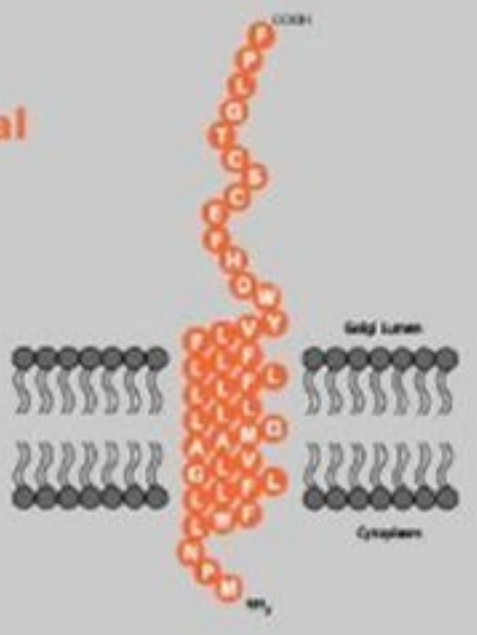
Monographs in Virology
Editor: H.W. Doerr
Vol. 23

Mechanisms of DNA Tumor Virus Transformation

Editor
L.J. Rosenthal



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Monographs in Virology

Vol. 23

Series Editor

H.W. Doerr *Frankfurt*

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Mechanisms of DNA Tumor Virus Transformation

Volume Editor

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24 figures and 2 tables, 2001

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Library of Congress Cataloging-in-Publication Data

Mechanisms of DNA tumor virus transformation / volume editor, Leonard J. Rosenthal.
p.; cm. – (Monographs in virology, ISSN 1422–2132; vol. 23)
Includes bibliographical references and indexes.
ISBN 3805572476 (hard cover : alk. paper)
1. Oncogenic viruses. 2. Viral cell transformation. 3. Viral carcinogenesis. I. Rosenthal, Leonard J. II. Series.
[DNLM: 1. DNA Tumor Viruses–physiology. 2. Neoplasms–etiology. 3. Oncogenic Viruses. QW 166 M486 2001]
QR201.T84 M43 2001
616.99′4071–dc21

2001038424

www.karger.com
Printed in Switzerland on acid-free paper by Reinhardt Druck, Basel
ISSN 1422–2132
ISBN 3–8055–7247–6

Contents

Foreword: Mechanism of Transformation

At the beginning of last century, scientific observations founded viral oncology. These observations defined transmissibility of avian leukemia in 1908 by Danish researchers Ellermann and Bang and soon afterwards of an avian sarcoma in chickens in 1911 by Peyton Rous in New York. These important discoveries were not appreciated at the time, and their impact on virology and medicine was not recognized for decades. Happily, Rous lived to be awarded the Nobel Prize in 1966, as the first nonagenarian to receive this honor, 55 years after his great discovery. This reflects the span of time that was needed to appreciate the full significance of Rous' findings and to accept the idea of viruses as causative agents in cancer by medical research community.

It has been 36 years since the isolation of Epstein-Barr virus (EBV), the first virus to be associated with a human tumor. The most recent human tumor virus isolated is another herpesvirus, human herpesvirus 8, which in 1994 was recognized to be associated with Kaposi's sarcoma. It is now clear that five virus types are involved in the causation of human cancer: papillomaviruses, retroviruses, herpesviruses, hepadnaviruses and flaviviruses. Approximately 15% of human cancer incidence can be attributed to virus infection, i.e., viruses represent the second most important risk factor (after tobacco consumption) for cancer development in humans. Only two RNA viruses (human T-cell lymphotrophic virus type I and hepatitis C) are considered as causative agents for human malignancies while most virus-induced tumors are attributed to infection with DNA viruses. Eighty percent of virus-induced malignancies are sequelae of infection by two DNA viruses, hepatitis B and human papillomavirus. Interestingly, there is a growing number of neoplasms ascribed to infection with another herpesvirus, EBV.

One of the major problems in proving that the association is causal or casual in human cancer is the high rate of infection in the general population,

given that there are geographical variations in infection rates. Several DNA viruses have been associated with human cancers and possessed transforming potential when tested in experimental models; however, a recent detailed epidemiologic observation failed to provide evidence for a causative role in human cancers. It is possible that these viruses may be important in a modulation of cellular pathways of already transformed cells by inducing/increasing their malignant potential. This may explain aggressive tumor growth observed in patients infected with herpesviruses such as human cytomegalovirus or herpes simplex virus type 2.

