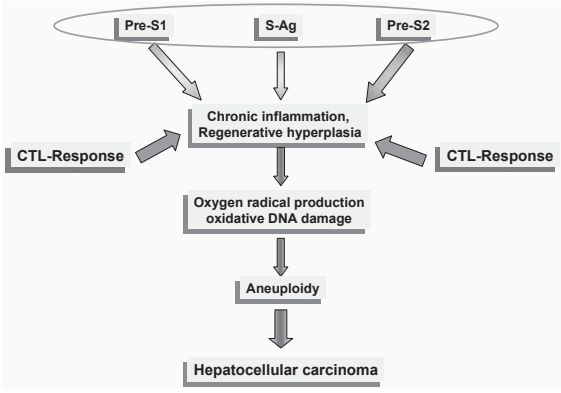

Fig. 6.7 Putative functions of HBs-antigens in the development of hepatocellular carcinomas.

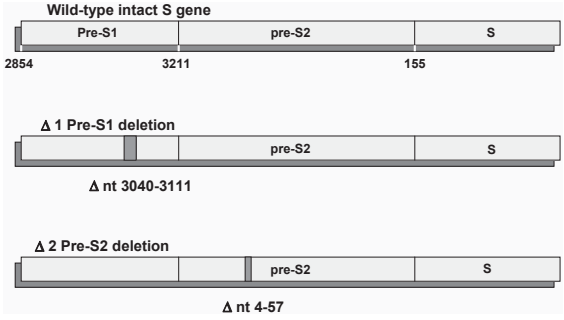

Fig. 6.8 Schematic outline of pre-S1 and pre-S2 deletions. (Modified from Hsieh, Y.H., et al., 2004.)

progression and HCC. This pre-S2 mutant also up-regulates cyclin A expression and induces nodular proliferation of hepatocytes (Wang et al., 2005). It emerges only in the late non-replicative phase of chronic HBV infection, and becomes the dominant gene product in hepatocytes (Hsieh et al., 2004). Both, pre-S1 and pre-S2 induce reactive oxygen species (ROS) in cells of the human liver cell line Huh-7 (Wang et al., 2003), though pre-S2 triggers a higher level of oxidative DNA damage. The two mutated S-antigen forms are outlined schematically in Figure 6.8.

Mutations in the X-linked *Hprt* gene in cells carrying the pre-S1 or pre-S2 mutant *HBS* genes are 4.5 to 6.2 times higher than in cells overexpressing the wild-type HbsAg (Hsieh et al., 2004). Thus, these two pre-S mutants reveal properties reminiscent of well-characterized oncogenes. Chromosomal imbalances occur already in cirrhotic nodules (Yeh et al., 2001).

6.1.7 Interaction of Hepatitis B Infection with Chemical Carcinogens (Aflatoxins and Alcohol)

A number of studies point to an interaction of HBV infections in hepatocarcinogenesis with chemical compounds that may induce liver damage upon repeated exposure (Wogan, 1992; Chen et al., 1997; Yu et al., 2000).

6.1.7.1 Alcohol

HCCs linked to alcohol consumption commonly occur after an initial development of liver cirrhosis. The main metabolite of alcohol, acetaldehyde, causes hepatocellular injury, which leads to increased oxidative stress and thus damages DNA (reviewed in Voigt, 2005). Alcohol, in addition, causes abnormalities in DNA methylation and may inactivate tumor suppressor genes. These effects of alcohol contribute to cancer development in chronically HBV-infected patients.

6.1.7.2 Aflatoxin

Early investigations on a synergistic role of aflatoxin exposure and concurrent HBV infections (reviewed in Kew, 2003) appeared from Swaziland (Peers et al., 1987) and China's Guangxi Province (Yeh et al., 1989). The authors of both studies concluded that simultaneous exposure to these agents was an important determinant for geographic variations in the incidence of liver cancer in these two regions. In transgenic mice, overexpressing the large polypeptide of HBV, the feeding of aflatoxin B1 resulted in rapid hepatocyte dysplasia and HCCs in comparison to unexposed littermates (Sell et al., 1991). Similarly, the feeding of hepatitis virus-infected woodchucks with aflatoxin B1 resulted in synergistic hepatocarcinogenesis (Bannasch et al., 1995). Four epidemiologic studies found a striking multiplicative carcinogenic effect of both agents (Ross et al., 1992; Qian et al., 1994; Wang et al., 1996; Lunn et al.,

1997). One study in Taiwan analyzed tumor tissue of HBV-infected patients by histochemical staining for aflatoxin B_1-N^7-guanine adducts and found that the positive patients were on average 10 years younger than those with adduct-negative cancers (Chen et al., 1992).

Approximately 60% of HCC patients from areas with high exposure to aflatoxin B1 reveal a guanine to thymine transversion at the third base of codon 249 of the *p53* gene (Hsu et al., 1991; Bressac et al., 1991). This was subsequently confirmed in epidemiologic studies in regions with high and low aflatoxin exposure (Ozturk, 1991; Eaton and Gallagher, 1994). There were some inconsistent reports, however. In Taiwan, in a first study codon 249 arginine to serine mutations were found in HbsAg-positive patients (Lunn et al., 1997), and in a second study in 36.3% of HBV-infected patients with liver cancer as compared to 11.7% of cancer patients without HBV markers (Wang et al., 1996). A meta-analysis of 49 published studies also failed to demonstrate any interaction between HBV and aflatoxin B_1 (Stern et al., 2001). In spite of these reports, there appears to be sufficient evidence for a synergistic interaction between persistent HBV infection and aflatoxin uptake in carcinogenesis. Whether the codon 249 transversion in *p53* of hepatocellular cancer patients originates indeed from aflatoxin exposure, or represents a marker of specific HBV infections, remains to be established.

The mechanism of interaction between HBV and aflatoxins is only partially understood: in HBV transgenic mouse lineages, specific cytochrome P450s (CYP 1A and CYP 2A5) are induced in association with liver injury (Kirby et al., 1994; Chemin et al., 1996, 1999; Chomarat et al., 1998). These effects were absent in lineages where liver injury was not associated with HBV transgene expression and occurred only in HBs, but not in HBx transgenic lineages (Chomarat et al., 1998; Chemin et al., 1999). Further studies are required to assess the significance of these observations.

Concomitant infection with hepatitis C virus which may occur within the same hepatocyte (Rodríguez-Íñigo et al., 2005) and diabetes mellitus are being discussed as additional risk factors (for reviews, see Hassan et al., 2002; Raimondo et al., 2005).

6.1.8 Mechanism of HBV-Mediated Oncogenesis

The previous sections have described specific functions of HBx and HbsAg in relation to events leading to liver cancer. A number of reports have speculated on a role of HBV DNA integration as cause of hepatocarcinogenesis. Integrated HBV sequences are found in approximately 80% of HBV-positive cancers (Brechot et al., 2000). The *HBx* gene is the most common ORF integrated into the host cell genome, where it becomes frequently mutated and rearranged (Huo et al., 2001). The integrated sequences possess a diminished ability to function as a transcriptional co-transactivator and as an NF-κB pathway activator. Yet, they are still able to bind to p53 and to abrogate p53-mediated apoptosis. In view of the fact that 20% of HBV-positive hepatocellular cancers are negative for HBV DNA integration, these observations may point to an auxiliary function of integrated HBx in malignant progression, although they do not support an essential role of this event.

A critical role for the integrational site of HBV DNA within the host cell genome has also been envisaged. Early observations claimed a rate of about 90% of integrated sequences in HBV-positive liver cancers, some of them without expression of HBsAg (Paterlini and Brechot, 1991). In contrast to observations made with WHV, no common integration site has been discovered. In some instances, however, integration has been noted in genes involved in cell proliferation, such as cyclin A2 (Wang et al., 1990), the retinoic acid receptor and steroid receptor genes (Dejean et al., 1986), in *SERCA1* and *TRAP1* genes (Graef et al., 1994; Pineau et al., 1996; Chami et al., 2000; Gozuacik et al., 2001), and into the human telomerase gene (Paterlini-Brechot et al., 2003). It should be of interest that a HBV pre-S-retinoic acid receptor beta chimera transforms erythrocytic progenitor cells *in vitro* (Garcia et al., 1993). Possibly related to this observation, the retinoid X receptor RXR alpha binds to and trans-activates the HBV enhancer (Huan and Siddiqui, 1992).

If one tries to summarize the available data, three prime factors appear to influence hepatocarcinogenesis in chronically HBV-infected patients: primarily functions of two viral genes, *HBx* and *HBs*, and in the latter case particularly mutated pre-S2, consistently act as mutagens by inducing oxidative stress within the infected cells and negatively interfering with DNA excision repair. These functions are in part initiated, augmented and amplified by the interaction of cytokines of CTLs. This chronic process of induced mutational events should eventually lead to necrotic changes and cirrhosis, and finally to the selection of clones with increased proliferative potential and invasive growth properties. The latency period, commonly in the order of 40 to 50 years after primary HBV infection, would find an explanation in this process, requiring a number of genetic modifications. The rare HCCs that arise already in childhood might find a reasonable explanation in pre-existing genetic modifications.

Based on these considerations, HBV-mediated carcinogenesis can be considered as a predominantly indirect process. On rare occasions, however, insertional mutagenesis may contribute more directly to cancer development.

6.1.9 Prevention and Control of HBV-Mediated Infections

6.1.9.1 Prevention

The discovery of HbsAg in the blood of infected carriers (Blumberg et al., 1967) paved the way for the development of hepatitis B vaccines. Initially, viral antigen was used and purified from the blood of HBs carriers (Buynak et al., 1976; Hilleman et al., 1983). This represented a major achievement in view of the absence of any suitable tissue culture system for virus propagation, and the lack of a neutralization test. Following the demonstration of the absence of infectious virus from the vaccine preparation in chimpanzees, and also of a protective effect against a HBV virus challenge, the clinical trials were commenced in 1975. Double-blind controlled clinical studies proved the protective efficacy of the vaccine (Szmuness et al., 1981; Francis

et al., 1982), and the plasma-derived vaccine was licensed in 1981, representing the first licensed viral subunit vaccine. Subsequently, with the advance of technology, recombinant yeast and bacterial expression systems were developed and the yeast antigen vaccine was licensed in 1986 as the first recombinant vaccine (McAleer et al., 1984; Hilleman, 1987).

Application of the vaccine within hours after birth protected 75–80% of infants born to HBV-positive mothers that otherwise would become viral carriers in 80–90% of cases (for a review, see Hilleman, 2003). Follow-up surveillance studies were conducted in Gambia, China, Italy, Taiwan, and Alaska (MacMahon and Wainwright, 1993; Kao and Chen, 2002). The vaccine has been clearly successful in preventing persisting HBV infections and in preventing fulminant hepatitis cases. The present follow-up is too short to arrive at strong conclusions concerning the preventive effect of this vaccine for HCCs, although a trend towards a reduction of this cancer has already become apparent in Taiwan (Kao and Chen, 2002).

It seems that approximately 5% of the overall population possesses a genetically encoded HLA constitution that precludes the development of an adequate T-cell response (Desombere et al., 1998). In addition, mutations in the surface antigen of HBV may occur, particularly in position 145 of the S-antigen, changing a loop in the protein against which the antibody is directed. This may result in children who become "immune carriers" (Carman et al., 1990; Wallace and Carman, 1997; He et al., 2001; Torresi, 2002).

The perspective of global application of this vaccine – which in all likelihood can be considered as a vaccine against a specific type of cancer – bears substantial promise for the future control of a common human cancer. If combined with another anticancer vaccine directed against high-risk papillomaviruses, it would probably protect against the development of a second common malignancy, cancer of the cervix (Báez-Astúa et al., 2005).

6.1.9.2 **Therapy**

Two chemotherapeutic drugs have been licensed to treat hepatitis B infections. These are the nucleoside analogues lamivudine and adefovir. Both drugs are relatively nontoxic, although some kidney toxicity has been reported (for reviews, see Nguyen and Wright, 2001; Chen et al., 2002; Pramoolsinsup, 2002; Conjeevaram and Lok, 2003; Jain and Fung, 2003). Some therapeutic successes have been achieved with both drugs, although mutations in the virus may lead to the development of drug resistance.

Interferon α has been a longstanding option for the treatment of hepatitis B, though the effectiveness of this treatment is limited, even when combined with chemotherapy (Hilleman, 2003). The activity of interferon can be increased to some extent by covalent linkage of a polyethylene moiety (pegylated interferon) (Hui et al., 2005; Maillard and Gollan, 2005).

Novel approaches, the use of single-stranded anti-sense DNA oligonucleotides, of ribozymes, small interfering RNA, and the use of double-stranded RNA for inter-

feron induction have not yet reached routine clinical application, and may be useful after solving the problems of their application (reviewed in Hilleman, 2003). The same appears to account for therapeutic vaccines which still require further research.

In general, success in the treatment of persistent HBV infections is still very limited and requires future attention of both basic and clinical research.

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7
Flaviviruses

7.1
Hepatitis C Virus

7.1.1
History

During the 1970s it became clear that a specific form of post-transfusion hepatitis could be caused by an unidentified agent, clearly different from hepatitis A and B (Feinstone et al., 1975). Prior to 1983, this unidentified agent had been transmitted experimentally to chimpanzees (Tabor et al., 1983). The first sequences of this putative agent were cloned and reported in 1989 (Choo et al., 1989), and subsequent studies showed the virus to be widely spread among human populations, to cause acute and chronic hepatitis infections, and also to have links with hepatocellular carcinoma (HCC) (for a review, see Hoofnagle, 2002).

It has been estimated that, globally, more that 170 million people are currently infected with hepatitis C virus (HCV) (Alter and Seeff, 2000). Since acute infections are frequently asymptomatic, early diagnosis is often difficult (Chisari, 2005). Approximately 70% of HCV-infected persons acquire a persistent and chronic infection, commonly associated with serious liver disease, such as liver cirrhosis and HCC. Today, HCV infections are the most common indication for liver transplantation, accounting for 40–50 % of liver transplants (Brown, 2005).

7.1.2
Epidemiology

High rates of HCV seropositivity have been noted in Japan, Spain, Hungary, Saudi Arabia, and Southern Italy (van der Poel, 1994). In Egypt, up to 19.2% of blood donors were found to be HCV-positive (Saeed et al., 1991; Hibbs et al., 1993). Seropositivity increases with age. In children the rate is commonly very low; for example, in rural Egypt 2.3% of children aged 1–5 years were positive, while in children aged 6–10 years the rate increased to 5.8%, and in those aged 15 years it was 9.7% (Hibbs et al., 1993).

Anti-HCV antibodies are often observed in family members of HCV-positive patients, suggesting intrafamilial transmission (Kiyosawa et al., 1990). About 10%

of infected mothers transmit the infection to their babies (Lin et al., 1994; Ohto et al., 1994). Predominant risk factors for HCV transmission are discussed in Section 7.1.4.

Hepatocellular carcinoma often develops in cirrhotic livers, but it may also arise in noncirrhotic organs. This cancer commonly arises two to four decades after the primary HCV infection (Liang and Heller, 2004), its occurrence being linked to a number of environmental, dietary and lifestyle factors, including alcohol consumption, aflatoxin exposure and, in particular, HCV infection (El-Serag, 2001). In addition, male gender, hepatitis B virus co-infection, household contact, acupuncture, tattooing- and transfusion-related HCV-transmission, and needle-sharing by injecting drug users predispose to a higher risk (Kao and Chen, 2000; Seeff, 2002).

Six major genotypes and more than 50 subgenotypes of HCV have been identified (Simmonds, 2004). These differ substantially not only in their geographic distribution, but also in the clinical course of the disease and in their response to therapy (Feld and Hoofnagle, 2005). Genotypes are defined here as differing from each other by 30–50% at the nucleotide level, whereas subtypes show differences of 10–30% (Bukh et al., 1995; Simmonds et al., 2005). Different isolates of the same subtype may differ by 5 to 15% (Hoofnagle, 2002). Genotype 1a is most common in Northern Europe and in the United States, while genotype 1b represents the most common form worldwide (El-Serag and Mason, 1999). Genotypes 2a and 2b also show a worldwide distribution, but are particularly common in Japan and in Northern Italy. Genotype 3 prevails in India, and genotype 4 is mainly found in Africa and the Middle East. The other genotypes 5 and 6 are rare, although 5 is more common in South Africa and 6 in Hong Kong and south-east Asia. A phylogenetic tree indicating areas of higher prevalence of the respective genotypes is shown in Figure 7.1.

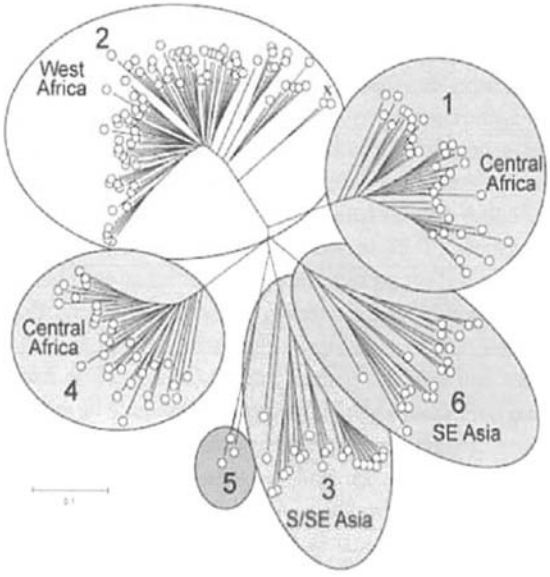

Fig. 7.1 Phylogenetic tree of hepatitis C virus genotypes and subtypes, together with indications of the prevalence of the respective infections. (Reprinted from Simmonds et al., *Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes.* Hepatology 42, 2005. With permission of Wiley-Liss, Inc, a subsidiary of John Wiley & Sons, Inc.)

The studies conducted to date indicate that HCV infections have been present for a long time in parts of Africa and Asia, and that the transmission pattern there has been different from that in Western and other non-tropical countries (Simmonds et al., 2005). Tattooing, skin scarifications, sexual contacts and pre- and perinatal transmissions seem to be the prime factors for transmission in some of the tropical areas. Blood transfusions, the use of nonsterilized needles and sharing of injection equipment most likely contributed to the spread of HCV infections in industrialized countries (Simmonds, 2001; Ndjomou et al., 2003). HCV genotype 1 appears to be most frequent among blood donors, hemophiliacs and hemodialysis patients, whereas genotype 3 was frequently found in cirrhotic patients and injecting drug users (Oliveira et al., 1999).

7.1.3
Viral Genome Structure, Transcription, Translation, Gene Functions, and Taxonomy

The HCV genome consists of 9.6 kb uncapped linear single-stranded RNA with positive polarity. Untranslated regions occur 5' and 3' which contain control elements for translation and replication (Sarnow, 2003; Lindenbach and Rice, 2005; Chisari, 2005). An uninterrupted open reading frame (ORF) codes for a single polyprotein of 3010 or 3011 amino acids, subsequently processed into structural and nonstructural proteins, cleaved by viral and host cell proteases (Lohmann et al., 1996; Penin et al., 2004). An outline of the HCV genome structure and derived proteins is shown in Figure 7.2.

Translation of viral RNA occurs entirely in the cytoplasm and depends on an internal ribosome entry site (IRES) located 5' in the genome (Spahn et al., 2001). The IRES sequence binds the 40S ribosomal subunit directly and induces mRNA-bound conformation in this subunit. After recruitment of the eukaryotic initiation factor eIF-3 and the ternary complex Met-tRNA-eIF-2-GTP, the 48S intermediate is formed which transits into the translationally active 80S complex (Ji et al., 2004; Otto and Pu-

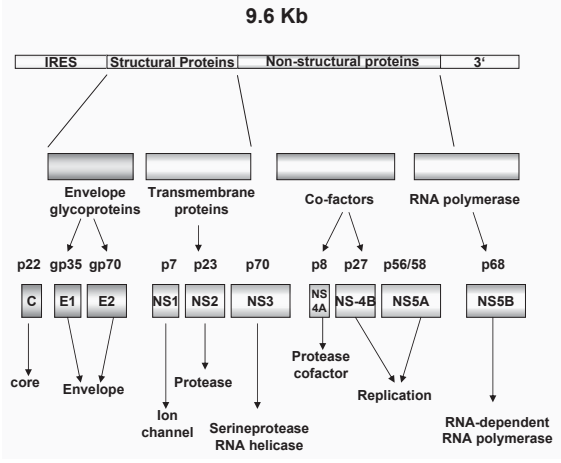

Fig. 7.2 Genomic structure of the hepatitis C virus genome and derived proteins cleaved by viral and cellular proteases.

glisi, 2004; Lindenbach and Rice, 2005). The translated large viral polyprotein is subsequently cleaved into 10 proteins: a basic core protein; two glycoproteins E1 and E2 and a small membrane protein; and p7, which probably functions as an ion channel (Griffin et al., 2003; Pavlovic et al., 2003). Additional nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B are responsible for regulating the life cycle of HCV.

The carboxy-terminal two-thirds of NS2 contain the catalytic triad of a cysteine protease (Lindenbach and Rice, 2005). The C-terminus of NS3 encodes a helicase which seems to function as a dimer (Serebrov and Pyle, 2004). NS5A interacts with cellular phosphorylases, generating different phosphoforms of this protein (Ide et al., 1997; Kim et al., 1999; Coito et al., 2004). An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus NS5A protein with cellular membranes (Brass et al., 2002).

NS5B encodes the RNA-dependent RNA polymerase and resembles the structure of a similar enzyme of bacteriophage φ6 (Butcher et al., 2001). For a detailed description of viral proteins and their functions, the reader is referred to the review by Lindenbach and Rice (2005).

During replication the mutation rate of HCV is high and amounts to 10^{-3} per nucleotide per generation (Levin et al., 2005). This results in an enormous accumulation of viral quasispecies in every persistently infected person, and also influences the immune response of the infected host.

Genome structure, translational properties and protein function classify HCV as a member of the Flavivirus family. It constitutes the sole representative of the genus hepacivirus (Robertson et al., 1998; Lauer and Walker, 2001). Thus far, it is the only member of the Flavivirus family to reveal oncogenic properties.

7.1.4 Infection, Transmission, and Viral DNA Persistence

Before the development of diagnostic tests for HCV, infection was transmitted through blood and blood-derived products, by hemodialysis, and by organ transplantation (Alter, 1997, 1999, 2002). Today, the majority of infections occurs in injecting drug users and their sexual partners (Alter, 1999). Sexual transmission appears to be of some importance, and inmates in correctional facilities frequently reveal a high degree of HCV positivity, whereas in the United States and many Western European countries approximately 2% of the general population is infected (Spaulding et al., 1999). Among HIV-infected individuals, up to 25% are coinfected with HCV (Choo et al., 1989). Besides transmission via blood products and sexual contacts, other – albeit less efficient – modes of transmission should exist, most likely via the digestive tract.

Approximately 10% of viremic mothers transmit HCV infections to their babies (Ohto et al., 1994), though the risk of transmission seems to be related to the extent of viremia in the mother. Mothers coinfected by HCV and HIV pose a much higher risk for HCV transmission to their newborns (Thaler et al., 1991; Novati et al., 1992).

In a typical infected person, about 10^{12} virus particles are produced each day, while chronically infected patients have viral loads in the range of 10^3 to 10^5 genomes per mL serum (Neumann et al., 1998; Lindenbach and Rice, 2005). As stated earlier, approximately 70% of infected persons become persistently infected, 20% of these will develop liver cirrhosis, and up to 2.5% will subsequently hepatocellular cancer (Bowen and Walker, 2005 a).

Since the acute infection is commonly mild, it remains frequently unrecognized. Prospective studies in humans at high risk for HCV exposure and experimental studies in chimpanzees revealed the control of acute primary viral replication by an expansion of antiviral $CD4^+$ and $CD8^+$ T cells (Bowen and Walker, 2005 b). Spontaneous resolution of HCV infection does not protect against later reinfections, but does reduce the risk for persistent infection upon re-exposure.

The studies on biological and molecular properties of HCV will be greatly facilitated by recent reports on the successful replication of this virus in tissue culture systems (Cai et al., 2005; Lindenbach et al., 2005; Zhong et al., 2005), with 10^5 infectious units per mL being produced within 48 h. The replication occurs in human hepatocyte-derived cells which had already previously been reported to permit low-efficiency replication of HCV (Kato et al., 2002, 2003). Replicon systems had been developed even earlier, though these neither allowed efficient replication nor produced infectious virus (Blight et al., 2000; Lohmann et al., 2001).

Recently, modulation of HCV RNA abundance by liver-specific microRNA has been described (Jopling et al., 2005). MicroRNA 122 is specifically expressed and abundant in human liver cells. This microRNA seems to facilitate the replication of viral RNA and may provide a clue for the hepatotropism of HCV infections.

A recent report on the association of HCV envelope proteins with exosomes and the association of CD81-carrying exosomes with viral RNA (Masciopinto et al., 2004) may point to a completely new mode of viral transmission, and may also explain some of the difficulties in finding HCV particles in infected tissues. Since similar reports appeared for specific retrovirus transmissions (see Chapter 8, Section 8.3), these observations, if confirmed, may also shed some light on the problems of immunologically clearing HCV infections.

7.1.5 Pathogenesis and Immune Interactions

7.1.5.1 Evasion of Host Defense Mechanisms

Infection with HCV triggers intracellular signaling which results in the production of interferons α and β (reviewed in Gale and Foy, 2005) via Toll-like receptors (Cook et al., 2004; Iwasaki and Medzhitov, 2004). The main components of this signaling are interferon (IFN) regulatory factor (IRF)-3 and nuclear factor κB (NF-κB) (Au et al., 1995; Lin et al., 1999; Richmond, 2002), which form an enhanceosome complex on the IFN-β promoter (Sen, 2001). NF-κB is also involved in the expression of chemokines and inflammatory cytokines (Tai et al., 2000). A large number of interferon-β-stimulated genes mediate the host response to virus infections (Der et al.,

1998). These result in the disruption of viral RNA translation and inhibition of viral RNA synthesis (Guo et al., 2001; Shimazaki et al., 2002; Gale and Foy, 2005).

Interferon-α is one of the induced genes which contribute to the maturation of immune effector cells and potentiates the synthesis of pro-inflammatory proteins (*Sen*, 2001). The expression levels of interferon-stimulated genes vary substantially in patients with chronic hepatitis C infection and cirrhosis (Smith et al., 2003). This shows that HCV infection is able to modify the host response and to evade a host cell-mediated control of this virus infection.

One of the cellular receptors binding double stranded (ds) RNA, and also of HCV, is the RNA helicase RIG-1 which signals the downstream activation of IRF-3 and NF-κB (Sumpter et al., 2005). Toll-like receptor (TLR) 3 is also activated by dsRNA and directs the activation of IRF-3 and NF-κB via a different pathway (for a review, see Gale and Foy, 2005).

Hepatitis C virus is able to interfere with these host cell control mechanisms and to evade them effectively. The NS3/4A protease functions counteract the IRF-3 activation and inhibit the interferon-β expression by blocking RIG-1 signaling and by cleaving the adaptor protein for TLR-3, TRIF, and thus impairing the function of TLR-3 (Foy et al., 2005; Li et al., 2005 a; Ferreon et al., 2005). The other function of NS3/A4 protease is the cleavage of nonstructural proteins from the HCV polyprotein during virus replication (De Francesco et al., 2000). A schematic outline of HCV pathogenesis is depicted in Figure 7.3.

Thus, HCV is using its protease activity, functioning as an essential component for viral replication, also to evade host cell control mechanisms by disrupting RIG-1 and TLR-3 signaling. This diminishes the function of two major pathways in interferon production (Li et al., 2005 b; Breiman et al., 2005). Overexpression of IKKε bypasses the HCV-mediated inhibition, restores transactivation of the IFN-β promoter, and impairs positive and negative replicative strands of the HCV replicon (Breiman et al., 2005).

Besides NS3/4A, NS5A antagonizes IFN-α functions (MacDonald and Harris, 2004; Zhang et al., 2005). This seems to be due to NS5A-mediated induction of IL-8

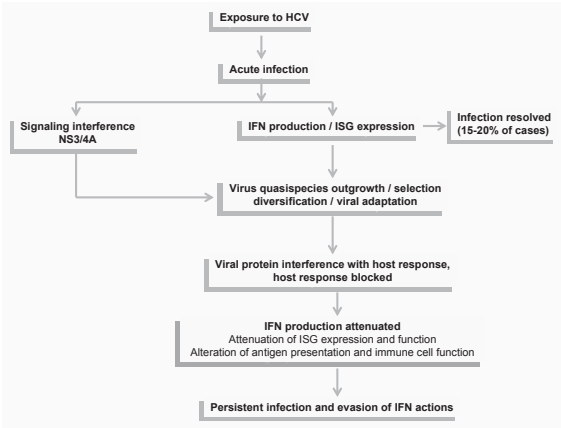

Fig. 7.3 Schematic outline of HCV pathogenesis. IFN: interferon; ISG: interferon-stimulated genes. (Modified from Gale and Foy, 2005.)

expression, a pro-inflammatory cytokine, which interferes with interferons (Khabar et al., 1997). In addition, HS5A and E2 proteins inhibit protein kinase R (PKR), apparently permitting evasion from the translation-suppressing functions of IFN and a PKR-mediated increased host response to infection (Gale et al., 1998; Taylor et al., 1999; Noguchi et al., 2001; Katze et al., 2002).

One other defense mechanism against host control results from the absence of a proofreading function of viral RNA-dependent RNA polymerase. This results in error-prone virus replication and creates closely related, but genetically different, quasispecies variants. It also influences the responsiveness to interferon therapy (Farci, 2001). Genetic variants occur at higher frequency during chronic infection and cirrhosis (Farci et al., 2000, 2002). A hotspot for genetic modifications appears to be a 40-residue interferon sensitivity-determining region of NS5A (Enomoto et al., 1996; Pascu et al., 2004; Schinkel et al., 2004).

7.1.5.2 **Host Immune Responses**

T cells play a central role in HCV control and clearance (for a review, see Bowen and Walker, 2005 a). The immunological memory does not protect against re-infection, although it does reduce the risk for viral persistence (Bowen and Walker, 2005 b).

Virus-specific antibodies commonly appear 7–8 weeks after infection (Pawlotzky, 1999). A major target to the antibody response is the hypervariable-1 region of the E2 envelope glycoprotein. Sequence variations in this region, occurring simultaneously with antibody seroconversion, determine the outcome of the infection in humans (Farci et al., 2000). The role of antibodies in resolving HCV infections is not entirely clear, since in some patients resolution may occur without detectable antibody response (Logvinoff et al., 2004; Meunier et al., 2005). HCV RNA genomes are detectable only a few days after infection, and reach the highest level 6–10 weeks later, irrespective of the outcome of infection (Abe et al., 1992; Beach et al., 1992; Alter et al., 1995).

Expansion of virus-specific $CD8^+$ T cells coincides with the immunopathological changes, monitored by elevated levels of transaminases in the serum (Cooper et al., 1999; Lechner et al., 2000; Thimme et al., 2002, 2003; Shoukry et al., 2003). Although higher concentrations of cytotoxic T-lymphocytes (CTLs) are found in chronically infected livers, they are not generally correlated with severity of disease (Nelson et al., 1997; He et al., 1999; Freeman et al., 2003). In chronic HCV infection, CTLs commonly target only a few epitopes (Koziel et al., 1992; Wedemeyer et al., 2002; Lauer et al., 2004). Occasionally, however, a broader spectrum is recognized during several years of persistent infection (Erickson et al., 2001; Kimura et al., 2005).

In chronically HCV-infected patients $CD4^+$ T-cell responses are low or not detectable (Diepolder et al., 1995; Takaki et al., 2000; Rosen et al., 2002). In resolved hepatitis C infection a broad specificity of virus-specific $CD4^+$ T-helper cell responses has been observed (Day et al., 2002). Antibody-mediated depletion of $CD4^+$ cells in immune chimpanzees, re-challenged with the same HCV strain, resulted in persistent infection Grakoui et al., 2003). These data point to the important role of $CD4^+$

T cells in the control of HCV infections. It seems that the interaction between the HCV core and the complement receptor gC1qR induces dysfunction of T lymphocytes via induction of the suppressor of cytokine signaling (SOCS) family members SOCS1 and SOCS3 through inhibition of the Jak/STAT pathway (Yao et al., 2005).

The production of the immune-suppressive cytokine interleukin 10 (IL-10) by a subset of $CD8^+$ T-cell lines derived from the liver of persistently infected patients provided a first hint for MHC class-I-restricted antigen-specific regulatory activity with the potential to suppress antiviral T cells (Koziel et al., 1995; Bowen and Walker, 2005 a). The observation that intrahepatic $CD8^+$ cells from persistently infected patients suppressed HCV-specific and IL-10-dependent in-vitro proliferation of liver-derived lymphocytes, lends further support to the virus antigen-specific suppression (Accapezzato et al., 2004).

By summarizing these studies, failure of $CD4^+$ T-cell responses predicts HCV persistence (Bowen and Walker, 2005 a). The HCV interference with innate immune mechanisms (see Section 7.1.5.1) may be linked with the defect in $CD4^+$ T-cell help. The role of $CD8^+$ T cells in resolving the HCV infection seems to depend on the effectiveness of viral interference with innate immunity. Yet, successes in the early therapy of HCV infection seem to be independent of broad $CD8^+$ T-cell responses (Lauer et al., 2005). Clearly, the present picture of host/virus immunological interactions is still incomplete and requires further investigation.

7.1.6 Role in Hepatocellular Carcinoma

7.1.6.1 Experimental Evidence for a Role of HCV in Liver Cancer

7.1.6.1.1 Evidence from Tissue Culture Studies and Transgenic Mice

The mechanism of HCV involvement in HCC is still not fully understood, although some progress has been made recently. The absence of integrated viral nucleotide sequences, or even of intranuclear episomal genomes, points more to an indirect involvement of this virus infection in the development of liver cancer. It seems, however, that – at least in the majority of HCV-linked HCCs – viral genomes persist within the cancer cells (Tang et al., 1995). Attempts to inhibit a specific gene function of the NS3 gene by recombinant intracellular antibodies also resulted in an inhibition of cell proliferation and loss of the transformed phenotype of NS3-transformed cells (Zemel et al., 2004). These last two results would be more compatible with a direct role of HCV infection in liver carcinogenesis.

Two approaches have attempted to contribute to the analysis of this question: first, measurements of the biological effects of transfection of individual viral genes of HCV into various tissue culture systems; and second, the implantation of these genes into transgenic mice. Carcinogenic effects have been reported for three individual HCV genes, but only two of these have repeatedly been shown to result in cell transformation and tumorigenicity of tissue culture cells and in transgenic animals.

Several reports claim malignant transformation of tissue culture cells by the HCV NS3 protein. NIH 3T3 cells transfected with the 5'-half cDNA of the NS3 serine protease sequence proliferated rapidly, lost contact inhibition, grew anchorage-independently in soft agar, and formed tumors in nude mice (Sakamuro et al., 1995). The transformed cells continued to harbor this viral DNA. These data were confirmed in another study in the human liver cell line QSG7701 (He et al., 2003). Sequence comparisons of NS3 clones isolated from hepatocellular carcinomas revealed unique changes at the vicinity of catalytic sites of this region. These were the insertion of a charged amino acid, or the substitution of a polar by a hydrophobic amino acid, or the substitution of a charged with a polar amino acid (Zemel et al., 2000). NS3 also transformed nontumorigenic rat fibroblasts to rapid and serum-independent growth, loss of contact inhibition, anchorage independence, and tumorigenicity for nude mice (Zemel et al., 2001). A mutation at the catalytic site (serine 139 to alanine) showed no transforming activity.

Studies on a potential oncogenic activity of other nonstructural proteins of HCV were less convincing: one report analyzed the cooperation of NS4B with Ha-ras and claimed a positive interaction (Park et al., 2000). The NS5A sequence has also been implicated in oncogenesis (Gosh et al., 1999; Lan et al., 2002), possibly acting by an inhibition of protein kinase R (Giminez-Barcons et al., 2005). NS5A-transgenic mice, however, exhibited no major histological change within the liver up to 24 months of age (Majumder et al., 2003). Thus, the involvement of these genes in liver carcinogenesis remains an open question.

Data on increased cell proliferation and transformation are more convincing for the HCV core protein. Two independent lines of core gene-transgenic mice developed hepatic steatosis early in life as a histological feature characteristic for chronic HCV infection. After 16 months, initially liver adenomas developed and subsequently also HCCs (Moriya et al., 1998). Additional reports support this observation (Naas et al., 2005). One report revealed malignant transformation of human Chang-liver cells (Shan et al., 2005), while the other showed that the HCV core protein aberrantly sequesters a nuclear transcription protein, designated as LZIP, in the cytoplasm, inactivates its function and potentiates cellular transformation (Jin et al., 2000). LZIP was suggested to serve as a cellular tumor suppressor factor targeted by the HCV core protein. In NIH 3T3 cells, the HCV core protein interacts directly with and activates STAT3 through phosphorylation of a tyrosine residue (Yoshida et al., 2002). This resulted in rapid proliferation, up-regulation of Bcl-XL and cyclin D1, anchorage-independent growth, and tumorigenesis. One other group reported the development of liver adenomas and malignant lymphomas in a line of transgenic mice at ages over 20 months (Ishikawa et al., 2003). In HCV transgenic mice treated with diethylnitrosamine, tumor progression was accelerated by the core protein-mediated suppression of apoptosis (Kamegaya et al., 2005). A cooperative interaction in the transformation of NIH 3T3 mouse fibroblasts and HCV core protein and hepatitis B X protein has also been noted (Jung et al., 2003). Hepatitis C virus replication in stably transfected HepG2 cells, where primarily core and NS5B proteins were detectable, resulted in growth in soft agar, and accelerated tumor growth in nude mice (Sun et al., 2004).

HCV core sequences isolated from liver tumors, but not from adjacent nontumor tissue, significantly reduce transforming growth factor (TGF)-β activity (Pavio et al., 2005). This appears to block the growth-inhibitory function of TGF-β. The effect is mediated by direct binding of the core protein to Smad3, preventing binding of the latter to DNA.

NF-κB is considered as a tumor promoter in inflammation-associated cancers (Pikarsky et al., 2004). Its activation is commonly triggered by tumor necrosis factor-α (TNF-α). HCV core protein, and also NS5A, activate NF-κB-dependent signaling and suppress TNF-α-mediated apoptosis in human cells (Marusawa et al., 1999; Miyasaka et al., 2003; Waris et al., 2003). The core protein associates with the TNF-R1-TRADD-TRAF2 signaling complex and synergistically activates the TNF-α induction of NF-κB (Chung et al., 2001; Yoshida et al., 2001). The core protein also associates with the TNF receptor-related lymphotoxin-β receptor and modulates the cytolytic activity of this receptor – ligand complex (You et al., 1999). Interestingly, HCV core protein also *trans*-activates the inducible nitric oxide synthase promoter via NF-κB activation (de Lucas et al., 2003). NF-κB also mediates the activation of cyclooxygenase-2 (Cox-2) and the product of Cox-2 activity prostaglandin E_2 (Waris and Siddiqui, 2005). This activation was sensitive to antioxidant and calpain inhibitors, underlining the role of HCV core protein in inducing oxidative stress. In contrast to hepatic cells, in macrophages HCV core protein suppresses NF-κB activation and Cox-2 expression by directly interacting with IκB kinase β (Joo et al., 2005). In macrophages, the core protein suppresses IL-12 and nitric oxide production that is critical for the induction of Th1 and innate immunity (Lee et al., 2001). Thus, these apparently opposing effects in hepatic cells and in macrophages seem to aid the virus in establishing its persistence. This interpretation is supported by the immune suppression and liver damage observed in HCV core protein transgenic mice (Soguero et al., 2002).

In NS5A-induced NF-κB activation, tyrosine phosphorylation of IκBα at tyrosine residues 42 and 305 induces the activation process (Waris et al., 2003). NS5A expression reduced the function of caspases 8, 9, and 3. Apparently, the inhibition of caspase 8 activation is the reason for the anti-apoptotic effect of NS5A protein (Miyasaka et al., 2003).

7.1.6.1.2 **Potential Oncogenic Functions of HCV Proteins**

The HCV core protein, which interacts with several cellular regulatory functions (Varaklioti et al., 2002; Yamanaka et al., 2002; Anzola, 2004), occurs in both an innate form (nucleotides 1–191) and in a processed mature form (nucleotides 1–173). The innate form is retained in the cytoplasm and enhances $p21^{WAF1}$ expression by activating p53, whereas the mature form persists in the nucleus and suppresses $p21^{WAF1}$ by a p53-independent pathway. The HCV core protein also interacts with p73 (Alisi et al., 2003; Benard et al., 2003). The interaction with p73α, but not with p73β, prevents growth arrest by the former. By interacting with these proteins, the HCV core protein can influence cell proliferation and apoptosis.

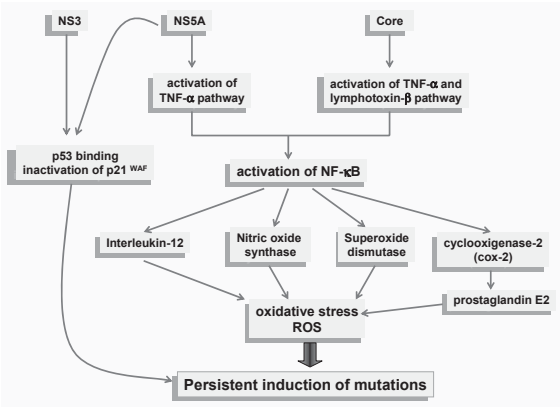

Fig. 7.4 Tentative scheme of potentially oncogenic functions of hepatitis C virus proteins.

NS3 protein is thought to form a complex with wild-type p53, and also may influence its functions (Ishido and Hotta, 1998). An additional report described inhibition of the promoter of $p21^{WAF1}$ by NS3 (Muramatso et al., 1997). The repression of this promoter seems to be due to the modulation of p53 activity. NS3 acts synergistically with the HCV core protein in mediating these functions. NS3 is stabilized by co-expression of NS4A, and the complex is directed to the endoplasmic reticulum (Wölk et al., 2000).

NS5A binds directly to p53 and co-localizes p53 in the perinuclear region (Lan et al., 2002). Intramolecular cleavage of NS5A around amino acid 150 may target one of the truncated proteins to the nucleus (Song et al., 2000). These post-translationally modified truncated forms of NS5A may act as transcriptional activators, although the individual functions are still poorly defined (Khabar and Polyak, 2002; Reyes, 2002). A tentative scheme of potentially oncogenic functions of HCV is illustrated in Figure 7.4.

7.1.6.1.3 Alcohol as a Cofactor in HCV-Mediated Carcinogenesis

Besides immunosuppression by HIV or co-infection with hepatitis B virus, alcohol consumption emerges as the most important cofactor for HCV-linked carcinogenesis. It has been stated that chronic alcohol consumption of more than 80 g per day for more than 10 years increases the risk for liver cancer approximately five-fold (Morgan et al., 2004). In decompensated alcohol-induced cirrhosis, the risk for HCC approaches 1% per year. Infection of the same individuals by HCV doubles the risk for liver cancer (Morgan et al., 2004). The prevalence of HCV infections is seven- to 10-fold higher in alcoholics in comparison to the general population, and up to 60% of HCV patients have a past history of alcohol abuse (Jamal and Morgan, 2003). In addition, alcohol abuse causes decreased response to interferon treatment of HCV patients and accelerates progression to liver fibrosis, cirrhosis and liver cancer (Safdar and Schiff, 2004). In HCV replicon-containing cells, alcohol increased HCV replicon expression in a concentration-dependent manner (Zhang et al., 2003). One function of alcohol seems to be activation of the NF-κB promoter. A specific inhibi-

tor of NF-κB, caffeic acid phenethyl ester, abolished alcohol-induced HCV RNA expression (Zhang et al., 2003). An additive activation of hepatic NF-κB by ethanol and HCV core protein has been described (Kim et al., 2001).

Thus, alcohol abuse emerges as the most important co-factor for liver cancer development in HCV-infected persons.

7.1.6.1.4 HCV – A Direct or Indirect Carcinogen: Does this Virus Fulfill Mediator Functions for Other Oncogenic Agents?

As highlighted previously, HCV exerts some functions which would be compatible with a more direct role in hepatocarcinogenesis. These include the frequent persistence of viral infection in carcinomatous tissue, and the use of recombinant antibodies in NS3-transformed cells which selectively inhibits proliferation of these cells. The significance of these observations appears questionable, however. Virus persistence is a poor indicator for a direct oncogenic role, and the sole description of a proliferation-inhibiting effect of recombinant antibodies requires further confirmation by other specific inhibitors of NS3.

The most persuasive evidence points to an indirect role of HCV infection in hepatocarcinogenesis. Tissue culture studies failed to demonstrate a direct growth-stimulating or immortalizing effect of specific HCV genes or complete viral genomes for primary human cells. Although murine and rat cells appear to be susceptible to transformation by HCV, these cells are also relatively susceptible to chemical and physical carcinogens (mutagens), and respond frequently with enhanced proliferation and transformation (Kuroki and Huh, 1993). Human cells transfected with HCV DNA or individual genes responding with increased carcinogenicity represent already immortalized lines or cells directly derived from liver tumors.

Combined with the studies in transgenic mice, the experimental evidence (even ignoring the vast body of epidemiologic studies) for a carcinogenic role of specific viral gene products emerges as increasingly compelling. It is particularly impressive for the HCV core protein expression, for the serine protease NS3 and, to a more limited degree, for NS5A. The described interaction of these proteins with p53, $p21^{WAF1}$ and p73 points to disturbances in mitotic checkpoint control and DNA repair. These observations found further support by an initial analysis for reactive oxygen species (ROS) and lipid and protein oxidation in HCV-positive patients (de Maria et al., 1996). Additional results show that superoxide dismutase is induced by HCV infection (Larrea et al., 1998), that mitochondrial injury, oxidative stress and antioxidant gene expression occur as the direct result of HCV core protein expression (Okuda et al., 2002; Korenaga et al., 2005), and that a differential contribution of NS5A and core protein results in oxidative and nitrosative stress generation (Garcia-Mediavilla et al., 2005). HCV infection activates the type II isoform of nitric oxide synthase, and thereby enhances DNA damage and mutations of cellular genes (Machida et al., 2004 a). Interestingly, ROS suppress HCV RNA replication, at least under tissue culture conditions (Choi et al., 2004). This may explain the relative paucity of HCV genomes observed in HCCs (Tang et al., 1995).

By summarizing these findings, it becomes increasingly likely that chronic DNA damage as a function of persisting HCV infections emerges as the main factor in HCV-mediated carcinogenicity. Nevertheless, it is clearly inappropriate to consider this interaction as a "hit and run" mechanism (Machida et al., 2004 b) since, at least in the majority of cancers, viral RNA continues to persist within the neoplastic cells.

These data do not exclude a mediator role of HCV for other carcinogenic agents. although to date no evidence has been produced to imply such function.

7.1.7 Role in Lymphoproliferative Diseases

7.1.7.1 Mixed Cryoglobulinemia

One of the most common lymphoproliferative extrahepatic manifestations of HCV infection is represented by cryoglobulinemia (reviewed in Sène et al., 2004). This condition is defined by circulating immunoglobulins that precipitate at cold temperatures and resolubilize when warmed. Mixed cryoglobulinemias with monoclonal components (type II), but also with only polyclonal immunoglobulins (type III) are frequently found in HCV patients (Pascual et al., 1990; Casato et al., 1991; Disdier et al., 1991; Ferri et al., 1991; Galli, 1991; Agnello et al., 1992). Cryoglobulinemia represents a clonal or polyclonal B cell proliferation accompanied by a small vessel vasculitis with vascular enluminal deposition of cryoprecipitates (Sène et al., 2004). Replication of HCV in B lymphocytes and neutrophils has been reported (Lerat et al., 1998; Crovatto et al., 2000; Fornasieri et al., 2000). HCV interacts with B cells via the CD81 receptor (Pileri et al., 1998). Lymphoproliferative conditions in HCV-infected patients are associated with lower expression of the CD81 receptor in peripheral B cells (Cacoub et al., 2003). It is likely that the expansion of clonal B-cell populations is enhanced by translocations of the Bcl-2, resulting in overexpression of this anti-apoptotic gene, as frequently observed in HCV-positive patients (Kitay-Cohen et al., 2000; Zignego et al., 2000, 2002). The core protein of HCV appears to be a constitutive component of cryoprecipitable immune complexes in type II mixed cryoglobulinemia (Sansonno et al., 2003).

Elevated serum levels of osteopontin are found in mixed cryoglobulinemia, and also in HCV-linked non-Hodgkin's lymphomas and in HCV-linked autoimmune disorders (Libra et al., 2005).

7.1.7.2 Splenic Lymphoma with Villous Lymphocytes

A second rare lymphoproliferation linked to HCV infections is splenic lymphoma with villous lymphocytes (for a review, see Sène et al., 2004). This condition is characterized by splenomegaly, pancytopenia, and clonal expansion of B cells with villous projections in the peripheral blood. The disease is closely related to immunocytoma and splenic marginal-zone non-Hodgkin's lymphoma (NHL). A multicentric French study pointed to the association between HCV infection and this

condition (Hermine et al., 2002). Patients who were successfully treated with interferon-α and ribavirin and remained negative for HCV RNA for up to 27 months, also revealed a complete hematological response. A relapse of the lymphoproliferative condition was accompanied by the reappearance of HCV RNA.

7.1.7.3 **Non-Hodgkin's Lymphoma**

The third clearly malignant condition, NHL, remains controversial in its links to HCV infections. Initial suspicions for a link arose from serological studies conducted in Italy, where between 9% and 42% of NHL patients were HCV antibody-positive in contrast to 1–9% of control groups (Ferri et al., 1994; Cavanna et al., 1995; Luppi et al., 1996; Mazzaro et al., 1996; Silvestri et al., 1997; Zignego et al., 1997). Similar data were reported from Japan, with 8% to 17% antibody-positive NHL patients in comparison to 1–7% of the control groups (Izumi et al., 1997; Mizorogi et al., 2000; Kuniyoshi et al., 2001). The majority of studies in non-Italian European countries and in North America, with one notable exception (Zuckerman et al., 1997), failed to confirm this result (Brind et al., 1996; McColl et al., 1997; King et al., 1998; Bauduer et al., 1999; Hausfater et al., 2001; Rabkin et al., 2002).