This book describes molecular mechanisms of cellular transformation of DNA viruses. Although a relevance of some viruses for human cancer remains elusive, the different DNA viruses utilize common strategies which may be important for development of tumors. The effector pathways, at least in part due to shared function of viral oncoproteins, are common not only to small oncogenic DNA viruses such as polyomaviruses and papillomaviruses, but also to large DNA viruses such as herpesviruses. These events may include activation of mitogen-activated protein kinases pathways, interaction of viral proteins with cellular tumor suppressor genes and effects on cell cycle progression or apoptosis. In the past, tumor virology, using model systems, has been the source of much of our fundamental knowledge of oncogenesis and basic cellular mechanisms (e.g. oncogenes were discovered in avian retroviruses; mRNA splicing was first described in human adenoviruses). Some of the novel findings introduced in this book confirm that tumor viruses retain their promise as tools for studying the basic mechanisms underlying neoplastic changes. On the other hand, special mechanisms of some viruses such as EBV, human herpesvirus 8 or human papillomavirus with a well-documented role in tumorigenesis are also considered in depth in the chapters which follow. From a medical point of view, the understanding of molecular mechanisms of virus-induced cellular transformation is an essential step for development of strategies for prevention and treatment of virus-associated tumors. In the absence of virus infection, the virus-induced cancers would be reduced by 95% with a significant reduction in morbidity and mortality.

There is still much to be learned. It is hoped that the contents of this book will give further help in understanding the mechanisms of DNA tumor virus transformation with special attention to virus/host cell interactions.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 1–27

JCV and BKV: Mechanisms of Viral Transformation

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The human polyomaviruses JC and BK are best known as opportunistic pathogens of the immunocompromised. JC virus (JCV) in particular, which causes a demyelinating disease of the central nervous system (CNS) termed progressive multifocal leukoencephalopathy (PML), has received considerable attention during the AIDS pandemic. Once considered rare, it was at one time estimated that 4% of all patients with AIDS would develop PML [1], which can be rapidly fatal, and consequently a significant factor in the mortality of the HIV-1 infected populace. However, even prior to the AIDS pandemic there had been considerable interest in JCV primarily because it was and still remains the only known human virus with tropism for the glial (oligodendrocytes and astrocytes) elements of the CNS. Moreover, the early realization that both JCV and BKV were members of the polyomaviridae family led numerous investigators to hypothesize that these viruses had oncogenic properties. This chapter will examine this literature, with particular focus on the mechanisms of JCV- and BKV-induced oncogenesis as well as those viral properties which result in cell type-specific transformation.

Historical Perspective

In 1958, Astrom et al. [2] described 3 patients with underlying malignancies of the hematopoietic system who died from an unusual demyelinating process of the CNS. The demyelination was multifocal in its spatial distribution, progressive with time and associated with bizarre morphological changes of the

cellular elements of the white matter. The authors speculated on the etiology of these changes, but without any supportive evidence, coined the descriptive term progressive multifocal leukoencephalopathy for this new entity. It is a tribute to the detail with which these cases were documented that this original description of the neuropathology seen in PML still remains the standard used for its diagnosis.

As more cases were reported in the decade that followed, it became clear that PML was almost always associated with an underlying malignancy or immunosuppressive illness, suggesting that an opportunistic infection or paraneoplastic syndrome was causing the demyelination. In 1965, ZuRhein and Chous [3] used electron microscopy to examine brain tissue from patients with PML and identified particles resembling papovaviruses in the enlarged nuclei of oligodendrocytes in the white matter. In 1971, Padgett et al. [4] successfully cultivated a papova-like virus from the brain of a patient with PML by passing the autopsied material onto cell cultures of human fetal brain tissue. The virus was labelled JC, after the initials of the patient whose tissue was used to isolate the virus. Based on its physical structure and size (40 nm nonenveloped icosahedral virion), JCV was categorized as a member of the genus *Polyomavirus* in the family *Papovaviridae,* along with SV40 and mouse polyomavirus.

In 1971, Gardner et al. [5] described the isolation of another human polyomavirus in the same issue of the *Lancet* in which Padgett's group described the isolation of JCV. It had been noted in 1970 that patients receiving renal allografts had a high incidence of warts, suggesting reactivation or primary infection with a papillomavirus. In 1970, patient B.K. underwent renal transplantation with a kidney donated by his brother. The patient subsequently developed ureteral stenosis and because a viral etiology was suspected, the urine was examined for the presence of any offending pathogens. Cytologic examination of epithelial cells found basophilic nuclear inclusions, which on electron microscopy were found to consist of 43.6-nm icosahedral virions, morphologically identical to the polyomaviridae. The virus, named BK, was subsequently isolated by passage of urine sediment onto African green monkey kidney cells (Vero cells). It was noted that viral propagation in these cultures was slow, requiring 1–3 months for any evidence of cytopathic effect. BKV has subsequently been propagated in human embryonic kidney (HEK), diploid lung fibroblasts (WI38) and human urothelial cells.

Soon after they were recognized as polyomaviruses, it was theorized that JCV and BKV could have oncogenic potential based on two lines of reasoning: (1) Other members of the *Papovaviridae* (SV40, polyoma) have oncogenic potential as demonstrated by the ability to induce tumors in several different mammalian species as well as their ability to transform cells in vitro. (2) PML was frequently found in the context of an underlying systemic malignancy.

Molecular Biology of JCV and BKV and Relation to Oncogenicity

Human polyomavirus oncogenesis is best understood in the context of the molecular biology of JCV and BKV. In this section we will examine the different stages of the viral life cycle and their role in viral transformation. Moreover, we will examine those elements of the viral genome which restrict viral transcription to certain cell types, which may account for the specificity of viral transformation.

Genomic Organization

The JCV and BKV genomes are both composed of a closed, circular, supercoiled DNA molecule. Sequencing of the entire genome and comparison with the map of SV40 have resulted in the map seen in figure 1. Using the conventions applied to SV40, the nucleotides were numbered in a clockwise orientation, commencing at the origin of replication. The organization of the genome is similar to SV40 and can be divided into two functional regions, early and late, based on the temporal expressions of the viral proteins. The proteins coded for in the early region are the first ones expressed in the viral life cycle and are continuously expressed throughout lytic infection. These two proteins, large T and small t, play critical roles in viral replication, transformation and transcription of the late region. Following the expression of the early phase proteins, viral replication occurs, which is in turn followed by the expression of the late region proteins. The late region proteins represent the capsid or structural proteins which will assemble into icosahedral viral particles. The origin of replication lies between these two regions, with the early region proximal or counterclockwise to the origin and the late region distal or clockwise. A third region, which lies between the early and late segments of the genome, just distal or clockwise to the origin of replication, contains the promoter-enhancer elements for both the early and late genes.

Regulatory Sequences

The regulatory sequences of JCV and BKV consist of the origin of replication and the *cis-acting* promoter-enhancer elements for both early and late transcription. These elements restrict the life cycle of JCV and BKV to certain species and cell types and hence are critical elements in determining which cells may be prone to malignant transformation by the polyomaviruses.