One meta-analysis considers HCV infections as risk factors for NHL with an estimated odds ratio (OR) of 10.8 (Gisbert et al., 2003). It is clear, however, that the overall risk for NHL is not high in HCV-infected patients, ranging between 0.2% and 2.49% (Ohsawa et al., 1999; Hausfater et al., 2000; El-Serag et al., 2002). HCV-associated lymphomas are, in the majority of cases, low-grade B-cell immunocytomas or follicular lymphomas (Sène et al., 2004).

Besides lymphomas, several other extrahepatic manifestations of HCV infection have been reported. Approximately one-third of patients with chronic HCV infections develop type 2 diabetes mellitus (Bahtiyar et al., 2004). The rate is particularly high in HCV-positive cirrhotic patients receiving liver transplants. Complications of mixed cryoglobulinemia, such as renal disease, neuropathy, Sjögren's syndrome, noncryoglobulinemic systemic vasculitis, porphyria cutanea tarda, and autoantibody production, among others, represent further manifestations (for reviews, see Mayo, 2003; Sène et al., 2004; Soule et al., 2005).

In attempting to summarize the role of HCV infections in lymphoproliferative disorders, it seems evident that persistent HCV infections result in specific lymphoproliferative diseases such as mixed cryoglobulinemia. The data relating to splenic lymphoma with villous lymphocytes also seem to be persuasive. The role of HCV in NHL is clearly not settled. The likely function of HCV as an indirect carcinogen, however, could argue in favor of its role in neoplastic changes induced in persistently infected B lymphocytes.

7.1.8 Prevention and Control

7.1.8.1 Vaccines

To date, an effective vaccine against HCV infection has not been developed, though some hope arises from observations in convalescent humans and chimpanzees who, in the majority of cases, are protected against reinfection by the same virus (Bassett et al., 2001; Weiner et al., 2001; Mehta et al., 2002; Lanford et al., 2004). It is difficult to prime $CD8^+$ CTLs with polypeptide subunit vaccines, though some responses have been obtained (Polakos et al., 2001; Pearse and Drane, 2005). The use of recombinant HCV envelope glycoproteins gpE1 and gpE2 as vaccine antigens appears to be somewhat more promising (Ralston et al., 1993; Houghton and Abrignani, 2005). In studies on chimpanzees, the highest responding animals were completely protected against HCV infection (Choo et al., 1994). One of the important questions to be resolved concerns the crossprotection against heterologous HCV strains. Vaccinated chimpanzees infected with heterologous HCV acquired acute infections but did not develop virus persistence, whereas in control animals the infection became chronic (Houghton and Abrignani, 2005). In contrast to these results, ISCOMATRIX adjuvanted NS3–4-5 core polyprotein failed to prevent chronic infection, although it elicited broad $CD4^+$ and $CD8^+$ T-cell responses in the absence of measurable antibodies to envelope glycoproteins (Polakos et al., 2001; Pearse and Drane, 2005).

Other approaches have used adenoviral, avipox, alphaviral and vaccinia viral vectors that are in part presently explored in various animals, including chimpanzees (Brinster et al., 2002; Perri et al., 2003; Pancholi et al., 2003; Abraham et al., 2004; Catalucci et al., 2005). However, the high mutation rate of HCV genomes poses a substantial problem, and may require different approaches for successful vaccination.

Similar to preventive vaccines, therapeutic vaccines have thus far not been successfully applied. A number of candidate vaccines are presently undergoing preclinical and clinical testing (for a review, see Houghton and Abrignani, 2005), but their efficacy remains to be established.

7.1.8.2 Therapy

The first beneficial effects in the therapy of HCV infections were seen with IFN-α (Hoofnagle et al., 1986). This led to a reduction of HCV RNA in the serum and, in some instances, also to a resolution of chronic infection (Lau et al., 1998). After 12 months of treatment sustained response was only obtained in 16–20% of cases (Di Bisceglie and Hoofnagle, 2002). Additional improvements have been achieved with concomitant treatment with ribavirin, which doubled the response to 35–40% (McHutchison and Poynard, 1999), and also with pegylated IFN-α, which provides a longer half-life and improved antiviral activity (Glue et al., 2000; Heathcote et al., 2000; Zeuzem et al., 2000; Lindsay et al., 2001).

The application of HCV-targeted drugs suffers from the rapid development of resistance of the virus under selective pressure. Resistance develops quickly against HCV enzyme inhibitors, and also against small interfering RNA (siRNA) (Tomei et al., 2005). The NS3–4 serine protease, a heterodimeric protease containing the amino-terminal domain of NS3 protein and the small NS4A cofactor, and the NS5B RNA polymerase emerge as the most suitable targets (De Francesco and Migliaccio, 2005). Some protease inhibitors are presently undergoing clinical tests and show promising results (for a review, see De Francesco and Migliaccio, 2005). Inhibitors of NS5B polymerase include several nucleoside analogues which induce premature termination of RNA synthesis, though only one of these has demonstrated antiviral activity in clinical trials (De Francesco and Migliaccio, 2005). Some non-nucleoside inhibitors of the viral polymerase have also entered clinical trials, but the chemical details of these have not been disclosed by the respective companies.

A very different approach is represented by the use of nucleic acid-based antiviral drugs, which target the IRES at the 5' end of the viral genome. This is conserved among all HCV genotypes. A ribozyme and an anti-sense oligonucleotide were submitted for initial clinical studies, but showed either adverse effects or limited efficacy (Foster, 2004; De Francesco and Migliaccio, 2005). RNA interference (RNAi) is also currently being investigated for antiviral activity, but clinical data are not yet available (Kapadia et al., 2003; Randall et al., 2003; Wilson et al., 2003; Yokota et al., 2003).

Immunomodulatory agents stimulating Toll-like receptors are also undergoing analysis for anti-HCV activity, and agonists for Toll-like receptors 9 and 7 have revealed some promising properties. The stimulation of dendritic cells with short synthetic oligonucleotides containing unmethylated CpG motifs flanked by specific sequences leads to the production of TNF-α, IL-12, and high levels of IFN-α (Schetter and Vollmer, 2004). In addition, B-cell proliferation and antibody secretion are stimulated. A 7-thia-8-oxoguanosine represents an agonist for Toll-like receptor 7 (Lee et al., 2003). This leads to the release of inflammatory cytokines and of high levels of IFN-α. To date, clinical trials with these compounds have appeared to provide encouraging results (De Francesco and Migliaccio, 2005).

Although the prospects for a targeted chemotherapy of HCV infection are good, at present the possible interference with this infection – particularly during chronic state – is still very limited. Hopefully, some of the clinical trials which are presently being conducted will change this therapeutic outlook.

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8
Retrovirus Family

Retroviruses have long been suspected to play a role in human cancers. They were initially identified as the causative agents of chicken sarcomas, murine mammary tumors and murine leukemias and lymphomas (see Chapter 1). Some retroviruses are acutely transforming viruses that evolved by capturing cellular genes during the course of virus replication, a process which resulted in the discovery of oncogenes (Stehelin et al., 1975). Their process of reverse transcription by the viral enzyme reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970), preceding integration into the host cell genome, coupled with the experimental use of this enzyme, permitted the analysis of complementary DNA (cDNA) and, along these lines, the accurate composition of spliced RNA sequences. Moreover, retroviruses served as models to study the early events in animal carcinogenesis, in particular in lymphoproliferative conditions.

Three types of retroviral infection have been identified in humans. The first type – infections with human immunodeficiency viruses, HIV-1 and HIV-2 – cause severe immunosuppression and contribute indirectly to carcinogenesis. These viruses are not described in this chapter, and the interested reader is referred to specific textbooks dealing with infectious diseases. The well-studied structure of HIV particles shows characteristics of most retroviruses, and is depicted in Figure 8.1.

The other two types of retroviral infections in humans are represented by the human T-cell leukemia retrovirus and by a heterogeneous group of viruses that entered the human germline, the endogenous retroviruses. These types of infections will be described here.

Presently, the retrovirus family contains seven to eight clades, and representatives of human retroviruses are found in the beta-, delta-, and gamma-retroviral clades. HIV belongs into the subfamily of lentiviruses. In addition, a human spumavirus has been described as human foamy virus (Epstein et al., 1974). This virus may, however, originate from nonhuman primates and be transmitted accidentally to humans, though no clear-cut evidence is presently available for human-interspecies transmission. The individual retrovirus clades with representative members are shown in Figure 8.2.

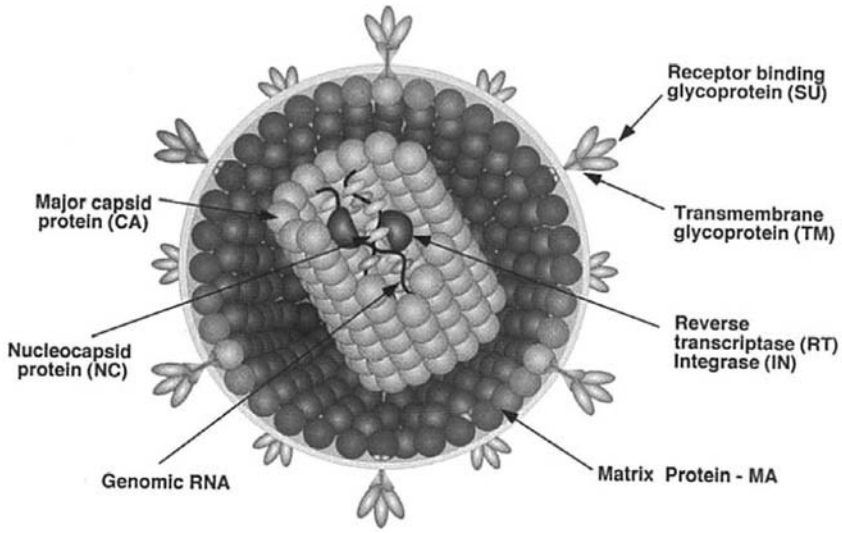

Fig. 8.1 The structural components of an HIV particle. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The DNA and RNA Reverse Transcribing Viruses, Retroviridae, 421. Copyright 2005, with permission from Elsevier.)

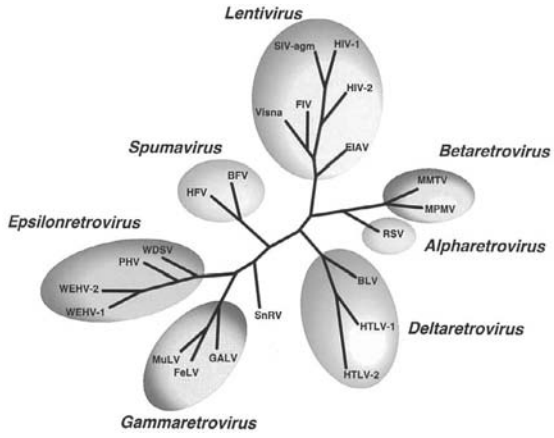

Fig. 8.2 The retrovirus family clades. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The DNA and RNA Reverse Transcribing Viruses, Retroviridae, 439. Copyright 2005, with permission from Elsevier.)

8.1
Human T-Lymphotropic Retrovirus (HTLV-1)

8.1.1
Historical Background

In 1979, a T-cell line was established by J. Minna, P. Bunn and A. Gazdar (quoted by Gallo, 2005) from a patient with a cutaneous T-cell lymphoma. From these cells a novel retrovirus, HTLV-1, has been identified (Poiesz et al., 1980). Prior to this discovery, in 1974 and 1977, Japanese researchers had described a specific T-cell leukemia in the coastal regions of Southern and Western Japan, with a remarkable clustering of cases in the Kyushu area (Yodoi et al., 1974; Uchiyama et al., 1977). The leukemic cells contained specific chromosomal markers and were cultivated *in vitro* (Miyoshi et al., 1979, 1980). The cells were found to contain a specific antigen which was detectable by indirect immunofluorescence tests with sera from adult T-cell leukemia (ATL) patients (Hinuma et al., 1981). Co-cultivation of these cells with normal lymphocytes resulted in immortalization of the latter (Miyoshi et al., 1981). In some of the cell lines a retrovirus was identified (Yoshida et al., 1982). The structure of the virus was determined by Seiki et al. (1983) and found to be identical with the Poiesz isolate.

8.1.2
Epidemiology and Transmission

Although HTLV-1 infections are most prevalent in the coastal regions of Southern Japan, virus carriers have also been noted in other regions of Asia, in the Caribbean, in South America, and in Africa (Blattner et al., 1982; Catovsky et al., 1982; Biggar et al., 1984; Merino et al., 1984; Saxinger et al., 1984). Besides Japan, focal areas of HTLV-1 infections occur in the South Pacific, in Iran, in Romania, in parts of West Africa, and in populations in the Western hemisphere, originating from endemic areas. ATL cases have also been observed in Italy (Manzari et al., 1985; Gradilone et al., 1986), New Guinea (Kazura et al., 1987), Israel (Ben-Ishai et al., 1985), the Arctic (Robert-Guroff et al., 1985), France, the United Kingdom (Taylor et al., 2005), and the United States (Blayney et al., 1983; Bunn et al., 1983). The number of HTLV-1 infected persons worldwide has been estimated at 10–20 million (de Thé and Bumford, 1993). In spite of a relative stability of the HTLV-1 genome during the course of evolution, strain differences among different geographical isolates (up to 9% of the nucleotide sequences) have been identified (Cassar et al., 2005). Phylogenetic studies permitted a classification in three well-defined subtypes: Cosmopolitan, Central African, and one found mainly in the Maroni Basin, French Guiana, and West Indies (Capdepont et al., 2005). A simian equivalent to HTLV-1 has been isolated from macaques and chimpanzees (Leendertz et al., 2004; for a review, see Franchini and Reitz, 1994).

Seroprevalence to HTLV-1 infections in endemic regions increases with greater age, and seropositivity is higher in females than in males (Blattner and Gallo, 1994).

Modes of transmission include the mother to infant route, and sexual as well as parenteral transmission. The breast milk of HTLV-1-positive mothers contains lymphocytes positive for HTLV-1 (Kinoshita et al., 1984). Thus, these infections occur mainly perinatally (Hino et al., 1985; Ando et al., 1987). Sexual transmission seems to occur more frequently from infected males to females with HTLV-1-infected lymphocytes in semen than from females to males (Brodine et al., 1992). Blood transfusions have also contributed in the past to HTLV-1 transmission (Sato and Okochi, 1986). Additional modes of transmission include needle sharing by intravenous drug users and male homosexual intercourse (Robert-Guroff et al., 1986; Bartholomew et al., 1987).

Only a small proportion of infected persons eventually develop ATL. After a latency period of several decades, approximately 6% of male and 2% of female carriers develop the disease (Taylor and Matsuoka, 2005). Risk factors for the development of ATL seem to include vertical or perinatal HTLV-1 transmission and increasing numbers of abnormal lymphocytes (Tajima, 1990; Hisada et al., 1998, 2001). The clinical picture of tropical spastic paraparesis, the HTLV-1-associated myelopathy (TSP/HAM) is mainly linked to infections at higher age, predominantly transmitted by sexual intercourse or blood products (Maloney and Blattner, 2003).

8.1.3 Viral Gene Organization and Gene Products

The HTLV-1 genome contains open reading frames (ORFs) typical for retroviruses, namely gag, pol, and env, and in addition a 3' region initially designated pX (Nicot et al., 2005). Several additional proteins are coded for by this region, among them two essential transcriptional and post-transcriptional regulators of viral gene expression, Tax and Rex proteins. A schematic outline of the genome organization and of virus-specific proteins transcribed from the genome is shown in Figure 8.3.

Following a short description of the structural proteins gag, pol, and env, and also of the virus-specific protease, the following sections will focus on proteins coded for by the X-region, which plays a clear role in HTLV-1-mediated oncogenesis and is profoundly engaged in regulatory processes of HTLV-1 and several cellular genes.

8.1.3.1 The gag Protein

The gag region is initially transcribed as a polyprotein precursor, and cleaved subsequently into the gag polypeptides, a 19-kDa matrix protein, the 24-kDa capsid protein, and a 15-kDa nucleocapsid protein (Copeland et al., 1983; Oroszlan et al., 1984). A post-transcriptional modification of p19 results in a covalently attached myristic acid tail at the amino terminus (Miwa et al., 1987). Myristoylation of the precursor 55-kDa gag protein emerges as a precondition for targeting it to the inner surface of the cell membrane (Hayakawa et al., 1992). Some immature precursors of the three major gag proteins are found in HTLV-1-infected cells (Cann and Chen, 1996).

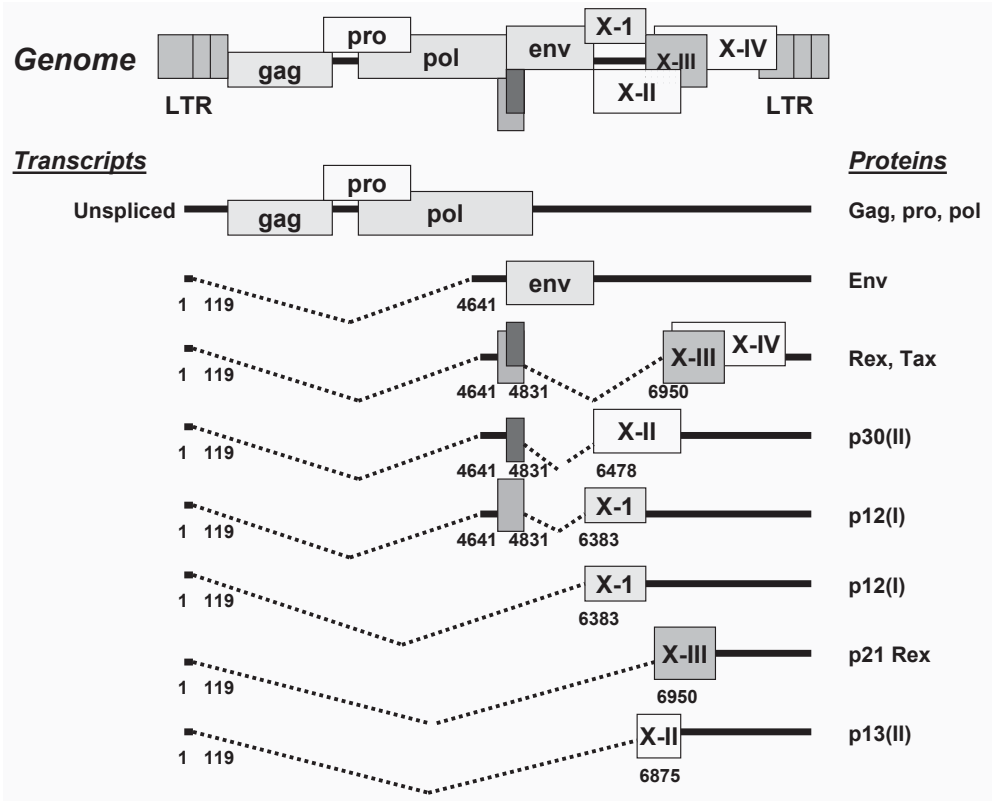

Fig. 8.3 Gene products of the HTLV-1 genome. (Modified from Nicot et al., 2005.)

8.1.3.2 HTLV Protease

The HTLV-1 protease is encoded by a region covering the 3' part of the gag and the 5' part of the pol ORFs. Ribosomal frameshifting as part of the gag polypeptide results in protease synthesis (Nam et al., 1988). This protease is responsible for processing of the mature gag products, and by autocatalyzation it generates the mature protease molecule (Kobayashi et al., 1991; Nam et al., 1993). The crystal structure of human T-cell leukemia virus protease has recently been unraveled (Li et al., 2005).

8.1.3.3 The Polymerase Protein

The pol region codes for an 896-amino acid product, including a ribosomal frameshifting event, resulting eventually in an 864-amino acid peptide (Nam et al., 1988). The 5' end of the protein is responsible for the reverse transcriptase activity, while downstream sequences contain the information for the integrase and RNaseH func-

tions. Divalent cations, most efficiently Mg^{2+}, are required for the reverse transcriptase function (Cann and Chen, 1996). The active HTLV-1 polymerase complex can exist as a p62/p49 heterodimer, formed by two cleaved products of the pol protein (Mitchell et al., 2006).

8.1.3.4 The env Protein

Depending on the cell line studied, the env glycoprotein is formed as a precursor protein of 61 to 69 kDa (Yamamoto et al., 1982; Lee et al., 1984; Schneider et al., 1984 a). The nonglycosylated precursor protein amounts to 54 kDa (Lee et al., 1984; Schneider et al., 1984 b). After cleavage of the precursor, a mature 46-kDa surface glycoprotein and a 21-kDa trans-membrane protein are formed, and Gp46 is shed from the surface of the cell. The conserved glycine-rich segment of the N-terminal fusion peptide of human T-cell leukemia virus type 1 transmembrane glycoprotein gp21 is a determinant of membrane fusion function and requires flexibility within the glycine-rich segment and hydrophobic contacts (Wilson et al., 2005).

8.1.3.5 The Tax Protein

The Tax proteins represent nonstructural regulatory proteins, occurring within the α-subgroup of retroviruses (for reviews, see Grassmann et al., 2005; Marriott and Semmes, 2005). These proteins are also required for viral replication, but stimulate at the same time proliferation of the infected host cells. In virus-producing cells, Tax acts as a transcriptional activator of the viral long terminal repeat (LTR). Tax does not bind directly to the promoter of the LTR but becomes linked with its N-terminus to the CREB protein which docks to the cyclic AMP-responsive motif of the LTR (Adya and Giam, 1995; Baranger et al., 1995). Tax represents a nuclear phosphoprotein, post-transcriptionally modified by ubiquitination (Chiari et al., 2004; Peloponese et al., 2004). Tax induces several cellular transcription factors, such as NF-κB, CREB, SRF and Ap-1 (Jeang, 2001; Azran et al., 2004), and also recruits transcriptional co-activators, such as CBP and p300 (Giebler et al., 1997; Bex et al., 1998; Harrod et al., 2000). The C-terminal activation domain of Tax attaches directly to the TATA-box-bound TBP protein (Caron et al., 1993), promoting the initiation of transcription and RNA polymerase elongation (Chung et al., 2003). Some of the early effects of Tax are shown schematically in Figure 8.4.

Tax expression profoundly modifies the cell cycle by accelerating the G_1-phase (Lemoine and Marriott, 2001). This seems to be due to transcriptional activation of cyclins E and D2. In addition, the levels of Cdk 4/6 and Cdk 2 are increased (Santiago et al., 1999; Huang et al., 2001). Tax binds directly to cyclins D3, D2, Cdk 4 and stabilizes cyclin D/Cdk4 complexes (Haller et al., 2002; Fraedrich et al., 2005). The additional binding to $p16^{INK4}$ prevents this cyclin-dependent kinase inhibitor from inactivating Cdk 4 and Cdk 6 (Low et al., 1997). As a result of binding to Cdk4, Tax promotes Rb phosphorylation and release of the transcription factor E2F, thus further

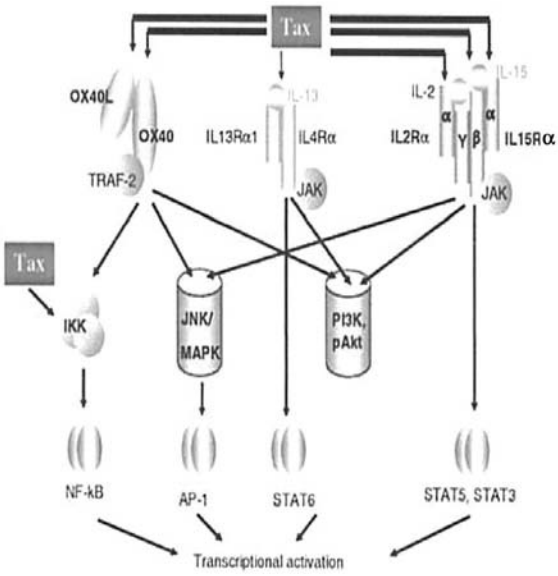

Fig. 8.4 Tax-induced pathways in stimulating cellular transcription. (Grassmann et al., 2005. With permission.)

facilitating cell-cycle progression (Lemasson et al., 1998; Ohtani et al., 2000). Moreover, Tax binds to the hypophosphorylated form of Rb and promotes its proteasome degradation (Kehn et al., 2005). Tax also constitutively activates NF-κB by forming a ternary complex of IKK/PP2A/Tax in which PP2A becomes inactivated (Carter et al., 2001; Fu et al., 2003).

Tax impairs the function of p53 (Mulloy et al., 1998; Pise-Masison et al., 1998). Tax-expressing cells fail to generate an appropriate p53-dependent response to DNA damage. Although Tax does not bind directly to p53, the effect seems to be due to a nuclear stabilization of transcriptionally inactive p53 (Cereseto et al., 1996; Tubakin-Fix et al., 2006), possibly due to a competitive binding of Tax and p53 to the ubiquitous co-activator CREB-binding protein CBP/p300, thus interfering with the p53 target gene activation (Suzuki et al., 1999 a; Van et al., 2001). Tax activates the $p21^{WAF1}$ gene independent of p53 (de la Fuente et al., 2000). In contrast to regular p21 functions, Tax-induced expression is associated with resistance to apoptosis (Kawata et al., 2003).

Tax also binds and interferes with functions of the Drosophila discs large tumor suppressor protein, hDLG (Suzuki et al., 1999 b), and in this function resembles the E6 protein of high-risk papillomaviruses (see Chapter 5). The repression of cyclin A by Tax may permit redundant DNA replication and contribute to aneuploidy (Kibler and Jeang, 2001).

Although Tax expression impairs the induction of human telomerase reverse transcriptase (hTERT), transduction of Tax into primary lymphocytes is sufficient to activate and maintain telomerase expression and telomere length when cultured in the absence of any exogenous stimulation (Sinha-Datta et al., 2004). Tax has a profound effect on a protein complex named shelterin, which includes the telomerase DNA-binding proteins TRF1, TRF2, and Pot1 (Escoffier et al., 2005). The down-reg-

ulation of hTERT transcription by Tax in HTLV-1 transformed or in Tax-expressing T lymphocytes is correlated with a significant increase of TRF2 and/or Pot1mRNAs. This interesting down-regulation of hTERT by a viral oncogene has been proposed also to contribute at an early phase of carcinogenesis to the intensive ploidy changes associated with the development of HTLV-1-associated malignancies (Escoffier et al., 2005).

De-novo expression of Tax causes accumulation of cells in G_2/M (Tyler et al., 2001; Liang et al., 2002; Haoudi and Semmes, 2003; Kino and Pavlakis, 2004). Tax forms a complex with Chk2 and Chk1, both of which are in the ATM/ATR signaling pathway and normally activate several downstream targets including p53 (Marriott and Semmes, 2005). The progression through mitosis by Tax seems to be delayed by its direct binding to, and premature activation of, APC^{clb2p}, which leads to improper cyclin B and securing degradation (Liu et al., 2003, 2005).

Tax suppresses nucleotide excision repair (Kao and Marriott, 1999). This inhibition correlates with the transcriptional activation of the proliferating cell nuclear antigen (PCNA) promoter (Ressler et al., 1997; Kao et al., 2000; Lemoine et al., 2000). Tax expression results in increased frequency of mutations within the cellular genome (Miyake et al., 1999), most likely as the result of a failing nuclear excision repair in response to environmental mutagens. Tax also suppresses the human DNA polymerase β promoter (Jeang et al., 1990); this enzyme is involved in base excision and mismatch DNA repair (Wilson et al., 1988). It has been shown directly that Tax expression specifically suppresses base excision repair of DNA (Philpott and Buehring, 1999). In addition, Tax also seems negatively to influence the repair of double-stranded DNA breaks (Ng et al., 2001). Apparently, the Ku80 protein plays an important role in the induction of micronuclei formation and Tax-mediated clastogenic activity, since Ku80-negative cells are protected from these aberrations (Majone et al., 2005). Thus, Tax expression is responsible for various forms of genetic instability.

8.1.3.6 **The Rex Protein**

Rex is a 27-kDA RNA binding protein that is essential for the splicing and transport of viral mRNA (Kashanchi and Brady, 2005). Although not required for cellular immortalization *in vitro*, Rex is indispensable for efficient viral replication, infection and spreading of the infection (Ye et al., 2003; Younis and Green, 2005). Rex influences the cytoplasmic levels of singly spliced and unspliced mRNA at the expense of doubly spliced mRNA (Hidaka et al., 1988). Rex expression leads to a reduction of splicing and to increased stability of mRNAs (Grone et al., 1996).

Rex proteins are found in multimers that are required for their function (Malim et al., 1990). A highly basic N-terminal RNA-binding domain, located within the first 19 amino acids, is essential for binding of Rex to the Rex-responsive element within the U and R regions of the 3' LTR (Seiki et al., 1988; Bogerd et al., 1991; Grassmann et al., 1991). The same domain serves as a nucleolus-targeting signal and mediates the transport of unspliced viral mRNA into the cytoplasm (Siomi et al., 1988; Nosaka et al., 1989; Bohnlein et al., 1991).

Another domain within residues 66–118 is important for the interaction with several cellular factors (Hope et al., 1991; Weichselbraun et al., 1992). It is also important for targeting Rex to the nuclear pore complex (Palmeri and Malim, 1996; Rehberger et al., 1997). Many of these cellular factors bind to the Rex-responsive elements in the 3' LTR and interfere with Rex binding. This accounts for the heterogeneous nuclear ribonucleoprotein A1, the splicing factor SF2, the exportin protein, CRM1, and the nucleolar protein B-23 (Dodon et al., 2002). Rex functions as a multimer (Malim et al., 1990), the nucleotides 54–69 being critical for multimerization (Weichselbraun et al., 1992).

The available data indicate that Rex plays an important role as a post-transcriptional regulator of HTLV-1.

8.1.3.7 **The p12(I) Protein**

This protein is encoded by either singly or doubly spliced mRNA by splicing from nucleotide 119 from the LTR to the splice acceptor at position 6383 (Ciminale et al., 1992; Koralnik et al., 1992, 1993). The doubly spliced product should yield a Rex/p12(I) hybrid protein, but expression of this message in HeLa cells leads only to p12(I) (Koralnik et al., 1992). p12(I) is found in the cellular endomembranes, in the endoplasmic reticulum, and in the Golgi apparatus (Koralnik et al., 1993; Ding et al., 2001; Johnson et al., 2001). This protein is not required for in-vitro replication of HTLV-1 (Derse et al., 1997; Robek et al., 1998; Albrecht et al., 2000), but contributes to infectivity *in vivo*. CTLs and serum antibodies from HTLV-1-infected persons recognize peptides within this protein (Dekaban et al., 2000; Pique et al., 2000).

The p12(I) protein is highly hydrophobic and contains four proline-rich (PXXP) Src homology 3 (SH3) binding domains (Franchini, 1995). It is able to form dimers (Trovato et al., 1999). Interestingly, this protein shows a 50% amino acid identity with part of the bovine papillomavirus (BPV) E5 protein (Franchini et al., 1993). It also cooperates with BPV E5 in a focus formation assay. Similar to E5, p12(I) binds to the 16-kDa subunit of the vacuolar ATPase (Franchini et al., 1993; Koralnik et al., 1995). It also interacts with the IL-2 receptor β and γ_c chains and decreases their surface expression (Mulloy et al., 1996). As a consequence of this interaction, p12(I) induces an increase in DNA binding and in transcriptional activity of STAT5 (Nicot et al., 2001). By STAT5 activation, p12(I) induces synthesis of IL-2 and contributes to the proliferation of infected T cells (Nicot et al., 2005).

p12(I) also influences T-cell activation and permits T cells to enter S-phase, even under conditions of suboptimal antigen stimulation, and induces a linker of activated T- cell-independent increase in intracellular calcium levels (Lewis, 2001). It has been suggested that p12(I) interferes with MHC presentation of viral peptides, as it binds to MHC class I heavy chains encoded by HLA-A2, -B7, and -Cw4 alleles (Nicot et al., 2005). This results in a failure of these MHC class I heavy chains to associate with β_2-microglobulin, in retransportation to the cytosol, and in degradation by the proteasome complex.

8.1.3.8 The p30(II) Protein

p30(II) represents a nucleolar protein, and is encoded by a doubly spliced mRNA, placing the Tax AUG in frame with ORF II (Ciminale et al., 1992; Koralnik et al., 1992). A CTL immune response and antibodies against this protein are observed in HTLV-1-infected individuals (Pique et al., 2000). P30(II) is a negative regulator of viral gene expression (Nicot et al., 2004). The autologous down-regulation of HTLV-1 expression seems to permit viral persistence within an immunocompetent host. Freshly isolated lymphatic cells from HTLV-1-infected individuals express only low or almost undetectable levels of viral antigens (Kinoshita et al., 1989; Gessain et al., 1991; Berneman et al., 1992; Yoshida, 2005).

p30(II) selectively reduces the level of Tax/Rex mRNA, but does not substantially affect the levels of Gag-Pol, Env, and p21Rex spliced mRNA (Nicot et al., 1993, 2004; Younis et al., 2004). The reduction is accompanied by nuclear accumulation of Tax/Rex mRNA which is bound specifically to p30(II). The trapping of Tax/Rex mRNA results in a reduction of productive viral replication.

p30(II) expression also affects several cellular transcription factors. It interacts with the transcriptional cofactors p300/CREB-binding protein. Overexpression represses cellular CREB-driven reporter gene activity, independent of Tax expression, whereas lower concentrations enhance the HTLV-1 LTR-driven reported gene activity, again independent of Tax expression (Zhang et al., 2000, 2001). p30(II) co-localizes with p300 in cell nuclei and binds directly to CBP/p300 in cells (Zhang et al., 2001). Other cellular genes affected by p30(II) are integrins and cadherins. Their expression is repressed, whereas the expression of genes involved in T-cell activation and apoptosis is increased (Michael et al., 2004). p30(II) also interacts with Myc-containing transcription complexes and transactivates the human D2 promoter (Awasthi et al., 2005). Co-expression of this protein with Myc enhances Myc-dependent cellular transformation of immortalized human fibroblasts. Thus, p30(II) may contribute to HTLV-1-mediated cell transformation.

8.1.3.9 The p13(II) Protein

p13(II) mRNA was initially detected in HTLV-1-positive cell lines and in patients with ATL (Berneman et al., 1992; Koralnik et al., 1992; Cereseto et al., 1997). The protein corresponds to the 87 C-terminal amino acids of p30(II), and is expressed from a singly spliced mRNA (Ciminale et al., 1992; Koralnik et al., 1992). p13(II) targets primarily the inner part of the mitochondrial membrane and becomes an integral protein at this site (Ciminale et al., 1999; D'Agustino et al., 2002). It induces marked changes in the morphology of mitochondria, which seem to swell. p13(II) increases mitochondrial permeability to small monovalent cations, particularly to K^+ (D'Agustino et al., 2002). The protein also negatively influences cell proliferation at high density and tumor growth *in vivo* (Silic-Benussi et al., 2004). In addition, the protein increases sensitivity to ceramide-induced apoptosis in T cells, although p13(II) itself did not induce apoptosis (Silic-Benussi et al., 2004; D'Agostino et al., 2005; Hiraragi

et al., 2005). The Fas ligand-induced apoptosis of T cells was also increased by p13(II).

8.1.4 Diseases Caused by HTLV-1 Infection

The most prominent disease that may develop as consequence of a persisting HTLV-1 infection is represented by ATL, which is discussed separately in the following sections. The other relatively prominent condition that may be caused by long-term persistence of this virus is HAM, or TSP. This condition was initially observed in the West Indies (Gessain et al., 1985), but occurs in all areas where HTLV-1 infections are endemic (Gessain et al., 1986; Osame et al., 1987; Rodgers-Johnson et al., 1988). The lifetime risk for the development of HAM/TSP in HTLV-1 infected persons has been estimated at $< 1\%$ (Kaplan et al., 1990).

Besides neurological symptoms, the cerebrospinal fluid of these patients contains antibodies to HTLV-1 and reveals lymphocytic pleocytosis. In the peripheral blood, the lymphocyte counts remain normal, but some lymphocytes reveal an atypical morphology (Osame et al., 1989; Furukawa et al., 1992).

Although a number of attempts have been made to link additional hematologic disorders to HTLV-1 infections, the reported data remain non-reproducible. One other rare complication of HTLV-1 infections is represented by chronic inflammatory arthropathy (Kitajima et al., 1991; Sato et al., 1991; Nishioka et al., 1993). Similar arthropathies have been observed in HTLV-1 transgenic mice (Iwakura et al., 1991). Uveitis has also been reported to be linked to HTLV-1 infections (Nakao et al., 1989; Mochizuki et al., 1992), and has also been observed in a rabbit infected with HTLV-1 (Taguchi et al., 1993).

ATL is also associated with an impairment of the cellular immune response (Hinuma et al., 1983; Capell et al., 1987; Taguchi and Miyoshi, 1989; Murai et al., 1990). This may cause problems with parasitic or other opportunistic infections (O'Doherty et al., 1984; Newton et al., 1992; Robinson et al., 1994).

8.1.5 Immune Response to HTLV-1 Infections

In contrast to the sequence variation, observed for example in HIV infections, and in spite of an error-prone reverse transcriptase, there is relatively little variation between different HTLV-1 isolates (Daenke et al., 1990; Kinoshita et al., 1991; Slattery et al., 1999). In HTLV-1-infected persons, more than 30% of peripheral mononuclear cells and more than 50% of $CD4^+$ cells represent proliferating provirus-containing cells (Cavrois et al., 1996; Etoh et al., 1997; Eiraku et al., 1998). In ATL patients the tumor cells are mostly of $CD4^+$ and $CD25^+$ mature T-lymphocyte phenotype (Uchiyama et al., 1977). HTLV-1 virions seem to be absent, and virus-specific mRNA or proteins are difficult to detect within those cells. Yet, an antibody response to HTLV-1, including IgM antibodies (Nagasato et al., 1991; Kira et al., 1992; Ishihara et al., 1994) and an activated CTL response, particularly to Tax (Jacobson et al.,

1990; Kannagi et al., 1991; Goon et al., 2004 a), point to HTLV-1 antigen expression. Indeed, a low level of HTLV-1mRNA has been detected in non-cultivated tumor cells and in lymph nodes of ATL patients (Kinoshita et al., 1989; Oshima et al., 1996). The inoculation of uncultured formalin-treated ATL-cells into naïve rats also resulted in the induction of a HTLV-1-specific T-cell response (Kurihara et al., 2005).

If explanted into tissue culture under appropriate conditions, $CD4^+$ cells from about 50% of HTLV-1-infected persons begin to express viral antigens starting 12 h after explantation (Hinuma et al., 1982; Hanon et al., 2000). The addition of $CD8^+$ T cells reduces the viral antigen expression in a dose-dependent manner. Thus, CTLs present in the $CD8^+$ T-cell preparations seem able to suppress Tax and other viral antigen expression (Bangham, 2003; Bangham and Osame, 2005). *In vivo*, CTLs appear to play a very active role in eliminating Tax-expressing cells (Vine et al., 2004). Thus, a very low Tax expression may permit escape of HTLV-1 provirus-carrying cells from immune surveillance mechanisms. This leaves the question open as to how HTLV-1 infections are transmitted to other susceptible T cells. It has been suggested that this may occur through cell contact-triggered "virological synapses" formed due to virus-induced polarization of the cytoskeleton (Igakura et al., 2003). However, an alternative route may exist via cellular exosomes containing the whole viral genome and being fused to previously uninfected cells (Duelli et al., 2005).

ATL-cells from approximately half of the patients do not induce viral antigens upon *in-vitro* cultivation (Kannagi et al., 2005). The HTLV-1 genome appears to be irreversibly silenced. It has been suggested that this is due to deletions in the 5' LTR region or within the gag/pol genes (Konishi et al., 1984; Tamiya et al., 1996).

Interestingly, there seems to exist no correlation between proviral load and CTL activity (Kubota et al., 2000; Wodarz et al., 2001). A slightly positive correlation appears to exist in TSP patients, who usually reveal a higher frequency of HTLV-1-specific CTLs (Elovaara et al., 1993; Greten et al., 1998). In contrast to TSP patients, who commonly reveal highly activated CTL activity, HTLV-1-specific CTLs are only rarely induced in ATL patients (for a review, see Kannagi et al., 2005). They are apparently present, but expand insufficiently (Arnulf et al., 2004), a finding which points to some immune suppression or tolerance in these patients.

Genetic polymorphisms influence the efficacy of the immune response to HTLV-1 (Nagai et al., 1998; Jeffery et al., 1999, 2000; Vine et al., 2002). One strong genetic determinant is an HLA-class I genotype; indeed, genes HLA-A*02 and HLA-Cw*08 were associated with a lower proviral load and a lower risk of TSP. It has been suggested that these data point to a particularly efficient CTL response in persons with those genotypes (Bangham and Osame, 2005). Minor sequence variations within HLA-A*02 were associated with significant differences in the prevalence of TSP in Kagoshima, Japan (Furukawa et al., 2000). Another genotype increasing the risk for TSP development is HLA-DRB1*0101 (HLA-DR1) (Usuku et al., 1990; Nishimura et al., 1991; Kitze et al., 1998; Sabouri et al., 2005).

Much less is known about the effects of natural killer (NK) cells, although HTLV-1-associated inflammatory conditions reveal a lower frequency of NK cells (Fujihara et al., 1991; Yu et al., 1991; Saito et al., 2003). Similarly, the role of $CD4^+$ (helper) T cells is also poorly understood, although they seem to recognize most commonly the env

protein, contrasting the preferential recognition of Tax by $CD8^+$ T cells (Goon et al., 2004 b).

8.1.6 Animal Studies

HTLV-1 effectively infects rabbits (Akagi et al., 1985; Lairmore et al., 1992), cynomolgus and squirrel monkeys (Nakamura et al., 1987; Murata et al., 1996; Kazanji, 2000) and, less efficiently, rats (Suga et al., 1991; Ibrahim et al., 1994).

Oral infection of rats results in virus persistence without measurable immune response (Kato et al., 1998), whereas intravenous routes of infection lead to strong antibody and T-cell responses (Kannagi et al., 2000). HTLV-1 infected rats appear to be a model for TSP, since immunosuppressed animals develop spastic paraparesis with degenerative spinal cord and peripheral nerve lesions several months following infection (Ishiguro et al., 1992; Kushida et al., 1993). HTLV-1 provirus was found in microglial cells and macrophages at lesional sites (Kasai et al., 1999). An ATL-like lymphoproliferative disease was established in adult nude rats after inoculation of HTLV-1 immortalized cell lines (Ohashi et al., 1999). Tax-specific small interfering RNA (siRNA) protected against this proliferative condition (Nomura et al., 2004).

Rabbits turned out to be suitable animal models to study HTLV-1 infection, although these animals rarely, if ever, show clinical symptoms (Lairmore et al., 2005). Rabbits can transfer the virus via blood, semen, or milk (Kotani et al., 1986; Uemura et al., 1987; Hirose et al., 1988; Iwahara et al., 1990). Rabbits also served as convenient models to study the development of antibodies against HTLV-1 (Cockerell et al., 1990), and to analyze env epitopes for protective immunization studies (Tanaka et al., 1994). Furthermore, they permitted an evaluation of the role of accessory proteins of the pX region for in-vivo infections (Collins et al., 1998; Bartoe et al., 2000; Silverman et al., 2004).

Experimental infection of nonhuman primates can be achieved by the inoculation of HTLV-1-transformed cell lines. Some of these animals may develop malignant lymphomas (Homma et al., 1984; Kanki et al., 1985), although the majority of the infections remain asymptomatic. Most experimental studies were conducted in squirrel monkeys and macaques. These animals also served for testing potential HTLV-1 vaccines (Nakamura et al., 1987) and hyperimmune globulin treatment (Murata et al., 1996; Akari et al., 1997).

ATL cells grow successfully in severe combined immunodeficiency (SCID) mice (Ishihara et al., 1992; Feuer et al., 1993), which succumb to lymphomas. Nonleukemic HTLV-1-infected cells only grow in these mice when their NK cell activity has been suppressed (Feuer et al., 1995). Transgenic mouse models have been used successfully to study the role of targeted tax gene expression and the respective host response. By using the HTLV-1 LTR promoter, the development of neurofibromas was observed (Hinrichs et al., 1987), while in other analogous experiments lymphocyte-mediated arthropathy developed (Iwakura et al., 1991; Yamamoto et al., 1993; Fujisawa et al., 1996). Tax expression under the control of the CD3-ε promoter-enhancer led to salivary and mammary adenomas (Hall et al., 1998). These experiments underlined the oncogene function of the *Tax* gene.

By targeting the mature T-cell compartment with the GzmB promoter, large granular lymphocytic tumors developed (Grossman et al., 1995). Cell lines derived from such tumors exhibited high levels of NF-κB expression and a wide variety of NF-κB target genes (Grossman and Ratner, 1997; Portis et al., 2001). The significance of these models is somewhat questionable in view of the different tumor type induced under these conditions in comparison to ATL.

Animal models are certainly useful in analyzing specific functions of viral genes. However, they have their limitations in cases where the mode of persistence or the induced clinical symptoms do not parallel the clinical situation in humans.

8.1.7 Mechanism of Cell Transformation by HTLV-1

The X-region of the HTLV genome carrying genes for the regulatory Tax proteins is unique among α-retroviruses. These proteins are required for productive viral infection, as well as for growth stimulation of infected lymphatic cells. Even in non-leukemic HTLV-1 positive patients, the infected cells expand clonally and persist for prolonged periods of time (Etoh et al., 1997; Gabet et al., 2000).

The virus enters CD4 cells via their GLUT1 (glucose transporter) receptor (for a review, see Manel et al., 2005). Other cell types can also be infected in patients, specifically $CD8^+$ lymphocytes, monocytes, and B lymphocytes (Koyanagi et al., 1993; Eiraku et al., 1998; Nagai et al., 2001; Grant et al., 2002). *In vitro* even non-lymphatic cells may become infected, such as primary human endothelial cells (Ho et al., 1984; Hoxie et al., 1984), microglial cells (Hoffman et al., 1992), and basal mammary epithelial cells (LeVasseur et al., 1998).

The primary factor for the stimulation of cell growth is the transcriptional activator Tax. This protein can be considered as a viral oncogene since it is able, in cooperation with Ras, to immortalize primary rodent cells which produce tumors when inoculated into nude mice (Pozzatti et al., 1990). In NIH 3T3 and Rat 1 cells, Tax antagonizes contact inhibition, and induces anchorage-independent growth in soft agar and nude mouse tumorigenicity (Tanaka et al., 1990). Tax-transformed cells also reveal an elevated resistance to apoptosis (Fujita and Shiku, 1995). Tax is also able to immortalize human T cells *in vitro*: if expressed from rhabdoviral (Grassmann et al., 1989, 1992) or retroviral vectors (Akagi and Shimotohno, 1993), T cells become immortalized, but depend on IL-2 for growth (Akagi et al., 1995; Rosin et al., 1998; Schmitt et al., 1998).

The analysis of Tax mutants permitted analyses of cellular factors required for immortalization. Interestingly, Tax mutants deficient for either interfering with CREB- or NF-κB pathways were still able to transform rodent fibroblasts (Smith and Greene, 1991; Yamaoka et al., 1996; Matsumoto et al., 1997). On the other hand, the serum responsive factor (SRF) was found to play a major role in the transformation of primary rat fibroblasts by Tax (Matsumoto et al., 1997). For immortalization of human lymphocytes, the CREB and/or SPF pathways had to be activated to clonally expand $CD4^+$ and $CD8^+$ T cells (Akagi et al., 1997; Rosin et al., 1998).

In Tax-immortalized cells, and also in ATL cells, Tax induces a multitude of changes in cellular gene expression and signaling cascades. The α-chain of the IL-2 receptor is up-regulated (Ballard et al., 1988; Ruben et al., 1988) which, jointly with β- and the γ-chain, forms the high-affinity IL-2 receptor (IL-2R) (Grassmann et al., 2005). In addition, the IL-2 gene is also activated by Tax (Hoyos et al., 1989; McGuire et al., 1993; Good et al., 1996). The hypothesis that Tax causes T-cell proliferation by an autocrine IL-2/IL-2R loop is weakened by Tax-immortalized cells, which still require the exogenous addition of IL-2 for proliferation. Besides IL-2, IL-15mRNA expression and its receptor, IL-15Rα is also elevated by Tax (Azimi et al., 1998; Mariner et al., 2001). In HTLV-1-transformed cells IL-13 is also up-regulated (Chung et al., 2003; Wäldele et al., 2004). Increased levels of IL-13 are also noted in Hodgkin's lymphoma cells. In addition to interleukins, the expression of one member of the tumor necrosis factor (TNF) family, OX40, is also increased (Pankow et al., 2000) and constitutively expressed in HTLV-1-producing cells, but not in resting T cells.

Several signaling cascades are influenced by the Tax activity. The phosphorylated signal transducers of activated T cells (STAT5 a and STAT5 b) are highly activated in HTLV-1-immortalized T-cell lines and in lymphocytes from ATL patients (Migone et al., 1995). The expression of TGF-β is also stimulated by Tax (Kim et al., 1990), but its signaling activity is negatively influenced by a Tax-mediated repression of the DNA-binding activity of transcription factors Smad3 and Smad4 (Mori et al., 2001; Arnulf et al., 2002; Lee et al., 2002). Another enzyme involved in signaling events influenced by Tax is the phosphoinositide 3-kinase (P13K) (Liu et al., 2001). One of this enzyme's downstream targets is Akt (Kelly-Welch et al., 2003), the pathway responsible for growth stimulation and anti-apoptotic activity.

The stimulation of various phases of the cell cycle by Tax and the interaction of Tax with p53 and Rb has been discussed previously. Overexpression of Tax, similar to other growth-promoting genes such as Myc, E2F, E7 and E1A, triggers apoptosis. At the same time, Tax possesses an anti-apoptotic effect which seems to be mediated by the transactivation of cellular regulators of apoptosis, such as Bcl-Xl and Bcl-2A1 (Tsukahara et al., 1999; Nicot et al., 2000; de la Fuente et al., 2003). Clearly, there is a fine balance between apoptotic and anti-apoptotic effects (as is also noted for other viral oncogenes), and this plays an important role in cell transformation by Tax. The long latency periods for ATL development indicate, in addition, that specific host cell modifications must take place prior to malignant conversion (see chapter on cellular interfering factors). The previously reported effects of Tax on DNA damage and genetic and chromosomal instability further underline the importance of host cell modifications prior to the onset of malignant transformation.

The available data provide sufficient evidence that HTLV-1 infection via Tax induction acts as a direct carcinogen for humans. In line with other human tumorvirus infections, viral oncogene expression *per se* is not sufficient for tumor induction. The latter requires changes in specific host cell genes which counteract the oncogenic function of the infectious agent.

8.1.8 Prevention and Therapy

The most effective mode of preventing HTLV-1 infections is the avoidance of breast-feeding in case of virus-carrying mothers. Although some attempts have been made in animals to develop vaccines against this infection, it is presently difficult to foresee their clinical application (for a review, see Lairmore et al., 2005). The comparatively low rate of mutations of this virus in relation to other retroviruses should render it a suitable candidate for vaccine development. The low number of infected persons globally, however, will be a restrictive factor for the interests of pharmaceutical companies engaged in vaccine production.

Although combination chemotherapy protocols have been developed for the treatment of ATL, the median survival time does not exceed 13 months (Yamada et al., 2001). Some ATL patients respond to anti-CD25 monoclonal antibody treatment (Waldmann et al., 1993), and a higher response rate has been achieved with the combination of azidothymidine and interferon-α, without however preventing a relapse (Gill et al., 1995; Hermine et al., 1995). A limited hope is presently based on allogenic human stem cell transplantation therapies leading to a longlasting complete remission in some ATL patients (Tsukasaki et al., 1999; Utsunomiya et al., 2001).

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8.2
Human T-Lymphotropic Retrovirus-2 (HTLV-2)

HTLV-2 was isolated from a T-cell line of a patient with hairy cell T-cell leukemia (Kalyanaraman et al., 1982). The line had been established four years earlier (Saxon et al., 1978). Epidemiological studies showed this infection to occur frequently in Central and West Africa (Gessain et al., 1993; Goubau et al., 1993), and to occur at a higher rate in native American populations throughout the continent (Hjelle et al., 1990, 1991; Lairmore et al., 1990; Heneine et al., 1991). Serological analysis revealed that this retrovirus is related to – but different from – HTLV-1, and it was therefore designated as HTLV-2. A second isolate was obtained four years later, again from a T-cell lymphocytosis (Rosenblatt et al., 1986). A few more isolates have been obtained subsequently.

Serological assays distinguishing between HTLV-1 and -2 infections revealed HTLV-2-positive sera among intravenous drug abusers in Great Britain (Tedder et al., 1984), and New York (Robert-Guroff et al., 1986). Within the same risk group, high incidences of HTLV-2 infections were noted in New Orleans (Lee et al., 1989; Rosenblatt et al., 1990). In several of these patients, the CD8+ T-cell counts were elevated, although the patients remained asymptomatic. Occasional observations noted spontaneous lymphocyte proliferation (Wiktor et al., 1991), mucosis fungoides (Zucker-Franklin et al., 1992), large granular lymphocyte leukemia (Loughran et al., 1992; Martin et al., 1993), and neurological complications corresponding to TSP (Harrington et al., 1993; Jacobson et al., 1993) in an occasional HTLV-2-infected person.

HTLV-2 shares approximately 70% of its nucleotides with HLTV-1, and encodes similar regulatory and accessory genes from pX regions ORFs (Feuer and Green, 2005). The availability of an infectious HTLV-2 clone (Chen et al., 1983 a) permitted earlier studies on HTLV gene structure and function than for HTLV-1 (Kimata et al., 1994; Derse et al., 1995).

HTLV-2 infections occur preferentially in $CD8^+$ cells (Miyamoto et al., 1991; Ijichi et al., 1992; Lal et al., 1993; Wang et al., 2000), although $CD4^+$ cells also become infected. In contrast to HTLV-1-immortalized lines, HTLV-2-transformed cells do not reveal an activated Jak/STAT signaling pathway (Lal et al., 1992; Mulloy et al., 1998).

HTLV-2 also immortalizes human peripheral blood T cells *in vitro* (Chen et al., 1983 b). As observed in HTLV-1 infections, Tax plays a decisive role in this process (Ross et al., 1996). The Tax activation of NF-κB and CREB/ATF is required for IL-1-independent T-cell proliferation (Ross et al., 1996).

The HTLV-1-encoded Tax (Tax-1) and the HTLV-2 Tax (Tax-2) share approximately 78% of amino acids, and both display characteristics of viral oncogenes (Feuer and Green, 2005). Similar to Tax-1, Tax-2 protects cells from Fas-mediated apoptosis (Zehender et al., 2001). Yet, both proteins differ in their transforming activities. They show differences in inducing cellular gene transcription (Ejima et al., 1993; Lal et al., 1993; Mori and Prager, 1996), and Tax-1 more effectively transactivates the viral LTR (Ye et al., 2003). The reduced transforming activity of Tax-2 seems to be due to the missing carboxy terminus of Tax-1 (Feuer and Green, 2005). Tax-2 also inhibits p53 function to a lesser extent than Tax-1 (Mahieux et al., 2000), and it is less efficient in transforming rat embryo fibroblasts *in vitro* (Endo et al., 2002). The tumorigenic potential of Tax-2-transformed lymphoid cell lines is also reduced in comparison to Tax-1-transformed cells (Feuer et al., 1995). There exists also a difference in intracellular localization between Tax-1 and Tax-2, the latter being found predominantly in the cytoplasm (Meertens et al., 2004).

The available data suggest that differences in the C-terminal part of Tax determine the higher pathogenicity of HTLV-1 in comparison to HTLV-2. Thus, the inconclusive evidence linking HTLV-2 infections with ATL-like lymphoproliferations seems to be determined by the C-terminal fragment of Tax. Interestingly, this part in Tax-1 contains the PDZ binding motif (Hall and Fujii, 2005). PDZ binding proteins play a key role in cell signaling and cell communication (Fanning and Anderson, 1999; Harris and Lim, 2001). Chimeric Tax-2, encoding the last 53 amino acids of Tax-1, regained increasing transforming potential for rat fibroblasts (Hirata et al., 2004).

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8.3
Human Endogenous Retroviruses

A large proportion of mammalian (including human) genomes originated from ancient transposable elements. DNA transposon-like structures and retroelements cover between 3% and 43% of the human genome (Deininger and Batzer, 2002; van de Lagemaat et al., 2003; Bannert and Kurth, 2004). DNA transposons are amplified without an RNA intermediate, while retroelements are transcribed into RNA and retrotranscribed by reverse transcriptase before they may re-enter the mammalian genome.

The retroelements can be subdivided into two major groups. One group, which is most abundant, carries no LTR, and the elements are usually 90 to 300 bp in length. These short interspersed elements (SINEs) are non-autonomous and seem to depend on long interspersed elements (LINEs) for their amplification (Weiner et al., 1986; Okada and Hamada, 1997). SINEs and LINEs will not be discussed here, but they are most likely derived from various tRNA genes (Daniels and Deininger, 1985; Deininger and Daniels, 1986) or the 7SL RNA gene (Ullu and Tschudi, 1984).

In this chapter, attention will be focused on a second group of human endogenous retroviruses (HERVs) which entered the human host by infecting germline cells. Most of these proviruses have acquired extensive mutations and deletions, and some have retained the coding capacity for functional proteins. The estimated percentage of such elements in the human genome amounts to slightly more than 8% (Bannert and Kurth, 2004).

The taxonomy of HERVs is based on the amino acid specificity of tRNA hybridizing to the primer binding site. The name is defined by adding its one-letter code as a suffix to the acronym HERV (Larsson et al., 1989; Bannert and Kurth, 2004). This approach leads to some confusion, as even distantly related HERVs may use the same tRNA. Clearly, there is a need for a detailed re-classification of these proviruses, preferentially on the basis of nucleotide comparisons.

Table 8.1 Classification of human endogenous retroviruses

Class	Related to	Human HERVs	Presence in nonhuman primates
Class I	γ-retroviruses (murine leukemia virus)	HERV-W HERV-H	Old and New World primates
Class II	β-retroviruses (mouse mammary tumor virus)	HERV-K	Only humans and chimpanzees (HERV-K-HML2 only in humans)
Class III	distantly related to spumaviruses	HERV-L HERV-S	Old and New World primates

Based on genome organization and sequence similarities, HERVs have been grouped into three classes (Griffiths, 2001):

- Class I HERVs are related to γ-retroviruses such as murine leukemia virus, and includes HERV-W and HERV-H.
- Class II HERVs are related to β-retroviruses which also harbor the mouse mammary tumor virus and several types of HERV-K.
- Class III HERVs are distantly related to spumaviruses, and include HERV-L and HERV-S.

Class I and class III HERVs have been discovered throughout the primate lineage, whereas many loci containing class II HERV-K proviruses are restricted to chimpanzees and humans. A summary of some characteristics of HERVs is shown in Table 8.1.

8.3.1 The Discovery of HERVs

The discovery of human endogenous proviruses relied initially on the detection of genome sequences related to animal retroviral elements (Repaske et al., 1983; Ono et al., 1987). Degenerate primers and chance observations made during the analysis of specific gene loci and chromosomal regions further contributed to a growing number of HERV-like sequences (Maeda, 1985; Harada et al., 1987). The availability of human databases has greatly facilitated the detection of HERV-sequences during recent years. The three major ORFs of retroviruses (gag, pol, and env) were maintained in only a small fraction of identified HERV sequences (Mayer et al., 1999; Griffiths, 2001; Turner et al., 2001).

Initially, typical retroviral structures were identified electronmicroscopically in the syncytial layer of full-term human placentas (Kalter et al., 1973; Vernon et al., 1974). Complete retroviral particles as well as specific antigens were also detected in human teratocarcinoma cell lines (Fig. 8.5) and in testicular tumors (Bronson et al., 1979; Boller et al., 1993, 1997). The latter particles were identified in teratocarci-

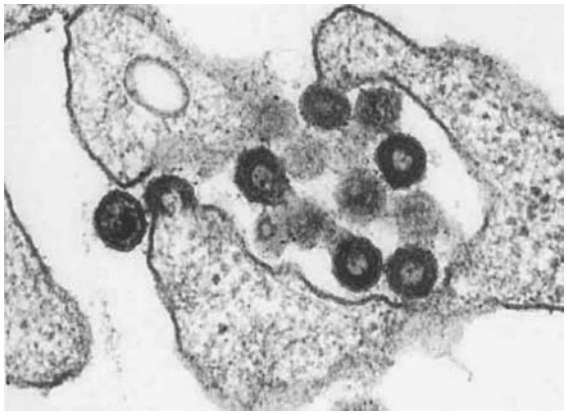

Fig. 8.5 HERV particles from a human teratocarcinoma line. (Illustration courtesy of K. Boller, Paul-Ehrlich-Institut, Langen.)

noma cells as HERV-K (Kurth et al., 1980). Cloning was first performed in 1981 (Martin et al.), since then endogenous retroviral particles belonging to the HERV-H and HERV-W families have also been identified (Perron et al., 2001; Christensen et al., 2003). Today, research in this field is actively growing, and is especially motivated by a possible role for reactivated HERVs in chronic inflammatory conditions, autoimmune diseases, and cancer.

8.3.2 Genome Organization and Transcription

8.3.2.1 HERV-K

HERV-K exists in a larger number of copies within the human genome (Bannert and Kurth, 2004). One subgroup (HERV-K HLM-2) is entirely human-specific (Medstrand and Mager, 1998; Buzdin et al., 2003). Approximately 113 elements of this DNA are present in the human genome, and eight to eleven of these are insertionally polymorphic (Belshaw et al., 2005). Fifteen of the HERV-K HLM-2 elements represent full-length genomes, while 98 are solely LTRs. It appears that the majority of the full-length elements were inserted relatively recently into the human genome, since their LTRs reveal a low level of mutational divergence (Turner et al., 2001; Belshaw et al., 2005). The HERV-K113 provirus is located on chromosome 19p13.11, and is also not completely fixed in the human population. It contains ORFs for all retroviral genes and remains an excellent candidate for a still active provirus in humans (Turner et al., 2001).

The structure of the HERV-K genome is outlined in Figure 8.6. Two types of HERV-K proviruses have been detected, differing by the presence or absence of a 292-bp sequence at the pol-env boundary (Löwer et al., 1993). Almost completely intact HERV-K proviruses were identified in human chromosome 7, and also reported as an allelic variant in the human population (Barbulescu et al., 1999; Mayer et al., 1999; Tönjes et al., 1999; Turner et al., 2001; Belshaw et al., 2005).

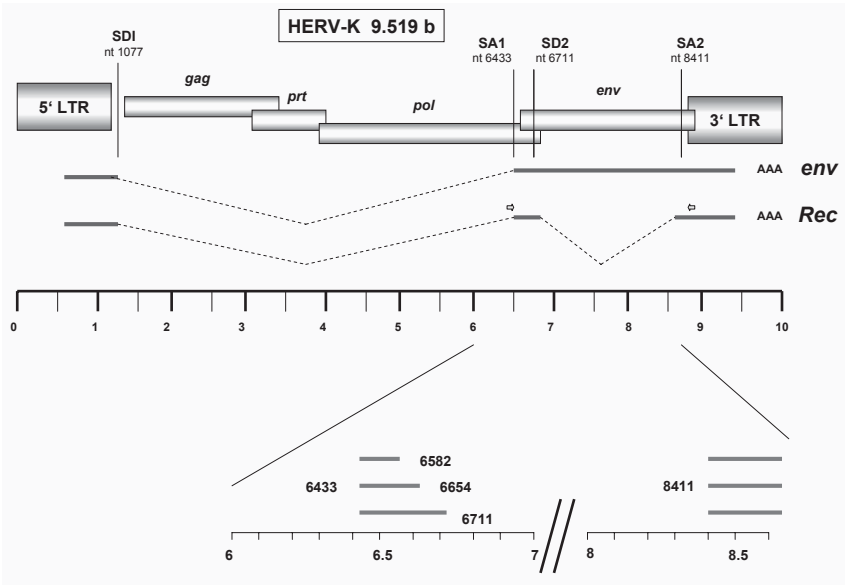

Fig. 8.6 Genomic organization of the HERV-K genome. (Modified from Mayer et al., 2004.)

A strong expression of HERV-K transcripts was noted in germ cell tumors which expressed all classical retroviral genes also as proteins (Sauter et al., 1995, 1996). In addition to these transcripts, within the *env* gene a 14kDa protein is expressed that shares 87 amino acids with the env protein and reveals additional 18 amino acids upstream from the 3' LTR in an ORF that differs from *env* (Löwer et al., 1995). Two additional smaller variants of this transcript have been also identified (Mayer et al., 2004); their structure is shown in Figure 8.6.

In addition, a 9-kDA protein, designated NP9, is produced is produced exclusively by HERV-K proviruses that contain the 292 bp deletion. Np9 shares the first 15 amino acids with the Rec and env proteins, while the C-terminal 59 amino acids are derived from the third (non-env, non-Rec) ORF. Np9 is expressed in various tumor tissues and transformed cell lines, but not in normal, nontransformed cells (Armbruester et al., 2002) (Fig. 8.7). Np9 interacts with the ligand of the Numb protein X (LNX) and can affect the subcellular localization of LNX. LNX has been reported to target the cell fate determinant and Notch antagonist Numb for proteasome-dependent degradation, thereby causing an increase in the transactivational activity of Notch. Np9 is unstable and is degraded via the proteasome pathway, whereas ectopic Numb can stabilize recombinant Np9. This may point to the possibility that Np9 is engaged in tumorigenesis through the LNX/Numb/Notch pathway (Armbruester et al., 2004).

The 14-kDa protein was termed Rec or K-Rev, and is only produced by those proviruses that do not contain the 292-bp deletion. The Rec protein appears to be functionally similar to HIV_{Rev} which exports unspliced HIV transcripts from the nucleus (Yang et al., 1999). It possesses an arginine-rich nuclear localization signal

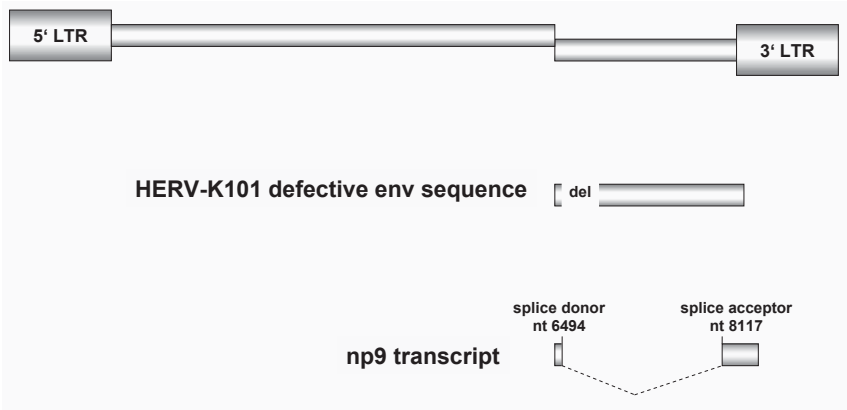

Fig. 8.7 Transcript of the *np9* gene in HERV-K 101. (Modified from Armbruester, V., et al., 2002.)

(Magin et al., 2000), and interacts with a cellular nuclear export factor (Crm1) and with the promyelocytic leukemia zinc finger protein (Boese et al., 2000).

Recent data indicate that HERV-K (HLM2) insertions result from reinfection rather than retrotransposition within germline cells (Belshaw et al., 2004), and that members of this family are likely to be infectious as well as insertionally active.

8.3.2.2 **HERV-H**

HERV-H is estimated to exist in about 1000 copies in the human genome (Wilkinson et al., 1994). These entered the human genome prior to the split of Old and New World monkeys (~30 million years ago), and expanded particularly in Old World primates (Anderssen et al., 1997; Mager and Freeman, 1995). The basic structure of the HERV-H genome corresponds to that of HERV-K. Most HERV-Hs are defective in one or several regions of their genome. HERV-H was defined based on histidine primer binding site (Mager and Henthorn, 1984). Based on the analysis of the pol region (~2800 bp), there are also variants that use phenylalanine tRNA as primer (Jern et al., 2004). Subsequently, Southern blots and LTR similarity also confirmed this classification (Anderssen et al., 1997; Wilkinson et al., 1994).

In teratocarcinoma cells producing retroviral particles, both HERV-K and HERV-H sequences are expressed (Löwer et al., 1993). Whereas the majority of HERV-H elements within the human genome reveals large deletions in pol and most of the *env* gene, 5–10% of them are full-length elements (Hirose et al., 1993; Wilkinson et al., 1993; Jern et al., 2002) and possess an almost complete env gene coding for transcripts that have been found in T-cell leukemia cell lines and lymphocytes from healthy blood donors (Lindeskog and Blomberg, 1997). Whereas gag and pol products are translated from full-length mRNA, env-encoded surface and transmembrane proteins are regulatory proteins and translated from spliced subgenomic mRNAs (Lindeskog and Blomberg, 1997). A rec/rev-like sequence, as detected in

HERV-K genomes, has also been described for HERV-H (Jern et al., 2004). A hybrid structure between the ORFs of HERV-H protease and HERV-E integrase and the HERV-E envelope surface glycoprotein was identified in six different human chromosomes, existing also in multiple copies in chimpanzees and gorillas, but not in orangutans or Old World monkeys (Lindeskog et al., 1998). This results in a relatively large part of HERV-E elements under the transcriptional control of HERV-H LTRs.

The most abundant subclass of HERV-H transcripts originates from a partially deleted subclass of these elements of 5.8 kb which is transcribed as ~5.6-kb unit length RNA and a ~3.7-kb spliced derivative (Goodchild et al., 1995; Kelleher et al., 1996).

8.3.2.3 **HERV-W**

The presence of extracellular particles with reverse transcriptase activity in leptomeningeal cells and monocyte cultures from patients with multiple sclerosis (Perron et al., 1989, 1991) resulted in intensive studies analyzing endogenous retroviruses possibly linked to the etiology of multiple sclerosis (MS). Retroviral sequences were partially cloned from the cerebrospinal fluid, plasma or cell culture supernatants of MS patients (Dolei, 2005), and expression of these sequences was demonstrated in both MS and normal brain tissues and in normal full-term placentas (Lefebvre et al., 1995; Blond et al., 1999). The isolated sequences belong to a multicopy endogenous retrovirus family now named HERV-W (Blond et al., 1999). The complete endogenous genome appears to be in a 10.4-kb genome sequence. Most – if not all – HERV-W sequences are defective for replication-containing mutations in one or more of the ORFs. Particle formation may thus result from *trans*-complementation events, supported by other HERV types.

Transcripts were obtained from placental tissue, consisting of three bands at 8, 3.1, and 1.3 kb. Probes from either gag, prt, pol and env recognized the 8-kb transcript, whereas the 3.1-kb transcript hybridized exclusively with the *env* probe (Blond et al., 1999). The protein coded for by *env* is highly conserved (Mallet et al., 2004), and deserves specific interest: it seems to serve an important function in the human host. The protein, syncytin, induces the formation of giant syncytia and seems to mediate placental cytotrophoblast fusion in placental morphogenesis (Mi et al., 2000). It can be considered as a specific marker of the human trophoblast (Malassine et al., 2005). Syncytin is synthesized as a glycosylated gPR73 precursor, which is cleaved into two mature proteins, a gp50 surface subunit and a gp24 transmembrane subunit. The intracytoplasmic tail is critical for the fusogenic phenotype (Cheynet et al., 2005). The crystal structure of the central fragment of syncytin-2 permitted a remarkable superposition with the structures of corresponding domains of present-day infectious retroviruses, in spite of a more than 60% divergent sequence (Renard et al., 2005).

8.3.2.4 Other Endogenous Human Retrovirus Genomes

Some additional sequences have been analyzed and assigned to HERV-A (Sugino et al., 1992), HERV-E (Medstrand et al., 1992), HERV-I (Maeda and Kim, 1990; Martin et al., 1997), HERV-L (Cordonnier et al., 1995; Benit et al., 1999), HERV-R (Kato et al., 1987, 1990; Andersson et al., 2002), HERV-S (Yi et al., 2004), and HERV-FRD (Blaise et al., 2004). All of these proviruses entered the human germline far back in our evolutionary history and, except for HERV-E, I, and L, they have been less intensively studied than the previously discussed members of these elements.

8.3.3 HERV Proteins and the Immune Response

Thus far, at least 16 ORFs coding for the env protein (six of them HERV-K) are apparently able to code for functional proteins (de Parseval et al., 2003). One of the env functions in forming syncytin in trophoblasts of the human placenta is discussed in Section 8.2.2.3 as a specific property of HERV-W env. Its fusogenic property is also shared by some other members of the HERV group (Blaise et al., 2003). Env may confer resistance to reinfection by the same or closely related agents by blocking the receptor for these events (Melder et al., 2003; Ponferrada et al., 2003). Endogenous Jaagsiekte sheep retrovirus blocks the entry of exogenous Jaagsiekte virus (Spencer et al., 2003). A similar effect could be attributed to gag functions, as the expression of a gag sequence of an endogenous murine retrovirus blocks infection by several murine leukemia viruses soon after entering the respective cell (Best et al., 1996).

The first report on antibody responses to proteins of endogenous retroviruses appeared in 1975 (Charman et al.), and was subsequently confirmed in animals and humans (Toth et al., 1985; Denner et al., 1996). Clearly, under certain conditions the anticipated immune tolerance of "self-antigens" breaks down. Antibodies to multiple epitopes of HERV-K env protein were even demonstrated in about one-third of healthy individuals and in approximately two-thirds of patients suffering from seminoma (Boller et al., 1997; Herve et al., 2002). Pregnant women also reveal elevated antibody titers twice as often as nonpregnant controls (Simpson et al., 1996; Boller et al., 1997) Inflammatory conditions seem to contribute to antibody development against HERVs, as for example identified in Sjögren's syndrome, multiple sclerosis (Clerici et al., 1999; Christensen et al., 2003), psoriasis (Moles et al., 2005), lupus erythematosus (Bengtsson et al., 1996), and rheumatoid arthritis (Hishikawa et al., 1997; Nakagawa et al., 1997).

Two other properties of specific HERV proteins deserve attention, namely immunosuppression and the potential function as superantigens. Cleavage of the env protein results in two components: the surface protein (gp70), and a transmembrane protein (gp15E) containing an immunosuppressive region (Larsson and Andersson, 1998). The immunosuppressive sequence from the Moloney murine leukemia virus LQNRRGLDLLFLKEGGLC is remarkably similar to the HERV-H19 sequence LQNRRGLDLLTAEKGGLC, and to similar sequences in HERV-R and HERV-E (Nelson et al., 2003). An experimental demonstration of immunosuppressive properties

of the HERV-H env was published by Mangeney et al. (2001). The immunosuppressive functions are advantageous not only for the virus, but also for the host as they should protect viral protein-expressing cells from an immune attack. At the same time, its expression in syncytiotrophoblasts of the placenta should protect the developing fetus from maternal immune responses (Venables et al., 1995; Swerdlow, 2000).

Superantigens elicit a strong primary T-cell response and are presented to T cells by major histocompatibility complex (MHC) class II complex on antigen-presenting cells (for reviews, see Herman et al., 1991; Huber et al., 1996). The superantigens bind solely to the Vβ portion of the T-cell receptor, bridging the T cell with the antigen-presenting cell (Jardetzky et al., 1994). This induces T cells to secrete cytokines that activate further T cells, resulting in a strong primary T-cell activation. Besides Rhabdoviridae and Herpesviridae, a retrovirus, mouse mammary tumor virus (MMTV), encodes a superantigen in its infective and its endogenous form (Dyson et al., 1991; Frankel et al., 1991; Marrack et al., 1991; Woodland et al., 1991). Since the HERV-K family is closely related to type B retroviruses, superantigen activity was analyzed and reported for the *env* gene of HERV-K18 (IDDMK$_{1,2}$22), located on chromosome 1 (Conrad et al., 1994, 1997; Stauffer et al., 2001; Sutkowski et al., 2001). ORFs, however, expressed from the putative superantigen region did not provide evidence for stimulation of human or murine T cells in a Vβ-specific pattern (Lapatschek et al., 2000). A few additional observations recently provided additional support for superantigen activity of HERV-K18: a superantigen activity previously described for Epstein–Barr virus (EBV) (Sutkowski et al., 1996) turned out to be due to the transactivation of HERV-K18 by EBV (Sutkowski et al., 2001). Subsequently, it was shown that the EBV latent membrane protein LMP2 A is sufficient for this transactivation and induction of HERV-K18 superantigen activity (Sutkowski et al., 2004). Although these data require further confirmation, such observations may contribute to novel insights into the potential pathogenesis of endogenous retrovirus reactivation. According to Meylan et al. (2005), negative thymocyte selection for HERV K18 superantigens constitutes a first checkpoint controlling peripheral tolerance compared with superantigen reactivity.

Interferons α and β are also able to strongly induce HERV-R and HERV-K18 expression in human vascular endothelial cells and in peripheral blood cells (Katsumata et al., 1999; Stauffer et al., 2001). This may have interesting implications in chronic inflammatory diseases, as discussed in the following section.

8.3.4 HERV: The Role in Human Tumors and Autoimmune Diseases

The difficulties in establishing a potential role of endogenous viral genomes for cancer development are substantial. The presence of retrovirus-like sequences comprising approximately 8% of the human genome renders it exceptionally difficult to pinpoint a transformation event to one of these sequences, especially, if insertional mutagenesis somewhat distant from the target gene results in transcriptional activation of the latter. A more direct proof could originate from specific infection or trans-

fection experiments resulting in proliferative modification of the respective target cells. In the absence of specific integration patterns, transformation by insertional mutagenesis must be an extremely rare event. The situation would be different, however, if specific viral proteins were to exert oncogenic functions.

The risk of insertional mutagenesis became evident in leukemia development of children receiving mouse leukemia virus vector-driven gene therapy for severe combined immunodeficiency (Hacein-Bey-Abina et al., 2003; Baum et al., 2004). In these patients, vector integration occurred in the proximity of the LMO2 gene and initiated enhanced cell proliferation. Oncogenesis also follows the high copy delivery of nonprimate lentiviral gene therapy to fetal and neonatal mice (Themis et al., 2005). It can almost be predicted that the transposition of endogenous retroviral genomes or infection after induction of retroviruses could lead to the same consequence.

A large number of reports have described an enhanced expression of proteins of endogenous retroviruses in human tumors. An insertional polymorphism of endogenous HERV-K113 and HERV-K115 retroviruses was reported in breast cancer patients that however, did not differ significantly from that in age-matched controls (Burmeister et al., 2004). The expression of spliced env and rec was found in 45% of metastatic melanoma biopsies and 44% of melanoma cell lines (Büscher et al., 2005). Neonatal melanocytes expressed only spliced rec, but no env.

Two endogenous retroviruses, SERPINB5 and a not fully characterized HERV-H, showed a high expression in colon adenomas and colon cancers, without significant expression in corresponding normal tissue (Wentzensen et al., 2004). Similarly, HERV-H was the only family expressed in cancers of the intestine, bone marrow, bladder, and cervix, and was highly expressed in cancers of the stomach, colon, and prostate (Stauffer et al., 2004) and in various cancer-derived cell lines (Yi et al., 2006). Expression of HERV-E in prostate cancer biopsies was suggested to serve as a novel marker for the early diagnosis of patients with prostate carcinomas (Wang-Johanning et al., 2003). The pol gene of the HERV-S family was reported to be more active in cancer cells than in other human tissues (Yi et al., 2004). Increased expression of HERV-K env transcripts was found in ovarian carcinomas, kidney cancer, and testicular carcinomas (de Parseval et al., 2003). Several studies analyzed the expression of endogenous retroviruses in leukemias and lymphomas and in cell lines obtained from these malignancies. A novel human endogenous retrovirus, HERV-H/F, was found in some B-cell lines of leukemic origin and in myeloid lines (Patzke et al., 2002). A HERV-K10-like *gag* gene was found to be overexpressed in six of eight leukemia samples (Depil et al., 2002). In megakaryocytes from patients with essential thrombocythemia, even HERV-K particles have been detected (Morgan and Brodsky, 2004). A less-characterized sequence highly homologous to human endogenous retroviruses was overexpressed in childhood acute lymphoblastic leukemia (Iwabuchi et al., 2004).

It remains an open question in virtually all of these studies as to whether the expression of proteins of endogenous retroviruses is a consequence of the malignant proliferation or contributed to the process of cell transformation. Similarly, increased seroreactivity against endogenous retroviral antigens, as observed for ex-

ample in seminoma and germ cell tumor patients (Löwer et al., 1995; Herbst et al., 1996; Sauter et al., 1996; Magin et al., 1999; Yang et al., 1999), suffer from the same difficulties in interpreting this as cause or effect of malignant growth.

Only one study has attempted to address the biological activity of a specific viral gene experimentally. Galli et al. (2005) showed that the *rec*-gene of HERV-K (related to the HIV *rev*-gene) in transgenic mice disturbed germ cell development and, at 19 months of age, induced changes reminiscent of a carcinoma *in situ*, as a precursor lesion of seminoma. If confirmed, this represents the first report of an organ-specific tumorigenesis by a human endogenous retrovirus gene in a transgenic mouse model.

Endogenous retroviral genomes in mice and man are readily induced by specific herpes infections. In murine systems, the first observations were published as early as the late 1970s by infecting or transfecting cells with partially UV-inactivated herpes simplex virus type 2 (Hampar et al., 1976; Boyd et al., 1978, 1980). This activation of murine type C viruses is not limited to transformed murine cells, but occurs also in nontransformed cells (Hampar et al., 1977). Herpes simplex virus infection also activates endogenous HERV-K and HERV-W retrovirus LTRs in human cells (Kwun et al., 2002; Lee et al., 2003). There are no reports on endogenous retrovirus activation by human herpesvirus type 6, yet, this agent – similar to herpes simplex virus infections – is able to up-regulate expression of an exogenous retrovirus infection, HIV-1, by inducing the LTR activity of that virus (Ensoli et al., 1989; Campbell et al., 1991; Garzino-Demo et al., 1996). The previously described activation of HERV-K expression by LMP-2 A of EBV (Sutkowski et al., 2004) reveals that herpesvirus infections are probably, to a large extent, able to activate endogenous retroviruses. A herpesvirus infection of chicken with Marek's disease virus seems to underline this statement: Marek virus DNA contains two integrated copies of avian leukosis virus, subgroup J (Isfort et al., 1992; Davidson et al., 2002). When activation of endogenous retroviruses plays a role in human carcinogenesis, herpesvirus infections may act as indirect carcinogens by activating those agents.

It is probably easier to analyze the role of endogenous retrovirus activation in the induction of autoimmune diseases. Although this will not be covered here in detail, the organ-specific strictly localized autoimmune disorders, such as MS (Christensen et al., 2000; Firouzi et al., 2003; Brudek et al., 2004), diabetes mellitus (Suenaga and Yoon, 1988; Badenhoop et al., 1996; Conrad et al., 1997; Marguerat et al., 2004), thyroiditis (Shiroma et al., 2001), and others such as Sjögren's syndrome and rheumatoid arthritis (Talal et al., 1990; Gaudin et al., 2000; Bessis et al., 2004) have been suspected to be linked to endogenous retrovirus activity. In MS, the haplotypes of human endogenous retrovirus HRES-1 LTR sequences differ from those of non-MS control groups (Clausen, 2003). Yet, none of the available data are presently conclusive.

8.3.5 The Trojan Exosome Hypothesis

In 2003 an interesting hypothesis was put forward by Gould et al., the Trojan exosome hypothesis. Basically, this hypothesis states that retroviruses may exploit a cell-encoded pathway of intercellular vesicle traffic, the exosomes, for biogenesis of retroviral particles and a low-efficiency but mechanistically important mode of infection. The hypothesis predicts that retroviral particles and exosomes contain a similar array of host cell lipids and proteins in the absence of retroviral env proteins. The theory was mainly derived from observations in HIV systems: HIV buds into endosomes in macrophages and dendritic cells (Blom et al., 1993; Raposo et al., 2002; Greene Nguyen et al., 2003). HIV strains, defective for the gene Vpu, bud into endosomes even in T cells (Klimkait et al., 1990; Gottlinger et al., 1993; Li et al., 1995). One other type of observation supports this hypothesis for HIV: env-independent infections have been well documented in different systems (Chalvet et al., 1999; Pang et al., 2000; Chow et al., 2002).

This model could explain a failure of adaptive immunity in controlling retrovirus infections and, since exosomes carry MHC/peptide complexes (Raposo et al., 1996; Escola et al., 1998; Clayton et al., 2001; Denzer et al., 2000; Wolfers et al., 2001), they may specifically target T cells. In this respect it may be interesting to note that the human CD46 lymphocyte surface antigen shares crossreacting antigenic epitopes with the envelope gp70 glycoproteins of gibbon ape leukemia viruses and Mason-Pfizer monkey virus primate retroviruses (Purcell et al., 1989), but is also a constituent of human immunodeficiency viruses (Montefiori et al., 1994). These authors speculate that a human endogenous retroviral sequence may partially or completely encode the CD49 antigen.

Exosomes have been discovered which carry the genetic information of hepatitis C virus (Masciopinto et al., 2004). Thus, it may also transpire that this novel mode of transporting even partially defective genomes to previously uninfected cells plays a very important role in the transmission of viral genomes which are unable to form infectious viral particles. This might represent a remarkably effective way of bypassing the immune surveillance system of the host. Recently, a very interesting example of Mason-Pfizer retrovirus transmission via exosomes was described where infectious viral RNA-containing exosomes infect human cells by cell fusion (Duelli et al., 2005). It is clear that this mechanism deserves further attention for its possible role in permitting the transmission of endogenous human viruses to different cell compartments and cell types.

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8.4
Gibbon Ape Leukemia Virus and Simian Sarcoma Virus

Although several different types of retrovirus infections have been noted in non-human primates – among them simian T-lymphotropic virus (STLV), simian immunodeficiency virus (SIV), simian type D retrovirus (SRV), and simian foamy virus (for a review, see Lerche and Osborn, 2003) – gibbon ape leukemia virus (GALV) and simian sarcoma virus (SSV) deserve a specific discussion. Besides STLV, GALV and SSV were both isolated from malignant proliferations (leukemias and sarcomas) in their native hosts, the gibbon ape and the woolly monkey, respectively (De Paoli and Garner, 1968; Theilen et al., 1971; Kawakami et al., 1972; Snyder et al., 1973; Todaro and Gallo, 1973; Gallo et al., 1978). The second reason originates from recent observations of sequences relatively closely related to these agents within the human genome, in part retaining the complete set of retroviral genes (see below).

GALV and SSV belong to the genus α of the retrovirus family, and are thus related to murine leukemia viruses and human endogenous virus types HERV-W and HERV-H. Several strains of GALV have been identified (Krakower et al., 1978; Reitz et al., 1979): the GALV-SF from gibbon lymphosarcomas (Kawakami et al., 1972; Snyder et al., 1973), GALV-S from a gibbon granulocytic leukemia (Kawakami and Buckley, 1974), GALV-H from an acute lymphatic leukemia in gibbons (Gallo et al., 1978), and also from frozen brain tissues of non-leukemic gibbons (GALV-Br) (Todaro et al., 1973). The woolly monkey SSV is closely related to these isolates, and shares 78% of amino acid identity with GALV-S (Ting et al., 1998). A cell-derived oncogene was not discovered within the isolates obtained from malignant proliferations of gibbons (Gelmann et al., 1982). It has, however, been discovered within the SSV genome (Dalla-Favera et al., 1981). A structural relationship of this sequence (v-sis) was subsequently discovered with human platelet-derived growth factor (PDGF) (Waterfield et al., 1983; Doolittle et al., 1983). This sequence is expressed in SSV-transformed cells (Deuel et al., 1983). The cellular homologue to v-sis, c-sis, was identified as one chain of PDGF (Josephs et al., 1984).

The viruses are apparently transmitted from viremic animals to their offspring either prenatally or postnatally (Kawakami et al., 1978). In prenatal infections – in contrast to postnatal exposure – large quantities of proviral DNA are commonly observed.

Studies on the biological activity of these viruses are still rare. SSV was able to induce tumors in marmoset monkeys (Wolfe et al., 1971), while a SSV isolate, supposedly originating from human leukemic cells, was shown capable of inducing tumors in these monkeys (Bergholz et al., 1977). The infection of young gibbons with GALV resulted in the development of chronic granulocytic leukemia with multifocal bone lesions and metastases after latency periods of 5–11 months (Kawakami et al., 1980). Gibbons infected at the age of 14 months developed persisting neutralizing antibodies to the virus and remained free of hematopoietic disease. Infection of human blood cells with either GALV or SSV led to enhanced induction of growth of B lymphoblasts (Markham et al., 1979).

A number of reports described the identification of SSV and GALV or the detection of antibodies directed against their antigens in human tumors or sera. Most of these studies were published during the 1970s or the early 1980s. One series of reports attempted to characterize a virus isolated from acute myelogenous leukemia (Gallagher and Gallo, 1975; Gallagher et al., 1975; Teich et al., 1975). At that time, GALV or SSV-related p30 antigens were demonstrated in peripheral white blood cells of humans with acute leukemia (Sherr and Todaro, 1975). Another report described the characterization of an RNA-directed DNA polymerase from human leukemic blood cells corresponding to the GALV-enzyme and being inhibited by antisera to reverse transcriptase from SSV (Mondal et al., 1975). A virus isolate from an AIDS patient with C-type virus characteristics was identified as a lymphocytopathic agent (Levy et al., 1984) and, after sequencing, as a new subtype of GALV (Parent et al., 1998). The demonstration of cytotoxicity of lymphocytes or antibodies against autologous tumor cells in patients with myeloid leukemia or preleukemic disorders was reported to be blocked by the gp70 antigen of GALV, and also by the corresponding protein of baboon endogenous retrovirus (Szabo et al., 1983). The close relationship of the human leukemia isolates to GALV and SSV, as initially demonstrated by tryptic digest mapping of peptides, raised the suspicion that they may have been derived from inadvertent contamination of the respective materials with gibbon and woolly monkey viruses (Fuqua and Naso, 1982). Reports demonstrating the presence of natural antibodies in 74–78% of sera from healthy humans to antigens of GALV (Aoki et al., 1976 a,b) added to the difficulties in interpreting the results of this period (see also Chapter 1).

It is interesting to note that virtually all isolates of GALV and SSV from human leukemic materials date back to more than 20 years ago. The data do not exclude an obviously extremely rare horizontal transmission of these nonhuman primate retroviruses to humans. It became clear, however, that neither GALV nor SSV sequences persist as endogenous genomes within the human germline. This seems to exclude a reactivation of such endogenous DNA in the course of leukemogenesis.

More recent studies point to the existence of a novel subgenus of human endogenous retroviruses most closely related to GALV and SSV. This sequence was apparently obtained from a patient with chronic myelogenous leukemia, and at present is only available in the human genome data bank (Xu and Zheng, 2003 a,b). The sequence represents a full-length retrovirus genome with the typical ORFs not interrupted by stop codons. At the nucleotide level it shares at various genomic sites between 54 and 66% of homology with the sequenced GALV isolate, and the individual ORFs show the following homology at the amino acid level: within the polymerase sequence of 47% for 1183 amino acids, for the gag sequence 34% for 506 amino acids, and for the env sequence 33% for 231 amino acids. The genomic organization of HVML is shown in Figure 8.8.

Our own studies revealed that at least three complete copies of this agent exist within the human genome (E.-M. de Villiers, R. Schmidt, and H. zur Hausen, unpublished results). Partial sequences are found in various chromosomal sites (see Fig. 8.9).

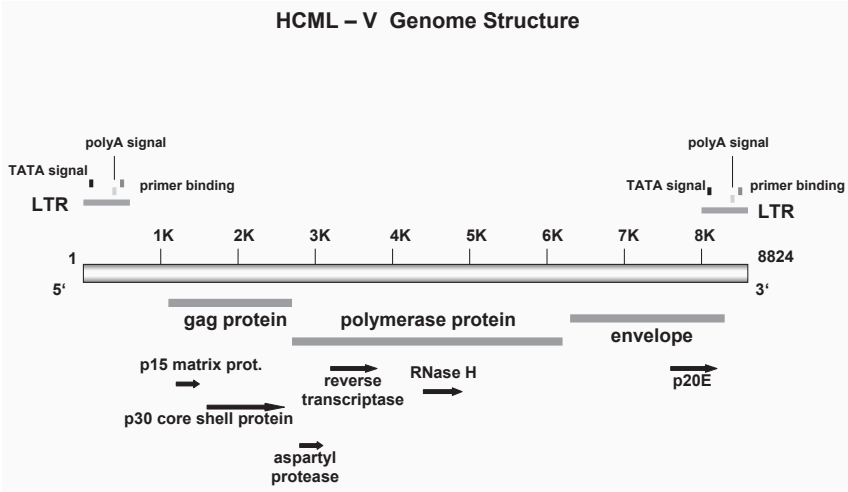

Fig. 8.8 HVML genome structure.

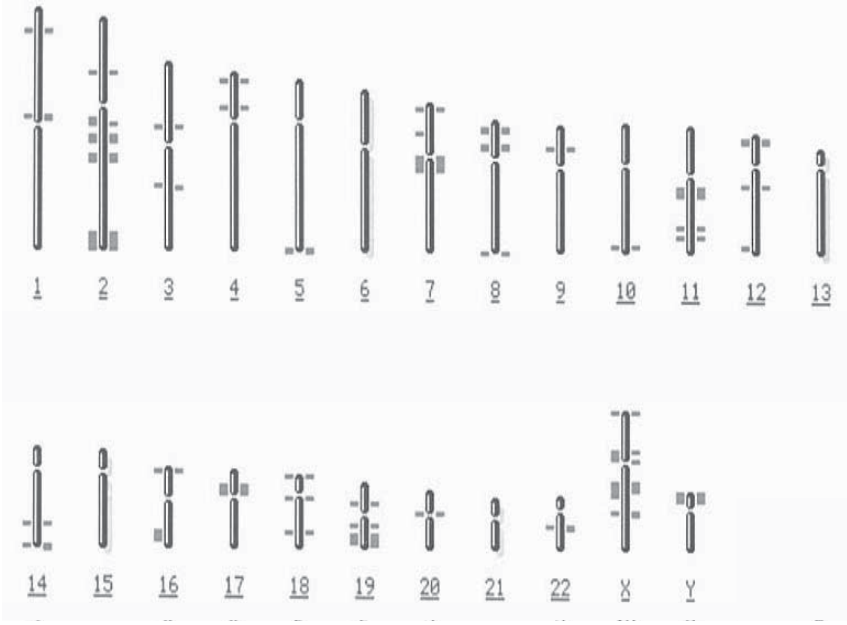

Fig. 8.9 Localization of HCML genomes in human chromosomes.

Besides HERV-K this seems to represent a second subgroup of human endogenous viruses which should be able to code for complete particles. The significance of these observations is presently difficult to assess. The original observation of the isolation of these GALV-related novel agents from a human chronic myelogenous leukemia, a condition which in gibbons is clearly induced by such infections, should create some interest in these or related agents.

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9
Other Virus Infections Possibly Involved in Human Cancers

9.1
Polyomaviruses (JC, BK, and SV40)

Members of the polyomavirus family represent small (~40 nm) non-enveloped viruses (Fig. 9.1) containing a circular double-stranded (ds) DNA genome of about 5200 base pairs. Open reading frames (ORFs) for one of these viruses (SV40) are shown in Figure 9.2.

To date, two human polyoma-type viruses have been isolated, and both have received designations with the initials of the patient from whom they were isolated: the BK virus was isolated in 1971 (Gardner et al.), and the JC virus within the same year by Padgett et al. Both of these agents are widely spread among all human populations, and cause persistent infections that may be reactivated under conditions of severe immunosuppression. In the latter case, specifically JC virus may cause progressive multifocal leukencephalopathy.

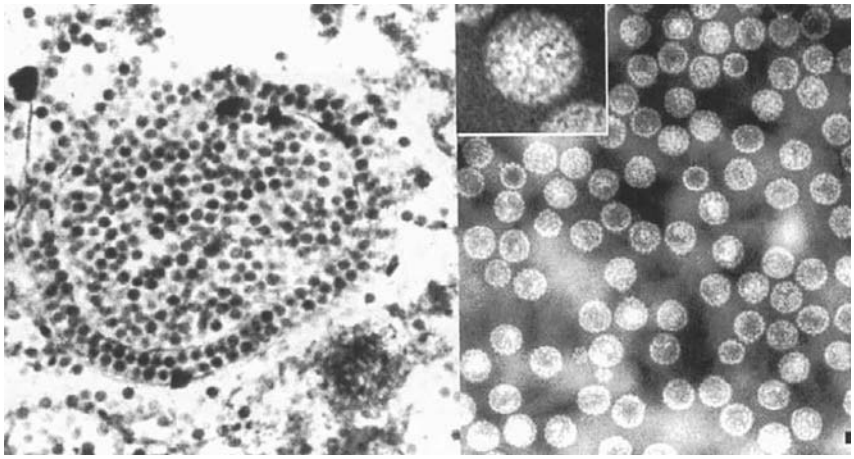

Fig. 9.1 Polyoma-type particles. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The Double Stranded DNA Viruses, Polyomaviridae, 232. Copyright 2005, with permission from Elsevier.)

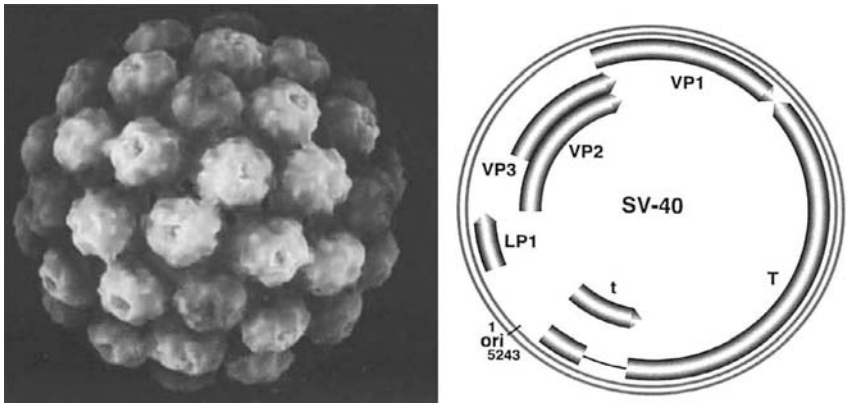

Fig. 9.2 Schematic representation of a polyoma virus particle and of the open reading frames within the SV40 genome. (Reprinted from VIRUS TAXONOMY, Eighth Report of the International Committee on Taxonomy of Viruses, Vol 1, Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (Eds.), Part II – The Double Stranded DNA Viruses, Polyomaviridae, 231. Copyright 2005, with permission from Elsevier.)

Several additional polyoma-type viruses have also been identified in nonhuman primates and other mammalian and avian species. Here, we will discuss only polyoma-type viruses in nonhuman primates. The first isolate in this group is the simian virus 40 (SV40), initially identified as a contamination of rhesus monkey kidney cells (Eddy et al., 1961, 1962). In addition to SV40, simian agent 12 is regularly found in baboons, while a novel polyoma virus was recently discovered in the feces of a chimpanzee (Johne et al., 2005). An additional polyoma-type virus was isolated from lymphoblastoid cell cultures of an African green monkey and labeled as B-lymphotropic polyomavirus (zur Hausen and Gissmann, 1979). A number of human sera contain antibodies neutralizing this virus, yet, attempts to isolate this or a related agent from human specimens thus far have been unsuccessful (Brade et al., 1981).

The majority of polyoma-type viruses are effective carcinogens. As a rule, with few exceptions, they do not induce tumors within their natural hosts but do this efficiently upon inoculation into newborn rodents. One of these viruses (JC) also induces gliomas upon intracerebral infection of adult owl monkeys (London et al., 1978).

It remains one of the most interesting – but poorly studied – questions as to how the natural host of these viruses inhibits the potential oncogenicity of these agents. Indeed, besides polyoma-type viruses a number of human adenovirus types have been identified which also represent effective carcinogens for newborn rodents, but have not been identified in any malignant human condition. The control for polyoma-type viruses can neither be solely immunological nor a question of permissiveness, since BK, JC, and SV40 viruses persist in their natural hosts despite a clearly measurable immune response. The suspicion that cells of the natural host succumb

to lytic infections by these agents and thus cannot be transformed, must also be incorrect, since at least JC virus infects human cells in tissue culture abortively without lysing or transforming them. The life-long persistence of some of these agents also argues against this hypothesis.

The evidence for a tumorigenic function of primate polyomaviruses in their natural hosts is at best poor (see below), although most of these virus types induce effectively tumors in the nonpermissive heterologous hosts. This led to the question of whether a reciprocal situation might exist for some animal viruses potentially infecting humans (*zur Hausen*, 2001). In this case, the nonpermissive human cells, not being adapted to the respective infection, may have failed to develop the necessary cellular interfering factors to cope with these then potentially transforming infections. Careful studies along those lines have not yet been conducted.