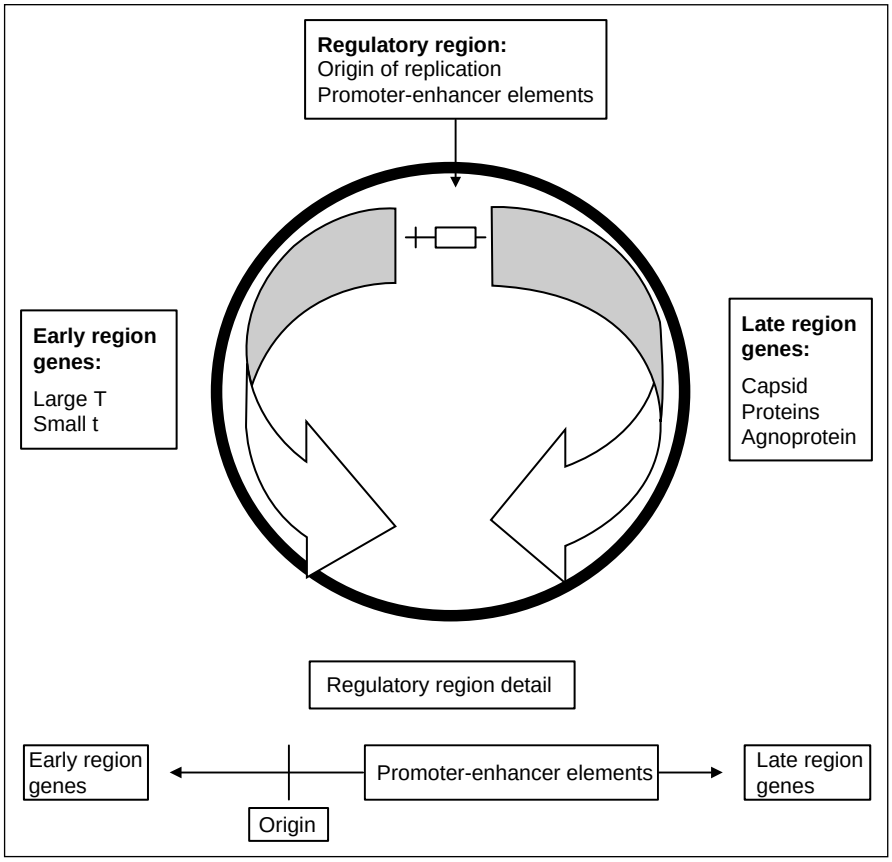

Fig. 1. Structure of JCV and BKV genome.

Origin of Replication

When compared with SV40, the nucleotide sequence of the origin of replications of both JCV and BKV were found to be highly conserved as seen in figure 2. Within these sequences, areas of dyad symmetry and palindromes are found in all three viruses. As will be discussed below, binding of T protein to the origin is critical for initiation of viral replication in the polyomaviruses. Two sites which have multiple copies of the T protein binding motif (5′-GAGGC-3′) have been identified in the JCV origin and have been termed sites I and II. This contrasts with SV40, which has three T protein binding sites (the third one is not shown in figure 2).

Studies that have compared JCV and SV40 replication have consistently found that JCV replication is less efficient and more restricted than that of

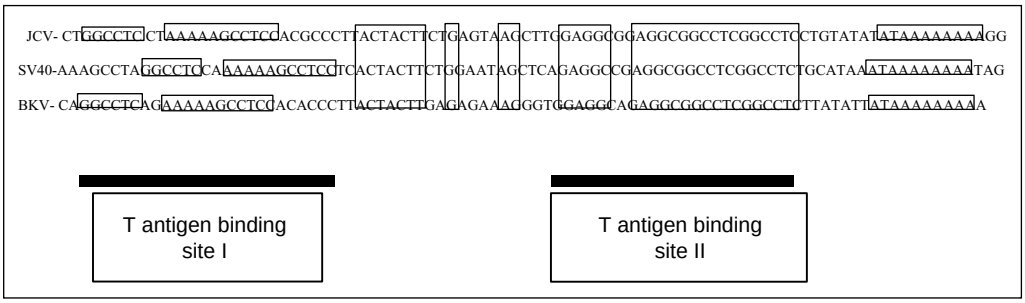

Fig. 2. Origin of replication of JCV, SV40 and BKV.

SV40. Even in the host cell of choice, the glial cell, JCV replication is not detectable until 5–7 days postinfection. Three elements in the replication process have been studied to account for this behavior.

(1) *The structure of JCV T protein and its interaction with the T protein binding sequences of the origin*. Both Lynch et al. [6] and Chuke et al. [7] constructed a series of SV40/JC chimeras in which the origin of replication of one virus was exchanged with that of the other virus. These studies demonstrated that JCV T protein functioned less efficiently than SV40 T protein in promoting viral replication at both the SV40 and JCV origin for at least two reasons: (a) reduced stability of JCV T protein relative to SV40 T protein and (b) weak binding of JCV T protein to site II of both JCV and SV40 origins. Tavis and Frisque [8] mutated the JCV T protein at ten different locations in its DNA binding domain to increase the homology of JCV T protein with SV40 T protein. Seven of these 10 mutants bound JCV DNA more efficiently than the wild type, indicating the importance of T protein conformation.

(2) *The structural conformation of nearby sites*. In 1988, Amirhaeri et al. [9] demonstrated that a region of the viral genome to the late side of the origin in the first TATA box (described below), had an unusual non-B, but right-handed, DNA conformation. It was later reported that mutations in a pentanucleotide sequence, AGGGA, found in this region markedly reduced replication mediated by JCV T protein [10, 11]. These two studies suggest that the tertiary structure resulting from the AGGGA element and the adjacent A/T-rich sequence influences the binding or replicating ability of JCV T protein.