During the past four decades, polyoma-type viruses have attracted intense attention, in particular the SV40 and murine polyomaviruses. This led to many early studies on the molecular biology of virus-induced oncogenesis, with the viruses serving as vector systems for gene technological experiments (Strayer et al., 2005), and last – but certainly not least – as potential human tumor viruses.

It is not the intention of this chapter to review the vast body of literature covering the molecular biology of these viruses and their transforming activities *in vitro* and *in vivo*. In this respect, the reader is referred to reviews by White and Khalili (2004), Ahuja et al. (2005), and Arroyo and Hahn (2005). This chapter will outline only those data related to human carcinogenesis and to the potential role of BK, JC, and SV40 viruses.

9.1.1 BK Virus

BK virus infections commonly occur early in childhood. In the vast majority of cases the primary infection is asymptomatic, but it may very occasionally lead to mild respiratory symptoms (Mantyjärvi et al., 1973; Goudsmit et al., 1982). Very rarely, the infection causes cystitis and kidney disease in children (Padgett and Walker, 1983; Saitoh et al., 1993). The virus persists apparently for the patient's lifetime, and may be reactivated during pregnancy, with slightly more than 3% of pregnant women excreting BK virus in the urine (Coleman et al., 1980). Under immunosuppression – for example in renal transplant patients or bone marrow transplant recipients – virus excretion occurs in 25–45% of cases (Hogan et al., 1980; Gardner et al., 1984; Andrews et al., 1988). Hemorrhagic cystitis is a common complication especially in allogeneic bone marrow recipients. This condition is regularly accompanied by – and probably caused by – the excretion of BK virus (Arthur et al., 1986; Apperley et al., 1987; Chapman et al., 1991).

9.1.1.1 Tumorigenicity of BK Virus in Experimental Animals

The immortalization of rabbit, rat, mouse, and monkey cells by BK virus infections was reported as early as the 1970s (Mason and Takemoto, 1977; Portolani and Borgatti, 1978; Seehafer et al., 1979). All immortalized cells produced the BK virus-specific T-antigen, commonly contained episomal virus DNA, and were not tumorigenic when heterografted into immunoincompetent hosts (nude mice).

The inoculation of BK virus into newborn rodents, however, resulted in tumors, with tumor development depending on the animal species, the site of inoculation, and on the amount of virus administered. Brain tumors were the most frequently observed, such as ependymomas in hamsters (Uchida et al., 1976; Corralini et al., 1977), choroid plexus papillomas (Greenlee et al., 1977), gliomas, neuroblastomas, insulinomas, neuroblastomas, nephroblastomas, osteosarcomas, fibrosarcomas, and lymphomas (Corralini et al., 1978, 1982; Yogo et al., 1980). It was noted at an early stage that histologically corresponding cancers in humans were devoid of detectable BK virus sequences and BK virus T-antigen (Grossi et al., 1981).

Transgenic mice containing the early BK virus regions developed liver and renal cell cancers at 8–10 months of age, and expressed viral mRNA within the tumor tissue (Small et al., 1986 a). Thymoproliferative disorders and lymphomas were induced in another set of experiments, besides renal adenocarcinomas (Dalrymple and Beemon, 1990). Transgenic mice carrying the HIV tat gene in addition to the BK early region developed skin leiomyosarcomas, squamous cell papillomas and carcinomas, adenocarcinomas of skin adnexa, glands, and B-cell lymphomas (Corallini et al., 1993).

9.1.1.2 Immortalization of Human Cells by BK Virus, and BK Virus in Human Cancers

Immortalization was also observed in BK virus-infected human kidney and human fetal brain cells, although the cells retained a semi-permissive state and continued to produce small quantities of infectious BK virus (Purchio and Fareed, 1979; Takemoto et al., 1979).

BK DNA has been reported in human neuroblastomas (Flaegstad et al., 1999), although neuroblastoma cell lines were negative for BK (Stolt et al., 2005). In addition, viral DNA was also reported in an occasional glioma (5/150) and in a single medulloblastoma (Huang et al., 1999). One report claimed the presence of episomal BK virus DNA in primary human brain tumors, in Kaposi's sarcoma and in cell lines from brain tumors, in Ewing sarcoma and in osteogenic sarcoma. Infectious BKV was rescued from several of these tumors and tumor cell lines by transfecting the total cellular DNA derived from such sources into human embryonic fibroblasts (Negrini et al., 1990). Since all these positive samples contained the DNA of one specific BK virus variant previously isolated from a tumor of the pancreatic islet by the same group, the risk of inadvertent contamination appears to be relatively high and, indeed, other groups were unable to confirm this finding (Weggen et al., 2000; Kim

et al., 2002; Rollison et al., 2005 a). There was also no serological indication for elevated BK virus antibody levels in patients with brain tumors (Rollison et al., 2003).

Thus, the presently available data provide no hint that BK virus is involved in human cancer development.

9.1.2 JC Virus

JC virus, similar to BK virus, is widely spread among all human populations, apparently persisting within the central nervous system for the patient's lifetime. The primary infection is commonly asymptomatic, but under conditions of severe immunosuppression it may cause a subacute fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML). Although during the past few decades PML has been mainly seen among elderly patients, the current AIDS epidemic has resulted in PML being identified in younger patients. Oligodendrocytes in PML lesions produce large quantities of the JC virus.

9.1.2.1 Tumorigenicity of JC Virus in Experimental Animals

An initial report described the transformation of primary hamster brain cells by JC virus or by viral DNA (Frisque et al., 1980). NIH 3T3 cells can be also transformed by JC, although at lower efficiency than with BK or SV40 DNA (Hayashi et al., 2001). In cell lines obtained from mice transgenic for the early region of JC virus, there was no T-Ag expression in mesenchymal fibroblasts. Instead, T-Ag-positive lines had characteristics consistent with a neural crest origin (Beggs et al., 1990). Primary brain cultures from the same animals contained many T-Ag-positive astrocytes, but no expression was detected in macrophages, epithelial cells, neuronal cells, or in oligodendrocytes. Thus, the strict tissue specificity of JCV T-antigen expression was maintained. This tissue specificity of early gene expression and function is determined by the JC virus promoter (Feigenbaum et al., 1992).

The virus effectively induces tumors upon inoculation into hamsters, mainly cerebellar medulloblastomas and plexus tumors (Nagashima et al., 1984). Intracranial injection of JC virus into newborn Sprague-Dawley rats resulted in brain tumors in the cerebrum, but not in the cerebellum. Most of the tumor cells were of an undifferentiated neuroectodermal nature (Ohsumi et al., 1986). One of the most interesting observations was the induction of brain tumors in owl monkeys at 18 and 25 months after intracerebral inoculation of JC virus (London et al., 1978). The first of these tumors represented one grade 3 to grade 4 astrocytoma, resembling a human glioblastoma multiforme; the second malignant tumor contained both, glial and neuronal cell types. A cell line established from one of the tumors continued to express the JC virus T-antigen (Major et al., 1984).

In mice, transgenic for the early region of JC virus, dysmyelination in the central nervous system was observed, but not in the peripheral nervous system (Small et al., 1986 b). Some of these animals also developed adrenal neuroblastomas (Small et al.,

1986 a). In another line of transgenic mice the development of massive, undifferentiated, solid mesenteric tumors of neural crest origin was noted, without obvious neurological symptoms (Franks et al., 1996; Krynska et al., 1997). Other animals developed tumors which closely resembled the human medulloblastoma/primitive neuroectodermal tumors (PNETs) in location, histologic appearance, and expression of marker proteins (Krynska et al., 1999 a). Approximately 50 % of the animals developed pituitary tumors by 1 year of age (Shollar et al., 2004), although a small subset developed solid masses arising from the soft tissues surrounding the salivary gland, the sciatic nerve, and along the extremities. Histologically, the tumors resembled malignant peripheral nerve sheath tumors, a rare type of neoplasm which occur in individuals with neurofibromatosis type 1 (NF1) (Shollar et al., 2004).

9.1.2.2 **Immortalization of Human Cells by JC Virus, and JC Virus in Human Cancers**

Primary human fetal glial cells can be transformed *in vitro* by JC virus infection and retain a partial permissivity for JC virus replication (Mandl et al., 1987). Few data exist on cell transformation in human systems, most likely due to the stringent cell tropism of the virus. Yet, transfection of BK virus DNA into human fibroblasts already infected with human cytomegalovirus results in pronounced replication of JC DNA (Heilbronn et al., 1993; Winklhofer et al., 2000).

In contrast to the paucity of reports on transformation of human cells by JC viruses, a larger number of publications claim a role for this virus in human carcinogenesis. Reports on the co-existence of cancerous lesions within PML foci raised the suspicion that JC virus may cause different types of brain tumor (Castaigne et al., 1974; Sima et al., 1983; Gullotta et al., 1992). In these studies, astrocytomas and gliomas were recorded, but unfortunately the tumors were not fully analyzed for JC virus DNA persistence or T-antigen expression, and no cell lines have been established from them. A few brain tumors, however, have been reported to be positive for JC virus DNA, RNA and T-antigen: one of these tumors was an oligoastrocytoma (Rencic et al., 1996). The tumor material consisted partly of an oligodendroglioma and partly of an astrocytoma, though the JCV T-antigen was only detected within the oligodendroglioma portion. In another observation, JCV DNA was identified in a pleomorphic xanthoastrocytoma of a nine-year-old child (Boldorini et al., 1998). A further report described JC virus DNA in 11 out of 23 childhood medulloblastomas (Krynska et al., 1999 b), with four of these tumors also expressing T-antigen in the nuclei of the cancer cells. One study suggested a role for the JC virus agnoprotein in the development of T-antigen-negative tumors (Del Valle et al., 2002); immunohistochemical analysis showed a cytoplasmic localization and widespread distribution of agnoprotein in the neoplastic cells in 11 of 20 samples (55 %). The JCV early gene product, T-antigen, was present in the nucleus of some – but not all – of the neoplastic cells. Other medulloblastoma samples that expressed agnoprotein had no sign of T-antigen expression. Among 32 medulloblastomas, 18 ependymomas, five choroid plexus papillomas, and seven pilocytic astrocytomas analyzed for the presence of JC virus DNA by Southern blot hybridization and direct sequencing, JC viral DNA

sequence was detected in only five ependymomas and one choroid plexus papilloma (Okamoto et al., 2005). In this study, immunohistochemistry revealed nuclear expression of the large T-antigen in one choroid plexus papilloma, but none of the medulloblastomas or pilocytic astrocytomas contained JC virus DNA. In another study, JCV DNA was found in nine out of 22 brain tumors, including eight astrocyte-derived tumors (seven glioblastomas and one astrocytoma) and one oligodendroglioma, and in two of 15 cerebrospinal fluid specimens with positive tumor tissue (one glioblastoma and one astrocytoma) (Boldorini et al., 2003). By using gene amplification techniques, Del Valle et al. (2001) demonstrated the presence of JC viral early sequence in 49 of 71 samples (69%) (oligodendroglioma, astrocytoma, pilocytic astrocytoma, oligoastrocytoma, anaplastic astrocytoma, anaplastic oligodendroglioma, glioblastoma multiforme, gliomatosis cerebri, gliosarcoma, ependymoma, and subependymoma). In this series, an immunohistochemistry analysis revealed expression of the JCV T-antigen in the nuclei of tumor cells in 28 of 85 (32.9 %) tested samples.

This impressive list of positive data was contrasted by some studies unable to detect JC virus DNA in brain tumors. For example, Arthur et al. (1994) failed to detect JC sequences in 75 glial tumors and tumor-derived cell lines; likewise, Herbarth et al. (1998) could not find JC virus DNA in 52 gliomas or tumor-derived cell lines. Similarly, in eight cases of medulloblastoma, Hayashi et al. (2001) did not find any evidence of JC viral DNA or expression of T-antigens; neither was any JC virus DNA noted in 15 primary medulloblastomas and five supratentorial primitive neuroectodermal tumors (Kim et al., 2002). Another study conducted between two separate laboratories found only three out of 225 brain tumors to be positive for JC virus DNA (Rollison et al., 2005 a).

The major problem in interpreting all of these results stems from the strict neurotropism of JC virus. It is difficult to exclude a passenger role of JC virus in DNA samples of, or even T-antigen-positive brain tumors, although at least some of the data appear suggestive of an etiological role. In order to analyze the question of causality more stringently, experiments are required to demonstrate the need for viral gene transcription and/or protein expression for maintenance of the malignant phenotype. The induction of polyploidy was noted in JC virus-infected cells (Neel et al., 1996), as well as increased chromosomal abnormalities in lymphocytes of patients with high antibody titers to JC antigens (Lazutka et al., 1996). This may suggest an indirect contribution of JC virus infections to carcinogenesis, though obviously additional studies are required to clarify the situation.

Some groups have reported the presence of JC viral DNA and T-antigens in a high percentage of samples from colorectal cancer and from normal colon mucosa (Laghi et al., 1999; Casini et al., 2005; Theodoropoulos et al., 2005). Another group was unable to confirm this finding in an analysis of 233 colorectal tumor samples (Newcomb et al., 2004), even though they were able to detect JC virus DNA in 70% of urine samples of such patients. This group found no evidence to indicate that JCV is the cause of genetic instability in colorectal cancer. Thus, the possible role of JC virus in colorectal cancer remains controversial and also requires further studies.

9.1.3 SV40

Since its discovery during the early 1960s, SV40 has been the prototype of a DNA tumor virus, besides mouse polyoma virus. It easily transforms a wide variety of cells in tissue culture and efficiently induces tumors when inoculated into newborn rodents. SV40 is present as a latent infection in rhesus monkeys and, at least at the time of the discovery of this agent, there was no evidence for SV40 infections in humans. A well-documented exposure of humans to this virus, however, occurred between 1955 and 1963 as the consequence of immunization with SV40 virus-contaminated poliovirus vaccines (Shah and Nathanson, 1976; Strickler and Goedert, 1998). In Europe and the United States, it is likely that several million people were vaccinated with this contaminated vaccine.

Since that time there have been many discussions on whether this inadvertent infection of humans with an established tumor virus might have contributed to human carcinogenesis. In particular, human cancers were studied which correspond to SV40-induced tumors in animal systems, such as a variety of brain tumors, including choroids plexus papillomas, mesotheliomas, and lymphomas.

9.1.3.1 Tumorigenicity of SV40 in Experimental Animals

It is not the intention of this section to review the extensive literature on tumor induction of SV40 in animal systems. Rather, the reader is referred to reviews by Shah (1996) and Ahuja et al. (2005). These authors also refer to the transforming properties of SV40 early proteins and the signaling pathway affected by the expression of these proteins. The infection into newborn mice, hamsters, rats, and other rodents results in tumor formation several months later. A remarkable chromosomal instability is induced by these infections as a function of T-antigen expression (for a review, see Lavia et al., 2003).

9.1.3.2 Immortalization of Human Cells by SV40, and SV40 in Human Cancers

The detection of SV40 sequences in human cancers is rendered difficult due to three technical problems:

- SV40 sequences, spanning almost the complete genome, are present in the majority of commercially used gene vector systems (Völter et al., 1997, 1998; Lopez-Rios et al., 2004; Vera and Fortes, 2004; Strayer et al., 2005). An example of the commonly used vectors that contain SV40 sequences is shown in Figure 9.3. Only a short stretch of 286 bp of the SV40 genome, 3' of VP 2 and VP3 and 5' of VP1, is not represented in any of more than 200 vector sequences present in the databanks (Fig. 9.3) (C. Völter and E.-M. de Villiers, unpublished results). Clearly, the frequent presence of

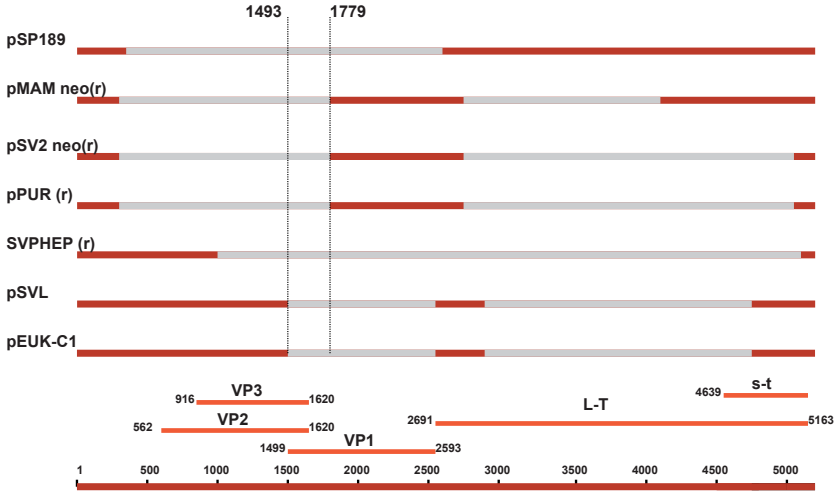

Fig. 9.3 SV40 sequences in some commonly used vector systems. The SV40 sequences are indicated in dark red. The corresponding open reading frames are colored light red. The scale at the bottom reveals the linearized SV40 DNA. (C. Völter and E.-M. de Villiers, unpublished results.)

SV40 sequences in vector DNA poses a substantial problem for possible contaminations (Lopez-Rios et al., 2004).

- A second problem stems from a high degree of homology between SV40 and JC viral DNA. This affects in particular the immunohistochemical detection of viral T antigens. Even a monoclonal antibody to SV40 large T-antigen labels a nuclear antigen in JC virus-transformed cells and in PML brain infected with JC virus (Stoner et al., 1988). Crossreactivity between these polyoma-type viral antigens seems difficult to avoid.
- A third problem is posed by the existence of an extensive homology between part of the SV40 T-antigen and human cellular DNA (Martini et al., 2002; zur Hausen, 2003). This is particularly problematic when polymerase chain reaction primers are used covering a region from nucleotides 4476–4104 (373 bp at the amino terminus) and from nucleotides 2774–2630 (145 bp at the carboxy terminus). These regions show a 97% homology with human genomic sequences in the telomeric regions of chromosomes 10 (191 of 195 bp) and 11 (340 of 348 bp) (Martini et al., 2002).

These considerations must be borne in mind during any analysis of the multiple positive findings of SV40 DNA in human tumors.

Initial observations of SV40 DNA sequences were made in brain tumors, including ependymomas and choroid plexus papillomas (Bergsagel et al., 1992), in five

meningeomas, in one astrocytoma, one oligodendroglioma, and one medulloblastoma (Krieg et al., 1981). A number of additional reports claimed the presence of SV40 DNA in brain tumors: Woloschak et al. (1995) found SV40 DNA in one human brain tumor and sequenced the viral genome. Several other groups reported a number of different brain tumors positive for SV40 DNA (Huang et al., 1999; Zhen et al., 1999; Kouhata et al., 2001; Malkin et al., 2001). The T-antigen region of SV40 was amplified from four ependymomas and three gliomas (Suzuki et al., 1997). Sequence analysis of two brain and one osteosarcoma SV40 sequences did not provide evidence for a "human-specific" SV40 sequence (Stewart et al., 1998). Two human glioblastoma cell lines were reported to contain both, BK virus and SV40 T-antigens (Martini et al., 1996).

Early and contemporary reports were unable to confirm these findings: Greenlee et al. (1978) failed to find SV40 T-antigen in human cerebral tumors. Even in northern India, where rhesus monkeys – the natural host of SV40 – are abundant, human brain tumors were found to be negative for SV40 DNA (Engels et al., 2002).

In addition to brain tumors, primarily methotheliomas, as well as osteosarcomas, renal cancer, thyroid carcinomas, lymphomas and Hodgkin's disease materials were reported to contain SV40 DNA. Human osteosarcomas were found to be positive for SV40 DNA sequences by several groups (Lednicky et al., 1997; Butel et al., 1998; Mendoza et al., 1998; Heinsohn et al., 2000; Yamamoto et al., 2000).

The first reports on SV40 DNA in human mesotheliomas appeared during the mid-1990s (Carbone et al., 1994, 1996), and several other positive reports followed shortly thereafter (Pepper et al., 1996; Galateau-Salle et al., 1998; Testa et al., 1998; Mayall et al., 1999; Shivapurkar et al., 1999; McLaren et al., 2000). SV40 genome-positive mesotheliomas were reported to have a poor prognosis (Procopio et al., 2000). One report described geographical differences for SV40-positive mesotheliomas that were found in the United States, but not in Turkey (De Rienzo et al., 2002). Another report noted that SV40-specific cytotoxic T cells had been generated from the peripheral blood of malignant pleural mesothelioma patients (Bright et al., 2002). One report from Japan indicated that SV40-positive mesotheliomas had occurred among a population which was unlikely to have been exposed to SV40-contaminated poliovirus vaccine (Jin et al., 2004). Furthermore, SV40 was reported to enhance the risk of malignant mesothelioma among people exposed to asbestos (Cristaudo et al., 2005). It should be noted that the listing provided here omits a larger number of reviews published on this topic.

Although at first glance impressive, an equally long list of negative findings contrast these reports: a number of epidemiological studies failed to find any increased incidence of mesotheliomas or other cancer in those populations exposed to SV40-contaminated poliovirus vaccines (Olin and Gieseke, 1998; Strickler et al., 1998, 2003; Engels et al., 2003; Rollison et al., 2004, 2005 a; Engels, 2005). Similarly, seroepidemiological studies analyzing populations previously exposed to contaminated vaccines for SV40 antibodies failed to detect evidence for persistent SV40 infections (Carter et al., 2003; Shah et al., 2004). Failure to detect SV40 T-antigens in mesotheliomas was published repeatedly (Pilatte et al., 2000; Simsir et al., 2001). In addition, a larger number of reports failed to confirm the presence of SV40 DNA in

mesotheliomas obtained from various regions of the world (Krainer et al., 1995; Strickler et al., 1996, 2001; Hirvonen et al., 1999; Mulatero et al., 1999; Emri et al., 2000; Hubner and Van Marck, 2002; Leithner et al., 2002; Manfredi et al., 2005). A critical discussion of positive data pointed to the problems linked to the finding of SV40 DNA in human tumors (Griffiths et al., 1998; Lopez-Rios et al., 2004).

An initial report showing the presence of SV40 DNA in human lymphomas, in Hodgkin's disease and in lymphadenopathies was published in 1998 (Martini et al., 1998). The same group had previously found SV40 sequences in the peripheral blood (Martini et al., 1996). These data were supported by other reports which also claimed SV40 sequences in a substantial percentage of blood cells from healthy donors (David et al., 2001). A surprisingly high percentage of SV40-positive non-Hodgkin lymphomas (NHL) and Hodgkin lymphomas was reported by Vilchez et al. (2002 a,b, 2005) and Shivapurkar et al. (2002, 2004). Several positive tumors were also found in Japan (Nakatsuka et al., 2003).

Initial data not fully compatible with these observations were published in 2003 (de Sanjose et al.). These authors failed to find serologic evidence for SV40 infections in lymphoma patients. In addition, epidemiological surveys provided no evidence for an increased risk of previously SV40-exposed persons for NHL (Engels et al., 2003, 2004; Rollison et al., 2005 b; Schuler et al., 2005; Thu et al., 2006). A large series of NHL (n = 152) from the United Kingdom turned out to be negative for SV40 DNA (MacKenzie et al., 2003). Other reports also failed to find SV40 DNA in human lymphomas (Montesinos-Rongen et al., 2004; Sui et al., 2005). An immunohistological search provided no evidence for the presence of SV40 T-antigen in a large series of NHL and Hodgkin's lymphomas (Brousset et al., 2004).

Sporadic reports of SV40 sequences in a larger number of additional tumors have been published, but remain as yet unconfirmed. Tests in cell lines obtained from bladder carcinomas, Hodgkin lymphomas, and Kaposi's sarcoma, as well as DNA from meningiomas and Kaposi's tumors were negative for any known polyoma virus DNA (Völter et al., 1997).

9.1.3.3 **Does SV40 Represent a Human Carcinogen?**

In comparison to other viral carcinogens discussed in this book, the data published on the role of SV40 in human cancers are at least disturbing. A large number of conflicting reports cover the three tumor localizations or major tumor types reported to be linked to this infection, namely brain tumors, mesotheliomas, and NHL. Neither does epidemiology support an increased cancer risk for the SV40-exposed population; nor does seroepidemiology point to an increased risk of the low number of SV40-seroreactive probands for cancers.

Given the limitations of the test systems discussed in the first section of this chapter, it becomes increasingly questionable as to whether this virus does indeed cause any proliferative disease in the human host. Possible pitfalls in SV40 DNA analysis have been pointed out succinctly by Lopez-Rios et al. (2004), but clearly these do not explain all the positive claims made until today. Moreover, the present author is not

convinced that the allocation of further funds into this problem will be of any help in this respect.

What remains therefore is one of the most intriguing questions: why human polyomaviruses and SV40 – all of which are equipped with potent oncogenes (with the possibility of rare exceptions) – do not represent tumorviruses for their natural hosts.

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10
Helicobacter, Chronic Inflammation, and Cancer

James G. Fox, Timothy C. Wang, and Julie Parsonnet

10.1
Discovery, Taxonomy, and Genomics

10.1.1
Discovery

10.1.1.1 Gastric Helicobacters

Despite the inevitable controversy that heralds any new discovery, *Helicobacter pylori* is firmly established as a human pathogen. The bacterium is found in the antral and fundic gastric mucosa of infected humans. The bacteria are always associated with histopathological lesions usually consisting of infiltrates of mononuclear cells and polymorphonuclear leucocytes. When the patient is treated with a regimen of antimicrobials known to eradicate *H. pylori*, the inflammation regresses over time. However, if the patient relapses and the organism is recultured, the gastritis is again observed.

Acute symptoms associated with natural infection also have been reported (Frommer et al., 1988). Initial *H. pylori* infections can cause profuse vomiting and epigastric pain associated with a marked acute inflammatory response. This inflammation changes to a more chronic type, although polymorphonuclear cells are still present. This active, chronic gastritis persists in most cases indefinitely and usually is not associated with symptoms.

It is now known that *H. pylori* is directly linked to peptic ulcer disease and, importantly, the World Health Organization (WHO) has listed *H. pylori* as a Class I carcinogen. While these diseases have been recognized for centuries, an infectious cause was not actively pursued until the latter part of the twentieth century. Prior to 1982, peptic ulcer disease was attributed to excessive acid or stress, while gastric cancer was linked to a variety of dietary factors documented by extensive epidemiological surveys. Both were known to be associated with chronic inflammation of the stomach, but this association remained unexplained. Nevertheless, the existence of gastric bacteria and a possible association with ulcer disease had been reported by investigators dating as far back as the nineteenth century.

Rappin in 1881 (Rappin, 1948), and Bizzozero in 1893 (Bizzozero, 1893) in more limited studies, are credited with the first observations of gastric spiral-shaped bacteria in animals (Balfour, 1906). The most extensive early investigation of bacteria in animal stomachs was that of Hugo Salomon in 1896 (Salomon, 1896) who observed spiral-shaped organisms in the stomachs of dogs, cats and the brown Norway rat, but failed to find bacteria in humans, monkeys, cattle, pigs, mice, pigeons, and crows.

Spiral-shaped bacteria were observed initially in the stomach of humans as early as 1874, and reported sporadically for the next several decades, sometimes in association with gastric carcinoma (Krienitz, 1906; Celler and Thalheimer, 1916).

In 1875, Bottcher and Letulle were able to demonstrate the presence of bacteria within the floor and margins of ulcers (Bottcher, 1874), and Jaworski in 1889 described the presence of spiral organisms in the sediment of gastric washings. In 1924, Luck detected the presence of urease activity within the gastric mucosa. These findings were confirmed by Conway and Fitzgerald, who also concluded that urease was produced endogenously by gastric epithelial cells and probably functioned as a mucosal protective agent to neutralize gastric acid (Modlin and Sachs, 1998).

In 1938, Doenges studied 242 human gastric autopsy specimens, and found "spirochaetes" in 43% of cases, but was unable to reach any conclusions because of the presence of significant autolysis (Doenges, 1939). He also concluded that the normal rhesus monkey harbored gastric bacterial after observing "spirochetes" in 100% of 43 animals (Doenges, 1939).

This observation was followed by a study in 1941 by Freedburg and Barron of 35 partial gastrectomy specimens, in which they detected "spirochaetes" in 37% of the samples analyzed (Freedburg and Barron, 1940). Even with the aid of silver-staining methodologies, these investigators found the organism extremely difficult to detect; nevertheless, they noted that the bacteria were more often associated with ulcers, both benign and malignant. Finally, during the early 1950s, Palmer conducted an extensive survey of 1140 gastric biopsy specimens which were examined histologically, but without the use of silver stains. Nonetheless, Palmer reported the finding that "…no structure which could reasonably be considered to be of a spirochaetal nature" (Palmer, 1954). Consequently, the previous reports of spiral bacteria were interpreted as oral contaminants which multiplied in postmortem gastric tissue. During the 1950s, the principle was established that because of the stomach's low pH, bacteria could not survive there, and the organ was, for the most part, considered to be sterile.

During the 1970s, upper endoscopy became established at many medical centers, leading to more frequent mucosal sampling of the gastric epithelium through endoscopic biopsy (Steer, 1975; Steer and Colin-Jones, 1975). In 1975, Steer and Colin-Jones reported the finding of bacterial closely related to the gastric mucosa in association with biopsy specimens showing gastritis, but not in biopsies from normal stomachs (Steer, 1975; Steer and Colin-Jones, 1975). The bacteria in their samples appeared to locate under the mucus layer and in close contact with surface mucous cells. They observed the presence of flagellae – "…at least one filum projecting from one end of the bacterium…" – and their ultrastructural studies actually revealed that

the bacteria were spiral, although this was not highlighted in their reports. They hypothesized that the polymorphonuclear leukocytes present in the mucosa may have migrated in response to the presence of these bacteria, and believed that they were not contaminants.

In 1984, Steer published scanning electron micrographs depicting curved and spiral bacteria in large numbers on the surface of the gastric epithelium and antrum, and in areas of gastric metaplasia in the duodenal bulb. The organisms were observed in 73% of patients with duodenal ulceration (Steer, 1984). Unfortunately, cultures only yielded *Pseudomonas aeruginosa*, a non-spiral bacterium, which was most likely a contaminant.

The observation that there were spiral bacteria present in the gastric mucosa was concurrently pursued in Western Australia by a pathologist, Dr. J. Robin Warren, at the Royal Perth Hospital. Warren had also observed for many years the presence of bacteria in the stomachs of gastritis patients, and in 1980 began compiling a series of cases in which he performed both hematoxylin and eosin (H&E) and silver stains. In 1982, he was joined in his studies by a gastroenterology fellow, Barry Marshall. In 1983, Warren reported his pathological studies, and his finding that "unidentified curved bacilli" were present in about half of all routine gastric biopsies, and were strongly associated with the presence of "active, chronic gastritis" (Marshall and Warren, 1984). He stated his belief that, "…these organisms should be recognized and their significance investigated". In an accompanying letter in the same issue of the *Lancet*, Marshall reported that he was able (after 34 previous failures) to culture the organisms (that bore some resemblance to campylobacters) on chocolate agar using *Campylobacter* isolation techniques, and that he identified as spiral bacteria. They were about 2.5 mμ in length but, unlike campylobacters, the gastric bacteria had up to five sheathed flagellae (Marshall and Warren, 1983). Although these initial studies did not address the possible pathogenic role of these bacteria, the authors concluded that "…they may have a part to play in other poorly understood, gastritis associated diseases (i.e., peptic ulcer and gastric cancer)".

Over the next few years the bacteria were isolated and cultured in several countries (United Kingdom, Holland, Germany, USA, Canada, Japan, and Peru), and characterized in much greater detail.

To demonstrate more convincingly that *H. pylori* could directly produce gastritis and/or symptoms (and thus fulfill Koch's postulates), Marshall ingested an isolate obtained from a 66-year-old man with nonulcer dyspepsia (Marshall et al ., 1985). Prior to self-inoculation, he underwent upper endoscopy. A biopsy taken from his gastric mucosa revealed no ulceration, gastritis, or evidence of infection. After premedication with cimetidine, Marshall dosed himself orally with ~10^9 colony-forming units (cfu) of *H. pylori*. Over the next few days, he had symptoms of indigestion, bloating, nausea, vomiting, headache, and irritability, and described his breath as "putrid". At 10 days post-inoculation, gastroscopy revealed active gastritis; however, at 14 days post-inoculation the symptoms resolved and gastroscopy indicated resolution of gastritis. Shortly thereafter, Arthur Morris, a young New Zealand gastroenterologist ingested 3×10^5 cfu of a different *H. pylori* strain, originally isolated from a 69-year-old woman with dyspepsia and chronic gastritis. Morris developed mod-

erate to severe attacks of epigastric pain, acute achlorhydria, and had evidence of histological gastritis (Morris and Nicholson, 1987). His symptoms evolved into a chronic dyspepsia that persisted despite three varied courses of antibiotics. Three years later, after a course of triple therapy consisting of bismuth/metronidazole/tetracycline (Morris et al. , 1991), *H. pylori* was eradicated and his symptoms and gastritis resolved.

10.1.1.2 **Enterohepatic Helicobacters**

Helicobacter cinaedi, previously classified as *C. cinaedi* (CLO-1 A), was first isolated from the lower bowel of homosexuals with proctitis and colitis (Totten et al., 1985; Vaira et al., 1990). Similar to *H. cinaedi* , *H. fennelliae* previously known as *C. fennelliae* (CLO-2), was first isolated from HIV-infected homosexuals with colitis and proctitis (Fennell et al., 1984; Totten et al., 1985).

Since then, an increasing number of *Helicobacter* spp. have been isolated from the intestinal tracts of rodents, birds, cats, dogs, primates, and humans (Lee et al., 1992; Stanley et al., 1993, 1994; Fox et al., 1994, 1995, 1996 a; Seymour et al., 1994; Ward et al., 1994 b; Franklin et al., 1996; Mendes et al., 1996; Shen et al., 1997). Several of these intestinal *Helicobacter* spp. appear to be part of the autochthonous microbiota of animal hosts (Gebhart et al., 1989; Mendes et al., 1996; Shen et al., 1997), while others have been implicated as etiological agents in diseases involving the gastrointestinal and reproductive tracts of infected hosts (Table 10.1). Indeed, intestinal *Helicobacter* spp. have been isolated from inflammatory lesions in the lower bowel of immunocompromised humans (Fennell et al., 1984) and mice (Cahill et al., 1997; Foltz et al., 1998; Fox et al., 1999 b; Franklin et al., 1999) and with chronic hepatitis (Ward et al., 1994 a,b; Fox et al., 1996 b; Franklin et al., 1998; Whary et al., 1998; Ihrig et al., 1999), hepatocellular carcinoma (Ward et al., 1994 b; Ihrig et al., 1999), biliary cancer (Matsukura et al., 2002), colon adenocarcinoma (Erdman et al., 2003 a,b), and cholecystitis (Franklin et al., 1996; Fox et al., 1998; Maurer et al., 2005).

Table 10.1 Non-*H. pylori* helicobacters isolated or identified by DNA analysis from humans (as of 2005).

Species	Other hosts	Primary site	Other sites	Reference(s)
"*H. rappini*"[a]	Sheep, dog, mice	Intestine	Blood (humans) Liver (sheep, humans[b]), stomach (dogs)	Archer et al., 1988; Kirkbride et al., 1985; Lockard and Boler, 1970; Sorlin et al., 1999; Weir et al., 1999
H. bilis	Mice, dog, gerbils	Intestine	Liver (mice, humans[b])	Fox et al., 1998; Matsukura et al., 2002

Table 10.1 (Continued)

Species	Other hosts	Primary site	Other sites	Reference(s)
H. canis[a]	Dog, cat	Intestine	Blood (humans) Liver (dog)	Foley et al., 1999; Fox et al., 1996 a; Shen et al., 2001; Stanley et al., 1993
H. cinaedi[a]	Hamster, rhesus monkey, dog	Intestine	Blood, soft tissue, joints (humans) Liver (monkey)	Flores et al., 1990; Fox et al., 2001; Gebhart et al., 1989; Orlicek et al., 1993; Tee et al., 1987; Vandamme et al., 1990
H. fennelliae	Dog, macaque	Intestine	Blood	Fennell et al., 1984; Kiehlbauch et al., 1994; Totten et al., 1985
H. ganmani	Mice	Intestine	Liver[b]	Tolia et al., 2004
H. pullorum[a]	Chicken	Intestine	Liver (chicken), humans[b]	Burnens et al., 1994; Stanley et al., 1994
H. canadensis	Geese	Intestine	NR	Fox et al., 2000 b
H. westmeadii	NR	NR	Blood	Trivett-Moore et al., 1997
H. winghamensis	NR	Intestine	NR	Melito et al., 2001
H. heilmannii[a]	Dogs, cats, monkeys, cheetahs, wild rats, swine	Stomach	NR	Heilmann and Borchard, 1991; Lockard and Boler, 1970; Otto et al., 1994; Sato and Takeuchi, 1982
H. felis[a]	Dogs, cats, cheetahs	Stomach	NR	Lavelle et al., 1994; Lee et al., 1988

[a] Some data suggest zoonotic potential.
[b] Organism identified by DNA analysis.
** NR, not reported.

10.1.2 Taxonomy

10.1.2.1 Gastric Helicobacters

The bacteria identified by Marshall and Warren were initially named *Campylobacter pyloridis*, because of their similarities to other *Campylobacter* species. The gastric or-

ganisms were observed to be microaerobic, Gram-negative, spiral-shaped bacteria that morphologically resembled bacteria of the *Campylobacter* genus. Given this similarity, these "campylobacter-like organisms" achieved official recognition in 1985 as *Campylobacter pyloridis* (Anonymous, 1985) and in 1987, the name was changed to *Campylobacter pylori*. A second small, rod-shaped bacterium that colonized primarily the inflamed antrum of ferrets was first cultured in 1985 by Fox et al. and named *C. pylori* ss. *mustelae* , later amended to *C. mustelae* (Fox et al., 1986, 1988 b, 1989). However, both the human and ferret gastric bacteria had a flagellar morphology that were distinct from bacteria in the *Campylobacter* genus (Marshall and Warren, 1984). The campylobacters had a single unsheathed flagellum, whereas the gastric bacteria were characterized by four sheathed flagella at one end, and the ferret organism had multiple bipolar sheathed flagella. In addition, the DNA:DNA homology, the 16 S RNA analysis and the cell wall components of the gastric bacteria differed substantially from those of *Campylobacter* spp. Finally, antibodies to the gastric bacteria showed little cross-reactivity with *C. jejuni* and other pathogenic *Campylobacter* species, indicating significant antigenic diversity. Marshall's and Warren's bacteria, and the bacteria isolated from ferrets by Fox, were therefore recognized in 1989 as belonging to a separate genus, and the organisms were renamed *Helicobacter pylori* and *H. mustelae*, respectively (Goodwin et al., 1989).

In terms of gastric helicobacters, *H. mustelae* was the second gastric organism to be isolated, characterized, and named (Fox et al., 1986, 1988 b, 1989). This organism is now linked to peptic ulcer disease and gastric adenocarcinoma, and to MALT lymphoma in ferrets (Fox et al., 1993 b, 1997; Erdman et al., 2003 a). *Helicobacter felis*, the third named gastric helicobacter was isolated from the gastric mucosa of both cats and dogs (Lee et al., 1988). "*Gastrospirillum hominis*", which has been renamed "*Helicobacter heilmannii*", has been observed to have a wide distribution and can be found in a large number of different hosts, including cats, dogs, pigs, cheetahs, nonhuman primates, and humans. In humans, "*H. heilmannii*" has been observed in association with chronic gastric inflammation, MALT lymphoma and rarely gastric adenocarcinoma, and thus is the only other gastric helicobacter (except for isolated case reports of *H. felis*) which has been associated with stomach diseases in human patients (Lee et al., 1993; Lee and O'Rourke, 1993; Fox, 1997). *H. acinonychis*, isolated from the stomachs of cheetahs, appears to be the most similar phylogenetically to *H. pylori* (Eaton et al., 1993). Another closely related organism, *H. cetorum*, has been isolated from the inflamed stomachs of dolphins and whales (Harper et al. , 2002, 2003).

10.1.2.2 **Enterohepatic *Helicobacter*** spp.

In 1984, a group of microaerobic *Campylobacter*-like organisms (CLOs) were isolated from rectal swabs of male homosexuals suffering from protocolitis and enteritis (Fennell et al., 1984; Totten et al., 1985). These bacteria could be broadly classified into three major DNA homology groups. One of these was *H. cinaedi*, previously classified as *C. cinaedi* (CLO-1 A) (Table 10.1). The second CLO2 was named

C. fennelliae, and the third (still unnamed) organism was classified as CLO3 (On and Holmes, 1995). These organisms were later classified as *Helicobacter* spp., in part because they had sheathed flagella, but primarily because of DNA/DNA hybridization data and 16 S ribosomal RNA (rRNA) analysis.

Over the past two decades, many additional members of the *Helicobacter* genus have been identified, which have included both gastric *Helicobacter* spp. and enterohepatic *Helicobacter* spp. Several of these enterohepatic helicobacters are linked with chronic inflammatory diseases of the bowel and liver, and in some cases are directly associated with gastrointestinal cancers. All together, over 26 novel species have been identified and assigned to the *Helicobacter* genus, mainly on the basis of 16 S rRNA sequencing data (Fox, 2002). Ribosomal RNA codes for proteins which facilitate protein synthesis, and these sequences have been highly conserved over the course of bacterial evolution. The degree of 16 S rRNA sequence similarity is thought to correlate closely with the ancestry of bacterial species, and bacteria with sequences that are more than 90% homologous to *H. pylori* have been assigned to the genus *Helicobacter*.

10.1.3 Genomic Analysis

10.1.3.1 *H. pylori*

The two *H. pylori* strains that have been sequenced *H. pylori* 26 695 and *H. pylori* J99 were isolated from a United Kingdom patient with gastritis in the early 1980s, and from a patient with duodenal ulcer living in the United States in 1994, respectively (Tomb et al., 1997; Alm and Noonan, 2001). Although the genome of 26 695 is 24 kb larger than that of J99 (1667 versus 1643), both possess a total (G+C) % of 39%. Both strains have similar average lengths of coding sequences coding density and contain two copies of the 16 S and 23 S-5 S rRNA loci in the same relative positions (Alm and Noonan, 2001).

Table 10.2 Prevalence of *H. pylori* infection in large (> 500 individual) population-based surveys (adapted from de Martel and Parsonnet, 2006).

Continent	Country	Year	Age group	No. of subjects	HP prevalence [%]	Reference
Studies in adults						
Europe	Helsinki, Finland	1990	> 70	618	65	Strandberg et al., 1997
	Germany	1987	18–89	1785	39	Brenner et al., 1999
	Leicestershire, UK	1997	21–55	1431	15	Stone et al., 1998

Table 10.2 (Continued)

Continent	Country	Year	Age group	No. of subjects	HP prevalence [%]	Reference
Studies in adults						
	East Anglia, UK	1992	20–44	841	22	Jarvis et al., 2004
	Bristol, UK	1996	20–59	10 535	16	Lane et al., 2002
	North England, UK	1998	40–49	7452	27	Moayyedi et al., 2002
	Glasgow, UK	1995	25–64	5749	68	Woodward et al., 2000
	Bruneck, Italy	1990	40–79	826	86	Mayr et al., 2000
	Italy	1996	35–74	930	45	Palli et al., 1993
	Northern Ireland	1986	26–64	3511	58	Murray et al., 1997
	Ankara, Turkey	1998	25–64	1089	77	Akin et al., 2004
Asia	Shandong, China	1997	35–69	3013	66	Brown et al., 2002
	Japan	1990	45–65	1322	81	Montani et al., 2003
	Kuala Lumpur, Malaysia	NA	19–54	548	26	Goh and Parasakthi, 2001
Oceania	Christchurch, New Zealand	1997	18–91	1045	21	Collett et al., 1999
Americas	Mexico	1987	20–90	5996	81	Torres et al., 1998
	United States	1991	≥ 20	7465	33	Everhart et al., 2000
Studies in children						
Europe	Leipzig, Germany	1997	6–7	2487	6	(Herbarth et al., 2001)
	Ulm, Germany	1996	5–8	945	13	(Rothenbacher et al., 1998)
	Pavia, Italy	NA	6–19	807	12	(De Giacomo et al., 2002)
	Sardinia, Italy	1997	5–16	2810	22	(Dore et al., 2002 a)
	Northern Ireland	1986	12–24	1231	29	(Murray et al., 1997)
Asia	Seoul, South Korea	NA	6–12	753	5	(Seo et al., 2002)

Table 10.2 (Continued)

Continent	Country	Year	Age group	No. of subjects	HP prevalence [%]	Reference
Studies in children						
Americas	Southern Andes, Colombia	1992	2–9	2801	69	(Goodman et al., 1996)
	San Juan Sacatepeques, Guatemala	1999	0–3	522	22	(Steinberg et al., 2004)
	Mexico	1987	1–19	5606	51	(Torres et al., 1998)
	United States	1991	6–19	2581	33	(Everhart et al., 2000)

In addition to allelic diversity, *H. pylori* strains also differ in their gene content. The two completely sequenced genomes of *H. pylori* strains 26 695 and J99 share only 94% of their genes (Alm et al., 1999), and 6–7% of the genes are unique for each strain (Tomb et al., 1997). A comparison of 15 strains with DNA microarrays showed that in this set of strains, 1281 of 1643 genes present on the array (combined from 26 695 and J99 genomes) were shared by all strains; however, only 22% of genes were present in some of the strains (Salama et al., 2000).

Indeed, it is increasingly evident that *H. pylori* has remarkable genetic variability and intraspecies diversity. Allelic diversity is so marked that nearly all unrelated isolates of *H. pylori* have a unique sequence when a fragment of several hundred base pairs fragments are sequenced from housekeeping or virulence genes (Kansau et al., 1996; Falush et al., 2003). This allelic diversity is the result of the combination of a high (mutator-type) mutation rate (Bjorkholm et al., 2001), a high frequency of recombination between strains during mixed colonization (Go et al., 1996; Suerbaum et al., 1998; Kersulyte et al., 1999), and the ability of *H. pylori* to integrate small fragments of exogenous DNA into its chromosome (Suerbaum et al., 1998; Falush et al., 2003).

To investigate how this variability arises, investigators recently compared the genome content of 21 closely related pairs of isolates taken from the same patient at different time points (Kraft et al., 2006). The comparisons were performed by hybridization with whole-genome DNA microarrays. Their analysis indicated that the great majority of genetic changes were due to homologous recombination, suggesting that adaptation of *H. pylori* to the host individual occurs principally through sequence changes rather than loss or gain of genes (Kraft et al., 2006).

10.1.3.2 *H. hepaticus*

H. hepaticus is currently the best studied of the enterohepatic *Helicobacter* species, a diverse group that comprises bacteria that colonize the intestinal tracts and/or livers of susceptible hosts and that includes several human diarrheal pathogens including *Helicobacter fennelliae* and *Helicobacter cinaedi* (Solnick and Schauer, 2001) (Table 10.2). DNA from enterohepatic *Helicobacter* species has been found in patients with hepatobiliary diseases including hepatocellular carcinoma and biliary tract cancer, but a causal role of these bacteria in human liver disease has not been firmly established (Fox et al., 1998; Solnick and Schauer, 2001).

H. hepaticus has many features in common with *H. pylori*: both persistently infect their hosts, leading to chronic inflammation, and in both cases this inflammation can progress to carcinoma (Suerbaum and Michetti, 2002). However, *H. hepaticus* does not colonize the stomach, but instead shares the same lower bowel habitat with *C. jejuni*, the most frequent bacterial cause of diarrhea in humans.

The complete genome sequence of *H. hepaticus* ATCC51449 was recently sequenced (Suerbaum et al., 2003). *H. hepaticus* has a circular chromosome of 1799146 bp, predicted to encode 1875 proteins. A total of 938, 953, and 821 proteins have orthologues in *H. pylori*, *C. jejuni*, and both pathogens, respectively. *H. hepaticus* lacks orthologues of most known *H. pylori* virulence factors, including adhesins, the VacA cytotoxin, and almost all *cag* pathogenicity island proteins, but has orthologues of the *C. jejuni* adhesin PEB1 and the cytolethal distending toxin (CDT). The genome contains a 71-kb genomic island (HHGI1) and several genomic islets, the G+C contents of which differ from the remainder of the genome. HHGI1 encodes three basic components of a type IV secretion system and other virulence protein homologues, suggesting a role of HHGI1 in pathogenicity. *H. hepaticus*, as well as other enterohepatic *Helicobacter* spp., also encodes for a CDT which plays an important role in cell cycle arrest (Chien et al., 2000; Young et al., 2000b; Taylor et al., 2003).

The flagellar biosynthesis system of *H. hepaticus* is similar to that of *H. pylori*, with genes encoding two flagellin types FlaA and FlaB under control of respective σ^{28} and σ^{54} promoters. Remarkably, there are two identical copies of *flaA*, including the promoter (HH1364 and HH1653), indicating a relatively recent duplication.

In contrast to *H. pylori*, with its very large number of restriction – modification systems, *H. hepaticus* has only two complete restriction – modification systems (HH238/239 and HH1050/1051). Like *H. pylori*, *H. hepaticus* is naturally transformable (Suerbaum et al., 2003).

10.2
Life Cycle, Specificity, and Virulence Determinants in Cancer Development

10.2.1
Epidemiology of *H. pylori*

Soon after the discovery of *H. pylori* by Marshall and Warren, serological tests for the bacterium were developed by a number of investigators, and proved useful for studies of its epidemiology. Studies worldwide verified the association of *H. pylori* infection with peptic ulcer disease, and follow-up studies showed unequivocally that triple antibiotic treatment of *H. pylori* resulted in decreased recurrences of peptic ulcer disease, and cure of ulcer disease in patients in whom *H. pylori* was eradicated (Graham et al., 1992; Hentschel et al., 1993; Sung et al., 1995; Van der Hulst et al., 1997). Moreover, while epidemiological investigations confirmed that *H. pylori* infection was more common in ulcer disease, these studies also revealed that infection was extremely common even in asymptomatic individuals, and that infection rates were significantly higher in underdeveloped countries. While *H. pylori* infection was found to be present by serological testing in 30–40% of asymptomatic individuals in the U.S., it was present in 70% or more of asymptomatic individuals in underdeveloped countries.

The association of *H. pylori* with chronic superficial gastritis was followed later by studies indicating that *H. pylori* gastritis may progress over several decades to chronic atrophic gastritis. This histopathological condition is a precursor of gastric carcinoma, and is characterized by a loss of specialized glandular tissue, including both parietal and chief cells (Kuipers et al., 1995) (Fig. 10.1). The association with this preneoplastic lesion, and the epidemiological parallel between *H. pylori* infection rates and gastric cancer prevalence, suggested a possible role for *H. pylori* in the pathogenesis of gastric cancer. Gastric cancer was known to be extremely prevalent in regions of the world (Peru, Mexico, Columbia, and parts of Asia) where virtually all adults were infected with *H. pylori*, and infection was commonly present early in childhood. Three prospective, case-controlled studies by three groups based on stored sera obtained between six and years prior to cancer diagnosis, showed clearly that *H. pylori* infection was significantly more common in gastric cancer patients compared to controls, with an odds ratio (OR) of approximately 4.0 (Forman et al., 1991; Nomura et al., 1991; Parsonnet et al., 1991). The studies by Forman et al. showed that the OR increased to approximately 9.0 when cancer cases were limited to those diagnosed more than 15 years after testing positive for *H. pylori* (Forman, 1996). Based on this epidemiological evidence, a Working Group of the International Agency for Research on Cancer (IARC) concluded that infection increased the risk of cancer, and classified *H. pylori* infection as representing a group I carcinogenic exposure (IARC working group on the evaluation of carcinogenic risks to humans, 1994).

In addition, *H. pylori* was strongly linked to other stomach diseases, including gastric MALT lymphoma, a rare disorder in which there is transformation of a clonal B-cell population within the gastric mucosa (Parsonnet et al., 1994). Finally, *H. pylori*

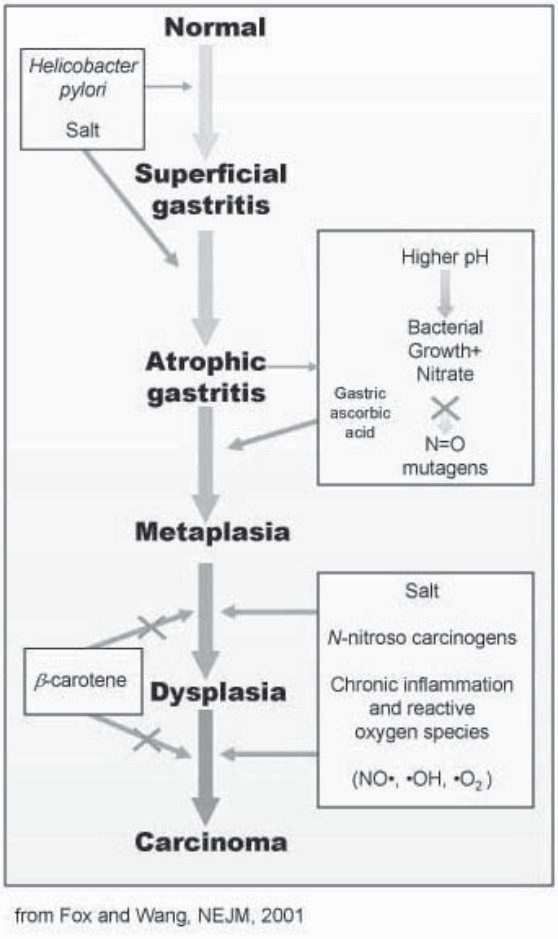

Fig. 10.1 Proposed Correa's cascade of pathologic events in gastric adenocarcinoma. An X through an arrow indicates inhibition of the process.

was also associated strongly with Menetrier's hypertrophic gastropathy (Bayerdorffer et al., 1994) and hyperplastic gastric polyps (Ohkusa et al., 1998).

Thus, *H. pylori* infection is extremely common, affecting approximately 50% of the world's population (Table 10.2) (Hunt, 1996). As with other enteric pathogens, however, the distribution of infection falls most heavily in the developing world. In poorer countries, *H. pylori* may be found in almost all adults and the majority of children by the age of 10 years. In contrast, *H. pylori* is now increasingly less common in the industrialized world, with studies from Europe, the U.S. and Australia indicating a decline in infection incidence of 25% per decade (Parsonnet et al., 1991; Roosendaal et al., 1997). Infection in children in these countries is now rare, except in immigrants from poorer regions of the world.

"*H. heilmannii*" ("*H. bizzozeronii*"), and to a lesser extent *H. felis*, colonize a small percentage of humans with gastritis, and peptic ulcer disease. Given that no environmental source for these bacteria have been recognized, pets have been impli-

cated in zoonotic transmission of the organisms. The eradication of "*H. heilmannii*" by antimicrobial therapy has also resulted in the resolution of gastritis and peptic ulcer disease (Heilmann and Borchard, 1991; Hilzenrat et al., 1995; Goddard et al., 1997). In addition, the recent observation that *H. felis* infection in INS/GAS transgenic and C57/BL mice induces gastric cancer adds credence to isolated case reports of "*H. heilmannii*"-associated gastric carcinoma (Morgner et al., 1995; Yang et al., 1995; Wang et al., 2000).

10.2.1.1 Transmission of *H. pylori*

H. pylori is most likely spread via human-to-human transmission. Humans appear to be the only natural host for the organism, and no significant animal reservoir of infection has been identified. Early reports suggested that gastric *Helicobacter*-like organisms (GHLO) colonized the gastric mucosa of pigs (Ho et al., 1991). However, others have failed to identify or isolate *H. pylori* in abattoir pigs from Brazil and Germany using serology and culture techniques (Rocha et al., 1992; Korber-Golze and Scupin, 1993). Studies to identify the pig as a natural reservoir for *H. pylori* have been complicated by the florid gastric microbiota because of the pig's copraphagic habits, making GHLO isolation attempts difficult. "*H. suis*", which is closely related to "*H. heilmannii*" type 1, has been identified in the stomach of pigs (Queiroz et al., 1990; Solnick et al., 1993; Mendes et al., 1994). Thus, detailed molecular analysis of *Helicobacter*-like organisms isolated from pigs continues to be required for identity, although at present there is no convincing evidence that pigs are a reservoir for *H. pylori*. Similarly, sheep have been incriminated as a source of *H. pylori*, but these data require further confirmation (Dore et al., 2001). Data have shown that cats from one commercial source were infected with *H. pylori* (Handt et al., 1994); however, most domestic cats do not appear to represent a significant vector for transmission of the infection. *Helicobacter pylori* and "*H. heilmannii*" are the most common species reported in monkeys. *H. pylori* has been recovered from two species of Old World macaques: rhesus (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) (Baskerville and Newell, 1988; Bronsdon and Schoenknecht, 1988; Dubois et al., 1994; Reindel et al., 1999). Rhesus macaques and Japanese macaques (*M. fuscata*) have been experimentally infected with human strains of *H. pylori* (Fukuda et al., 1992; Fujioka et al., 1993; Shuto et al., 1993). Based on biochemical, phenotypic and molecular analysis (and in limited studies using molecular techniques), it is believed that *H. pylori* isolated from macaques is highly related or identical to isolates from humans. However, their limited numbers and minimal direct contact with humans renders them an unlikely source for human infection.

It has been difficult to demonstrate the presence of *H. pylori* in the environment, in contrast to most other enteric bacteria. Although water has also been implicated as a source of *H. pylori* infection (Klein et al. , 1991; Goodman et al., 1996; Brown, 2000) and the organism has been amplified (and very rarely cultured) from water (Hulten et al., 1996; Lu et al., 2002), the epidemiology of infection is not consistent with water being a prominent mode of transmission in most parts of the world. The

possibility has been raised that a contaminated water supply could serve as a source of *H. pylori* infection, and *H. pylori* DNA has been detected in water samples from Lima, Peru using PCR techniques (Hulten et al., 1996). The municipal water supply (versus well water) was incriminated as an important source of *H. pylori* infection, irrespective of whether the families were of high or low socioeconomic status (Hulten et al., 1996). However water sources were linked within the neighborhood of residence and variables including population density, family age distribution, household density, and frequency of drinking untreated water were not considered (Hulten et al., 1996). Interestingly, epidemiological data collected concerning 684 children residing in the southern Colombian Andes indicated that there was a strong association of infection with swimming in rivers or streams a few times a year (Goodman et al., 1996). In another cross-sectional study, *H. pylori* infection was best predicted by childhood living conditions such as lack of fixed hot water supply (Mendall et al., 1992). Studies in southern China showed no correlation to exist between fecal contamination (using fecal coliform counts) of the water supply and the prevalence of *H. pylori* infection. However, it was determined that most subjects boiled their drinking water, irrespective of origin, and stored the water in vacuum flasks, prior to ingestion. Based on these results, the authors concluded that water was not an important source of transmission of *H. pylori* in this region of China (Mitchell et al., 1992 a).

A large epidemiological study in 1815 young adult Chileans aged under 35 years showed an association of ingestion of uncooked vegetables to increased *H. pylori* infection (Hopkins et al., 1993). These authors speculated that contamination of vegetables by raw sewage could have played a role in *H. pylori* transmission. Confounding factors such as measuring socioeconomic status on a dichotomized scale without taking into consideration that three of the 14 items – water supply, sewage disposal and indicators of residential crowding – are directly related to disease transmission (Hopkins et al., 1993). In the Colombian study, children who frequently consumed raw vegetables in general – and lettuce in particular – were more likely to be infected with *H. pylori* (Goodman et al., 1996). Children eating lettuce several times a week had a higher risk of infection (Goodman et al., 1996).

There has been a great deal of speculation regarding whether the coccoid form of *H. pylori* might play a role in the organism's survival outside its host (Jones and Curry, 1990; Mai et al., 1991; Bode et al., 1993 a,b). Experimental data indicate that *H. pylori* coccoid forms can survive for more than one year in river water, and that *H. pylori* could be cultured at 10 days from river water at 40 °C (Karim and Maxwell, 1989; West et al., 1990). *H. pylori* can also survive in milk for several days (Karim and Maxwell, 1989), implying that milk contaminated with feces containing *H. pylori* could potentially be infectious to humans. In order to test definitively the relevance of the coccoid forms, *in-vivo* experiments must be performed – that is, coccoid (unculturable) forms should be inoculated into a suitable animal model to ascertain whether indeed they are infectious. Until this is accomplished the importance of coccoidal forms in transmission of the organism from host to host will be unknown.

Declines in *H. pylori* prevalence over time are presumed to be due to corresponding improvements in sanitation and hygiene. *H. pylori* is most readily transmitted in

environments where many people share living space (Teh et al ., 1994; Goodman et al., 1996). Because household crowding has been so consistently linked with infection, it has been presumed that *H. pylori* is transmitted from person to person. In support of this hypothesis, *H. pylori* has been cultured from saliva, diarrheal stools and, most consistently, from vomitus, of infected hosts (Leung et al., 1999; Parsonnet et al., 1999). Moreover, several studies have indicated that the organism is transmitted to close contacts when an infected host develops gastroenteritis (Mitchell et al., 1992 b). Thus, poorer countries, which have crowded households and higher incidence of gastroenteritis, would also be more likely to sustain transmission.

Although *H. pylori* DNA has been detected by RT-PCR in dental plaque, there are few earlier reports of viable organisms within the oral cavity, and thus an oral source of transmission is still debated (Shames et al., 1989). Recent data however, indicate an increasing number of publications where *H. pylori* has been identified by culture or PCR from dental samples (Dowsett and Kowolik, 2003; Umeda et al., 2003; Matsuda and Morizane, 2005). Also, cats colonized with *H. pylori* had the organism cultured from their saliva (Fox et al., 1996 c).

H. pylori has been detected within the feces in both children (Thomas et al., 1992) and dyspeptic adults (Kelly et al., 1994), and ability to culture *H. mustelae* in ferrets colonized with *H. mustelae* have supported the notion that fecal-oral transmission, particular during the acute achlorhydric stage (Fox et al., 1992, 1993 a). Since early childhood (age $<$ 3 years) appears to be the most frequent age of acquisition of infection, and diarrhea is particularly frequent in this age group, a fecal-oral route seems plausible. For example, *H. pylori* was cultured from the feces of humans administered a cathartic (Parsonnet et al., 1999). Nevertheless, definitive evidence that fecal-oral transmission occurs routinely is still lacking, and many questions remain regarding the mechanisms involved in acquisition of *H. pylori* infection (Cave, 1997).

10.2.1.2 **Age of Acquisition**

In populations of lower socioeconomic status, infection with *H. pylori* most frequently occurs in childhood. Cohort studies of young children in the U.S. border region with Mexico, immigrants from Latin America in California, Alaskan native Americans and in Peru indicate that children have infection rates that are threefold those of adults in the same communities (Parsonnet, 1995). Several investigators report that young children are infected recurrently, losing infection and then acquiring it again until it eventually becomes a persistent infection. These conclusions, however, are confounded by imperfect sensitivity and specificity of diagnostic tests. For example, a breath test which has 95% specificity would, by chance alone, yield a false new infection rate of 5% with serial testing. In one study that required PCR confirmation of positive stool antigen testing, 46% of transiently positive stools were positive for *H. pylori*, indicating that some true transient infections do occur (Haggerty et al., 2005). Regardless of the frequency of transient infection, however, in high-risk regions of the world, sustained infection typically begins before age 10 years, and often before age 5 years. In industrialized regions of the world, infection

is acquired more evenly among age groups. In that respect, *H. pylori*'s epidemiology parallels that of other enteric infections – that is, *Shigella* and hepatitis A – which cause childhood infections in the developing world, but in areas of low incidence, are less age-discriminatory. Regardless of age at onset, because *H. pylori* infection persists throughout life, its prevalence universally increases with age. For unknown reasons, men also have somewhat higher prevalence of *H. pylori* than women (de Martel et al., 2005).

The duration of *H. pylori* infection and age of acquisition have also been linked to variability in *H. pylori* disease outcome. Unfortunately, these risk factors have been difficult to prove observationally because the time of *H. pylori* acquisition is rarely known. Through analogy to other tumors, however, this idea gains credence. Studies of experimentally induced cancers indicate that chronic inflammation results in random CpG mutations that accumulate over time, increasing risk for cancer with duration of inflammation. Such CpG mutations have been observed in gastric tissue of individuals with *H. pylori* infection, suggesting that the longer infection persists, the greater the probability of accumulating the deleterious mutations that lead to cancer (Shibata et al., 2002). In humans, however, only indirect evidence supports the duration/age hypothesis (Blaser et al., 1995).

As described above, *H. pylori* is genetically diverse. This diversity is so informative to have been used to geographically map human migrations (Falush et al., 2003). Because *H. pylori*'s genetic variability also influences disease pathogenesis (see below), the geography of strain type informs disease epidemiology (Covacci et al., 1999). In broad epidemiological strokes, virtually all strains of *H. pylori* in Korea and Japan contain the pathogenicity island (PAI), whereas in Europe and the U.S., the proportion is closer to 60%. In northern Europe, most strains of *H. pylori* have a *vacA* signal peptide sequence of the s1a genotype, whereas in Iberia (and Latin America), s1b predominates; in both regions the m2 genotype of the *vacA* middle sequence exceeds m1. In contrast, in Asia, *vacA* typically is of s1c type with m2 exceeding m1. In Egypt, the less virulent s2 genotype predominates (Van Doorn et al., 1999). Within countries, there are also population differences in infecting strains. For example, in California, strains with the PAI are more common in blacks and in immigrants than in other populations (Parsonnet et al., 1997b). It is not clear how much these regional differences in bacterial genotype prevalence influence disease incidence.

Once *H. pylori* infection occurs, it persists in the stomach for the host's life, or until advanced preneoplasia in the stomach makes the gastric environment inhospitable to further colonization. Typically, the host will die never knowing the infection was present. In a considerable number of individuals, however, one of four adverse outcomes might occur: gastric ulcer, duodenal ulcer, gastric adenocarcinoma, or gastric MALT lymphoma. As would be expected for a major risk factor for disease, the epidemiology of these diseases parallels *H. pylori*'s epidemiology (although MALT lymphoma is too rare and too poorly reported to monitor trends over time).

10.2.1.2.1 Epidemiology of Gastric Cancer and *H. pylori*

Gastric cancer was the leading cause of cancer death in the U.S. and Western Europe through the mid-twentieth century, with incidence rates in men in 1930 exceeding 45/100 000 per year. Over the century, the incidence of gastric cancer has plummeted, however. In the U.S., the age-adjusted incidence rates between 1998 and 2002 was 8.8/100 000 per year (Ries et al., 2005). Despite the aging of the U.S. population (the majority of gastric cancers occur in the elderly), the National Cancer Institute estimates that less than 1% of U.S. residents born now would be expected to develop stomach cancer in their lifetime. This mortality rate of 4.5/100 000 per year represents a tenfold drop since 1930; mortality rates have continued to decline 25% during the past decade. Unfortunately, due to the late stage at which most tumors are discovered, however, the case-fatality rate remains high, with a five-year survival of only 23% and 11 500 people in the U.S. dying of stomach cancer in 2005.

Worldwide, there is great variability in gastric cancer rates (Ferlay et al., 2004) (Fig. 10.2). In 2002, the age-adjusted incidences of gastric cancer in European men ranged from 7.9/100 000 in Denmark to 41.9/100 000 in Belarus. In Latin America

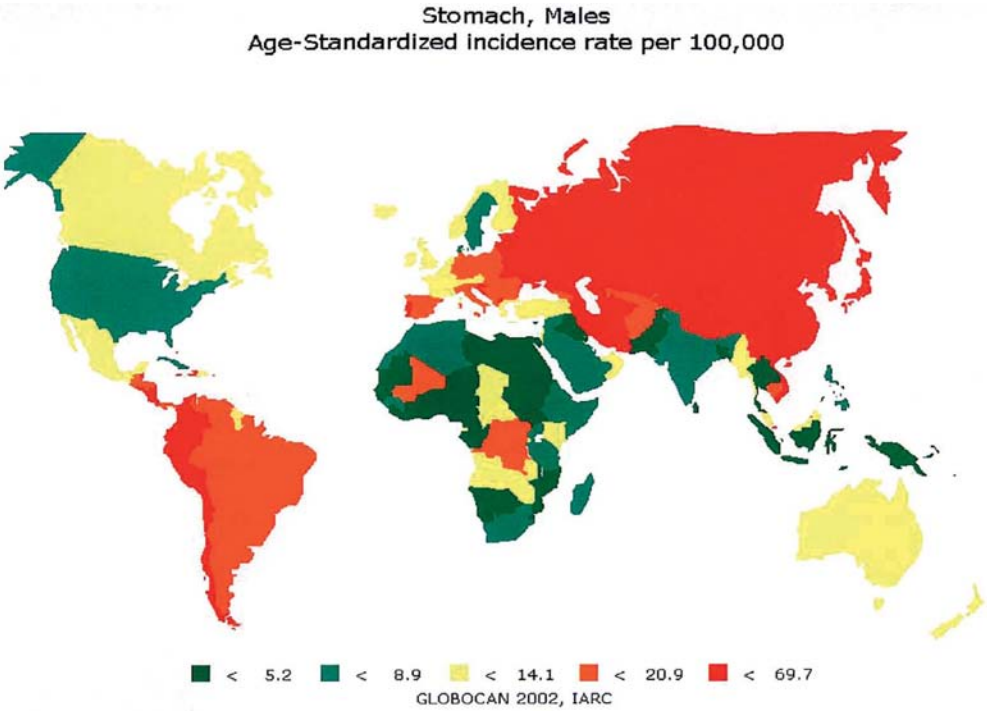

Fig. 10.2 Age-standardized incidence of stomach cancer worldwide demonstrates variability within regions (from Ferlay et al., 2004). Although low reported low rates of cancer in Africa have led some to pursue physiological mechanisms for the "African enigma", incomplete cancer registries and limited cancer diagnostics in some countries may make comparisons misleading.

and East Asia, rates of stomach cancer remain consistently high, with peak rates in men in Chile (46.1/100 000) and Korea (69.7/100 000), respectively, and lowest rates in Mexico (13.1/100 000) and Mongolia (39.2/100 000), respectively. Other regions of the world have considerable variability in cancer rates, although registries are of inconsistent coverage and quality to make broad conclusions (Parkin et al., 1999). These geographical patterns are similar for men and women, although in any given location men consistently have twice the rates of cancer as women.

Overall, IARC data indicate a decline in gastric cancer incidence and mortality in virtually every country in which cancer registries monitor trends. In, Japan, where gastric cancer is infamous for its high incidence, rates are also declining dramatically – in some areas by almost 50% between 1966 and 2001 (Fig. 10.3) (Osaka Cancer Registry, 2004). Despite this unplanned, international "triumph" (Howson et al., 1986), gastric cancer remains a blight in much of the world. In the year 2001, 522 000 men and 328 000 women died from stomach cancer, making it the second leading cause of cancer death worldwide (Table 10.3). Even within low-risk countries, certain racial/ethnic groups retain higher rates of disease than others. In the U.S., cancer statistics indicate that gastric cancer remains the sixth leading cause of cancer death among Asians, the eighth among Native Americans, and the tenth among Blacks and Hispanics (U.S. Cancer Statistics Working Group, 2005). In Malayasia, gastric cancer is more common among Chinese and Indians, and less common in Malays (Kandasami et al., 2003). Furthermore, although worldwide gastric cancer mortality rates declined 5% between 1985 and 1990, due to aging of the world's population the number of cases actually increased 6% (Parkin et al., 1999).

Table 10.3 Estimated cancer deaths globally (2000), at the most common organ/tissue sites (from Parkin et al., 2001).

Cancer	Male	Female	Both sexes	%
Lung	810 000	293 000	1 103 000	17.8
Stomach	405 000	241 000	647 000	10.4
Liver	384 000	165 000	549 000	8.8
Colon/rectum	255 000	238 000	492 000	7.9
Breast	0	373 000	373 000	6.0
Esophagus	227 000	111 000	338 000	5.4
Cervix uteri	0	233 000	233 000	3.8
Pancreas	112 000	101 000	213 000	3.4
Prostate	204 000	0	204 000	3.3
Leukemia	109 000	86 000	195 000	3.1

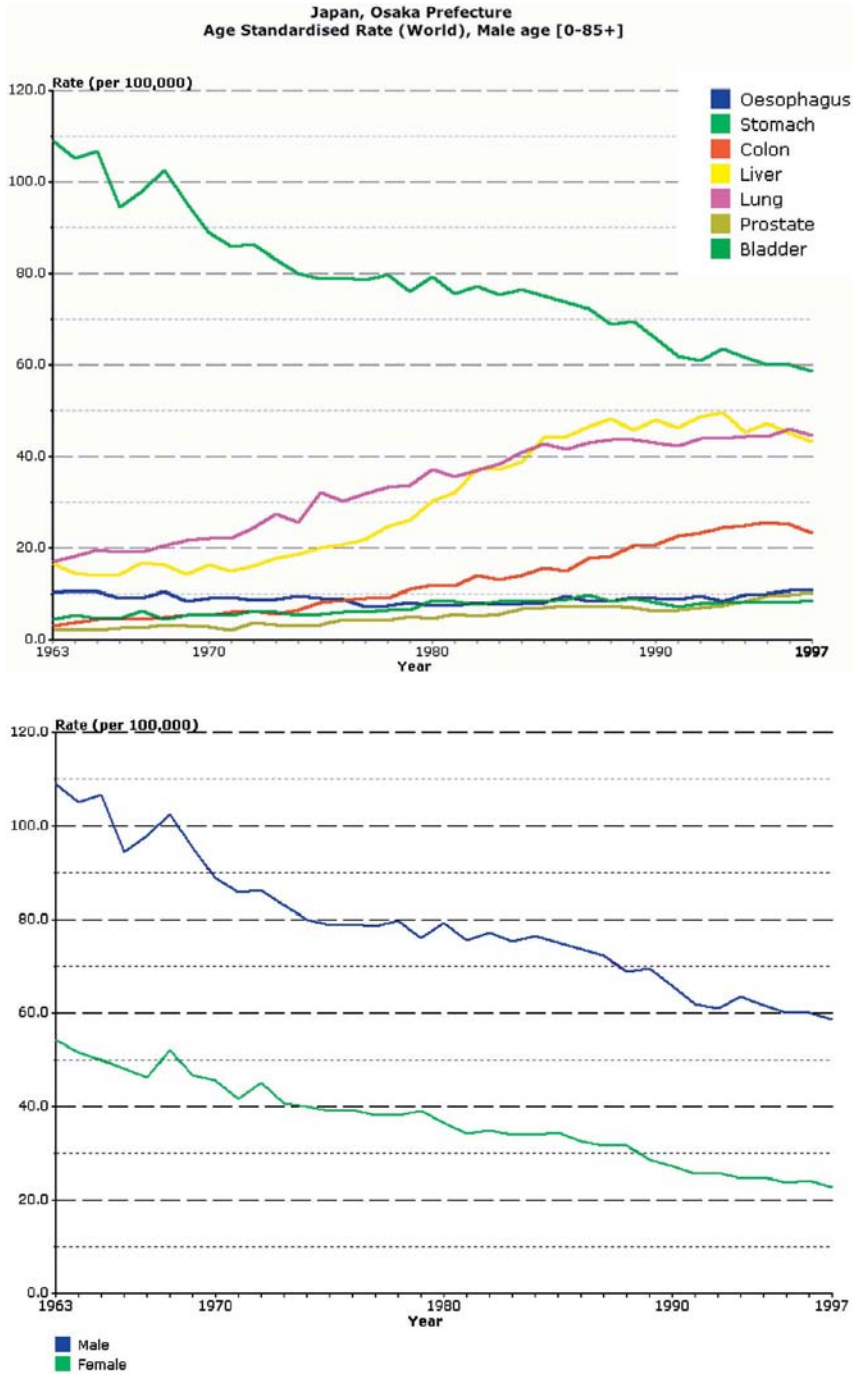

Fig. 10.3 Cancer rates have declined dramatically in many parts of the world, including Japan. Graphs representing cancer incidence rates in Japan adjusted to the world population (Osaka Cancer Registry, 2004).

It is well established that gastric cancer is preceded by chronic and acute gastric inflammation or gastritis (see Section 10.2.6). During the early 1980s, Marshall and Warren, as well as many other investigators, established that *H. pylori* was the preeminent cause of this gastritis, making it a leading suspect in gastric carcinogenesis. Subsequently, hundreds of epidemiological studies have been conducted to investigate this possible link. The strongest among these studies were those with prospective types of designs. These include nested case-control studies with greater than 10 years of follow-up (summary OR for gastric cancer = 5.9) and a prospective study in Japan which, after eight years of follow-up found cancer only in *H. pylori* -infected subjects (infinite OR) (Helicobacter and Cancer Collaborative, 2001; Uemura et al., 2001). The strong associations observed led the International Agency for Cancer Research to categorize *H. pylori* as a definite cause of cancer in humans, its strongest risk factor category (International Agency for Research on Cancer, 1994). In fact, *H. pylori* must be considered among the strongest risk factors identified for any cancer. Based on the OR above, and the high prevalence of infection, it can be crudely estimated that in high-risk areas, the proportion of gastric cancers attributable to *H. pylori* exceeds 80%.

Given the continued high rates of gastric cancer worldwide and the high attributable proportion, understanding and modulating *H. pylori*'s role in carcinogenesis remains imperative. As studies of cancer prevention continue, another critical issue being addressed is why a small minority of infected hosts (estimated at $<5\%$) develop gastric cancer, while the great majority do not. Among theories being actively pursued are variability in bacterial genetics, variability in host genetics, age at acquisition of infection, diet, co-infection with other pathogens, and noninfectious environment exposures (Menaker et al., 2004). Of these, the strongest support for a role in outcome exist for bacterial and host genetics (Blaser, 2002; Basso and Plebani, 2004).

Several bacterial genes impart higher risk of cancer than others. A number of investigators have observed that *H. pylori* which contain the PAI of genes confer a higher risk for gastric cancer than those that do not (Parsonnet et al., 1997a; Enroth et al., 2000). Accumulating data suggest that monitoring for *H. pylori* by a single method such as a serological test markedly underestimates the prevalence of *H. pylori* infection. A Swedish study used not only a conventional ELISA but also used Western blot analysis for antibodies against the CagA protein of *H. pylori*, the presence of which signifies prior or active infection. A high prevalence of infection in patients with gastric cancer was found, and a much higher OR was associated with *H. pylori* infection (Ekstrom et al., 2001). Also, in a recent study conducted in Germany (Brenner et al., 2004), the investigators applied three exclusion criteria to minimize bias against possible clearance of *H. pylori* during progression of the disease, and documented that this increased the OR of noncardia gastric cancer from 3.7 [95% confidence interval (CI) 1.7–7.9] to 18.3 (95% CI 2.4–136.7) for any *H. pylori* infection, and from 5.7 (95% CI 2.6–12.8) to 28.4 (95% CI 3.7–217.1) for *H. pylori* $CagA^+$ strains-infected patients (Brenner et al., 2004).

A meta-analysis that included 16 studies with 2284 cases and 2770 controls has recently confirmed the increased risk of gastric cancer in patients infected with $cagA^+$

H. pylori. Infection with the latter strains increased the risk for gastric cancer over the risk associated with *H. pylori* infection alone (2.87- and 2.28-fold, respectively (Huang et al., 2003).

Nevertheless, the almost universal prevalence of PAI-positive strains in some parts of the world can obscure this finding. The association between the PAI and cancer is particularly manifest for the "intestinal type" of gastric cancer that retains morphological similarity to intestinal tissue. PAI-positive strains have also been closely linked to p53 mutations found in intestinal cancers (Shibata et al., 2002) (Table 10.4). The diffuse type of gastric cancer, though still strongly associated with *H. pylori*, has equal association with PAI-positive and -negative strains (Parsonnet et al., 1997 a). Additionally, polymorphisms in the vaculolating cytotoxin (*vacA*) gene of infecting *H. pylori* strains may impart a higher risk for malignancy. Among the polymorphisms studied, the s1 and m1 *vacA* alleles (these alleles are associated with augmented inflammation) have been most consistently linked to a higher cancer risk (Bravo et al., 2002; Figueiredo et al., 2002; Garza-Gonzalez et al., 2004; Perez-Perez et al., 2005). Host genetics also play a role in disease outcome. Numerous studies have now confirmed El-Omar's landmark report which indicated that the host's interleukin (IL) 1β genotype and the genotype of the IL-1β endogenous receptor antagonist (IL-1 RN) influenced disease outcome (El-Omar et al., 2000). In case-control studies of *H. pylori*- infected subjects with and without cancer, biallelic C/C

Table 10.4 Genetic changes in gastric adenocarcinoma.[a]

Changes	Gene	Frequency [%]
Suppression/Loss	P53	60–70
	FHIT	60
	APC	50
	DCC	50
	E-cadherin	< 5
Amplification/Up-regulation	COX-2	70
	HGF/SF	60
	VEGF	50
	c-Met	45
	AIB-1	40
	β-catenin	25
	K-sam	20
	Ras	10–15
	c-erb B2	5–7
Microsatellite instability (MSI)		25–40
DNA aneuploidy		60–75

[a] Data from Kounouras et al. (2005).

at either base 31 or 511 increases the risk of cancer 2.5-fold compared to the T/T alleles. Similarly, the presence of an allele of IL-1 RN with only two tandem repeats increases the cancer risk. These findings have been reproduced in other, but not all, countries (Perez-Perez et al., 2005). Combinations of these detrimental alleles increases the risk of cancer further (Figueiredo et al., 2002). Host tumor necrosis factor (TNF)-α and IL-10 allelic variation has also been linked to disease, though with somewhat smaller and more variable effect sizes (Perez-Perez et al., 2005); in conjunction with a PAI+ *H. pylori* infection, this effect is even more marked (El-Omar et al., 2003). In addition, several reports have implicated HLA genotypes as either detrimental or beneficial in protecting against cancer, though no consistent pattern has emerged.

Prior to the discovery of *H. pylori*, diet had been studied extensively as a cause of gastric cancer. In 1997, a panel of experts met to review the entirety of dietary data and to weigh the risk of cancer associated with foods. Foods were categorized as convincing, probable, possible, and insufficient risk factors for malignancy. For stomach cancer, only a few items were considered convincing or probable in their association with disease: these included salt (convincingly deleterious) and fruits, vegetables, and vitamin C (probably protective) (World Cancer Research Fund, 1997). A number of other factors were deemed to have no probable relationship with stomach cancer, including alcohol, black tea, coffee, and nitrates from vegetables. The panel did not consider dietary factors in conjunction with *H. pylori* infection however, and data in this area are slim. Data on salt are particularly unconvincing. Although salt has been identified as a risk factor for gastric cancer in a population with relatively high *H. pylori* prevalence (Tsugane et al., 2004), no study has compared the effects of salt in *H. pylori*-infected and uninfected humans. In animal models, salt has variable effects on carcinogenesis with *H. pylori*, accelerating progression of preneoplasia in infected C57/BL6 mice but not B6129 or hypergastrinemic INS-GAS mice (Fox et al., 1999a, 2003a; Rogers et al., 2005)

Some of the most intriguing cofactors of gastric cancer that are being actively pursued are other infectious agents. With advanced gastric atrophy consequent to *H. pylori* infection, gastric acid secretion diminishes. This loss of acid permits colonization of the gastric mucosa by nitrosating bacteria that can convert nitrates to mutagenic *N*-nitrosamines. In contrast, another co-infection – intestinal helminth infection – may diminish cancer risk by limiting host inflammatory responses. This is accomplished by shifting the normal proinflammatory Th1 response induced by *H. pylori* to a noninflammatory Th2 response promoted by the helminth infection. In children, in Colombia and South Africa, investigators have observed higher levels of Th2-dependent IgG1 to *H. pylori* than in children without helminths (Mitchell et al., 2002; Whary et al., 2005). Physiologically, persons with *H. pylori* in conjunction with *Schistosoma mansoni* infection, have been found to have reduced apoptosis and cell proliferation than persons with *H. pylori* alone (Elshal et al., 2004). It has been postulated that these effects explain the paradoxically low rates of gastric cancer in impoverished areas with high prevalence of *H. pylori*. Alternative explanations, however, such as low overall life expectancy and limited cancer registries and diagnostics, have not been excluded as reasons for the "African enigma". Epstein–Barr

virus (EBV), which is a known risk factor for a rare form of gastric cancer – lymphoepithelioma-like carcinoma – has not been shown to interact with *H. pylori* to increase cancer risk.

10.2.1.2.2 **Gastric Non-Hodgkin's Lymphoma**

Primary gastric non-Hodgkin's lymphoma (NHL) is a rare disease. In studies from Europe, North America and the Middle East, this tumor affected a mere 0.2 to 0.9 per 100 000 population per year (Ullrich et al., 2002; Bani-Hani et al ., 2005). Incidence rates increase with age, with gastric lymphomas being vanishingly rare before the age of 30 years (Claviez et al., 2005). They are also somewhat more common in men than in women. Although rates of gastric lymphoma are quite low, the stomach is the most common extranodal site for NHL.

Gastric lymphomas may be of several histological types, the most common of which are marginal-zone B-cell lymphomas arising from lymphoid follicles known as mucosal-associated lymphoid tissue (MALT) and diffuse large B-cell lymphomas. The proportions in each category vary from study to study, probably due both to aggressiveness in diagnosis and the skill of the pathologist. Because MALT is a known consequence of *H. pylori* infection (30% of infected persons have MALT; Marshall and Windsor, 2005), interest in infection as a cause of MALT lymphoma naturally followed its discovery. The first case-series revealed well over 92% prevalence of *H. pylori* in 110 patients with gastric lymphoma (Wotherspoon et al., 1991), while one early study reported the regression of lymphoma with *H. pylori* eradication (Wotherspoon et al., 1993). In an epidemiological survey of 33 American and Swedish gastric lymphoma cases and matched controls, antecedent *H. pylori* was associated with a 6.3-fold increased risk of gastric lymphoma; the great majority of these lymphomas were diffuse large-cell lymphomas (Parsonnet et al., 1994). The most compelling data linking *H. pylori* and cancer, however, are derived from treatment trials. In these studies, early-stage gastric MALT lymphomas show remarkable response to *H. pylori* eradication, with 80% of patients going into complete remission (Stolte et al., 2002). Unfortunately, approximately 5% will relapse annually after achieving remission. Those who do relapse or have recurring *H. pylori* infection are more likely to have t(11 : 18) rearrangements of the immunoglobulin heavy chain than those not relapsing (Liu et al., 2002; Wundisch et al., 2005). Moreover, among those patients who remain in remission for an extended period, approximately 30% will have evidence of gastric lymphocyte monoclonality on molecular analysis. Thus, although there is clinical evidence that *H. pylori* provides a proliferative drive to lymphocytes, longer-term follow-up is necessary to assess the true "cure".

Why gastric lymphoma occurs in a vanishingly small proportion of *H. pylori*-infected hosts remains a mystery. As with gastric cancer, host polymorphisms in genes related to inflammation – specifically IL-1 L receptor antagonist and glutathione S-transferase genes –may play a role in outcome (Rollinson et al., 2003). Unlike gastric cancer, however, microbial polymorphisms have only been weakly linked to lymphomagenesis (Witherell et al., 1997; Delchier et al ., 2001).

"*H. heilmannii*" has also been associated with primary gastric low-grade lymphoma in humans (Regimbeau et al., 1998; Morgner et al., 2000). Similar to *H. pylori*-associated lymphoma, clinical remission of the lymphoma was noted in five patients after antibiotic eradication of the gastric *Helicobacter* (Roggero et al., 1995; Hussell et al., 1996; Morgner et al., 2000).

10.2.2 Bacterial Factors Responsible for Cell Specificity and Virulence

Helicobacter pylori is highly adapted to survive in the highly acidic environment of the stomach. In addition, the organism has specific tropism for gastric epithelial cells. Thus, in addition to colonizing the stomach, the organism can be found in areas of gastric metaplasia that occur in the duodenum, in Barrett's esophagus (Borhan-Manesh and Farnum, 1993), and even in the jejunum (within Meckel's diverticulum) and in isolated gastric metaplasia of the rectum, though in the latter two cases this appears to be a rare event (Hunt, 1996; Hill and Rode, 1998). Within the environment of the stomach, the organism is actively motile and free-living, existing just beneath the mucus layer of the stomach overlaying the gastric epithelial cells. Motility is clearly important for colonization of the stomach; this ability to colonize the gastric milieu is achieved through the organism's spiral shape and the unipolar flagella which allow movement through and below the mucus layer of the stomach. A recent elegant study performed in gerbils, indicates that *H. pylori* utilizes the gastric mucus pH gradient for precise spatial orientation in the mucus layer of the stomach (Schreiber et al., 2004).

For the most part, *H. pylori* are not invasive organisms, and the vast majority of the bacteria exist in an nonadherent, extracellular, mucous environment, which may account for many of the difficulties in immune clearance by the host, or antibiotic eradication. A small number of organisms have been shown to adhere to gastric epithelial cells (perhaps ~10%), and rare organisms may be found intracellularly, suggesting actual invasion (Wilkinson et al., 1998; Semino-Mora et al., 2003; Oh et al., 2005). Adherence of *H. pylori* to the gastric epithelium is a complicated process which most likely involves a number of surface receptors including Lewis B-binding adhesin, BabA (Ilver et al. , 1998) (which binds to surface mucus cell receptor Lewis B histoblood group antigen, and Lewis X, a glycosphingolipid which recognizes and binds to Sab A (sialic acid binding adhesin) of *H. pylori* (Mahdavi et al., 2002). The distribution mirrors the expression of trefoil factor 1, which also serves as binding factor for *H. pylori*, as does Muc5 AC (Linden et al., 2002; Clyne et al., 2004). In addition, *H. pylori* has been found to adhere preferentially, within the gastric units, to the surface mucous cells of the gastric pits, and generally not to adhere to the mucous neck, parietal or chief cells (Falk et al., 1993). However, the organism is able to invade deeply within the gastric glands, particularly within the non-acid-secreting mucosa of the gastric antrum (Thomsen et al., 1990).

The bacterial factors which allow for persistence of *H. pylori* within the gastric lumen are still being investigated, but several critical factors have been determined. Some of this information derives from animal models and the creation of *H. pylori*

isogenic mutants. The most critical factor which allows *H. pylori* survival is the abundant production of urease, enabling hydrolysis of urea to ammonia and carbon dioxide. *H. pylori* synthesizes extremely high quantities of urease, which in total contribute to over 5% of the bacterial protein (Mobley, 1997). The generation of urease, among other effects, assists in neutralizing gastric acidity and allows the organism to withstand the low pH of the stomach. *H. pylori* is an acid-tolerant neutrophile (Rektorschek et al., 1998), and isogenic mutants of *H. pylori* which are deficient in urease are unable to colonize the gastric mucosa of the gnotobiotic piglet (Eaton et al., 1991). Motility by *H. pylori* is also critical to the organism's survival, and isogenic mutants of *H. pylori* lacking flagella have also been unable to colonize the gnotobiotic piglet (Eaton et al., 1992, 1996). *H. pylori* also produces a number of enzymes (e.g., phopholipase A2) which are involved in the breakdown of the surfactant layer overlying the gastric epithelium. Isogenic mutants of the gene *pldA*, which encodes for an outer member phospholipase, are also unable to colonize mouse models of *Helicobacter* infection.

The two genetic loci linked with virulence that have received the greatest amount of study are the *cag* locus and the *vacA* gene which encode for the vacuolating cytotoxin. The vacuolating cytotoxin first described by Leunk et al. (1988) induces a vacuolating effect in several cell lines, including gastric cell lines. The vacuolating toxin of *H. pylori* has been identified and characterized as a secreted protein; isogenic mutants lacking the toxin do not induce a vacuolating effect on cell lines (Harris et al., 1996; Smoot et al., 1996).

It has been established that there is considerable genetic diversity in *Vac* alleles, the most extensively studied having been the s1/m1 *vacA* allele. This typically encodes vacA protein which has a high level of vacuolating cytotoxin activity *in vitro* (Atherton et al., 1995). Other forms of vacA are associated with either a lower cytotoxin activity or no activity at all (Atherton, 2002). In one study, Tox^- *H. pylori* strains did not induce gastric cell damage *in vivo*, whereas Tox^+ *H. pylori* strains caused gastric damage in a mouse model (Marchetti et al., 1995). Oral dosing of vacA also caused damage to gastroduodenal epithelia, including superficial erosions (Telford et al., 1994; Ghiara et al., 1995). Results in gerbils and gnotobiotic piglets dosed experimentally with wild-type toxigenic strains of *H. pylori* or their null mutants, showed no differences between gastric lesions produced by both strains, however (Eaton et al., 1997; Wirth et al., 1998). Nevertheless, it is important to remember that although ca. 50% of *H. pylori* strains are Tox^-, they are still capable of inducing gastritis in humans (Cover, 1996; Gebert et al., 2003).

Recent data with direct relevance to the role of vacA in pathogenesis and the persistence of *H. pylori* infection indicate that VacA efficiently blocks the proliferation of T cells by inducing a G_1/S cell cycle arrest (Gebert et al., 2003). These authors concluded that VacA interfered with the T-cell receptor/IL-2 signaling pathway at the level of the Ca^{2+}-calmodulin-dependent phosphatase, calcineurin. Nuclear translocation of the nuclear factor of activated T cells (NFAT), a transcription factor which acts as a global regulator of immune response genes, was abrogated. This manifested in down-regulation of IL-2 transcription. The immunosuppressive drug FK506 partially mimicked vacA activities; thus, they likely shared a mechanism

which could account for a local host immune suppression. These data could in part explain the often life-long chronicity of *H. pylori* infections (Gebert et al ., 2003). It is interesting to note that *C. jejuni*, as well as several enterohepatic helicobacters, produces a CDT which also inhibits cell cycle progression in epithelial cells, including T cells (Whitehouse et al. , 1998; Chien et al., 2000; Young et al., 2000 b).

The cytotoxin-associated (*cagA*) gene was initially considered a prerequisite for vacuolating toxin activity due to the high degree of correlation between the presence of cagA and the ability of *H. pylori* to express the toxin (Tummuru et al., 1993). However, subsequent data showed that isogenic mutants of cagA still were still able to produce the toxin (Tummuru et al., 1994; Crabtree et al., 1995; Xiang et al., 1995).

The *cagA* is now known to be part of the cag locus, considered an important virulence determinant. This 40-kb DNA fragment is considered to be a PAI, and consists of a cluster of genes (more than 25). This PAI is more commonly found in *H. pylori* strains isolated from peptic ulcer and gastric cancer patients (Censini et al., 1996).

A functional *cag* PAI is required for the internalization of CagA within gastric epithelial cells. Once internalized, CagA becomes phosphorylated by members of the Src family of kinases, implicated in other malignancies. The phosphorylated CagA then activates SHP2 (a eucaryotic phosphatase), as well as ERK (a member of the MAPK family). This process results in morphological derangement of epithelial cells (Higashi et al., 2002 b; Tsutsumi et al., 2003). More recently, investigators have studied the possible biological activities of CagA proteins in relation to tyrosine phosphorylation motifs (TPMs). Interestingly, the number of TPMs in *H. pylori* strains in patients from Western countries varies, as does the number of motifs resulting in variability in SHP2 binding affinity. This contrasts with the TPMs of *H. pylori* strains isolated from patients living in eastern Asian countries, wherein the TPMs match the consensus SHP2 binding site, resulting in increased SHP2 binding and cell derangements similar to that noted when cells are stimulated by growth factors (Higashi et al., 2002 a). These differences in TPMs between Western and Eastern strains may in part explain the marked differences in gastric cancer noted in these different geographic regions.

It is proposed that the cag PAI is involved in the delivery of effector molecules into host cells (Odenbreit et al., 2000; Stein et al., 2000). Other cag PAI-related proteins are also required for the activation in gastric epithelial cells of NF-κB, which plays a key role in host immune responses and inflammation (Keates et al., 1997; Sharma et al., 1998).

Strains of *H. pylori* with the cag PAI (Covacci et al., 1993), the so-called *H. pylori* type I, versus *H. pylori* type II strains which lack the cag island (Xiang et al., 1995), are capable of inducing IL-8 expression and tyrosine phosphorylation of a 145-kDa protein from gastric epithelial cells (Crabtree et al., 1994; Segal et al., 1997). Furthermore, isogenic mutants lacking numerous genes of the *cag* PAI abolish these *in-vitro* effects (Tummuru et al., 1995; Censini et al., 1996; Segal et al., 1997). Importantly however, *H. pylori* isogenic mutants lacking either cagA, cagF or cagN failed to abolish IL-8 production.

Recently, investigators have shown that *H. pylori* was recognized by epithelial cells via Nod1, an intracellular pathogen-recognition molecule with specificity for peptidoglycan of Gram-negative bacteria (Viala et al., 2004). These authors demonstrated that Nod1 detection of *H. pylori* depended on the delivery of peptidoglycan to host cells by the *H. pylori* type IV secretion system, encoded by *cag* PAI. Consistent with the involvement of Nod1 in host defense, Nod1-deficient mice were more susceptible to infection by *cag* PAI-positive *H. pylori* than were wild-type mice (Viala et al., 2004). Thus, it appears that Nod1 may represent a key molecule in the innate immune sensing of *cag* PAI-positive *H. pylori* by epithelial cells. Nevertheless, it remains unclear how such cells might sense and respond to *H. pylori* that do not possess a *cag* PAI, or in which the *cag* PAI is non-functional (Philpott et al., 2002).

The recent sequencing of the entire genome of two strains of *H. pylori* will allow investigators to explore more fully the virulence factors already described, as well as to determine the presence of others (Tomb et al., 1997; Alm et al., 1999).

10.2.3 Host Factors Playing a Role in Gastric Diseases

Although worldwide, half of the population is infected with *H. pylori*, only a small percentage of individuals (e.g., 1–3%) progress to gastric cancer. There has been extensive debate as to whether bacterial or host factors can account for the diverse outcomes (asymptomatic, duodenal ulcer or gastric cancer) of *H. pylori* infection. While bacterial factors likely play an important role in disease pathogenesis, a true "carcinogenic strain" has not been identified, and the bulk of evidence suggests that host factors are paramount in determining progression to gastric cancer. As detailed below, murine models of *Helicobacter* infection indicate that certain inbred murine strains (e.g., C57 BL/6) are highly susceptible to cancer progression, while other inbred strains (e.g., BALB/c) are very resistant to gastric adenocarcinoma and its precursor lesions. In addition, *Cag*-negative strains such as *H. felis* appear to be as carcinogenic (if not more so) in mice as *Cag*-positive strains. In every model system studied, the progression to gastric cancer correlates strongly with the intensity and type of immune response, which is strongly governed by host genetic factors.

During the past few decades, a number of studies have indicated a strong role for genetic factors in gastric cancer. Gastric cancer risk is increased up to threefold in individuals with a first-degree relative with gastric cancer, and 10% of cases appear to exhibit familial clustering. While *H. pylori* infection also runs in families, family history remains a risk factor even after control for *H. pylori* infection. Nevertheless, only a small part of the familial clustering of gastric cancer could be attributable to known family cancer syndromes.

The search for other genetic factors began with the observation that relatives of patients with gastric cancer had a higher prevalence of atrophy and hypochlorhydria (see Table 10.3). This increased prevalence of atrophy was limited only to those patients who were also infected with *H. pylori*, suggesting (hypothetically) that genetic factors led to more intense immunity to *H. pylori*. In addition, the search for genetic factors also built upon the observation that patients who progressed to atro-

phy and cancer appeared to secrete lower levels of acid compared to patients with duodenal ulcer disease. Thus, the initial genetic study of families with an increased incidence of precancerous changes focused on IL-1β, a well-known proinflammatory cytokine that is also a powerful inhibitor of acid secretion. The IL-1β gene cluster includes both IL-1β and IL-1RN, its naturally occurring receptor antagonist. The study conducted by El-Omar et al. of a Caucasian population from Poland and Scotland showed that individuals with the IL-1β-31*C or -511*T and IL-1RN*2/*2 genotypes had a two- to threefold increased risk of developing atrophy and gastric cancer in the setting of *H. pylori* infection (El-Omar et al., 2000). This was quickly confirmed by Portuguese and Japanese groups, who also showed a strong association between IL-1 gene cluster (IL-1β and IL-1RN) proinflammatory polymorphisms and an increased risk of gastric cancer (Machado et al., 2001; Furuta et al., 2004). The basic finding that an increased risk for gastric cancer is associated with proinflammatory IL-1β polymorphisms has been confirmed in many populations, including those in China, Taiwan, Mexico, Korea, Germany, and Brazil, as recently reviewed (Furuta et al., 2004; Palli et al., 2005; Perez-Perez et al., 2005), although a smaller number of studies have not found any such association. Later studies by El-Omar combined genetic polymorphisms in TNF-α and IL-10, resulting in a high-risk genotype with a 27-fold or greater risk of gastric cancer (El-Omar et al., 2003). A study by Machado and colleagues also noted the importance of the TNF-α-308 polymorphism, and found that a combination of IL-1 and TNF-α gene polymorphisms markedly increased the risk for atrophy and gastric cancer (Machado et al., 2001). More recent studies have identified the -251 T allele in the IL-8 promoter as being significantly associated with an increased risk of gastric cancer (Lee et al., 2005; Taguchi et al., 2005). These observations provide strong evidence for host genetics in determining the progression to gastric cancer, and have strengthened the connection the inflammatory response and gastric carcinogenesis.