(3) *Host cell factors necessary for replication*. Feigenbaum et al. [12] demonstrated that host cell range is restricted at the level of replication as well as the transcriptional level (discussed below). They found that JCV DNA could replicate in a variety of primate cell lines in which T protein (either JCV or SV40 T protein) was expressed. This contrasted with nonprimate cells in which

no replication was detectable, even in the presence of T antigen. They concluded that a host cell factor in primates was essential for replication mediated by T proteins. One possible factor was suggested in a study which found that binding of NF-1 to the enhancer of JCV stimulated DNA replication [13]. The stimulation of DNA replication could be abolished by introducing mutations within the NF-1 binding site. Cheng and Kelley [14] showed that insertion of an NF-1 binding site near the origin of DNA replication in SV40 stimulated replication of SV40 minichromosomes in vivo and in vitro. Interestingly, the stimulation of JCV DNA replication by NF-1 binding could only be demonstrated in vivo and not in vitro. This observation led Sock et al. [13] to suggest a possible role for NF-1 in determining chromatin structure. There are apparently three clusters of protein binding sites or domains within the JCV regulatory region (discussed in detail in the section below on gene expression). Each cluster or domain contains at least one NF-1 binding site. Sock et al. [13] found that there was a stepwise decrease in the relative rate of JCV DNA replication as each of the domains was deleted.

Promoter-Enhancer Elements

The other elements in the regulatory region are the promoters and enhancers, found on the late side of the origin. In the prototype MAD-1 strain of JCV, these elements are duplicated and found in tandem repeats (fig. 3). While both BKV and SV40 also have tandem repeats of promoter-enhancer elements in their respective regulatory regions, they differ from JCV in both sequence and organization. The TATA box, which positions the 5′ end of mRNA, is within the tandem repeats of JCV, in contrast to SV40 and BKV in which the TATA box is a separate element.

Unlike the rest of the viral genome, the promoter-enhancer elements are hypervariable among the different JCV isolates. Most of the original isolates of JCV were passaged through cell culture prior to sequence analysis, which suggested that the variability seen was introduced in vitro. This phenomenon was best characterized by the infection of HEK cells with the MAD-1 strain of JCV which resulted in an adapted JCV strain markedly different at several points in the genome, including the promoter/enhancer elements [15]. However, direct cloning of the viral genome from a number of PML patients has demonstrated that regulatory region variability is common in vivo.

The first JCV isolate to be sequenced was MAD-1 [16], which has two 98-base-pair (bp) repeats in the regulatory region, as seen in figure 3, and has been termed the prototype sequence. JCV has subsequently been sequenced from the brain [17, 18], kidney [19], urine [18] and peripheral lymphocytes [20, 21] of both PML patients and normal subjects and demonstrated a striking heterogeneity. The different isolates have been broken down into two groups