10.2.4 Environmental Factors

While almost all patients infected with *H. pylori* develop some degree of chronic active gastritis, most will remain free of symptoms or adverse consequences to their health and well-being. Prior to the recent focus on host genetic polymorphisms, a number of studies investigated the role of diet and other environmental cofactors in contributing to the development of gastric cancer. Migration studies in particular point to the importance of the local environment in dictating gastric cancer risk, and while much of this environmental risk was likely due to *H. pylori* infection, the infection rates alone do not account for the vast geographical differences in gastric cancer rates in Japan compared to Africa and other countries. Thus, a recent assessment regarding the role of diet, parasite co-infection and bacterial overgrowth will be discussed in the following sections.

10.2.4.1 **Diet**

Several decades ago, there was a tremendous interest in the relationship between dietary composition and the overall risk of gastric cancer. Epidemiological studies had pointed to a possible risk associated with diets high in salt and nitrates, and low in fresh fruits and vegetables. The greatest attention was given to the effect of high nitrate intake, under the notion that nitrates can be reduced to nitrite and then react with other nitrogenated substances to produce *N*-nitroso compounds which are known carcinogens. However, large prospective cohort studies failed to confirm an increased risk (van Loon et al., 1998). A high salt intake has been even more strongly linked epidemiologically to gastric cancer risk, and has been shown in some animal studies to induce more severe gastric lesions. However, long-term murine studies have not demonstrated any acceleration of gastric cancer in *Helicobacter*-infected mice (Rogers et al., 2005). The protective effect of a high consumption of fresh fruits and vegetables led to a number of chemoprevention studies involving treatment with antioxidants such as selenium, beta-carotene, alpha-tocopherol, and ascorbic acid. However, these studies have also been largely inconclusive. A recent meta-analysis confirmed this general lack of efficacy, with the possible exception of selenium (Bjelakovic et al., 2004). In long-term interventional studies, such as that conducted by Correa and colleagues, the beneficial effects of antioxidants, while present at six years of follow-up, were not evident at 12 years of follow up, in contrast to the effects of antibiotic eradication which were long-lasting (Mera et al., 2005).

10.2.4.2 **Co-Infection**

While most of the focus has been on diet as a cofactor for gastric cancer, a number of recent studies have pointed to parasitic co-infection as possibly important in modulating cancer risk. Gastric atrophy and cancer have been clearly linked to a vigorous Th1 immune response to *H. pylori*. In contrast, many parasitic infections, particularly infections with intestinal helminths, lead to a strong Th2 immune response. The "hygiene hypothesis" has suggested that improvements in sanitation leading to decreased parasitic infections has led to an increase in Th1 immune-mediated diseases. In addition, it was postulated by Fox et al. that the African enigma could be due in part to a higher level of parasitic infections in some areas of the world (Fox et al ., 2000 a). Childhood infection with Th2-polarizing helminthic infection, in areas of the world where climate and poor sanitation favor the life cycle and transmission of parasites, could in theory promote a Th2 response to *H. pylori* . In order to test this as a possible mechanism, Fox's group carried out a co-infection study in mice, which demonstrated amelioration of gastric atrophy co-infected with *H. felis* and *Heligmosomoides polygyrus*, a murine intestinal nematode (Fox et al., 2000 a). The decrease in risk for gastric atrophy in mice dually infected with *H. felis* and the nematode was supported by a shift in the Th1-biased response to *H. felis* toward a Th2-like phenotype of gastritis. Co-infection with *H. polygyrus* also led to a marked increase in anti-inflammatory cytokines IL-4, IL-10 and TGF-β, and lower levels of proinflammatory

interferon (IFN)-γ, TNF-α and IL-1β. More direct experimental evidence that T-cell immune responses lead directly to preneoplastic changes in the gastric mucosa have come from the *H. felis* model of adoptive transfer gastritis, whereby adoptive transfer of C57 BL/6 splenocytes or $CD4^+$ lymphocytes or Th1 cells to immunodeficient recipients results in enhanced gastritis and decreased bacterial colonization after *H. felis* infection (Mohammadi et al., 1997; McCracken et al., 2005). In addition, studies by Yamori et al. have employed a noninfectious gastritis model employing mice transgenic for T-cell receptors specific to ovalbumin, and using the adoptive transfer of T cells, with or without deficiency of IL-4, IFN-γ or IL-12, and injections of ovalbumin into the gastric mucosa (Yamori et al., 2004). These studies demonstrate clearly that antigenic activation of Th1-type T cells infiltrating into the gastric mucosa enhance dysregulated apoptosis and epithelial cell turnover.

Support for this Th1/Th2 paradigm as the best explanation for geographic variations in gastric cancer rates has come from measurement of IgG subclass responses as a surrogate marker for polarization of T-helper cell function (Mitchell et al., 2002). These studies suggested that adults in undeveloped areas of the world tend to show predominantly Th2-associated IgG2 response to *H. pylori*, rather than the Th1-associated IgG2 responses observed in developed countries. Thus, the host immune response to *H. pylori* infection in an African population differs from that observed in subjects from developed countries (such as Germany) (Mitchell et al., 2002). More recently, the Th2 responses to *H. pylori* have been linked more directly to intestinal helminth infections. In a study of children living in two different regions of Columbia, Whary et al. showed that there was a higher rate of intestinal helminthiasis and higher Th2-associated IgG1 responses to *H. pylori* in children from the region (Tumaco) showing lower gastric cancer rates (Whary et al., 2005). Overall, parasitic co-infection is likely to be strongly protective against gastric cancer development, recognizing that other factors are also important.

10.2.4.3 **Bacterial Overgrowth**

While *H. pylori* is clearly the major trigger leading to chronic gastritis and the development of gastric atrophy, *H. pylori* colonization actually declines in patients with severe hypochlorhydria. At the time of diagnosis of gastric cancer, *H. pylori* infection is often difficult to appreciate or detect, even with multiple methodologies. Thus, questions have been raised as to whether *H. pylori* is important in the advanced stages of preneoplasia, or simply a trigger for the development of gastric atrophy. Indeed, reports from several groups have shown that in mice, bacteria other than *H. pylori*, such as *Acinetobacter iwoffii*, can induce atrophic gastritis (Rathinavelu et al ., 2003). Furthermore, there is evidence from a number of model systems that, in general, gastric cancer is preceded by a long period of achlorhydria, and reduced gastric acid consistently predisposes the stomach to colonization by bacteria and inflammation. The number and diversity of bacterial organisms in the stomach rises as the intragastric pH rises, and bacterial overgrowth in theory leads to increased formation of *N*-nitroso compounds. Thus, gastrin-deficient mice

housed in a conventional (non-SPF) facility develop bacterial overgrowth, chronic gastritis, and progress to gastric cancer (Zavros et al., 2002, 2005). Similar antral tumors develop in *H. felis*-infected wild-type mice, again after longstanding achlorhydria and after *Helicobacter* organisms are nearly undetectable (Cai et al., 2005). Consequently, one can hypothesize that the primary role of *H. pylori* in gastric cancer is in the induction of atrophic gastritis and hypochlorhydria, leading to bacterial overgrowth. While acid-suppressing drugs can result in bacterial overgrowth and possibly increased formation of *N*-nitroso compounds, there is currently no supportive evidence for an increased risk of gastric cancer in patients receiving these medications, particularly in the absence of *H. pylori* infection. Recent studies have shown beneficial effects of antibiotic eradication regimens on gastric cancer progression in *H. pylori*-infected patients (Wong et al. , 2004); however, the possibility must be considered that the beneficial effect of antibiotics might be as much from the eradication of non-*H. pylori* organisms as it is from *H. pylori* eradication.

10.2.5 Natural History and Stages of Infection

Helicobacter pylori, as the second most common chronic bacterial infection in humans, infects almost half of the world's population. However, the natural history of this infection is extremely variable and the vast majority of infected individuals – perhaps 75% or more – will remain asymptomatic and experience no adverse consequences from the infection. Thus, with respect to gastric cancer, it is clear that *H. pylori* infection on its own is not sufficient, and that other factors – bacterial, host or unknown cofactors – are required. Infection is typically acquired early in life, either through a fecal-oral or oral-oral route of transmission, followed by a long quiescent phase where there is a chronic gastritis of variable intensity and minimal symptoms. Peptic ulcer disease tends to develop when patients are in their twenties and thirties, whereas gastric cancer arises several decades or more later. Perhaps only 10–15% of infected patients will develop peptic ulcer disease, while the risk of gastric cancer is estimated at approximately 1% (though it may be 3% or more in Japan and other high-risk countries). Interestingly, patients who develop duodenal ulcer (DU) disease appear to be somewhat protected from gastric cancer, with a lower overall risk of developing malignancy of the stomach (Hansson et al., 1996). This is thought to be due to a higher basal level of acid secretion, which in some instances could be related to a low-expressing IL-1β genotype. The notion that patients with DU are protected has been supported by endoscopic studies, where such patients had a low risk of cancer progression, while those with non-ulcer dyspepsia had a relatively high risk (e.g., 5%) of progression (Uemura et al., 2001). Based on the results of this and other studies, it has been suggested that the location and severity of gastritis could be predictive of the risk of progression to gastric cancer, with antral gastritis alone representing a lower risk while "corpus gastritis" (gastritis involving the body and proximal portions of the stomach) represented a greater risk. In any case, it appears that progression to atrophy/cancer, duodenal ulcer, or asymptomatic status represent relatively distinct pathways for long-term infected patients.

H. pylori has been associated with an increased risk of both "intestinal-type" and "diffuse-type" gastric cancer – two histological types of gastric cancer according to the Lauren classification. While little is known regarding the histological progression leading to diffuse gastric cancer, that leading to intestinal-type gastric cancer has been studied for several decades and has been organized into a series of discrete steps known as the Correa pathway (Correa, 1995) (see Fig. 10.1). According to this model, intestinal-type gastric cancer arises from chronic *Helicobacter* infection, through a multistep pathway involving stages of superficial gastritis, atrophic gastritis, metaplasia, dysplasia and finally carcinoma (Fox and Wang, 2001). While the terms "atrophy" and "intestinal metaplasia" have been confusing and at times used synonymously, accumulating evidence suggests that intestinal metaplasia (IM) is less consistently associated with gastric cancer than gastric atrophy. Gastric atrophy – defined in the corpus as loss of specialized cell types such as parietal and chief cells – has also been recognized as being associated with replacement of the stomach by pseudopyloric metaplasia (El-Zimaity et al., 2002), also known as mucous metaplasia or spasmolytic polypeptide-expressing metaplasia or SPEM (Schmidt et al., 1999). Accumulating data suggest that SPEM is more strongly associated than IM with gastric cancer, and is more likely to be the precursor lesion (Halldorsdottir et al., 2003). At the present time, it is difficult to determine precisely what histological stage constitutes the "point of no return" for gastric cancer progression. While human studies suggest that, at the stage of atrophy/metaplasia, the lesion is only partially reversible and cancer not preventable with antibiotic eradication (Wong et al., 2004), studies in mice (see below) have suggested that atrophy/SPEM can regress significantly with antibiotic eradication (Cai et al., 2005).

10.2.6
Chronic Inflammation and Cancer

While a number of factors are likely involved in the predisposition and progression to gastric cancer, it is clear that chronic inflammation is a paramount feature that links gastric cancer to many other types of cancer. The link between inflammation and cancer dates back several millennia, but is commonly attributed to Virchow who in 1863 first demonstrated the abundant presence of leukocytes in neoplastic tissues. Epidemiological studies show a clear association between chronic inflammatory conditions and subsequent malignant transformation of the tissues, and the gastrointestinal tract is commonly affected with inflammation associated neoplasia (Philip et al., 2004). *Helicobacter pylori*-associated gastritis is perhaps the best example of a more widespread association, but one that has generated remarkable new insights into the mechanisms of carcinogenesis. Chronic inflammation is typically derived from acute inflammation, but in fact is qualitatively different from the more acute process. Indeed, several studies have suggested that while chronic inflammation predisposes to cancer, acute inflammation may actually protect the host from cancer (Philip et al., 2004). Some of the factors that distinguish chronic inflammation, particularly in the setting of *Helicobacter*-associated gastritis, are highlighted in the following sections.

10.2.6.1 Reactive Oxygen/Nitrogen Species

One of the hallmarks of chronic inflammation, such as *H. pylori*-associated gastritis, is an increase in oxidative stress induced by reactive oxygen species (ROS) such as superoxide anion O_2^-, hydroxyl radical OH•, and H_2O_2, are generated in cells as physiological byproducts of electron transfer reactions and arachidonic acid metabolism (Hocker et al., 1998). The inflamed stomach is also characterized by increased production of nitric oxide (NO) through up-regulation of the inducible nitric oxide synthase (iNOS); NO subsequently reacts with superoxide to form a variety of nitrosated species that can exert oncogenic effects. Indeed, it has been recognized that *H. pylori* arginase, encoded by rocF, competitively inhibits NO production in macrophages by consuming l-arginine (Viala et al., 2004). In the case of gastritis, the majority of ROS and NOS is likely generated by infiltrating inflammatory cells such as neutrophils and macrophages, but ROS and NOS can also be produced by epithelial cells and *H. pylori* itself. For example, spermine oxidase is induced in gastric epithelia by *H. pylori* which, via generation of H_2O_2 by oxidation of polyamines, can cause apoptosis and DNA damage (Xu et al., 2004).

While ROS and NOS can be elevated in acute pathological states, greater damage is associated with long-term increases in ROS and NOS associated with chronic inflammation. While the host does have a complex antioxidant system (catalase, superoxide dismutase, glutathione peroxidase, and thioredoxin) with which to battle the oxidative stress, over time this protective system can be overcome or depleted, leading to disorders such as peptic ulcer disease and gastric cancer.

Although oxidative stress can have cytotoxic effects, and function as a trigger for programmed cell death (apoptosis), it can also modulate the expression of a variety of genes involved in immune and inflammatory responses (such as NF-κB), as well as the expression of growth factor-regulated genes (Hocker et al., 1998). Oxidative stress can result in up-regulation of a variety of cytokines, adhesion molecules, cyclooxygenase (COX)-2 and angiogenic factors (such as VEGF).While there are likely many downstream targets, the most important target of ROS and NOS is DNA (Toyokuni et al., 1995). ROS and nitrosating species such as NO can induce several kinds of DNA damage, including strand breakage, base modification and DNA–protein crosslinking; however, the end result is the likelihood of mutation of DNA. In the classical model linking inflammation and cancer, the combination of increased cell damage and turnover, increased cell proliferation, and increased mutagenesis are sufficient to lead to initiate and promote cancer formation (Parsonnet, 1993). Indeed, many chemoprevention trials employing antioxidants have attempted to lever this model to inhibit or prevent gastric cancer development by reducing ROS and NOS. While antioxidant supplementation has had mixed results, studies in mouse models involving overexpression of thioredoxin-1 (TRX-1), as a redox-active protein involved in scavenging ROS, has been successful. Transgenic mice overexpressing TRX-1 showed decreased oxidative stress and decreased atrophic gastritis compared to wild-type mice, thereby validating to a large extent the importance of ROS in gastric preneoplasia (Kawasaki et al., 2005). Similarly, $iNOs^{-/-}$ knockout mice on a C57 BL background infected with *H. felis* have a predominant Th2 immune response with only a modest inflammatory response (Ihrig et al., 2005).

10.2.6.2 Epithelial Cell Proliferation and Apoptosis

While chronic inflammation clearly has many procarcinogenic effects, a critical factor in the cascade leading to gastric cancer is likely the induction of cellular apoptosis in the gastric mucosa. An altered rate of apoptosis and proliferation has for many years been considered an important biomarker for increased risk of neoplasia. The observation that *H. pylori* infection is associated with increased apoptosis was first reported in human patients by Moss et al. (1996), and this has since been confirmed by many groups. Increased apoptosis secondary to gastric *Helicobacter* infection was shown for the first time by Wang et al. in the *H. felis*-infected C57BL/6 mouse (Wang et al., 1998). These findings were confirmed by others in both the mouse model and in a Mongolian gerbil model (Peek et al., 2000). Importantly, in the *Helicobacter* mouse model, mucosal apoptosis occurs over a narrow time window and is associated closely with a marked decline in parietal and chief cells in the fundic mucosa. In addition, this apoptotic phase is followed by rebound hyperproliferation and the appearance of metaplasia.

While *H. pylori* may induce apoptosis in part through direct interactions with epithelial cells, the organism is largely confined to the gastric pits, while the apoptotic zone clearly extends deeper into the neck region and beyond the gastric glands. In addition, a number of studies have pointed to the effects of inflammatory cytokines such as TNF-α, IFN-γ, and IL-1β that can all have proapoptotic effects. However, studies by Houghton et al. have pointed to the Fas/FasL system as being primarily responsible for the induction of gastric epithelial apoptosis. *H. pylori* infection is associated with increased expression of Fas receptors – due in part to upregulation by IL-1β and TNF-α – and an influx of T lymphocytes that express FasL (Houghton et al., 2000a). *Helicobacter* infection of Fas-deficient mice did not result in any significant increases in apoptosis, proliferation or atrophy, underscoring again the link between the induction of apoptosis and the proliferative response (Houghton et al., 2000b).

Mucosal apoptosis may be very important in extending or amplifying the inflammatory response, and likely plays a role in the recruitment of cells from the circulation. In addition, many – if not most – carcinogens appear to induce apoptosis early on in the tissues most susceptible to the development of cancer. In the case of *H. felis*-infected C57 BL/6 mice, this field of increased apoptosis extends through the isthmus/progenitor cell zone of the fundic mucosa (Wang et al., 1998). The implications of apoptosis in the stem cell zone of the stomach, which would lead to stem cell deficiency or failure, have not previously been considered.

10.2.6.3 Role of Specific Cytokines

Another important feature of chronic inflammation – which may distinguish it from acute inflammation – is the sustained elevation of specific proinflammatory cytokines. In patients with chronic *H. pylori* infection, the increase in local levels of chemokines and cytokines may be quite marked, and may on their own result in

specific effects as well as contributing to the recruitment and activation of immune cells. Much remains to be discovered regarding the pathways leading to initial immune recognition and antigen presentation by *H. pylori*, but it seems clear that the initial interaction involves the innate immune system. Macrophages and monocytes respond to *H. pylori* and other gastric helicobacters through the Toll-like receptor-2 (TLR2) (Mandell et al., 2004). A role for other Toll-like receptors, such as TLR4, 5, and 9, has been suggested but remain less well established. Signaling through TLR2 leads to NF-κB activation and release of early proinflammatory cytokines, such as IL-1β, followed later by activation of an adaptive immune response, which in the case of *H. pylori* is typically of a Th1-polarized cytokine pattern. The importance of a Th1 immune response in the induction of gastric cancer is discussed above. In any case, it is clear that the development of gastric atrophy, metaplasia, dysplasia and cancer is largely the result of the immune response to gastric *Helicobacter* infection, rather than representing direct effects of the bacteria. Immunodeficient mice, including both RAG-deficient mice and T-cell-deficient mice, are protected from *Helicobacter*-dependent atrophy and dysplasia, despite high levels of colonization (Roth et al., 1999; Houghton et al., 2002). Mice that are susceptible to gastric cancer, such as C57 BL/6 mice, have very strong Th1 immune responses, characterized by high levels of cytokines such as IFN-γ, TNF-α, IL-1β and IL-6 (Fox et al., 2000 a). This has led to the hypothesis that much of the preneoplastic epithelial changes that arise in the gastric mucosa are due to increases in these proinflammatory cytokines. This hypothesis has been supported by the findings of several recent studies which have involved the infusion of proinflammatory cytokines. First, Cui et al. presented a study in which they examined the effect of short-term infusions of cytokines on the gastric mucosa of two strains of mice (C57 BL/6 and BALB/c). The cytokines – which were given using Alzet osmotic pumps at equimolar doses of 5 mg kg^{-1} per day (IL-1β), 10 mg kg^{-1} per day (TNF-α), and 20 mg kg^{-1} per day (IFN-γ) for two weeks along with bovine serum albumin (BSA) controls – reproduced many of the epithelial changes observed with chronic *Helicobacter* infection. The infusion of cytokines, including IL-1β, resulted in a reproduction of the atrophy model and in measurable increases in apoptosis as measured by caspase-3 staining, decreased parietal cell number and induction of mucous metaplasia (Cui et al., 2003). Interestingly, the infusion of cytokines had no significant effect in BALB/c mice, which were previously shown to be resistant to *H. felis*-dependent gastric atrophy (Wang et al., 1998). In more recent studies, Kang et al. have also reported that infusion into mice of IFN-γ recapitulated the mucous metaplasia observed in *H. felis*-infected mice (Kang et al., 2005). More recent preliminary studies suggest that transgenic overexpression of IL-1β can induce gastric atrophy and neoplasia in the absence of *Helicobacter* infection (Tu et al., 2005). Taken together, these results strongly support a model in which progression to gastric cancer is primarily a cytokine-driven and immune-mediated disease.

Recent studies by Mueller et al. using laser capture microdissection and transcriptome analysis of chief, parietal, and mucus epithelial cells in *H. pylori*-infected mice indicated that the mucous cell of the infected stomach orchestrated a complex interaction (Mueller et al., 2004, 2005 a). Interestingly, neither chief nor parietal cell

genes were deregulated at either two time points of the experiment – that is, at 2 and 28 wks post *H. pylori* infection. The mucous cell plays a role in sampling and sensing the gastric environment as well as initiation of host defense responses. Cytokines, chemokines and genes involved in antigen processing were up-regulated in the mucous cell in *H. pylori* -infected mice (Mueller et al., 2005 a). In the mucus-producing cell, the authors found strong evidence for a proinflammatory response: the cytokines IL-1β, TNF-α, and IFN-γ or their downstream target genes, as well as the two chemokines, granulocyte macrophage colony-stimulating factor and RANTES, were up-regulated (Mueller et al., 2005 a). The mucous cell transcriptome was also highly enriched for cytoskeletal and cell-junctional proteins – factors that probably play an important role in maintaining cell polarity and forming a tight barrier that protects against the host gastric contents. Most genes in the tumor suppressor category were repressed, compared with uninfected mice, suggesting that tumor suppression was dampened. These data support the proposal that, at least in the timeframe of the experiment, *H. pylori* affects growth control and apoptosis as early as the first week of infection (Mueller et al., 2004, 2005 a).

10.2.6.4 **Link to T Cells and Macrophages**

The link between gastric cancer and specific types of cytokines again points to the importance of specific immune cell subsets in the pathogenesis and progression of cancer. In the case of gastric cancer, as well as many other types of solid cancer, the key culprits appear to be macrophages and T cells. The data regarding T cells has been discussed above, and relates to the critical role played by IFN-γ-producing $CD4^+$ T cells in *Helicobacter*-dependent atrophic gastritis. The predominant immune cell present in *H. pylori*-gastritis is the Th1-polarized $CD4^+$ T cell, and in murine models, transfer of this cell enhances gastritis while deletion of IFN-γ inhibits progression to preneoplasia (Mohammadi et al., 1997; Itoh et al., 1999; Sawai et al., 1999). $CD4^+$ T cells have also been shown to play a key role in immune enhancement of skin carcinogenesis (Daniel et al., 2003). However, while these studies demonstrate that $CD4^+$ T lymphocytes are essential, they do not provide that they are the only cell type involved in the initiation and progression of gastric cancer. Indeed, growing evidence supports a key role for other immune cell populations, particularly macrophages.

As suggested above, macrophages are the main source of growth factors and cytokines as the site of inflammation, and macrophages have a central role in not only the initiation of inflammation but also in the resolution of inflammation. Recent studies indicate that the primary cells expressing IL-8 and IL-1β – macrophages and related myeloid lineages such as tumor-associated macrophages (TAM) – play a critical pathogenic role in many tumors. These inflammatory cells of the innate immune system are recruited to the tumor microenvironment, where they contribute to the regenerative "niche" (Pull et al., 2005) and exert protumorigenic effects (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Accordingly, in many human tumors the infiltration of macrophages is associated with a poor prognosis.

Evidence from Pollard's group indicates that infiltration with macrophages promotes both the development of breast cancers and their eventual spread to other sites in the body (Lin et al., 2001). The elimination of macrophages using CSF-1 null mice showed that macrophage recruitment is important for progression of mammary gland tumors, since invasive growth and metastasis were significantly attenuated (Lin et al., 2001; Wyckoff et al., 2004). A number of studies now suggest that macrophages promote the growth and invasiveness of cancer cells via NF-κB (Hagemann et al., 2005). In addition to enhancing vascularity and growth, these macrophages may suppress local T-cell responses (Kusmartsev and Gabrilovich, 2005). Another downstream target of IL-1β, the cytokine IL-6, is mainly produced by myeloid cells during the promotion stage of colon cancer (Greten et al., 2004). Macrophages appear to be particularly important in cancer progression, because of their production, in addition to IL-1β and IL-8, of many factors that can promote cancer, including reactive oxygen intermediates, growth factors, and metalloproteinases. Numerous studies have shown a correlation between macrophage infiltration and tumor vascularity and progression, including studies in gastric cancer (Ohno et al., 2003). In a transgenic model of preneoplasia involving overexpression of both COX-2 and mPGES-1 in the gastric mucosa, Oshima et al. showed that prostaglandin E_2 enhanced the recruitment of tissue macrophages and demonstrated more directly a link between macrophage activation and preneoplasia of the stomach (Oshima et al., 2004, 2005).

10.2.6.5 **Bone Marrow Stem Cell Recruitment**

While chronic inflammation leads to up-regulation of many chemokines and cytokines, and recruitment to the stomach of a number of immune cell populations, only recently has attention focused on the recruitment of progenitor cell populations. Studies conducted by Wang and colleagues led to observations that gastric cancer in the *H. felis*-infected C57 BL/6 mouse is preceded by the development of mucous metaplasia and expansion of an aberrant cellular lineage that expressed TFF2 (Wang et al., 1998). This lineage, termed SPEM for spasmolytic polypeptide-expressing metaplasia, is distinct from the normal mucous neck cells of the stomach, but clearly gives rise to the dysplasia and cancer that later develops in the stomach (Schmidt et al., 1999; Lee et al., 2003). Transcriptional profiling of this SPEM population, obtained using laser capture microdissection of C57 BL *H. felis*-infected mice, indicated the presence of transcripts such as Xist that were not normally expressed in adult epithelial tissues, but were normally expressed early in embryonic development and in stem cells (Nomura et al., 2004).

Classically, epithelial cancers such as gastric carcinoma are believed to originate from the transformation of tissue stem cells. However, based on the unusual nature of this metaplasia, our laboratory set out to test the hypothesis that gastric cancer might originate from a circulating bone marrow-derived stem cell (BMDC) population (Houghton et al., 2004). The model used was a lethally irradiated wild-type C57 BL/6 mouse transplanted with tagged bone marrow from donor mice. These in-

cluded transplantation with gender-matched or gender-mismatched bone marrow from C57 BL/*6 JGtrosa26* (ROSA 26) mice that express a non-mammalian beta-galactosidase enzyme, C57 BL/6 J-*beta-actin-EGFP* (GFP) mice, or C57 BL/6 J controls.

In the initial studies, transplantation was carried out with ROSA26 donors, and BMDCs were tracked with X-gal staining. Under the usual experimental conditions used of lethal irradiation and rescue with bone marrow transplantation, long-term BMDC engraftment in the stomach was observed to be an extremely unusual event. In addition, acute injury or selective gastric cell loss was not sufficient for engraftment of the stomach. ROSA26-transplanted C57 BL/6 mice with ulcers generated by cryoinjury or submucosal acetic acid injections showed no evidence of mucosal engraftment by BMDCs (Houghton et al., 2004). Similarly, complete parietal cell loss induced by the chemical compound DMP777 (a protonophore with specificity for acid secretory membranes) led to proliferation and regeneration but not to BMDC engraftment in the stomach. Acute short-term (3 weeks) *H. felis* infection, which is associated with a severe, acute inflammatory response, was also insufficient. However, chronic (20–52 weeks) *H. felis* infection led to substantial BMDC engraftment which was revealed by X-gal staining of epithelial cells in the body and corpus of the stomach, and also by immunocytochemistry for *E. coli*-specific beta-galactosidase. The vast majority – if not all – of the metaplasia, dysplasia and cancer was shown to be derived from BMDCs. Double-label immunofluorescence studies showed that the metaplastic and dysplastic BMDCs were clearly epithelial in origin, as indicated by the expression of cytokeratins (CK19) and the absence of CD45 expression. The donor origin of the cancers in these mice was thus demonstrated using a variety of methodologies, including: (i) X-gal staining; (ii) immunocytochemistry for *E. coli* beta-galactosidase; (iii) immunofluorescence of GFP; (d) immunostaining for GFP; (iv) laser capture microdissection and PCR for the Rosa26 transgene; and (v) Y-FISH for the donor-specific Y chromosome in gender-mismatched transplants. Finally, this process did not seem to involve fusion, as evidenced by the presence of a 2 N DNA content and a single X and single Y chromosome (Houghton et al., 2004).

Overall, these data have suggested a new paradigm for the development of adenocarcinoma of the stomach, and a new explanation for the link between chronic inflammation and cancer (Houghton and Wang, 2005). In this model, the carcinogenic stimulus –in this case a combination of *H. pylori* infection and the associated inflammatory response – results in recruitment and engraftment of BDMCs to the gastric mucosa, where they give rise over time to metaplasia, dysplasia, and cancer. The model encompasses a number of discrete steps that include: (i) inflammation-mediated alterations in the gastric stem cell niche; (ii) damage or failure of endogenous tissue stem cells; (iii) mobilization into the circulation of progenitor cells; (iv) recruitment and engraftment of BMDCs into the gastric stem cell niche; and (v) continued proliferation and aberrant differentiation of BMDCs in the altered niche.

10.3 Prevention of *H. pylori*-Induced Cancer

10.3.1 Interrupting Transmission in Children

Clearly, epidemiological data strongly support the key role that socioeconomic conditions play in determining the prevalence of *H. pylori* infections in various countries. Thus, personal hygiene, sanitary standards and reduction of high-density living conditions combine to reduce transmission of *H. pylori* in children.

Prophylactic vaccine strategies have been proposed as an ideal method to interrupt *H. pylori* transmission in children. The feasibility of this approach has been proven in mouse models, but successful vaccine candidates have not been forthcoming despite considerable effort.

10.3.2 Treatment Strategies of *H. pylori* in Populations at Risk

With respect to dietary antioxidants, some observational data in humans suggest that ascorbic acid and beta-carotene can protect against cancer in *H. pylori*-infected hosts (Ekstrom et al., 2000). Intervention studies, however, have been less promising. In one study, the six-year administration of supplemental ascorbic acid and beta-carotene prevented progression of preneoplasia in less than 20% of infected subjects immediately after supplementation was completed; however, these benefits were not maintained six years later (Correa et al., 2000; Mera et al., 2005).

The recognition that *H. pylori* represented a significant risk factor for gastric cancer raised the possibility that antibiotic treatment might reduce the overall risk of the condition. Decision analysis studies indicated that, if *H. pylori* eradication could reduce gastric cancer risk by 30% or more, a strategy of screening and treating patients for *H. pylori* infection could in theory be cost-effective (Parsonnet et al., 1996). However, the critical question continues to be whether *H. pylori* eradication can reduce gastric cancer risk, and at what stage the histopathological progression is reversible. Initial studies from Japan involving *H. pylori* eradication in patients who had undergone partial gastrectomy for early gastric cancer suggest a reduction in risk of recurrent gastric cancer (Uemura et al., 1997). The study by Uemura et al. (2001) was one of the first long-term, prospective studies of infected and uninfected patients with dyspepsia who underwent endoscopy and in whom the development of gastric cancer was the end point. It is important to note that *H. pylori* infection was assessed by three different methods. In this study of 1526 Japanese patients, gastric cancer developed in approximately 3% of the infected patients but in none of the uninfected patients. The risk was increased in all subgroups of patients (those with gastric ulcers, hyperplastic polyps, and nonulcer dyspepsia) except for patients with duodenal ulcers. Although the study was limited by histological analysis of only two gastric-biopsy specimens at each time point, the risk of gastric cancer was also clearly related to specific histological features found on the initial endoscopy, which

included severe gastric atrophy, corpus-predominant gastritis, and intestinal metaplasia (Uemura et al., 2001).

One of the most tantalizing and provocative findings in the study by Uemura and colleagues is that eradication of *H. pylori* prevented or delayed the development of gastric cancer. However, the question of who should undergo *H. pylori* eradication remains unresolved. The ideal treatment for nonulcer dyspepsia has been hotly debated, with the focus largely on the efficacy of anti-*H. pylori* therapy for the relief of upper abdominal symptoms (McColl et al., 1998; Talley et al., 1999). Uemura et al. suggest that patients with nonulcer dyspepsia have the highest risk of cancer (4.7%), and it can now be argued that these patients should have anti-*H. pylori* therapy on the basis of the risk of cancer alone. However, most patients with chronic *H. pylori* infection have no symptoms, which raises questions about the need for population-based screening for infection or predictive gastric pathological features. Complete resolution of this question will require large randomized controlled trials with long-term follow-up, most likely carried out in countries where gastric cancer rates are high. One such randomized clinical trial of *H. pylori* eradication as cancer prevention has been completed to date. Conducted in China, the results were inconclusive. Over 7.5 years (an interval that may have been too short), *H. pylori* eradication did not prevent cancer, although the trend was towards some diminution in cancer risk (Wong et al., 2004). In a subset of those without preneoplastic lesions, *H. pylori* eradication appeared to provide a significant reduction in cancer. Unfortunately, since the group without preneoplasia is at relatively low risk of cancer, this finding provides little insight into cancer prevention. Studies of *H. pylori* 's effects on preneoplastic conditions themselves are conflicting (Hojo et al., 2002). Acute inflammation has typically been deemed reversible, whereas intestinal metaplasia has not. Moreover, atrophic gastritis variably regressed. The most promising study evaluated the effects of *H. pylori* eradication 12 years after randomized treatment or placebo (Mera et al., 2005). In that study, the benefits of *H. pylori* eradication on preneoplasia score accrued over time, suggesting that shorter-term studies were inadequate to discern potential benefits.

The approach of widespread eradication of *H. pylori* for the purpose of reducing gastric cancer risk has been complicated by recent speculation that *H. pylori* may actually be protective against gastroesophageal (GE) junction tumors (Blaser, 1999 a,b). The prevalence of *H. pylori* has clearly been declining in most developed countries such as the U.S., in concert with declining rates of well-differentiated, intestinal adenocarcinomas. However, the rates of cancers involving the esophagus or gastric cardia, the so-called GE junction cancers, have been rapidly increasing over the past decade. Eradication of *H. pylori* was shown in some studies to result in increase rates of GE reflux, a known factor in the pathogenesis of Barrett's esophagus and esophageal cancer (Labenz et al., 1997; Vicari et al., 1998). In addition, reports from one group have indicated that infection with *H. pylori*, particularly $cagA^+$ strains, was inversely associated with GE junction cancers, suggesting that it may be protective (Chow et al., 1998). Thus, many questions remain with respect to the role of *H. pylori* in specific gastric diseases and the precise recommendations regarding diagnosis and treatment of *H. pylori* in asymptomatic patients.

10.4
Animal Models

10.4.1
Animal Models for *Helicobacter*-Induced Gastric Cancer

10.4.1.1 Gerbil

The gerbil has been shown to have particular relevant features which can be used to address the question of the potential of *H. pylori* to induce gastric cancer. Gerbils were first reported as a model for experimental *H. pylori* infection in 1991 (Yokota et al., 1991). Subsequently, Japanese investigators noted intestinal metaplasia, atrophy, and gastric ulcer in gerbils after experimental infection with *H. pylori* (Hirayama et al., 1996; Honda et al., 1998 a). In one study, acute gastritis with erosions of the gastric mucosa occurred shortly after infection, whereas gastric ulcers, cystica profunda, and atrophy with intestinal metaplasia were observed at 3–6 months after *H. pylori* infection (Honda et al., 1998 a). Following these findings, two separate research groups have noted that gerbils infected with *H. pylori* from periods ranging from 15 to 18 months develop gastric adenocarcinoma (Honda et al., 1998 b; Watanabe et al., 1998) (Table 10.5).

In one study, gerbils were observed for up to 62 weeks, and 37 % were found to develop adenocarcinoma in the pyloric region (Watanabe et al., 1998). The gastric cancers were clearly documented histologically. Vascular invasion and metastases were not observed; it is possible, however, that they may develop with longer periods of observation. In another report, *H. pylori* induced adenocarcinoma at 15 months post inoculation, but the authors did not record metastases or vascular invasion (Honda et al., 1998 b). Interestingly, the development of cancer is preceded by a cystica profunda; this is an invagination of atypical glands into the submucosa and is considered by some to be a premalignant lesion. The histological progression in the gerbil closely resembled that observed in humans, in terms of the early appearance of intestinal metaplasia, well-differentiated histological patterns of the gastric malignancy, and antral location of the gastric cancers. As for the association with gastric ulcers and gastric cancer in humans, the gerbils also had gastric ulcer disease in conjunction with gastric cancer (Hansson et al., 1996). The development of metaplasia with production of predominantly acid sialomucins was associated with tumor development. Whether the cancers arose directly form these metaplastic cells is unknown, but the tumors clearly originated deep in the gastric glands, in close proximity to these metaplastic cells (Watanabe et al., 1998).

Although most of the tumors in this *H. pylori* gerbil model originated in the pyloric region of the stomach, significant changes in the oxyntic mucosa consistent with chronic atrophic gastritis were seen (Watanabe et al., 1998). Glandular tissue in the gastric body and fundus were atrophied and replaced by hyperplastic epithelium of the pseudopyloric type. The differences between this lesion and gastric atrophy in human patients is that the gerbil corpus is not “thinner” consequent to the pseudopyloric hyperplasia (Wang et al., 1998; Watanabe et al., 1998). Thus, the diagnosis of

Table 10.5 Summary of key rodent models of infectious gastrointestinal and liver cancer.[a]

Rodent	Infectious agent/ transgene	Tumor	Comment
C57 BL mice	*H. felis*	Gastric adenocarcinoma	Natural gastric pathogen, but lacks *cag* and *vacA*
INS-GAS FVB mice[b]	*H. felis* and *H. pylori*	Gastric adenocarcinoma	Constitutive hypergastrinemia promotes tumorigenesis
Mongolian gerbil	*H. pylori*	Gastric adenocarcinoma	Closely mimics human disease, but few reagents
BALB/c mice	Several *Helicobacter* spp.	Gastric MALT lymphoma	Usually requires 18–24 months
Genetically engineered mice: IL-10-, IL-2-, $G\alpha_{i2}$-, Muc2-, Smad3$^{-/-}$, etc. -deficient; especially on 129 Sv background	„Endogenous microbiota“ or *H. hepaticus*, *H. bilis*	Lower bowel carcinoma	Bacteria in endogenous microbiota models not well defined; *H. hepaticus* or *H. bilis* reliably induces disease
Lymphocyte-deficient mice: SCID or Rag$^{-/-}$; especially on 129 Sv background	„Endogenous microbiota“ or *H. hepaticus*	Lower bowel carcinoma	Often used for adoptive transfer studies; *H. hepaticus* induces tumors in untreated Rag2$^{-/-}$ mice
Transgenic mice[b]	HBV or HCV transgene(s)	HCC	Prove tumorigenic potential of viral gene products; adoptive transfer or inducible gene strategies required for hepatitis
A/JCr and other mice[b]	*H. hepaticus*	HCC	Natural murine pathogen induces chronic active hepatitis and HCC

[a] Data modified from Rogers and Fox (2004).
[b] Male predominant. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MALT, mucosal-associated lymphoid tissue; PAI, pathogenicity-associated islands.

atrophy is not involved with the thickness of the mucosa but rather with the loss of oxyntic (parietal and chief) cell populations within the gastric glands. Growing evidence suggests that the parietal cell may regulate key differentiation decisions within the gastric glands, and ablation of parietal cells using transgenic technology (Li et al., 1996) or their loss to infection with *H. felis* and *H. pylori* infection leads to altered glandular differentiation and neck cell proliferation as well as to changes in gastric acid and gastrin physiology.

The expansion of an aberrant neck cell (“regenerative hyperplasia” or “pseudopyloric hyperplasia”) in the gerbils is similar to that observed in the *H. felis* mouse

model. This lineage has been shown to be spasmolytic polypeptide (SP) -positive (Wang et al., 1998), and this SP-positive lineage also develops in *H. pylori*-associated gastric cancers in humans (Schmidt et al., 1998). The loss of oxyntic glandular tissue in response to *H. pylori* infection suggests that the gerbil becomes achlorhydric before the development of gastric cancer. Although the precise effects of chronic *H. pylori* infection on gastric acid secretion are not known, serum gastrin levels in the *H. pylori* infected gerbil are increased.

The unusual susceptibility of this animal species to gastric cancer using a fairly standard *H. pylori* strain underscores once again the overriding importance of host factors in determining the outcome from *Helicobacter* infection (Wang and Fox, 1998). It is important to note that many laboratories – particularly those outside of Japan – have been unable to document the carcinogenic potential of *H. pylori* in the gerbil model. Recently, however, investigators in the U.S. using *H. pylori* strain B128 serially passaged in gerbils documented rapid onset gastric adenocarcinoma in gerbils. This study serves to highlight the importance of bacterial factors as well as host susceptibility in cancer induction (Franco et al., 2005).

10.4.1.2 **Mouse**

As detailed above, most of the major advances in our understanding of gastric cancer pathogenesis have been derived from studies with mice. In particular, much insight has been derived from the *Helicobacter*-infected mouse, first described by James Fox and Adrian Lee (Lee et al., 1990). While a number of other animal models have been developed for both gastric cancer (e.g., MNNG-induced cancer in rats) and for *Helicobacter*-mediated infection (ferret, gerbil, gnotobiotic pig, primate, etc.), the mouse model has clear advantages with respect to their small size, cost, ease of infection, reproducibility, and especially the power of genetic manipulation. The *Helicobacter*-infection model has been combined now with a variety of inbred strains, transgenic and knockout mice to assess the relative contribution of host versus non-host factors in the preneoplastic process. Indeed, the combination of genetic manipulation in the setting of initiation and promotion by the authentic carcinogen (gastric *Helicobacter*) makes this model, in many respects, the best cancer model for study.

In the early investigations by a number of groups, it became clear from studies with inbred mice that progression to preneoplasia was largely determined by the host response. For example, the C57BL/6 inbred mouse strain responded to *H. felis* infection with a robust Th1 immune response, in contrast to the BALB/c inbred strain which showed a predominant Th2 response (Mohammadi et al., 1996; Sakagami et al., 1996; Wang et al., 1998). Consequently, C57BL/6 mice showed rapid progression to atrophy and metaplasia with declining colonization levels, while BALB/c mice maintained high numbers of organisms with minimal epithelial injury or atrophy. Additional studies employing RAG and SCID mice and T-cell-deficient mice pointed to the importance of T-cell responses (Roth et al., 1999), while the use of cytokine knockout mice showed *H. pylori*-induced mucosal inflammation to

be Th1-mediated and exacerbated in IL-4 but not IFN-γ gene-deficient mice (Smythies et al., 2000).

However, while genetic manipulation of the various cytokine genes clarified many aspects of the immunopathogenesis of the disease, the unmanipulated C57BL/6 mouse has proved surprisingly robust as a model for cancer development. Interestingly, many of the early *H. felis* infection studies included observations periods that extended up to one year but not beyond, and described changes of atrophy and metaplasia, but not more advanced pathology. The presence of dysplasia and invasive carcinoma in the *H. felis*-infected C57BL/6 mouse was first described by Fox and Wang (Fox et al., 2002 b), when the observation period was extended out to 14 months. More recently, progression to antral carcinomas has been reported in mice in which the model has been extended out to 22 months (Cai et al., 2005). Indeed, in many of the models of gastric cancer, a long period of achlorhydria, associated with presumed bacterial overgrowth, appears to be the common denominator in mouse models of antral carcinoma (Zavros et al., 2005).

As described above, the C57BL/6 mouse model of *Helicobacter* infection has been very useful for the study of dietary cofactors (such as salt) and for manipulation of the Th1-Th2 immune response. However, another factor is likely to be the particular *H. pylori* strain, and this question has been more difficult to address using the wild-type C57BL/6 mouse. The wild-type mouse can be infected with a few "mousefied" *H. pylori* strains. The mouse commonly used strain has been the *H. pylori* Sydney strain, first described by Lee in 1997 (Lee et al., 1997). However, most *H. pylori* strains – including the Sydney strain – have shown less consistent patterns of colonization, and for the most part have proven less carcinogenic in the C57BL/6 mouse (Thompson et al., 2004), although a recent study has for the first time demonstrated high-grade dysplasia in C57BL/6 × 129 S6/SvEv (B6129) mice infected for 15 months with *H. pylori* SS1 (Rogers et al., 2005). Nevertheless, the *H. felis*-infected C57BL/6 mouse has proven for the most part to be the superior model.

A role for bacterial factors also has been demonstrated using *Helicobacter*-infected transgenic mice. The first *Helicobacter*-dependent mouse model of gastric cancer was reported by Wang et al., who showed that *H. felis* infection of insulin-gastrin (INS-GAS) transgenic mice led to the rapid appearance of atrophy, metaplasia, dysplasia and gastric cancer (Wang et al., 2000). The model has proved ideal as an accelerated model of carcinogenesis. The model leads to accelerated gastric cancer in part due to the effect of elevated circulating levels of amidated gastrin which bind to CCK-B receptors to activate a histamine pathway, leading to altered growth and immune responses (Takaishi et al., 2005), and to relatively low levels of glycine-extended gastrin which may inhibit progression to preneoplasia (Cui et al., 2004). This INS-GAS mouse model has been used to demonstrate that cagE is likely an important virulence factor, since deletion of this gene (in the B128 *H. pylori* strain) delayed the progression to carcinoma (Fox et al., 2003 b). Previous studies have also demonstrated the requirement for cagE in inducing gastric injury during the early states of *H. pylori* infection in gerbils (Israel et al., 2001).

These results are also consistent with a report demonstrating that *H. pylori* isogenic *cagE* mutant strains retain the ability to induce expression of host genes that

may function in cellular recognition events (Guillemin et al., 2002). The authors co-cultured either a wild-type *H. pylori* strain or an isogenic *cagE*$^-$ mutant with gastric epithelial cells. Both strains induced expression of several mediators of injury, including IFN-γ, plasminogen, endothelin-1, and trefoil factor 1, as well as a number of signal transduction genes (Guillemin et al., 2002). However, these genes were not induced by an *H. pylori* mutant strain that lacked the *cag* island (Guillemin et al., 2002), indicating that host cells respond differently to strains lacking the entire *cag* island and those with a defective secretory system (e.g., *cagE*$^-$). These data raise the hypothesis that the *H. pylori* B128 *cagE*$^-$ mutant strain still harbors portions of the *cag* secretion apparatus that allow it to engage in intimate contact with host cells and to elicit a proinflammatory response (Fox et al., 2003 b).

While a number of other very useful murine model of gastric cancer have been developed (Judd et al., 2004; Rogers and Fox, 2004; Zavros et al., 2005), it is likely that the role of *Helicobacter* infection – and the associated immune response – will be critical in mimicking precisely the cancer pathways most relevant to human disease.

10.4.1.3 **Ferret**

H. mustelae persistently infects the inflamed mucosa of ferrets, and colonization of *H. mustelae* in ferret stomachs occurs shortly after weaning (Fox et al., 1988 a). In addition, Koch's postulates have been fulfilled; that is, oral inoculation of *H. mustelae* into naive ferrets not infected with *H. mustelae* induces a chronic, persistent gastritis analogous to the gastritis associated with *H. mustelae* in naturally infected ferrets. Ferrets have also been used successfully as a suitable model to study the pathogenesis and epidemiology of *Helicobacter*-associated chronic gastritis and gastric cancer (Fox et al., 1990). The histopathological changes observed closely coincide in topography with the presence of *H. mustelae*. In the oxyntic mucosa, inflammation and *H. mustelae* are limited to the superficial portion, and as such is classified as a superficial gastritis. In the distal antrum the inflammation involves the full thickening of the mucosa, and is given the term "diffuse antral gastritis", as described in humans. *H. mustelae* are noted in the distal antrum at the surface, the superficial portion of the glands, and within the gastric pits. In the proximal antrum and the transitional mucosa, the element of focal glandular atrophy and regeneration is observed in addition to those lesions described in the distal antrum. There is also deep focal *H. mustelae* colonization of multiple antral glands. *Helicobacter*-associated gastritis in the ferret model has many similarities with the human disease, and contributes considerably to the interpretation of chronic gastritis in humans. The lesions observed in the distal antrum and the oxyntic mucosa closely resemble the diffuse antral gastritis observed in humans which, like the ferret model, is usually accompanied by superficial gastritis of the corpus. This clinicopathological entity underlies the duodenal ulcer syndrome (Fox et al., 1990; Correa, 1992), whereas histologically the proximal antrum and the transitional zone of *H. mustelae*-infected ferrets represent early stages of the multifocal atrophic gastritis of humans; this histological presentation is often linked to gastric ulcer and gastric carcinoma (Fox et al., 1990; Correa,

1992). The importance of these premalignant lesions associated with *H. mustelae* infection is highlighted by the report that naturally occurring pyloric adenocarcinoma has also been linked to *H. mustelae* in ferrets (Fox et al., 1997).

Chronic *H. pylori* infection can cause an increase in cell proliferation (Brenes et al., 1993), and many studies have indicated that abnormal cell proliferation and maturation are related to both the development and progression of neoplasia (Lipkin and Higgins, 1988). The effect of *H. mustelae* infection on gastric epithelial proliferation was studied in ferrets colonized with *H. mustelae* and specific pathogen-free (SPF) ferrets not infected with *H. mustelae* (Yu et al., 1995). PCNA-expressing gastric epithelia in the antrum and the body regions were significantly increased in the *H. mustelae*-infected ferrets versus the SPF ferrets ($P < 0.001$) (Yu et al., 1995). Comparison of the histopathology of infected ferrets indicated that PCNA positivity correlated with the histological severity of gastritis. *H. mustelae*-infected ferrets had significantly higher grades of inflammation than SPF ferrets, which had normal gastric mucosa.

In a study to define the *H. mustelae*-infected ferrets as an animal model of *H. pylori*-associated gastric cancer using MNNG (Fox et al., 1993 b), young female ferrets were given a single oral dose of MNNG (50–100 mg kg^{-1}) to ascertain whether they would develop adenocarcinoma of the stomach. Age-matched unmanipulated *H. mustelae*-infected control animals were included for comparative purposes. Nine of 10 ferrets dosed with MNNG had invasive gastric adenocarcinoma of the antrum diagnosed at necropsy (29–55 months after dosing), whereas none of the control ferrets had developed gastric cancer, when examined at an average of 63 months after the initiation of the study. The large number of gastric adenocarcinomas in the ferrets in this study may be partially due to *H. mustelae*-associated gastritis present in 100% of the MNNG-treated animals. These results indirectly implicate *H. mustelae* in MNNG-induced gastric cancer in the ferret. This hypothesis is supported by a study in our laboratory where a group of *H. mustelae*-free SPF ferrets dosed with a single dose of 100 mg kg^{-1} MNNG did not develop gastric cancer by 3.5 years post dosing (J.G. Fox, unpublished results).

The ferret stomach closely resembles that of the human stomach in its anatomy, histology, and physiology (Fox, 1988). The structure of the ferret gastric mucosa at the cellular level is remarkably similar to that of humans. Ferrets secrete gastric acid and proteolytic enzymes under basal conditions and, like humans infected with *H. pylori*, *H. mustelae*-infected ferrets have hypergastrinemia (Perkins et al., 1996). The ferret also is similar to humans in its biochemical processing of beta-carotene, making it an important model for studying the anticancer properties of this compound (Wang et al., 1992). Most recently, the *H. mustelae*-infected ferret has been used to demonstrate the protective effects of lycopene supplementation in smoke-induced changes in cell proliferation, p53, and apoptosis in the gastric mucosa (Liu et al., 2006).

10.4.2 Animal Models of MALT Lymphoma

10.4.2.1 *H. felis*-Induced MALT Lymphoma

Mucosal-associated lymphoid tissue (MALT) low-grade lymphoma (MALToma) in *H. felis*-infected BALB/c mice has been reported. These mice did not develop lymphoma until more than 20 months after infection, almost the entire life span of a mouse (Enno et al., 1995). This has particular relevance with the association of *H. pylori* and MALToma in humans (Wotherspoon et al., 1993). A genetic event has been postulated to initiate the transition from gastritis to low-grade MALToma (Calvert et al., 1995). Humans have an approximately balanced κ:λ ratio which facilitates the distinction of lymphoma from gastritis. In humans, MALToma has been distinguished from severe gastritis by evaluating clonality using κ and λ antigen markers or immunoglobulin gene probes (Wotherspoon et al., 1993; Savio et al., 1996). One disadvantage of studying the mouse gastric MALToma is the disparate 20:1 κ:λ ratio in mice, which has made comparisons to the human situation difficult. Monoclonal lesions confirmed by phenotype and genotype have regressed after antibiotic therapy for *H. pylori*, suggesting that some of these lesions may actually represent a transition to neoplasia (Savio et al., 1996). The *H. felis*-infected mouse model has helped to answer these fundamentally important questions.

These authors have used the BALB/c model to further dissect the role of *Helicobacter* spp. (*H. pylori*, *H. felis*, and *H. heilmannii*) in gastric lymphomagenesis (Mueller et al., 2003). They showed that the initial step in lymphoma development is marked by infiltration of reactive lymphocytes into the stomach and the initiation of a mucosal immune response. Using the microarray analysis of both the mucosa and lymphoma element of gastric tissue (employing laser capture microdissection), they reported on the molecular markers of both of these processes, including genes coding for the immunoglobulins and the small proline-rich protein Sprr 2A. In their model, a progression of the disease is characterized histologically by the antigen-driven proliferation and aggregation of B cells and the gradual appearance of lymphoepithelial lesions with increased expression of genes previously associated with malignancy, including the laminin receptor-1 and the multidrug-resistance channel MDR-1 (Mueller et al., 2003). The transition to destructive lymphoepithelial lesions and malignant lymphoma was identified by an increase in transcription of a single gene encoding calgranulin A/Mrp-8. The transcript was expressed in both the mucosal and the lymphocytic fraction, being more abundant in the mucosal tissue. Because calgranulin mainly functions extracellularly (Donato, 2001), the authors suggest that investigating the localization of the corresponding protein will be useful in shedding light on its function in MALT lymphoma development (Mueller et al., 2003).

In a more recent report using the same model, the authors have provided insight into why some *Helicobacter*-induced MALTomas relapse after apparent remission following antibiotic treatment (Mueller et al., 2005 b). They addressed the role of antigenic stimulation in the pathogenesis of the lymphoma by experimental infection

with *H. felis* in BALB/C mice, followed by antibiotic eradication therapy and subsequent reinfection. Antimicrobial therapy was successful in 75% of mice, and led to complete histological but not "molecular" tumor remission. Although the lymphoepithelial lesions disappeared and most gastric lymphoid aggregates resolved, transcriptional profiling revealed the long-term mucosal persistence of residual B cells. After experimental reinfection with *Helicobacter*, there was a rapid recurrence of the lymphomas. These tumors were more aggressive than the original lymphomas, having higher proliferation and a more aggressive phenotype of the tumor (Mueller et al., 2005 b). Immunophenotyping of tumor cells revealed massive infiltration of lesions by $CD4^+$ T cells, which express CD28, CD69, and IL-4 but not IFN-γ. These data suggested that tumor B-cell proliferation was driven by Th 2-polarized, immunocompetent, and activated T cells. The number of dendritic cells in the follicles also predicted the success of antibiotic therapy in the regression of the tumor (Mueller et al., 2005 b).

10.4.3 Animal Models for Enterohepatic *Helicobacter*-Induced Cancer

10.4.3.1 *Helicobacter hepaticus*-Induced Liver Cancer

A bacterium was identified in the livers of A/JCr mice with hepatitis, hepatic adenomas, and hepatocellular carcinoma (HCC) during a long-term carcinogenesis study conducted at the National Cancer Institute in 1992 (Ward et al., 1994 a). A Gram-negative bacterium isolated from these mice with diseased livers was characterized and named *H. hepaticus* (Fox et al., 1994). This organism causes a persistent infection and is prevalent in mouse colonies, both commercial and academic, throughout the world (Weghorst et al., 1989; Foltz et al., 1995; Shames et al., 1995). In addition to A/JCr mice, *H. hepaticus* also causes hepatitis in BALB/cCr, SJL/NCr, SCID/NCr, C_3 H/HeNCr, $B_6C_3F_1$ mice and AXB recombinant strains. Like AJ/Cr mice, *H. hepaticus* infection in B6C3F1, AXB and AB6F1 mice also causes liver cancer. The ecological niche of *H. hepaticus* is the lower bowel, and fecal-oral transmission is the suspected natural route (Fox et al., 1996 b). The liver lesion of mice with natural *H. hepaticus* infection progressively increases in severity.

Similar to the lesions found in acute viral hepatitis of humans, the early inflammation in livers of mice infected with *H. hepaticus* consisted of lobular infiltrates comprised predominantly of macrophages variably accompanied by fewer neutrophils and/or lymphocytes. Lobular inflammation was accompanied by spotty or confluent coagulative necrosis of hepatocytes. Portal/periportal infiltrates were comprised chiefly of mixed T and B lymphocytes as well as unlabeled lymphocytoid, presumably natural killer (NK), cells. Up-regulated expression of iNOS and COX-2 within inflammatory foci was demonstrated in both leukocytes and hepatocytes by immunohistochemistry. Immunohistochemical analysis reinforced the striking similarities in inflammatory patterns and progression between susceptible mice infected with *H. hepaticus* and human patients suffering from acute viral hepatitis (Ferrell et al., 2002).

Random and perivenular lobular necroinflammatory lesions, with spotty or confluent hepatocellular coagulative necrosis, precede lymphocyte accumulations in the portal triads in infected A/JCr mice (Ward et al., 1994 a,b; Fox et al ., 1996 b; Rogers et al., 2004). Random scattered acidophil bodies, representing apoptosis of individual hepatocytes, may be seen without overt inflammation. Kupffer and hepatic stellate (Ito) cell hyperplasia accompany lobular lesions, and oval cell proliferation with neocholangiole formation is a common sequel to portal inflammation, especially in cases of interface hepatitis (Ward et al., 1994 a; Ihrig et al., 1999; Rogers et al., 2004). It is not known whether hepatic lesions are solely attributable to bacterial invasion or whether soluble antigens or toxins gaining access from the lower bowel via the portal circulation might also contribute (Fox et al., 1996 b). Using BrdU, increased liver cell proliferation associated with *H. hepaticus* was detected (Fox et al., 1996 b). There was an age-associated increase in cell proliferation in infected animals that was not seen in uninfected control mice and was more pronounced in infected male than female mice (Fox et al., 1996 b). Increased hepatocyte proliferation indices in *H. hepaticus*-infected male mice is consistent with the observation of increased hepatomas and hepatocellular carcinomas observed in *H. hepaticus*-infected aged A/JCr male mice (Ward et al., 1994 a,b; Franklin et al., 2001). Importantly, we have induced liver lesions experimentally by intraperitoneal or oral inoculation of *H. hepaticus* into A/JCr but not C57BL mice (Ward et al., 1994 a; Ihrig et al., 1999). Persistent infection of germ-free female outbred mice with *H. hepaticus* also induced chronic hepatitis, albeit of milder severity than that seen in males and in one mouse, HCC (Fox et al., 1996 d).

Using Affymetrix gene chip microarray we recently compared uninfected versus *H. hepaticus*-infected A/JCr male mice at 3, 6, and 12 months, and also compared infected males with and without severe liver lesions (Boutin et al. , 2004). As expected, a large number of transcriptionally up-regulated genes in the livers of mice with moderate-severe hepatitis were related to inflammation. In agreement with immunohistological demonstration of histiocytic lobular hepatitis, many up-regulated genes were attributable to Kupffer cells and recruited monocyte/macrophages including MARCO, MHCII, lysozyme, LY-6, LY-117, CCR5, macrophage-expressed gene 1, and LAPTM5. Innate immunity and acute-phase responses were represented by numerous genes including serum amyloid A, and very prominently by lipocalin 2 (NGAL/24p3), and other lipocalins including orosomucoid and retinol-binding protein (Boutin et al., 2004). Lipocalins bind lipophilic molecules including lipopolysaccharide (LPS) and other bacterial cell wall products, and also are involved in mucosal immunity, chaperoning, lymphocyte apoptosis, and male scent expression (Cavaggioni and Mucignat-Caretta, 2000; Devireddy et al., 2001; Xu and Venge, 2000). Evidence of active antigen processing was documented by increases in proteasome, Ia invariant chain, cathepsin S, and MHC II transcripts. The type 1 immune response demonstrated by serum antibody subtype analysis, and documented previously (Whary et al., 1998), was corroborated by microarray analysis including increases of numerous IFN-γ-inducible GTPases, proteins, and transcripts, as well as STAT1, leukocyte-specific protein, and others. Many endogenous hepatic enzymes were significantly down-regulated in mice with lesions, suggesting

decreased hepatocellular functionality in the diseased liver. Interestingly, male mice with severe disease versus both uninfected controls and infected males without disease, had significantly decreased levels of hydroxysteroid dehydrogenase-5, δ<5>-3-β, the hepatic enzyme responsible for inactivating the potent androgen dihydrotestosterone. This could explain in part the gender effect. Other enzymes specifically associated with steroid metabolism were also up-or down-regulated in males with disease versus those without (Boutin et al., 2004). There is significant overlap of function in enzymes involved in sex-steroid modification and bile acid synthesis, raising two potential pathogenetic mechanisms for disease outcomes in the livers of male mice (Pirog and Collins, 1999; Schwarz et al., 2000).

10.4.3.2 *Helicobacter bilis*-Associated Hepatitis

Helicobacter bilis has been isolated from the intestines and livers of inbred mice aged 18 months or more. This organism, like *H. hepaticus*, is strongly urease-positive (Fox et al., 1995), but morphologically it is more fusiform than spiral in shape. It measures 0.4 × 4–5 μm in size, but has overlapping periplasmic fibers similar to those of "*H. rappini*". The livers of *H. bilis*-infected inbred mice had multifocal hepatitis. The severity of the liver lesions was strain-dependent, with CBA/CA having the most severe hepatitis, followed by DBA/2 mice, and the least severe being C57BL/6 mice. The BALB/c mice had no inflammatory lesions in the liver. *H. bilis*-associated hepatitis is also observed in outbred CD mice (Fox et al., 2004). A bacterium with this morphology has also been observed in the livers of rats experimentally infected with *Fasciola hepatica* (Foster, 1984). Unlike *H. hepaticus*, *H. bilis* has an extended host range, having been isolated from the feces of dogs, cats, and gerbils, as well as being incriminated in cholecystitis and liver cancer in humans (Fox et al., 1998; Fox, 2002; Matsukura et al., 2002). Immunogenic proteins present in *H. bilis* have also been identified in *H. hepaticus* and *H. pullorum* (Ge et al., 2001; Kornilovs'ka et al., 2002). These immunodominant proteins may be useful for the development of the serodiagnosis of these helicobacters in both humans and animals.

10.4.3.3 *Helicobacter hepaticus* and Lower Bowel Cancer

While an extensive review of animal models of inflammatory bowel disease (IBD) is beyond the scope of this chapter, it is increasingly apparent that enterohepatic *Helicobacter* spp. – particularly *H. hepaticus* and *H. bilis* – play a key role as inflammatory mediators in the onset and progression of colitis in a number of mouse models. *H. hepaticus* and *H. bilis* persistently colonize the lower bowel of mice (Fox et al., 1995, 1996 d; Maggio-Price et al., 2005) and presumably reach the liver via the portal circulation, although direct retrograde migration via the common bile duct has not been disproven. The immune response to *H. hepaticus* infection appears to be Th1-mediated, since splenocytes produced large amounts of IFN-γ when stimulated with *H.*

hepaticus antigens (Whary et al., 2000). Normally, humans and other animals remain hyporesponsive or tolerant to their own enteric flora. In patients with IBD, tolerance is disrupted as individuals become "hyper-responsive" to endogenous enteric antigens (Duchmann et al., 1995). IL-10 is a Th2 cytokine with anti-inflammatory and Th1-suppressive effects, and the lack of IL-10 manifests predictably as an unopposed Th1 immune response (Berg et al., 1996; Kullberg et al., 1998). The IL-10$^{-/-}$ mice reared conventionally (it is highly probable that these mice were infected with *Helicobacter* spp.) present with enterocolitis (Kuhn et al., 1993) and lower bowel cancer (Berg et al., 1996). Rearing IL10$^{-/-}$ mice in SPF facilities restricted the inflammation to the colon (Berg et al., 1996; Kullberg et al ., 1998), whereas in germ-free IL-10$^{-/-}$ mice there is no lower bowel inflammation. Thus, enteric bacterial antigens including *H. hepaticus* or *H. bilis* contribute to the induction of colitis (Kullberg et al ., 2002; Maggio-Price et al., 2005) and, more recently, the induction of colon cancer in RAG mice, $TGFB_1$ and Smad3$^{-/-}$ deficient mice (Engle et al., 2002; Erdman et al., 2003 a; Maggio-Price et al., 2006).

The CD4^{+}CD45^{+}Rb^{hi} model of *H. hepaticus*-induced colitis in SCID or RAG mice is well established, and has been duplicated in a number of laboratories (Cahill et al., 1997; Powrie et al ., 1997). Monoinfection of germ-free SCID mice or outbred mice with either *H. hepaticus* or *H. muridarum* and their ability to induce enterocolitis also illustrates the proinflammatory capabilities of these helicobacters (Fox et al., 1996 d; Jiang et al., 2002).

This model has been further defined in the *H. hepaticus*-infected 129 Rag mouse model which documents the progression of colitis to lower bowel adenocarcinoma (Erdman et al., 2003 a,b). Importantly, this model of lower bowel cancer fulfills all of the histological criteria for a diagnosis of colonic adenocarcinoma outbred by a panel of experts (Boivin et al., 2003). The progression of *H. hepaticus*-induced lower bowel cancer can be modulated by subsets of T cells; T-regulatory cells abrogate inflammation and cancer, while T-effector cells CD4^{+}CD45^{+} Rb^{hi} accelerate the process (Erdman et al., 2003 a,b).

Observations that colorectal cancer can be reduced in patients receiving oral aspirin therapy or various other anti-inflammatory medications indicate the apparent role of inflammation in the pathogenesis of these tumors. Using the Apc$^{min/+}$ mouse model of colon polyps, we have demonstrated that T-regulatory cells (CD4^{+}CD45^{+}Rb^{low}) can actually induce the regression of intestine polyps in this model (Erdman et al., 2005). Surprisingly, when the Apc$^{min/+}$ Rag$^{-/-}$ mouse C57BL/6 model infected with *H. hepaticus* or administered proinflammatory lymphocytes (CD4^{+}CD45^{+}Rb^{hi}), both intestinal and mammary tumors are statistically increased in number (Roa et al. in press).

10.5
Virulence Determinants of Enterohepatic *Helicobacter* spp.

10.5.1
H. hepaticus is a Tumor Promoter in the Liver

A/JCr has a low incidence and multiplicity of liver tumors, and their susceptibility to *H. hepaticus*-induced hepatitis and development of HCC has provided a unique opportunity to dissect *Helicobacter*-associated tumorigenesis. Hepatocyte proliferation is strongly linked to tumor promotion, and presumably is the result of *H. hepaticus*-induced chronic inflammation; it is in part responsible for the increased rates of hepatocellular tumors in male A/JCr mice (Fox et al ., 1996 b; Nyska et al., 1997; Ihrig et al., 1999). There is also evidence for the presence of elevated levels of oxidative stress in *H. hepaticus*-associated hepatitis, there being a time-dependent increase in 8-oxo-2'-deoxyguanosine in the liver of *H. hepaticus*-infected A/JCr mice compared to uninfected, age-matched controls (Sipowicz et al., 1997 a). The source of the ROS was determined to be the hepatocytes. Immunohistochemistry revealed an increased number of cells expressing cytochrome P450 (CYP), and co-localization of formazan and the 2A5 isoform of the enzyme (CYP2A5) suggested a possible mechanism for the production of ROS. In addition, immunohistochemistry for glutathione S-transferase indicated that the hepatocytes were attempting to produce increased amounts of reduced glutathione (GSH). GSH is involved in protecting cells from killing by NO and by ROS, and both *de-novo* synthesis of GSH and reduction of oxidized glutathione (GSSG) are important responses to increased oxidative stress (Luperchio et al., 1996). The oxidative stress associated with *H. hepaticus* infection may result in an induction of lipid peroxidation and the generation of malondialdehyde. The latter reacts with deoxyguanosine in DNA and results in the formation of the cyclic pyrimidopurinone *N*1, *N*2 malondialdehyde – deoxyguanosine (M_1dG) adduct. This adduct can cause mutations that may ultimately lead to liver carcinogenesis. Higher levels of M_1dG were detected in the liver DNA of *H. hepaticus*-infected A/JCr mice compared to controls, with levels increasing from 3 to 12 months post infection. There was a significant age-dependent increase in the level of M_1dG in the caudate and median lobes of the A/JCr mice relative to control mice (Singh et al., 2001).

A central problem with these two studies is that a single biomarker may not be representative of the chemistry occurring at sites of *H. hepaticus*-induced inflammation. Future studies should include utilization of a series of DNA damage products as biomarkers of inflammation, including nitrosative deamination products of DNA bases (2'-deoxy-xanthosine, -oxanosine, -uridine, - and inosine), the etheno adduct of dA (believed to arise from lipid peroxidation products), and M_1G as well as sensitive methods to quantify abasic sites and strand breaks in cells.

Strain differences in *H. hepaticus* susceptibility also suggest a mechanism such as tumor promotion, because it is known that tumor promotion in the mouse liver is influenced strongly by genetics of the host (Diwan et al., 1986; Watanapa and Watanapa, 2002). A tumor promotion mechanism is also supported by a lack of mu-

tagenic response in the Ames' assay, as well as a lack of demonstrated mutations in H-, K-, and N-ras and p53 tumor suppresser genes in liver tumors of A/JCr mice infected with *H. hepaticus* (Canella et al., 1996; Diwan et al., 1997; Sipowicz et al., 1997 b). When male A/JCr infant mice infected with *H. hepaticus* were given a single intraperitoneal dose of NDMA they developed a statistically higher incidence of hepatocellular adenomas at 31–36 weeks and 51–64 weeks post infection when compared to uninfected A/JCr mice similarly treated with the carcinogen (Diwan et al., 1997). There was also a fourfold increase in multiplicity of adenomas at 31–36 weeks post infection, and a fivefold increase in both incidence and multiplicity of carcinomas after 50 weeks. These data indicate that *H. hepaticus* not only stimulates growth of tumors from initiated cells, but also enhances progression to malignancy (Diwan et al., 1997). All of these factors in combination suggest that *H. hepaticus* exerts a tumor promotion effect in the liver of A/JCr mice.

10.5.2 *H. hepaticus* Increases ROS and Intestinal Tumors

Gpx double knockout mice lack the two isoforms of Gpx expressed in the intestinal tract, and therefore are deficient in their ability to scavenge ROS (Chu et al., 2004). When these knockout mice are infected with *H. hepaticus* they develop a significantly higher incidence of intestinal tumors when compared to their uninfected counterpart (Chu et al., 2004).

10.5.3 *H. hepaticus* Pathogenicity Island

Genome sequence analysis of *H. hepaticus* revealed the presence of a genomic island with low G+C content that comprises 70 kb of sequence and 71 predicted genes. The island, in part, comprises elements that suggest a role for it in virulence. This island, termed *H. hepaticus* genomic island 1 (HHGI1), comprises three genes that encode homologues of components (VirB10, VirB4, and VirD4) of a type IV secretion system (T4SS). HHGI1 also contains a gene with homology to *Vibrio cholerae hcp*, which encodes a secreted protein co-regulated with the *V. cholerae* hemolysin, a gene cluster (HH244 to HH251) with significant homology to clusters of genes of unknown function on the small chromosome of *V. cholerae* (VCA0107 to VCA0115) and the *Yersinia pestis* genome. Unlike many pathogenicity islands, HHGI1 is not associated with a tRNA gene and not flanked by direct repeats. However, it contains a prophage P4-like integrase gene (HH0269), a feature that has been found in several pathogenicity islands. In the same study, we established by genome comparisons with a whole-genome DNA microarray that while all strains that had been associated with liver disease, including the sequenced strain 3B1 (ATCC 51449), contained the complete island, many *H. hepaticus* isolates lack parts of the island or even all HHGI1 genes. Significantly, none of these HHGI1-defective strains had caused liver disease in the mice from which they had been isolated. Taken together, these data suggested that HHGI1 might be involved in virulence of *H. hepaticus*. We there-

fore selected two *H. hepaticus* isolates, HhNET and HhG, with partial or complete deletions of the island and compared their virulence with that of the sequenced strain that harbors the complete island (Suerbaum et al., 2003).

We have shown that A/JCr mice infected with strains of *H. hepaticus* (HhG and HhNET) lacking all or part of a 70-kb genomic putative PAI have less-severe *Helicobacter*-associated liver disease than mice infected with the strain Hh3B1, which contains a complete HHGI1 island. While the study clearly demonstrates that isolates of *H. hepaticus* with different genomic contents differ significantly in their potential to cause liver disease in A/JCr mice, further experiments with isogenic mutants lacking the island will be required to firmly prove the role of the HHGI1 island in these differences, and attempts to construct series of such mutants are under way in our laboratories (Boutin et al., 2005).

10.5.4 Cytolethal Distending Toxin (CDT)

Candidate virulence determinants in enterohepatic *Helicobacter* species (EHS) have begun to be identified. *H. hepaticus* and a subset of the other EHS (*H. cinaedi*, *H. pullorum*, *H. bilis* and *H. marmotae*) each produce CDT (Chien et al., 2000; Young et al., 2000a,b), a potential virulence factor elaborated by a heterogeneous group of pathogenic bacteria including *C. jejuni* and other *Campylobacter* species, certain *E. coli* strains, *Shigella dysenteriae*, *Haemophilus ducreyi*, and *Actinobacillus actinomycetemcomitans*. CDT induces progressive cell enlargement and eventual death in cultured mammalian cells. Cytotoxicity is accompanied by G_2/M cell cycle arrest. In all bacterial species (including *Helicobacter* spp.) in which CDT activity has been demonstrated, three linked genes (*cdtA*, *cdtB*, and *cdtC*) encode cytotoxic activities. CdtB has position-specific homology to type I mammalian DNases and exhibits nuclease activity *in vitro* (Lara-Tejero and Galan, 2000; Elwell et al., 2001). Mammalian cells treated with CDT have evidence of the activation of DNA repair mechanisms (Li et al., 2002; Hassane et al., 2003). There is evidence that CdtA, CdtB, and CdtC form a tripartite AB_2 toxin, with CdtB as the active A subunit and CdtA and CdtC forming a heterodimeric B subunit (Lara-Tejero and Galan, 2001).

To investigate the role of CDT in the pathogenesis of *H. hepaticus*, transposon mutagenesis was used to generate a series of isogenic mutants in and around the *cdtABC*gene cluster. An *H. hepaticus* transposon mutant with a disrupted *cdtABC* coding region no longer produced CDT activity. Conversely, a transposon insertion outside of the cluster did not affect the CDT activity. An examination of these mutants showed that CDT represents the previously described granulating cytotoxin in *H. hepaticus*. Challenge of C57BL/6 IL-$10^{-/-}$ mice with isogenic *H. hepaticus* mutants revealed that CDT expression is not required for colonization of the murine gut at 6 weeks post infection. However, a CDT-negative *H. hepaticus* mutant had a significantly diminished capacity to induce lesions in this murine model of inflammatory bowel disease (Young et al., 2004).

A cdtB-deficient *H. hepaticus* isogenic mutant (HhcdtBm7) was recently generated in our laboratory and characterized for colonization parameters in four

intestinal regions (jejunum, ileum, cecum, and colon) of outbred Swiss Webster (SW) mice. Inactivation of the *cdtB* gene abolished the ability of HhcdtBm7 to colonize female mice at both 8 and 16 weeks post infection (wpi), whereas HhcdtBm7 colonized all four intestinal regions of three of five males at 8 wpi and then was eliminated by 16 wpi. Wild-type *H. hepaticus* was detected in the corresponding intestinal regions of both male and female mice at 8 and 16 wpi (Ge et al., 2005).

Infection with wild-type *H. hepaticus* at 8 wpi was associated with significantly increased mRNA levels of ileal and cecal IFN-γ in females between wild-type *H. hepaticus*-infected and sham-dosed females. This was not noted in female mice infected with the mutant Hhcdt Bm7. In contrast, the mRNA levels of IFN-γ were significantly higher in the colon and trended to be higher in the cecum in HhcdtBm7-colonized male mice versus the sham-dosed controls at 8 wpi. In addition, mRNA levels of ileal IFN-γ were significantly higher in control females than males at 8 wpi. There were significantly higher Th1-associated immunoglobulin G2 a (IgG2 a), Th2-associated IgG1 and mucosal IgA responses in mice infected with wild type *H. hepaticus* when compared to HhcdtBm7 at 16 wpi. Colonic IL-10 expressions at 16 wpi were significantly lower in both female and male mice colonized by wild-type *H. hepaticus*, or in males transiently colonized through 8 wpi by HhcdtBm7 versus control mice. These lines of evidence indicate that: (i) *H. hepaticus* CDT plays a crucial role in the persistent colonization of *H. hepaticus* in SW mice; (ii) SW female mice are more resistant to *H. hepaticus* colonization than SW male mice; (iii) there was persistent colonization of wild-type *H. hepaticus* in the cecum, colon, and jejunum, but only transient colonization in the ileum of female mice; and (iv) *H. hepaticus* colonization was associated with down-regulation of colonic IL-10 production (Ge et al., 2005).

10.6
Enterohepatic Helicobacter spp.: Are they Co-Carcinogens?

Almost 10% of people worldwide are infected with either hepatitis B virus (HBV) or hepatitis C virus (HCV). The estimated prevalence of HCV infection is 170 million individuals, or 3% of the global population (Kane, 1995), while the number of carriers of HBV exceeds 350 million or 6% of all people. More than one million people die from HBV-associated liver failure and cancer every year (Mast et al., 1999). About 70% of individuals exposed to HCV become chronically infected, and of those 5–10% will develop fatal cirrhosis or cancer (Mast et al., 1999). Because most HCV carriers are unaware of their infection status, the true prevalence of HCV-associated disease may exceed current estimates (Poovorawan et al ., 2002). Chronic viral hepatitis of either type B or C greatly increases the risk of HCC in humans. Indeed, the vast majority of HCC diagnoses worldwide are made in people who are seropositive for HBV, HCV, or both (Ferrell et al., 2002). Although studies using HBV- and HCV-transgenic mice clearly demonstrate that viral gene products can induce tumors *a priori* (Koike et al., 2002), most humans infected with HBV or HCV are exposed to

additional agents known independently to increase the risk of HCC. Known toxins which initiate or promote HCC include the fungal-derived food contaminant aflatoxin B, alcohol abuse, and a large array of environmental and industrial chemicals (Ferrell et al., 2002). Animal models of human viral hepatitis also demonstrate additive risk for tumor development when exposed to toxins (Slagle et al., 1996; Madden et al., 2001). However, it is crucial to note that most studies involving animal models of viral hepatitis have not accounted for the possibility of confounding due to co-infection with hepatobiliary helicobacters. In recent years, enterohepatic *Helicobacter* spp. have been identified in most animal species used to model viral hepatitis, including woodchucks, mice, and nonhuman primates (Fox, 1997; Fox et al., 2001, 2002 a). To date, the only proven nonviral infectious causes of human liver tumors are biliary trematodes including *Opisthorchis viverrini* and *Clonorchis sinensis* , which increase the risk of cholangiocarcinoma (Watanapa and Watanapa, 2002).

Until recently, the potential role of bacterial infection in hepatobiliary carcinogenesis went largely unexplored. In the hepatobiliary system, an intriguing association between gall bladder cancer in Chilean women and *Helicobacter* spp. was uncovered by our group (Fox et al., 1998). This association has been reinforced by studies in our laboratory which demonstrate the pivotal role that certain enterohepatic helicobacters play in the development of cholesterol gallstones (a precursor to gall bladder cancer and noted in all patients in our Chilean study) in C57L mice fed a lithogenic diet (Maurer et al., 2005). Observational and case-control studies performed by numerous others worldwide have documented significant associations between hepatobiliary disease, including cancer, and the presence of *Helicobacter* spp. (Nilsson et al., 2000; Fan et al., 2002; Fukuda et al., 2002; Leong and Sung, 2002; Matsukura et al., 2002). Of importance, recent evidence implicates hepatobiliary *Helicobacter* spp. in severe HCV infection outcomes, including HCC (Ponzetto et al., 2000; Dore et al., 2002 b). A review of the recent literature concluded that there is strong evidence pointing to a role for human helicobacters in hepatobiliary neoplasms, but that clinical studies have been hampered by a shortage of appropriate control specimens as well as reliable biomarkers of enterohepatic *Helicobacter* infections (Leong and Sung, 2002). Intriguingly, very high rates of human HCC are reported in south-east Asia, where hepatobiliary helicobacters have also been identified (Kullavanijaya et al., 1999; Fukuda et al., 2002; Leong and Sung, 2002).

Because human studies are necessarily limited in scope, animal models can provide valuable tools for establishing etiological relationships and mechanistic pathways in the complicated step-wise progression of infection, inflammation, tissue damage, and neoplasia. There is therefore a need to characterize murine models of human co-infection with HBV, HCV or liver parasitic infections and hepatobiliary *Helicobacter* spp. Although *Helicobacter* spp. and human hepatitis viruses represent broadly divergent classes of pathogens, there are striking similarities in serum biochemical abnormalities and liver lesions between humans with viral hepatitis and mice with *H. hepaticus*, which suggests that there are similarities in the types of immune responses generated by both infections (Rogers Fox 2004). Serum transaminase activities are increased in both, though not necessarily correlated with individual disease severity. Initial random and perivenular lobular necrogranuloma-

tous histological activity precedes portal lymphocyte aggregations and interface hepatitis (Rogers et al., 2004). Hepatocellular degeneration can be manifested by hydropic and steatotic vacuolar degeneration, apoptosis, and coagulative necrosis in both diseases. Because the disease patterns are similar, it is hypothesized that HBV or HCV and hepatic *Helicobacter* infections in humans could result in an additive or synergistic fashion resulting in more severe and rapid hepatitis and tumor induction than would be induced by either agent alone (Rogers A., Fox J.G. unpublished observations).

Results from these studies may reveal heretofore unrecognized synergism between virus, bacterium, and host immunity, and suggest new therapeutic targets for the prevention and intervention of chronic hepatitis and cancer.

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11
Parasites and Human Cancers

11.1
Schistosoma Infections

Early suspicions that specific parasite infections play a role in human cancer development were discussed in Chapter 1. In 1905, Goebel postulated a causal association between *Schistosoma haematobium* infection and bladder cancer.

A total of five species of *Schistosoma* trematodes (Fig. 11.1) infect humans: *Schistosoma haematobium, S. mansoni, S. japonicum, S. mekongi,* and *S. intercalatum.* The first three represent the most important organisms, and have long been considered risk factors for cancers of the gastrointestinal tract and of the liver (Inaba et al., 1984; Chen and Mott, 1989).

Fig. 11.1 A pair of *Schistosoma* parasites. The female is kept tightly linked within the deep ventral groove or schist of the male, where it resides permanently. (SPL / Agentur Focus.)

It has been estimated that 600 million people are at risk for schistosomiasis (IARC, 1994), and that within 74 countries about 200 million are currently infected (WHO, 1993). The vast majority of human infections are due to *S. mansoni* and *S. haematobium*.

The life cycle of *Schistosoma* infections is complex (Fig. 11.2). Adult worms may persist for up to 30 years in the vesical plexus of the urinary bladder (*S. haematobium*) or in mesenteric veins (von Lichtenberg, 1987). The median life span of schistosomes amounts to three to six years (Anderson, 1987). All schistosomes produce large numbers of eggs which are excreted into the urinary bladder (*S. haematobium*) or the intestines and excreted with urine or feces. Retained eggs survive for a further three weeks and cause most of the pathological manifestations (Warren, 1978). Released embryonated eggs hatch in water and liberate the miracidium larvae. In the infected snail, sporocysts are formed from which several hundred daughter sporocysts are produced. These migrate to the digestive and reproductive tract of the snail and produce cercariae, which may infect humans. Once the cercaria has penetrated the human skin, it metamorphosizes to a "schistosomulum"; this occurs as the cercaria enters a lymphatic vessel or capillary. Further morphologic changes lead to a schistosomulum, which enters the lung and subsequently the left side of the heart (Wilson, 1987). After a few recirculations, the schistosomulum reaches the portal vein system, where a further metamorphosis occurs. Here, the mature worms pair and migrate to the final sites in the vesical plexus of the bladder (*S. haematobium*) and mesenteric veins.

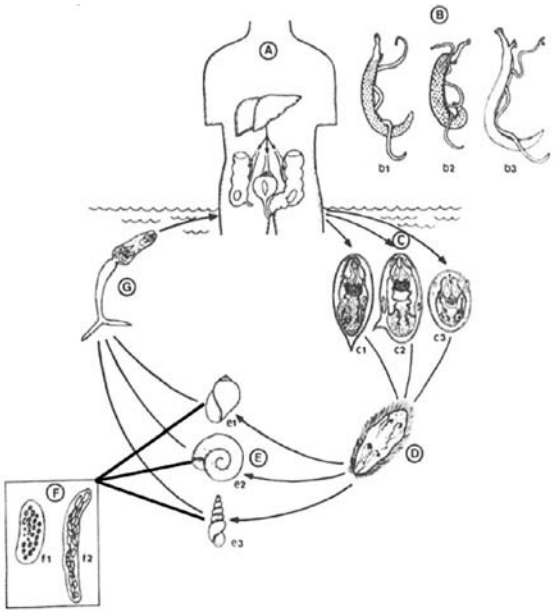

Fig. 11.2 Life cycle of *Schistosoma* infections. Illustrations b1–b3 show adult *S. haematobium*, *S. mansoni*, and *S. japonicum*, respectively. Embryonated eggs (c1–c3) of the three species, respectively, are also shown. The larvae (miracidium) infect as an intermediate host the snail species *Bulinus* sp. (*S. haematobium*), *Biomphalaria* sp. (*S. mansoni*), and *Oncomelania* sp. (*S. japonicum*), respectively. F shows the intramolluscum stage, and G a cercaria. (WHO, The Control of Schistosomiasis. Second Report of the WHO Expert Committee (WHO tech. Rep. Ser. 830), Geneva, 1993.)

11.1.1 Epidemiology

The geographic distribution of *Schistosoma* infections depends in turn on the geographic distribution of their respective intermediate snail hosts. *S. mansoni* is widely spread and occurs in 52 countries in Africa, the Eastern Mediterranean, South America, and the Caribbean (*IARC*, 1994). *S. haematobium* shares many of these areas, but does not occur in the Americas (Fig. 11.3).

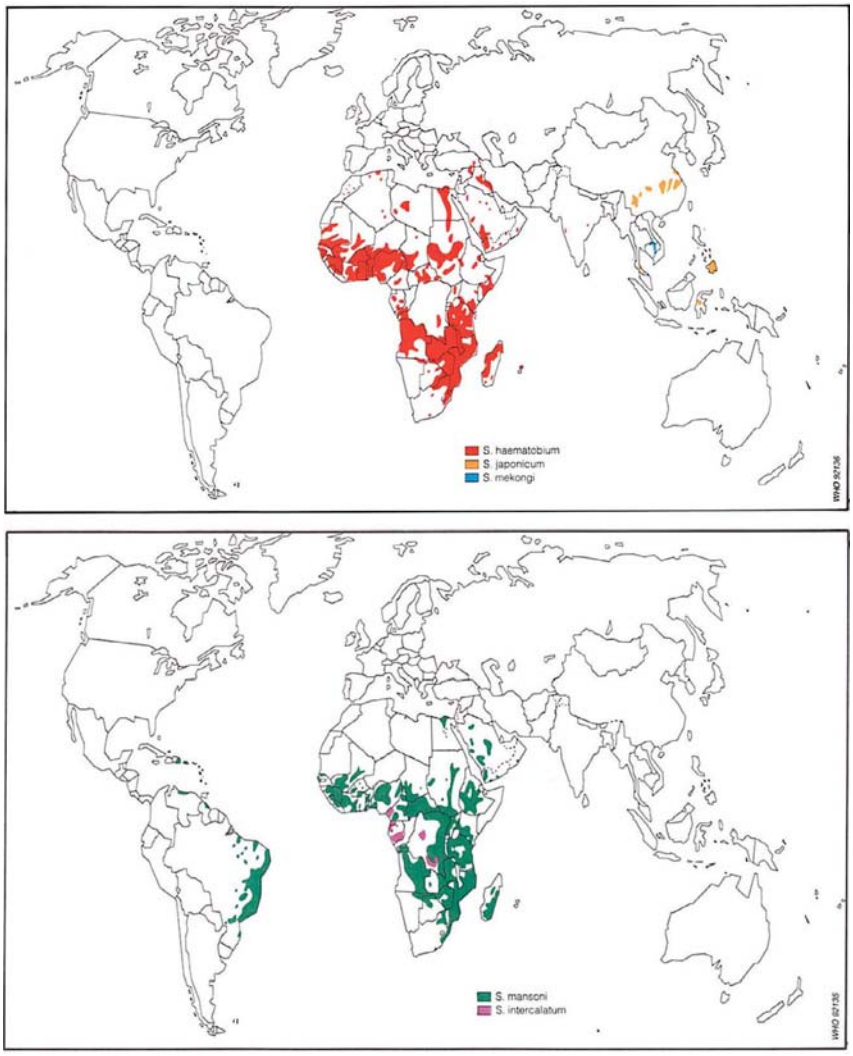

Fig. 11.3 Geographic distribution of *S. haematonium*, *S. japonicum*, and *S. mekongi* (upper panel), and of *S. mansoni* and *S. intercalatum* (lower panel). (WHO, The Control of Schistosomiasis. Second Report of the WHO Expert Committee (WHO tech. Rep. Ser. 830), Geneva, 1993.)

The main risk factor for acquiring schistosoma infections is contact with contaminated water (Jordan and Webbe, 1993), though genetic factors also seem to play a role in patient susceptibility (Abel et al., 1991). In hyperendemic regions for *S. haematobium* infection, such as lower Egypt, Zambia, and Malawi, the peak of bladder cancer incidence occurs at about the age of 45 years (El Bolkainy et al., 1981; Lucas, 1982; Elem and Purohit, 1983). In these countries cancer of the bladder is one of the most prevalent carcinomas. In non-endemic areas for schistosomiasis, bladder cancer occurs most frequently in age groups above 60 years.

A clear-cut relationship between *S. mansoni* infections and human cancer has not yet been established. Moreover, the rates of incidence for colorectal cancer in Africa do not differ significantly between endemic and non-endemic regions (Parkin, 1994).

In spite of a few positive correlations with liver cancer and cancers of the esophagus and stomach (Liu et al., 1983; Xu and Su, 1984; Chen et al., 1990; Guo et al., 1993) for areas with high rates of *S. japonicum* infections, the available data do not seem to permit firm conclusions to be made. An earlier study did not identify the reported associations (Inaba, 1984). Major problems in interpretation arise from the high prevalence of other potentially tumorigenic infections within the same areas, including *Helicobacter pylori*, hepatitis B and C, and Epstein–Barr virus.

Thus, *S. haematobium* emerges as the only well-documented infection linked to human cancer, and this will be discussed later. *S. haematobium* has been linked specifically to squamous cell carcinomas of the bladder (Lucas, 1982; Al-Fouadi and Parkin, 1984). In fact, in areas where males provide most of the agricultural labor (e.g., in the Nile Delta), the male:female ratio of bladder cancer cases may rise to 12 : 1 (Makhyoun et al., 1971). In other areas, where women perform the majority of agricultural tasks, the male:female ratio may fall to 1 : 1 or 1 : 2 (Keen and Fripp, 1980). In Egypt, a ratio of 70 : 25 : 5 has been reported for squamous cell carcinomas, transitional cell carcinomas, and adenocarcinomas (Khafagy et al., 1972). whereas in western countries the ratio is 5 : 94 : 1 (Mostofi, 1956).

11.1.2 Experimental Studies in Animals

A number of experimental studies with *S. haematobium* have been conducted in mice, rats, hamsters, opossums, and nonhuman primates (for a review, see IARC, 1994), though the data in relation to cancer induction are inconclusive. Among nonhuman primates, the exposure of capuchin monkeys to 1000–2000 cercariae resulted in papillary hyperplasia of the bladder in six of nine animals, and focal nodular hyperplasia in two animals (Kuntz et al., 1978). The infection of five baboons with 1000 cercariae, followed 2.5 years later with the chemical carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, led to polyploidy hyperplasia of the urinary tract in four animals, and to endophytic papillary hyperplasia of the ureter in one animal (Hicks et al., 1980; Hicks, 1982). In contrast, baboons treated only with the chemical carcinogen did not develop any lesions.

In *S. japonicum* infections, the induction of cancer by known chemical carcinogens is increased (Ishii et al., 1994), though many of the relevant experiments date back more than 30 years and are the results presently difficult to interpret.

One interesting aspect has been discovered by analyzing *S. japonicum* and *S. mansoni* genomes for host cell sequences following passage in mice (Imase et al., 2003). Mouse MHC class I sequences and a type A retroviral sequence were detected in the cercarial DNA of both organisms by blot hybridization; however, this observation requires further confirmation. The presence of a larger number or retrotransposons within the *Schistosoma* genome has been noted in genomic analyses (DeMarco et al., 2005).

11.1.3 *Schistosoma* Eggs and Cancer

In areas of hyperendemic *S. haematobium* infections, schistosoma eggs are regularly found in bladder tumors (el-Bolkainy et al., 1981; Lucas, 1982; Al-Adnani and Saleh, 1983; Elem and Purohit, 1983). Normally, these eggs escape in the host's excretions, but if they are retained in the host tissue they elicit immunopathological reactions (Perrin and Phillips, 1988; Perrin et al., 1989), with delayed-type hypersensitivity granulomatous reactions developing. These granulomata, which may by far exceed the size of the egg, can lead to early obstructive lesions in the lower end of the ureter. Fibrotic responses may follow, resulting in obstructive changes (WHO Report, 1987).

In this respect it remains remarkable that adult worms persist in the venous system without being recognized by the immune system of the host. No inflammatory responses are detectable at the site of their persistence. Immunity against schistosomes is mainly active against invading larvae in the skin, and anti-disease immunity, which controls abnormal fibrosis in tissues invaded by schistosome eggs (Dessein et al., 2004). Interleukin (IL)-13 in the skin and interferon (IFN)-γ in the liver seem to represent the key players in protective immunity against schistosomes. These roles relate to the high anti-fibrogenic activities of IFN-γ and to the unique ability of IL-3 in Th2 priming in the skin and in the mobilization of eosinophils in tissues (Dessein et al., 2004).

Unlike the adult trematodes, however, the eggs are recognized by the host, and sensitized lymphocytes, inflammatory cells, and antibodies each contribute to the granulomatous process (King and Mahmoud, 1992). Since *Schistosoma* egg excretion is dependent on an intact host CD4 T-helper (Th) cell response to egg antigens, eggs may have evolved to be highly immunogenic and capable of inducing potent Th responses (Pearce, 2005). The egg-induced Th response is unusual in that it is highly Th2-polarized. The selective pressure on the host to mount a Th2 response against eggs is apparent in the fact that Th2 response-defective mice develop acutely lethal disease when infected with schistosomes (Pearce, 2005). Activation of Toll-like receptor 3 was recently implicated as one of the primary events responding to immunological stimulation by *Schistosoma* eggs (Aksoy et al., 2005). Activation of this receptor resulted in the activation of NF-κB and the positive regulatory domain III-I site from the IFN-β promoter.

It seems that the inflammatory responses directed against the *Schistosoma* eggs contribute in particular to bladder cancer development in *S. haematobium* infections. It remains, however, unresolved as to what extent other factors might also contribute. Interactions with additional infections or chemical carcinogens seem also to contribute.

11.1.4 Interactions of *Schistosoma* with Other Infections, and Chemical Factors

Early suspicions that *Schistosoma* infection might interact with papillomavirus infections were mainly based on an increased incidence of cancer of the cervix in regions of high rates of *Schistosoma* infections (Petry et al., 2003). However, in the case of bladder cancer no evidence was found for any interaction with human papillomavirus (HPV) (Cooper et al., 1997).

A relatively high prevalence of bacteriuria was found in young men with schistosomiasis, and low levels of *N*-nitroso compounds were present in all specimens (Hicks et al., 1982). When the groups were subdivided on the basis of the ability of their bacterial flora to reduce nitrate to nitrite, significantly higher levels of *N*-nitroso compounds were found in *S. haematobium*-infected individuals also infected with nitrate-reducing bacteria by comparison either with uninfected controls ($p < 0.0005$) or with those infected with non-nitrate-reducing bacteria ($p < 0.001$).

Significant increases in the excretion of volatile *N*-nitroso compounds were found in *S. haematobium*-infected patients, with a mean excretion of 19.2 ± 21 μg per day of *N*-nitroso-dimethylamine (NDMA). In the control group, NDMA was detected at concentrations (mean ± SD) of 0.27 ± 0.47 μg per day (Mostafa et al., 1994). These data point to the formation of potentially carcinogenic nitrosamines in *Schistosoma*-infected patients which may either arise from metabolic processes of the trematodes or from concomitant bacterial infections. Since *Escherichia coli* contains the enzyme nitrate reductase, this infection may contribute to higher nitrite levels (El-Aaser et al., 1982; Bonnefoy and Demoss, 1994; Blasco et al., 2001). An enzyme known to be involved in activating carcinogens, namely β-glucuronidase, has been detected in high concentrations in *E. coli*-infected bladders of patients with schistosomiasis (El-Aaser et al., 1982).

Inflammatory cells, such as neutrophils, macrophages, and eosinophils, are an important endogenous source of oxygen radicals. Stimulation of these cells by tumor promoters or by foreign agents (parasites, bacteria, etc.) causes the release of reactive oxygen species (ROS). Studies on ROS formation were mainly conducted in *S. mansoni* egg-induced granulomatous inflammation (Chensue et al., 1984), or in sporocyst-carrying snails (Connors et al., 1991). In granulomatous inflammations, the formation of ROS is favored, including superoxide ions, hydrogen peroxide and hydroxyl radicals, all of which may induce lipid peroxidation (Facundo et al., 2004). In snails, a high rate of production of ROS emerges as a protective mechanism against *Schistosoma* infection, and also determines the susceptibility to infection (Bender et al., 2005). Apparently, IL-4 plays a protective role by controlling the tight regulation of the generation of reactive oxygen and nitrogen intermediates in the liver (La Flamme et al., 2001).

11.1.5
Mechanism of *Schistosoma*-Induced Cancers

Mechanistic events leading to bladder cancer in *S. haematobium* infections are still poorly understood. Clearly, these parasites have an indirect impact on the induction of malignant proliferations. In all likelihood, carcinogenic metabolites produced by concomitant infections cause mutational events at the sites of granulomatous proliferations surrounding persisting *Schistosoma* eggs. Inflammatory reactions at these sites result in the production of reactive oxygen and nitrogen intermediates which add to the mutational modifications.

Although a number of genetic modifications have been noted in cellular oncogenes in *Schistosoma*-associated bladder cancers (Ramchurren et al., 1995), no characteristic genetic changes were found in these tumors.

11.1.6
Control and Therapy

Several modes for the control of schistosomiasis are possible, with health education, the provision of safe water supplies and sanitation and the avoidance of contaminated water emerging as the most clear and effective measurements. Unfortunately, chemical elimination of the snail intermediate host causes adverse effects, in almost all circumstances, for other organic forms of life within the environment.

Effective drugs are available for treatment. Praziquantel, (2-cyclohexylcarbonyl)-1,2,3,6,7,11 b-hexa-hydro-2H pyrazino[2,1a]isoquinolin-4-one, belongs to a series of very effective antischistosomal compounds (Gönnert and Andrews, 1977). The discovery of praziquantel has been a breakthrough for the treatment of patients infected with schistosomes (Chen, 2005). Praziquantel is usually administered as a single oral dose and has no or only mild and transient side effects. The drug is highly efficacious against *S. japonicum*, whether in patients with acute and chronic stages of the infection, among subjects with extensive hepatosplenic involvement, or in patients with other complicated disease forms. Chemotherapy with praziquantel also plays a role in the transmission control of schistosomiasis, although the interruption of subsequent transmissions cannot be achieved by chemotherapy alone (Chen, 2005).

Nonetheless, by using integrated control measures, the eradication or substantial reduction of *Schistosoma* infections have been achieved in several countries (for a review, see IARC, 1994).

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11.2
Infection with Liver Flukes (*Opisthorchis viverrini, O. felineus, Clonorchis sinensis*)

The three *Opisthorchis* liver flukes represent food-borne members of the Trematode family. They establish chronic infections within the smaller intrahepatic bile ducts, more rarely in the pancreas and the gall bladder of fish-eating mammals and humans (IARC, 1994).

The life cycle of these trematodes is even more complex than that of *Schistosoma* trematodes. Infection occurs by consumption of raw or undercooked fish. The metacercaria evade from the cyst in the duodenum, enter the ampulla of Vateri, and finally reach the smaller bile ducts of the liver. They can also be found in pancreatic ducts and exceptionally within the gall bladder (Hou, 1955; Sithithaworn et al., 1991). After about one month, the adult worms have matured and produce large quantities of eggs, which are released via the bile duct into the feces. The average life span of the worm is about 10 years, but occasionally more than 25 years may be reached (Attwood and Chou, 1978). The life cycle of these trematodes is depicted in Figure 11.4. Although only a small number of snails as the first immediate host become infected in a water pond or stream, commonly 100% of the local fish subsequently carry the metacercaria (Brockelman et al., 1986). More than 80 species of Cyprinidae fish and 13 species of other fish families may serve as second intermediate hosts (Komiya, 1966; Vichasri et al., 1982; Rim, 1986; Joo, 1988).

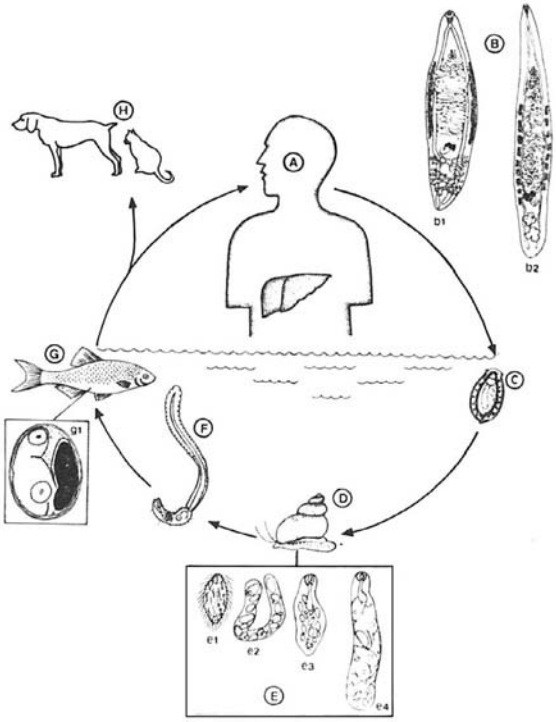

Fig. 11.4 The life cycle of liver flukes. Adult liver flukes in the bile duct (b1 = *Clonorchis sinensis*; b2 = *Opisthorchis viverrini*). C represents an embryonated egg. D is the first intermediate host, *Bithyria* sp. E represents the intramolluscan stages (miracidium e1, sporocyst e2, mother redia e3, daughter redia e4). F shows a cercaria and G the second intermediate host (cyprinoid fish). From the fish muscle (when eaten), the metacercaria (g1) reaches the final host (dog, cat, or human). (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 61 (Schistosomes, liver flukes and Helicobacter pylori). Lyon, France: IARC, 1994.)

11.2.1 Epidemiology

The worldwide distribution of the three liver fluke species is illustrated in Figure 11.5. Infections are mainly found in China, Korea, Laos, Thailand, Vietnam, Russia, Ukraine, and parts of Eastern Europe. In 1994, the World Health Organization (WHO) estimated that about 17 million people were infected by these trematodes. Of these subjects, seven million are thought to be infected by *Clonorchis sinensis*, nine million by *O. viverrini*, and 1.5 million by *O. felineus*.

Opisthorchis viverrini infections are most prevalent in North-East and those northern provinces of Thailand that border Laos (Jongsuksuntigul and Imsomboon, 1997). It is also common in the low lands of Laos (Giboda et al., 1991; Pholsena et al., 1991), although the total number of infected persons is difficult to estimate. *Opisthorchis felineus* is mainly found in Western Siberia and also along the Volga-Kama river basin (Iarotski and Be'er, 1993). Infections are also noted in the Ukraine and Kazakhstan.

Clonorchis infections are distributed throughout China, notably in regions where raw or undercooked fish is consumed (Chen et al., 1994). The infection is also frequent in Hong Kong and Taiwan (Hou et al., 1989; Chen, 1991), but it has been largely eliminated in Japan (Chen et al., 1994). Northern Vietnam and the Amur River basin in Russia also represent regions with a high rate of infections.

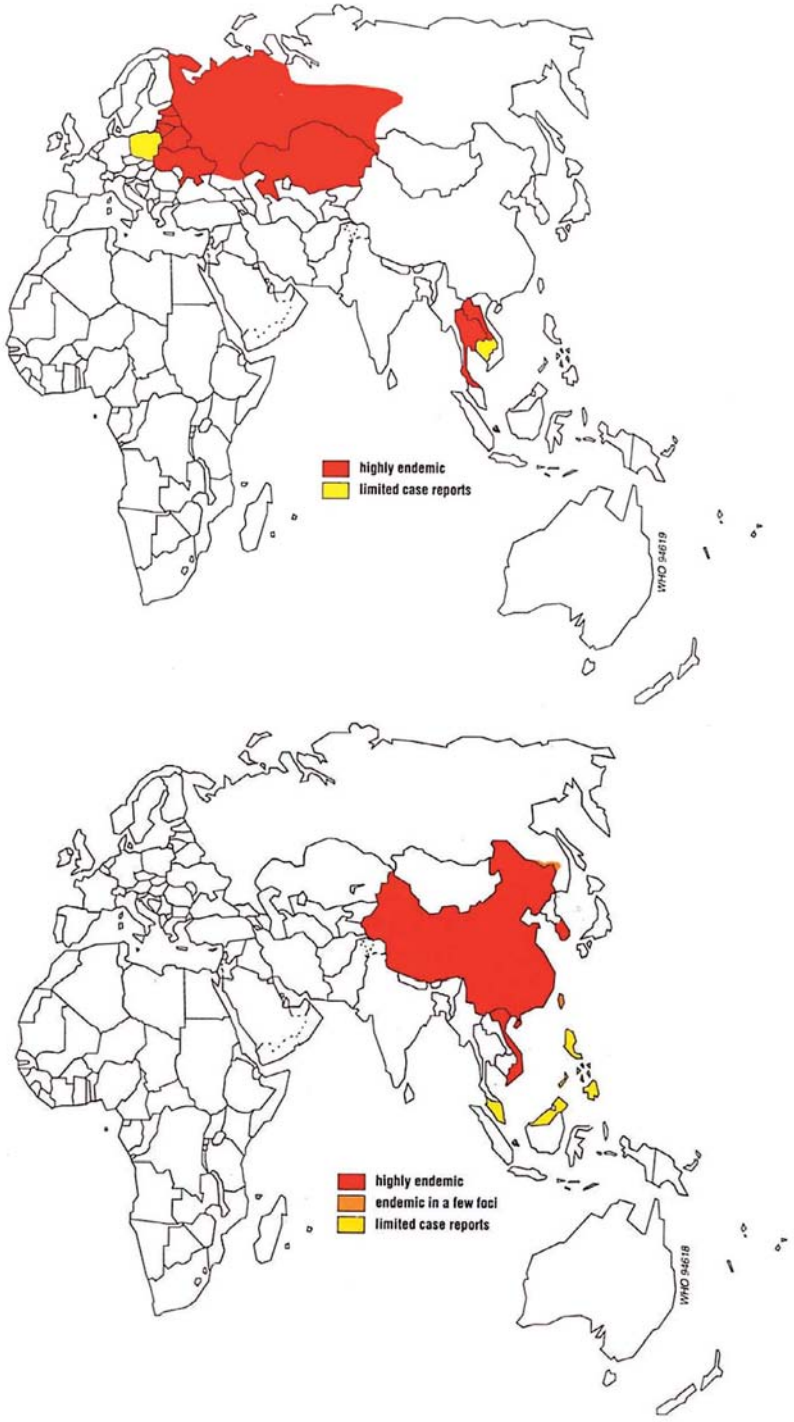

Fig. 11.5 Worldwide distribution of *Opisthorchis viverrini* and *O. felineus* (upper panel), and of *Clonorchis sinensis* (lower panel). (WHO, Control of Foodborne Nematode Infections (WHO Tech. Rep. Ser.), Geneva, 1994.)

The sole risk factor for these three infections is the consumption of raw or incompletely cooked fish, sometimes moderately or completely fermented, smoked or salted fish (IARC, 1994). Infection may occur in young children, but peaks among late teenage groups (Upatham et al., 1982; Sithithaworn et al., 1991).

11.2.2 Immune Response

A significant degree of humoral and cell-mediated immune responses to the parasite can be detected both in patients as well as in animal models (Wongratanacheewin et al., 2003). The patients' IgG levels appear to correlate with the *Opisthorchis* egg count, and decrease after treatment. A number of antigens of *Clonorchis* and *Opisthorchis* have been defined (Choi et al., 2003); the frequency and intensity of immunoblot reactions against these antigens were positively correlated with the intensity of the liver fluke infection. Even egg-negative residents of an opisthorchiasis-endemic area possess high levels of anti-*Opisthorchis* antibodies (Akai et al., 1995 a). A serological crossreactivity exists with antigens of other parasitic trematodes and possibly a broader spectrum of parasites (Sakolvaree et al., 1997). Serum antibodies persist for prolonged periods of time, even after successful chemotherapy of the fluke infections (Akai et al., 1995 b).

The immune response does not seem to influence the worm burden or the egg output, since T-cell-deprived *Opisthorchis*-infected animals produced the same egg counts as immunocompetent controls (Flavell and Flavell, 1986). The parasites survive high levels of parasite-specific immunoglobulins in both, serum and bile (Wongratanacheewin et al., 1988). The reasons for the escape of these trematodes from immunosurveillance in spite of an active immune response against parasite-specific antigens presently remain unclear.

High levels of antibodies have been observed in *Opisthorchis*-infected individuals with hepatobiliary disease and cholangiocarcinoma (Poopyruchpong et al., 1990; Haswell-Elkins et al., 1991; Mairiang et al., 1992).

11.2.3 Role of Liver Flukes in Human Cancer, and Studies in Animals

The geographic coincidence of *Opisthorchis* and *Clonorchis* infections and cholangiocarcinomas is striking. In hyperendemic regions of Thailand, the incidence rate of these tumors may reach 84.6 and 36.8 per 100 000 per year in men and women, respectively (Srivatanakul et al., 1991 a; Parkin et al., 1993). Outside of Thailand, the incidence of choriocarcinomas varies only to a limited degree (0.1 to 4.8 per 100 000 per year).

The infection of Syrian golden hamsters with metacercariae of *O. viverrini* results in acute inflammatory reactions, but usually not in cholangiocarcinomas (Flavell and Lucas, 1982, 1983). Only exceptionally were such tumors noted (Thamavit et al., 1993). The concomitant or subsequent administration of specific chemical carcinogens resulted, however, more frequently in cholangiocarcinomas. Treatment of *O.*

viverrini-infected hamsters with *N*-nitrosodimethlyamine resulted in cholangiocarcinoma induction within 23 weeks, whereas the animals receiving exclusively the chemical carcinogen did not develop tumors (Thamavit et al., 1978). Similar observations were made in other studies with the same compound (Flavell and Lucas, 1983; Thamavit et al., 1988) and with *N*-nitrosodiethylamine (Thamavit et al., 1987).

Increased endogenous nitrosation was reported in *O. viverrini*-infected patients (Srivatanakul et al., 1991 b; Haswell-Elkins et al., 1994; Satarug et al., 1996, 1998), as well as in infected hamsters (Oshima et al., 1994). It is presently unclear whether these data were the direct result of the parasitic infection, or that they originated from concomitant bacterial infections.

Cholangiocarcinomas have also been noted in *Clonorchis sinensis*-infected cats (Hou, 1964), and in a C. sinensis-infected dog (Hou, 1965).

11.2.4 Mechanism of Carcinogenicity

Opisthorchis viverrini infection induces inflammation in and around the bile duct, leading to cholangiocarcinoma in humans. In cells of the RAW 264.7 macrophage line treated with an extract of *O. viverrini* antigen, the expression of Toll-like receptor 2 (TLR-2) was induced (Pinlaor et al., 2005). This treatment also resulted in a dose-dependent induction of NF-κB, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). These results suggest that *O. viverrini* induces inflammatory response through the TLR2-mediated pathway, leading to NF-κB-mediated expression of iNOS and COX-2 (Pinlaor et al., 2005).

As discussed in Section 11.1 for *Schistosoma*-linked carcinogenesis, liver flukes in all likelihood also act as indirect carcinogens. The trematode-induced inflammation with concomitant induction of reactive NOS might be sufficient to induce mutational events which eventually lead to the outgrowth of a malignant cell clone (for reviews, see Gentile and Gentile, 1994; Oshima and Bartsch, 1994). Moreover, the available experimental data obtained from infected animals stress the syncarcinogenic potential of other carcinogens, in particular of nitrosamines.

11.2.5 Control and Therapy

Improved sanitation and health education, and persuading people not to eat raw fish, can prevent infection and reinfection (Sornmani et al., 1984; Saowakontha et al., 1993).

As in *Schistosoma* infection, a single dose of praziquantel is effective in killing the trematodes in over 90% of cases (Vivatanasesth et al., 1982; Chen et al., 1983; Viravan et al., 1986). Cured patients are, however, immediately thereafter susceptible to reinfection (Upatham et al., 1988), since they fail to develop an immune response.

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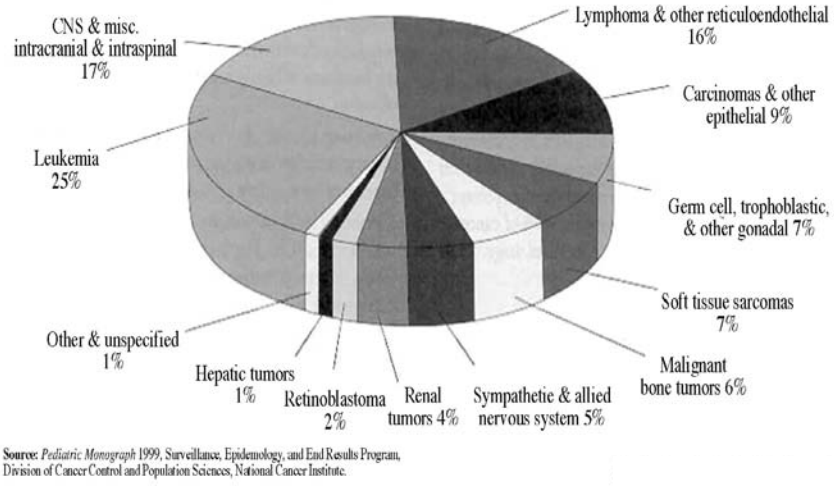
12
Cancers with a Possible Infectious Etiology

12.1
Leukemias and Lymphomas

An increased risk for leukemias for persons exposed for prolonged periods to X-irradiation or exposure to radioactive irradiation is well established. A broad spectrum of additional risk factors has been reported for non-Hodgkin lymphoma (NHL) (reviewed by Fisher and Fisher, 2004), and most of these seem also to account for acute lymphoblastic leukemias and Hodgkin's disease. Yet, the etiology of spontaneously occurring human leukemias and lymphomas remains presently poorly understood. In the past, some hints have pointed to a possible involvement of infectious agents, particularly in childhood leukemias and lymphomas. In the United States, these two malignancies constitute approximately 41% of childhood cancers; the distribution of childhood cancers in the United States is shown in graphically Figure 12.1.



Source: *Pediatric Monograph* 1999, Surveillance, Epidemiology, and End Results Program, Division of Cancer Control and Population Sciences, National Cancer Institute.

Fig. 12.1 Distribution of childhood cancers within the United States (ages 0–19 years). (Pediatric Monograph 1999, Surveillance, Epidemiology, and End Results Program, Division of Cancer Control and Population Sciences, National Cancer Institute.)

A number of specific chromosomal translocations have been identified in various types of human leukemias and lymphomas (for a review, see Greaves and Wiemels, 2003), commonly involving transcription factors. Their induction, together with several epidemiological observations published during the past three to four decades, still require an explanation. Infectious agents have been repeatedly suspected to be involved in the etiology of leukemias and lymphomas, yet specific infectious agents have been identified in only a relatively small percentage of these neoplasias. Epstein-Barr virus (EBV) occurs in approximately 50% of B-cell lymphomas arising under immunosuppressive conditions, preferentially also in the endemic form of Burkitt's lymphomas and in a small subset of T-cell lymphomas. As outlined in previous chapters, human herpesvirus type 8 contributes to rare primary effusion lymphomas, and HTLV-1, a retrovirus, is responsible for adult T-cell leukemias occurring in endemic in coastal regions of Southern Japan. Indirect evidence points to a role of a bacterial infection (i.e., *Helicobacter pylori*) in specific gastric lymphomas, the MALT-tumors.

Thus, although some causative agents have been identified, there is still no explanation available for the majority of these malignant proliferations. Indeed, for certain of the virus-positive tumors, such as EBV-positive Burkitt's lymphoma within the African tumor belt, an explanation of the epidemiological pattern poses substantial problems.

12.1.1 Epidemiological Data

Epidemiological data are based on changes in cancer incidence, case/control and prospective studies, as well as on space-time clustering of leukemias and lymphomas. A general decline in cancer incidence has been noted in the United States, most likely related to a reduction in smoking. In contrast to the general trend, the incidence of NHL, and also of melanomas, non-melanoma skin and liver cancers, has increased substantially between 1991 and 1995 (McKean-Cowdin et al., 2000). In fact, the SEER statistics reveal approximately twice as many cases of NHL in the year 2000 when compared to 1973 (SEER, 2003) A slight increase was also noted within the same period for acute lymphoblastic leukemia (Figs. 12.2 and 12.3). The rate of other leukemias and of Hodgkin lymphomas remained more or less constant during that period.

The dramatic increase in NHL incidence has mainly been observed in developed parts of the world, although the AIDS epidemic resulted in a sharp rise of these lymphomas also in developing countries (Devessa and Fears, 1992). In the United States, the incidence in Caucasians was approximately 30% higher than in African Americans up to the year 2000 (Fig. 12.2). The rate of all other leukemias and lymphomas, with the exception of chronic myelogenous leukemia, was lower in socially less privileged populations than in affluent societies (SEER, 2003).

A larger number of publications appeared during the past 25 years, describing space-time clustering of childhood leukemias and lymphomas. In 1981, Hamadeh et al. analyzed a possible clustering of leukemias, Hodgkin's disease and other lym-

phomas in Bahrein by comparing 125 cases with matched controls. These authors found a trend for some clustering of cases in urban areas. Districts far away from large urban centers of higher socioeconomic status revealed an excess risk of childhood acute lymphoblastic leukemia (ALL) (Alexander et al., 1990; Pearce et al., 2004). A larger number of these studies were conducted in Great Britain (Alexander, 1992; Alexander et al., 1993; Knox, 1994; Gilman and Knox, 1995; Gilman et al., 1999; Birch et al., 2000; McNally et al. 2002), showing evidence for space-time clustering of ALL and Hodgkin's lymphoma. Similar data were also reported from Sweden (Gustafsson and Carstensen, 2000), Greece (Petridou et al., 1996), Italy (Magnani et al., 2003), from the EUROLOCUS project carried out in 17 defined geographic regions of Europe (Alexander et al., 1998), from Israel (Chen et al., 1997), from San Francisco (Philippe, 1999), and from Hong Kong (Alexander et al., 1997; Chan et al., 2002). Spatial clustering was found particularly for childhood leukemias in children aged up to 4 years (Petridou et al., 1996; Alexander et al., 1997). Local increases in areas of high population mixing have also been described (Kinlen et al., 1993; Kinlen, 1995). Negative results for time-space distribution of childhood leukemia was reported in two small studies from the Netherlands (van Steensel-Moll et al., 1983) and from New Zealand (Dockerty et al., 1999).

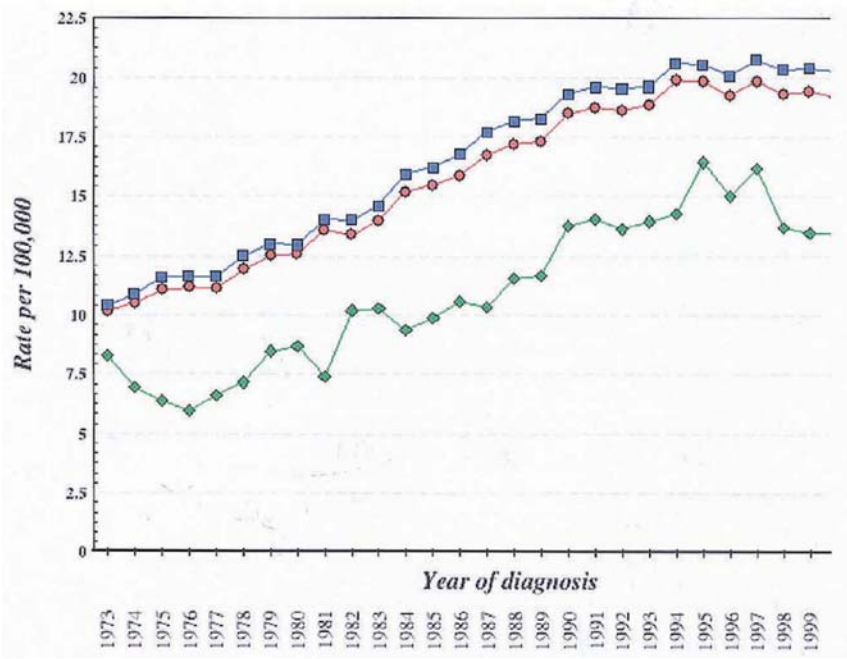


Fig. 12.2 SEER statistics for 1973–1999 for non-Hodgkin lymphoma. Red line: all races. Blue line: white. Green line: black. (National Cancer Institute, SEER Program. Statistics for 1973–1999. http://canques.seer.cancer.gov/)

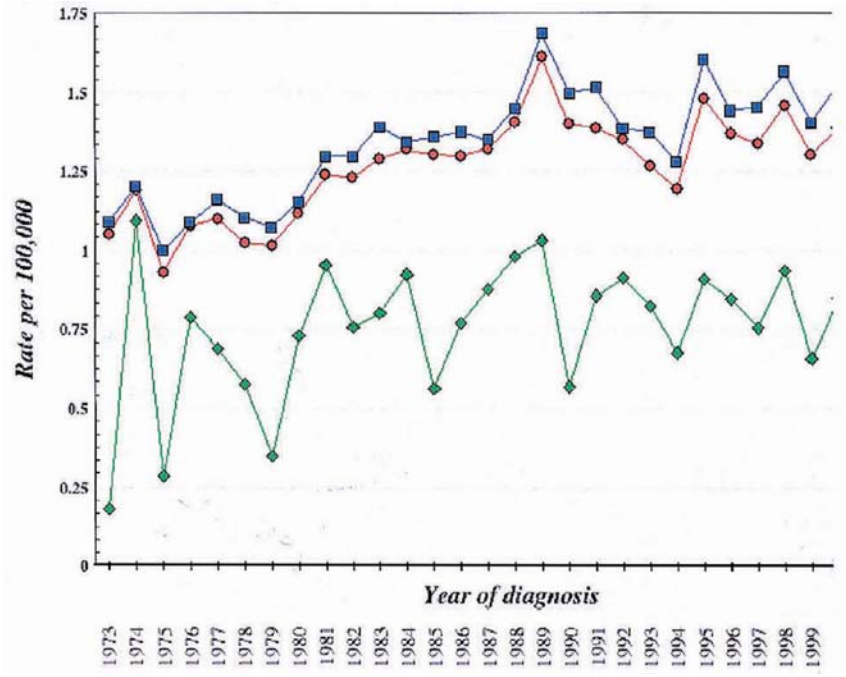


Fig. 12.3 SEER statistics for 1973–1999 for acute lymphatic leukemias. Red line: all races. Blue line: white. Green line: black. (National Cancer Institute, SEER Program. Statistics for 1973–1999. http://canques.seer.cancer.gov/)

The vast majority of all the reports observing space-time clustering of childhood ALL interpret these findings as indirect evidence for a transmissible agent (Alexander, 1992; MacMahon, 1992), and that such transmissible agents may share some of the epidemiological characteristics of herpesviruses (Alexander, 1993). There exist additional observations which have been interpreted as indirect evidence pointing in the same direction. This concerns several risk factors that have been identified, linked particularly with ALL and NHL. These involve agricultural occupations (Milham, 1971; Blair et al., 1985; Saftlas et al., 1987; La Vecchia et al., 1989; Eriksson and Karlsson, 1992; MacMahon, 1992) and contact with cattle (Pearce et al., 1986 a,b; Assenato et al., 1995; Metayer et al., 1998; Fritschi et al., 2002; Becker et al., 2004). Several authors also report an elevated risk for these neoplasms in butchers, abattoir workers and meat cutters (Johnson et al., 1986; Pearce et al., 1988; Metayer et al., 1998). Recently, a case-control study involving 5900 subjects from seven European countries, after adjusting for smoking, revealed an increased risk for lung cancer after exposure to meat aerosols and even more so after exposure to live animals (Durusoy et al., 2006). An increased risk for colon cancer after red meat consumption was attributed to the formation of DNA adducts of O6-carboxymethyl guanine (Lewin et al., 2006). Since exposure to live animals reveals an even higher risk, at least for lung cancer, this interpretation does not seem to hold up for this malig-

nancy and has been interpreted in favor of an infectious agent (Durusoy et al., 2006). Additional incriminated factors were occupational exposure to pesticides, industrial paints, and solvents, hair dyes, the leather industry (Scherr et al., 1992; Pearce and Bethwaite, 1992), and forestry work (Feychting et al., 2001). The latter study failed to confirm pesticides and paints as risk factors for leukemias. There are no clear-cut data available pointing to a significant effect of specific paternal occupations and subsequent development of childhood leukemias. Increased birth weight seems to represent another risk factor for developing acute lymphoblastic leukemia during the first four years of life, as shown in several publications (Kaye et al., 1991; Murray et al., 2002; Ou et al., 2002; Hjalgrim et al., 2004). The risk for ALL was also higher among children of older mothers and fathers (Dockerty et al., 2001).

Interestingly, a number of protective factors have been reported to reduce the risk for these hematological malignancies. Several epidemiological studies demonstrate a protective effect of whole-day care of children in the age group 0 to 4 years, and of intermittent infections in this time period for leukemias (van Steensel-Moll et al., 1986; Greaves and Alexander, 1993; Petridou et al., 1993; McKinney et al., 1999; Infante-Rivard et al., 2000; Perrillat et al., 2002; Jordan-Da Silva et al., 2004), lymphomas (McKinney et al., 1987), Hodgkin's disease (Pfaffenbarger et al., 1977; Chang et al., 2004), and neuroblastomas (Menegaux et al., 2004). Other studies, unable to confirm this directly, quoted significant effects of other socioeconomic parameters increasing the risk for the respective diseases, such as parameters suggestive of a protected environment in early childhood in the case of leukemias (Roman et al., 1997; Smith et al., 1998; Pearce et al., 2004), or a higher level of education in Hodgkin's lymphoma (Serraino et al., 1991). Two reports could not find any association with day care and early infections (Neglia et al., 2000; Rosenbaum et al., 2000). A marked inverse association of leukemia risk with birth order was first noted in 1966 (Stark and Mantel, 1966; Shaw et al., 1984; Dockerty et al., 2001; Hjalgrim et al., 2004). Correspondingly, children living in more crowded households had a substantially lower risk than children born into less crowded homes (Murray et al., 2002). Three reports also found an inverse association between alcohol intake and NHL risk (Nelson et al., 1997; Chiu et al., 1999; Morton et al., 2005). Very recently, a case-control study involving 6305 children (aged 2–14 years) without cancer and 3140 children with cancer (diagnosed in the United Kingdom, 1991–1996), of whom 1286 had ALL, showed that increasing levels of social activity were associated with consistent reductions in risk of ALL (Gilham et al., 2005). Interestingly, even children with non-ALL malignancies (mainly central nervous system tumors, other leukemias, NHL and Hodgkin's lymphoma) revealed a similar pattern. The greatest reduction for risk of ALL was seen in children who attended formal day care during the first three months of life. The established risk, as well as protective factors, are summarized in Figures 12.4 and 12.5.

It has been argued that there exists a biological heterogeneity of childhood leukemia, and that a family of related malignancies is derived from different stem cells in the hematopoietic hierarchy (Greaves, 1999). This would render it *a priori* unlikely that even a well-defined hematologic subtype has an exclusive etiology (Greaves, 2000). This seems to account for environmental chemical and physical

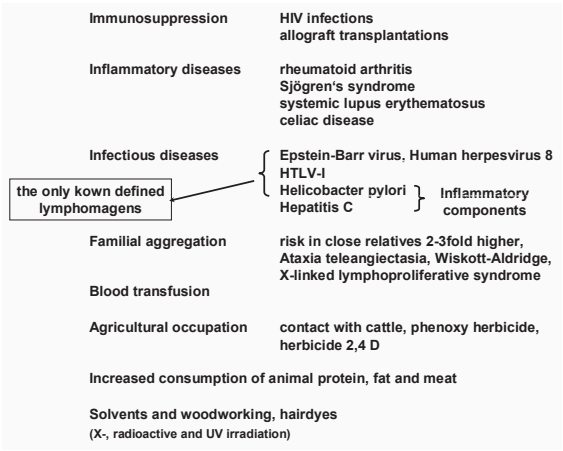


Fig. 12.4 Reported risk factors for non-Hodgkin lymphoma.

Fig. 12.5 Reported protective factors against childhood leukemias, lymphomas, and Hodgkin's disease.

carcinogenic factors. Among infectious agents, EBV is an excellent example to demonstrate that the same agent is able to infect and transform cells at different stages of differentiation (see Chapter 4, Section 4.3.1).

The observed protective effects of early infections and whole-day care and the observations on space-time clustering of childhood leukemias and lymphomas stimulated speculations arguing for an infectious etiology of childhood hematopoietic malignancies (Greaves, 1988, 1997; Alexander, 1992, 1993; Greaves and Alexander, 1993; Kinlen et al., 1993; Kinlen, 1995; Alexander et al., 1998; Smith et al., 1998; Pearce et al., 2004). Two hypotheses have been developed along these lines: Greaves (1988, 1997; Greaves and Alexander, 1993) speculated that delayed exposure to common infections results in an increased risk for childhood leukemia, especially of the pre-B acute lymphoblastic leukemia. According to Greaves' view, a first step takes place during pregnancy, where the occurrence of a specific chromosomal translocation leads to the appearance of a rearranged B-cell precursor clone (Greaves and Wiemels, 2003). As a second event, Greaves postulated an unspecified common infection and labeled it the "delayed infection hypothesis" (Greaves, 2000). According to this hypothesis, the pattern and timing of infection in early life would be of critical importance in relation to the developmental programming of the immune system. Early infections educate the immune system and result in an expansion and elimination or suppression of T-cell subsets or clones. In the absence of early exposures,

the immune system may remain un-modulated and un-educated. Later infections may result in abnormal immunological responses that increase the risk for leukemia.

Kinlen developed a different interpretation: he hypothesized that mixing of a population of low exposure to a putative leukemogenic agent (anticipated for rural residences with low population density) with another population originating from urban areas previously highly exposed to the incriminated agent, could promote an epidemic of the relevant infection (Kinlen et al., 1993, Kinlen, 1995). Examples would be represented by oil drilling or industrial plant development in rural areas with a sudden influx of people from urban areas. Kinlen noted under these circumstances a higher than expected incidence of childhood leukemias. The lack of an increase in childhood leukemias in the context of massive evacuations of mothers and children during the Second World War (MacMahon, 1992) could, however, argue against these considerations.

An additional hypothesis speculating on an infectious origin of childhood leukemias and lymphomas was developed based on some virologic data (zur Hausen and de Villiers, 2005). This was triggered by the analysis of a possible role of a recently discovered virus family, the so-called TT viruses (Nishizawa et al., 1997), in human malignant proliferations. This virus group reveals a remarkable heterogeneity of genotypes (Okamoto et al., 2002; Peng et al., 2002) varying in genome size between 2100 and 3800 nucleotides in a single-stranded circular genome (Takahashi et al., 2000; Okamoto et al., 2002). A substantial variation also exists in the amino acid composition of putative proteins derived from the various open reading frames (ORFs) of the virus. TT viruses are widely spread among all human populations (Abe et al., 1999; Itoh et al., 1999; Jelcic et al., 2004). They replicate preferentially in bone marrow cells (Okamoto et al., 2000), and cells of the hematopoietic system act as a reservoir for these viruses (Takahashi et al., 2002).

Initial attempts to detect TT viruses resulted in a high rate of detection of viral DNA in human cancers, particularly in gastrointestinal tumors, in breast and lung cancer, as well as in leukemias and multiple myelomas (de Villiers et al., 2002). However, these experiments could not exclude a contamination of the tumor material with TT virus-positive cells of lymphatic origin. In addition, it was disturbing that TT virus-DNA was not detected in tissue culture lines derived from tumor types with a high TT virus presence.

Surprisingly, the analysis of a single heavily infiltrated spleen biopsy from a patient with Hodgkin's lymphoma showed 24 different TT virus genotypes (Jelcic et al., 2004). The cloned viral DNA revealed a remarkable heterogeneity, particularly within two viral clades: one contained substantial variations within three regions of the long ORF, whereas several clones of the second clade had stop codons within the same ORF, resulting in the formation of new ORFs. The variation in genotypes points to a remarkable heterogeneity and instability of TT virus genomes.

Recently, a short stretch of TT-like viral DNA was found in several cell lines of lymphatic origin and derived from other tumors: EBV-negative lines derived from Hodgkin's lymphoma and of other leukemic and lymphatic origin were found to contain this DNA (zur Hausen and de Villiers, 2005; E.-M. de Villiers et al., unpub-

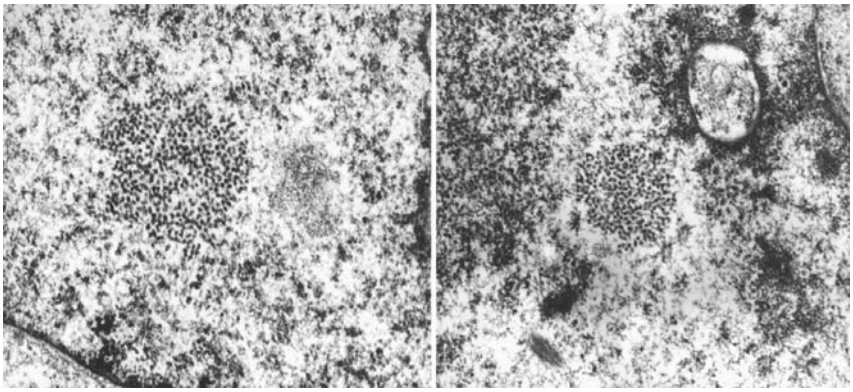


Fig. 12.6 Virus-like intranuclear particles within a cell of the Hodgkin's lymphoma line L1236 (left) and a Jurkat cell (right).

lished results). This sequence was cloned with the aid of consensus primers and identified to represent the most highly conserved region of the TT virus genome (Peng et al., 2002). Several of these cell lines contained nuclear virus-like particles in very rare occasional single cells, which corresponded in size with TT viruses (Fig. 12.6). Some of the structures revealed also tubular forms, suggesting cylindrical structures or an abnormal aggregation of proteins to specific areas of cellular chromatin (Fig. 12.6) (H. zur Hausen and R. Schmidt, unpublished data). It is not clear whether these particles have indeed a viral origin or may represent nuclear speckles (Huang and Spector, 1992).

The use of primers to amplify the newly identified TT virus-like sequences occasionally resulted in the amplification of sequences involved in translocation events (e.g., *TEL-AML*) or of sequences of the dgl tumorsuppressor gene coding for a protein which interacts with the APC protein (Makino et al., 1997). These results formed the basis for a hypothesis which proposed a novel mechanism for the possible involvement of infectious events in the etiology of hematopoietic malignancies, and for a novel role of specific viruses in human carcinogenesis (zur Hausen and de Villiers, 2005).

12.1.2 The Target Cell Conditioning Model

According to this model, the initial event potentially leading to leukemia and lymphoma development is triggered by a ubiquitous virus infection acquired either prenatally or perinatally. Transplacental or perinatal infection results in virus persistence, as observed in previous studies for established TT virus genotypes (Saback et al., 1999; Goto et al., 2000; Matsubara et al., 2001). The expansion of latently infected cells during the course of a newborn child's development in the first year of life (Peng et al., 2002) should result in an increasing number of infected cells and, correspondingly, to a steadily increasing viral load. For TT viruses it has been reported that viral concentrations increase with age (Saback et al., 1999). Existing par-

tial homologies of this viral DNA with host cell genes frequently engaged in translocations of leukemic or lymphoma cells should increase the risk for specific translocations during the course of viral and cellular DNA replication proportional to the number of viral genome-positive cells. Occasionally, such translocations are also found in healthy individuals, as for example described for the lymphoma-associated translocation t(14;18) (Limpens et al., 1995). Intermittent infections by other viruses (e.g., influenza, measles, rubella, mumps or other respiratory or gastrointestinal infections) result in interferon synthesis, blocking the production of persisting agent and thus reducing the respective viral load. Interferon treatment indeed reduces for instance the TT virus level, as described by Akahane et al. (1999). Frequent infections in early childhood consequently lead to a low load of the incriminated agent and to a reduced level of virus persistence in hematopoietic cells. This in turn should reduce the risk for specific translocations. Absence or a low number of early childhood infections should have the opposite effect and increase the respective risks; this is outlined schematically in Figure 12.7.

Second events, caused either by further translocations or by infection with specific viruses (possibly as yet unknown herpesviruses) mediate the conversion to a malignant phenotype. This is exemplified in the case of EBV-positive cases of Hodgkin's disease, where expression of EBNA-1, LMP-1 and LMP-2 of EBV seems to provide the "second hit". The infection of previously "conditioned target cells", conditioned by the induction of specific translocations, provides here the basis for malignant conversion. It seems to be an interesting footnote that reported roseola and/or fever and rash during the first year of life reduced the risk for ALL (OR = 0.33 [95% CI 0.16, 0.68]), whereas tonsillitis during the period 3–12 months before the reference date increased the risk (OR = 2.56 [95% CI 1.22, 5.38] (Chan et al., 2002). Infectious mononucleosis and apparently also some HHV-8 infections frequently start with tonsillitis-like symptoms (Veltri et al., 1975; Ryan et al., 2004; Chen et al., 2004).

This model does not necessarily relate to TT viruses of TT-related agents. Endogenous retroviruses, activated during the prenatal or perinatal period, would also fit into this model. It readily explains the protective effect of intermittent infections in early childhood occurring at higher frequency in whole-day care conditions. Thus, a highly protective environment during this time period in affluent societies emerges

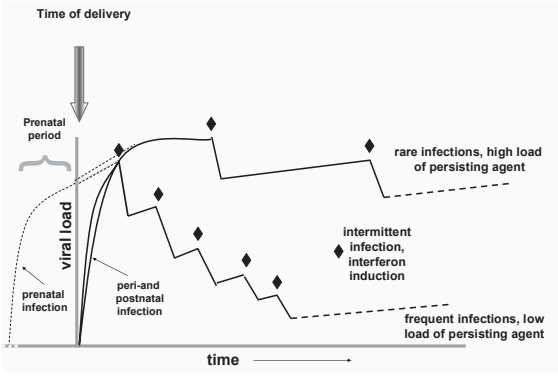


Fig. 12.7 Scheme of the target cell conditioning model hypothesis.

as a risk factor for hematological malignancies and could explain (and even predict) the observed increase in those malignant conditions in such populations.

Another aspect originates from the observed higher risk of agricultural occupations and exposure to cattle. It is possible that at least some genotypes of TTV-like agents found in human malignancies or tumorviruses of bovine origin are shed by cattle or are transmitted to humans by beef products (dairy products, meat, leather materials or close contact with cattle) (zur Hausen, 2001). They may also establish persistent infections in humans. For example, TT virus sequences have been reported in 25% of the cows analyzed (Leary et al., 1999).

Remarkable differences exist in the epidemiology of individual types of lymphomas and leukemias; this is most pronounced for the pattern of Burkitt's lymphomas. These tumors occur as an early childhood disease within the African equatorial "tumor belt" and are mainly EBV-positive. Outside of this region, Burkitt's lymphoma is rare and the majority of tumors are EBV-negative. It could be postulated that a specific agent endemic in these high-risk Burkitt's lymphoma regions is involved in preferentially inducing the c-*myc*/immunoglobulin gene rearrangements. At least in theory, it could be transmitted from wild animals (e.g., wild buffalos, specific antelopes) to cattle, sheep or goats in common grazing areas where Burkitt's lymphoma is endemic. Persistent infections of humans with such agents, followed by severe EBV infections, would lead to malignant conversion of modified cell clones. Indeed, emerging evidence points to a role for specific EBV genes in the maintenance of the malignant phenotype of EBV-positive Burkitt's lymphoma cells (Kennedy et al., 2003; Sheng et al., 2003). Outside the high-risk regions, similar translocations may be mediated by related agents at a much lower frequency. Support for this speculation could be derived from differences in the breakpoint of the c-*myc* gene at chromosome 8q24 between endemic and sporadic cases of Burkitt's lymphoma (Shirimizu et al., 1991; Ambinder and Griffin, 1991). A previously discussed alternative interpretation implying holo-endemic *Plasmodium falciparum* malaria as the first triggering event (Burkitt, 1962; Magrath, 1990) is difficult to reconcile with the existence of regions with a high prevalence of malaria and low Burkitt's lymphoma incidence in south-east Asia.

Although the model presented here is a hypothesis, it could explain some of the epidemiological characteristics of various hematological malignancies which have been poorly understood until now. Moreover, it provides a model for a novel mechanism by which persistent infections may contribute to the development of human cancers. It could support previous speculations on a possible role of transmission of tumor-inducing agents from farm animals and pets to humans (zur Hausen, 2001). Models should stimulate further experimentation and hopefully promote a better understanding of the etiology of these frequently fatal childhood malignancies. The numerous epidemiologic data quoted before demand an explanation. They are suggestive for a role of infectious events in these malignancies.

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12.2
Human Breast Cancer

A number of viruses have been reported to be involved in human breast cancer, among them Epstein–Barr virus, cytomegalovirus, and various papillomavirus types. The published data have not been confirmed in other studies and, thus, remain difficult to interpret.

For more than 30 years reports have been appearing which claiming the presence of an agent which is closely related to mouse mammary tumor virus (MMTV) in human breast cancer patients and in high-risk populations for breast cancer. The non-Mendelian maternal inheritance of mammary cancer in high-incidence laboratory strains of mice suggested, at an early stage, the involvement of an extrachromosomal agent in the etiology of this cancer (Staff of the Roscoe B. Jackson Memorial Laboratory, 1933). Bittner (1936) subsequently showed that an agent was passed from the mother to offspring with the milk. Offspring from low-incidence mothers foster-nursed on high-incidence mothers developed mammary cancer at a higher rate (DeOme, 1940). A detailed electron microscopic description of the virus particles followed in 1948 (Porter and Thompson), and the virus was later identified as a member of the retrovirus family.

One interesting aspect which came out of MMTV research was that its horizontal transmission via milk was accompanied by germline Mendelian inheritance (Bentvelzen, 1975). Viral genomes persist as endogenous DNA in more than 25 copies per mouse genome (Kozak et al., 1987). Another interesting observation was the detection of preferred sites for MMTV integration in the genomes of mammary tumors after exogenous infection (Nusse and Varmus, 1982). Direct stimulation of MMTV expression by hormones was suggested by a high number of MMTV particles in mammary tumors from late pregnant and lactating infected mice when compared

to tumors from nonlactating infected mice (Hairstone et al., 1964). Subsequently, multiple glucocorticoid response elements were discovered within the long terminal repeat (LTR) promoter regions of MMTV (Chandler et al., 1983; Payvar et al., 1983).

The pathway of MMTV from the infected gut to the mammary glands has been clarified during the past two decades. Manifestation of the infection in B lymphocytes of intestinal Peyer's patches leads to transfer of the virus to T lymphocytes which eventually transfer the virus to mammary epithelial cells (Tsubara et al., 1988). The MMTV 3' LTR sequence encodes a superantigen that orchestrates the multiplication of T and B lymphocytes, resulting in an amplification of virus-producing cells capable of delivering the infection to the mammary gland (for reviews, see Matzuzawa et al., 1995; Ross, 1998). Immunosuppression thus, as the most interesting consequence of these observations, does not facilitate MMTV-mediated mammary carcinogenesis but has a slight protective function (Fig. 12.8). This is in remarkable contrast to most other virus-caused cancers, the emergence of which is commonly enhanced under conditions of immunosuppression (see Chapters 4, 5, and 7). Since the incidence of human breast cancer is also under hormonal control and is not increased under immunosuppression, these data might suggest an analogous mechanism in the development of the human malignancy.

During the late 1960s and the 1970s, a number of reports appeared claiming the detection of MMTV-like particles in women from families with a history of breast cancer and in ethnic groups with a high breast cancer incidence (Moore et al., 1969, 1971; Chopra et al., 1973). A simultaneous detection test permitting the demonstration of reverse transcriptase activity linked to supposedly viral 60–70 S RNA genomes was also reported to be positive for specific fractions of human milk and breast tumors (Schlom et al., 1971, 1972; Axel et al., 1972; McGrath et al., 1974). Subsequent studies produced conflicting results (Wooding, 1972; Calafat and Hageman, 1973), while one epidemiologic survey did not reveal any differences in breast cancer rates between breast- or bottle-fed babies (Henderson, 1974). For a few years no additional data were obtained in support of an exogenous virus hypothesis in human breast cancer.

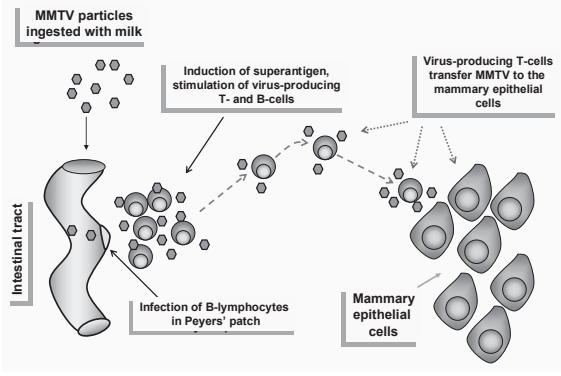

Fig. 12.8 Schematic outline of mouse mammary tumor virus (MMTV) transfer to the mammary gland epithelial cells, which may be transformed after specific integration of the viral genome.

Since MMTV persists as an endogenous agent, and endogenous human retroviruses were discovered which belonged to the same retrovirus subfamily as MMTV (HERV-K) and also possessed a glucocorticoid-responsive element in the LTR region, interest in a possible role for these genomes in human breast cancer was resurrected during the late 1980s. Particles containing HERV-K-related sequences are released from a human breast cancer cell line T47-D (Ono et al., 1987; Patience et al., 1996; Seifarth et al., 1995, 1998), their production is up-regulated by glucocorticoids, the sequences in the particles are highly defective, and the *env* gene is not transcribed. Serologic studies provided ambiguous data with HERV-K antigens (Vogetseder et al., 1993; Boller et al., 1997), although one report referred to MMTV-related antigen in particularly aggressive breast cancers of Tunisian women (Levine et al., 1984). Thus, a relationship between endogenous retrovirus expression and human breast cancer is far from being established.

In recent years there have been several reports of bona fide MMTV sequences in a high percentage of human breast cancers (Wang et al., 1995, 1998). Initially, a 660-bp sequence with 98% homology to the MMTV *env* gene was amplified from 40% of breast cancer biopsies, but from only 2% of normal donors. In 66% of positive samples the env sequence was also transcribed (Pogo et al., 1999; Liu et al., 2001; Ford et al., 2003; Faedo et al., 2004). Even a MMTV-like superantigen coded for by the MMTV-like LTR was described in human breast cancer (Wang et al., 2004). Infection of human mammary cells by MMTV has been demonstrated in tissue culture studies (Katz et al., 2005; Indik et al., 2005). Other groups have been unable to confirm the presence of MMTV-like sequences in human breast cancer (Zangen et al., 2002; Mant et al., 2004).

Although the evidence linking human breast cancer to infectious events remains scarce, it should be re-emphasized here that there exists in one other aspect a striking similarity to MMTV-linked mammary carcinoma development in mice: this is the remarkable absence of increased breast cancer incidence under conditions of immunosuppression. One report has even described a protective effect for breast cancer development in organ xenograft recipient women maintained under long-term immunosuppression (*Stewart et al.*, 1995). The reason is well established for the MMTV system, where immunosuppression reveals a protective effect. For humans, however, further studies should establish similar effects in human breast cancer.

12.3 Other Human Cancers Possibly Linked to Infectious Events

Several cancers develop on the basis of chronic inflammation; these seem to include prostate cancer, thyroid cancer, cancers developing in chronic skin ulcerations, and many others. The evidence for specific events in the development of these tumors is presently non-existent, and so will not be discussed here. One possible exception is that cutaneous lymphomas rarely develop at sites of chronic *Borrelia burgdorferi* infections (for reviews, see Grange et al., 2002; Bogle et al., 2005). In view of the

frequency of *Borrelia* infections these complications must be exceedingly rare, while the possible mechanism is poorly understood.

Research into infectious causes of human cancer has been more than rewarding during the past 40 years. Nonetheless, it would be very surprising if further links between specific infectious agents and human malignancies were not identified during the next few years.

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