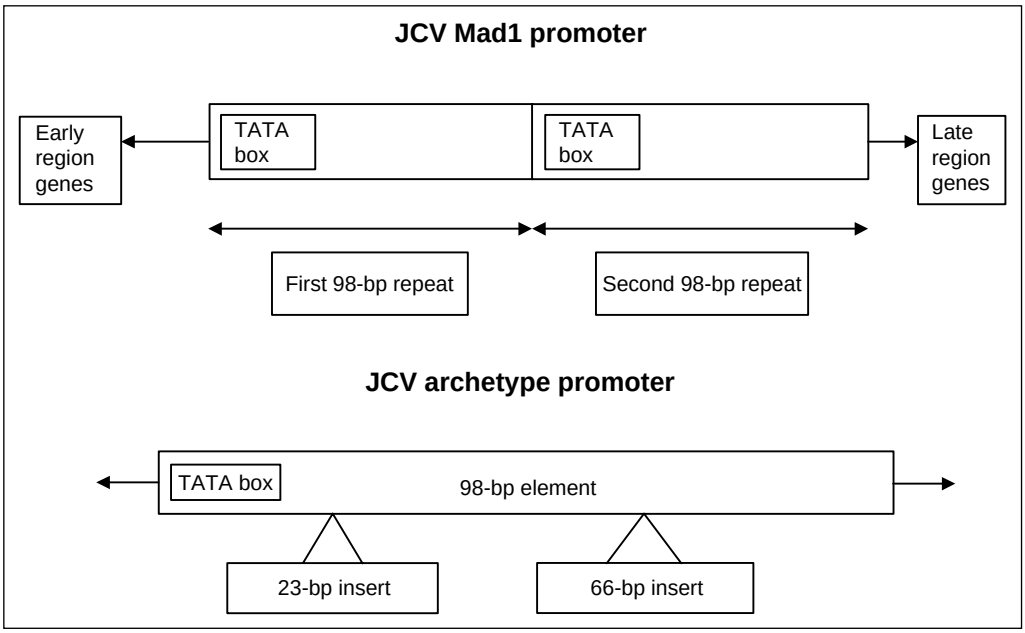

Fig. 3. Structure of the JCV Mad1 and archetype promoter.

termed type I and type II genomes. Type I have a duplicated TATA box and two 98-bp repeats, e.g. MAD-1. Type II JCV genomes have a GC-rich 23-bp insertion at nucleotide 36 and have deleted the TATA box in the second tandem repeat. Furthermore, the type II isolates differ from one another in the length of the enhancer repeats. The MAD-4 isolate is the only exception to this schema. It has a deletion in the second TATA box but lacks the 23-bp insert. As will be discussed, MAD-4 is oncogenic in both hamsters and primates. Both type I and II genomes have been isolated from various tissues from PML patients and normal individuals. In any one given patient it has been reported that (a) the same isolate was found in both brain and urine, (b) the isolate in brain differed from that in kidney and (c) more than one isolate was found in the brain and/or urine.

In 1990, Yogo et al. [22] cloned a JCV regulatory region from immunocompetent individuals which could not be classified as either type I or type II. As seen in figure 3, it consists of a single 98-bp enhancer element with the 23-bp insert and a second 66-bp insert. No duplication of the enhancer elements was present. Of note is that several years earlier, Dorries [19] had also isolated this same strain from the kidney of a PML patient. This isolate was of particular

interest in that all the elements found in both the type I and type II variants could be found in this strain and it was postulated that all isolates originated from this 'archetypal' strain. JCV isolates with this archetypal regulatory sequence have since been found in the urine of normal individuals, renal transplant patients, pregnant women and AIDS patients, all from widely separated geographical locations, suggesting that this archetype is distributed worldwide.

Regulatory Elements – BKV

BKV isolates, like JCV, also have been categorized into two classes based on the structure of the regulatory elements: archetype and rearranged elements. Gardner's initial isolate consisted of three tandem repeats in the enhancer element. In contrast, other isolates (DIK, WW and AS) have a linear arrangement with no repeats. It was argued that the isolates with the linear arrangement were the wild-type virus found in vivo, while the isolates with more than one tandem repeat represented laboratory-adapted strains of virus, the rearrangements acquired as part of the in vitro life cycle. Negrini et al. [23] and Rubinstein and Harley [24] independently demonstrated that the regulatory elements of non-passaged BKV isolates did not contain any tandem repeats, consistent with an archetypal strain. Rubinstein et al. [25] further went on to demonstrate that passage of archetypal BKV in tissue culture resulted in (1) deletions ranging in size from 38 to 91 bases and (2) amplification/duplication of the enhancer elements, confirming the archetype theory. It has since been argued that the rearranged enhancers may confer transcriptional cell specificity. BKV-IR, which was initially isolated from a human insulinoma, is a particularly striking example [26]. Sequencing of the enhancer element directly from the tumor was identical to the enhancer structure of the passaged virus, suggesting that the rearranged element occurred in vivo and may have changed the biology of BK such that it resulted in a pancreatic tumor.

Watanabe and Yoshiike [27] demonstrated that experimental deletion of two of the 68-base-tandem repeats in the BKV regulatory region resulted in a mutated virus which transformed rat cells far more efficiently than the wild-type (Gardner prototype) BKV. This was further borne out by analysis of pm522, a BKV isolate from a pineocytoma which was also found to have a deletion of two of the repeats. This particular isolate is highly tumorigenic and results in insulinomas when injected into rodents. This mutation results in prolonged T protein expression in BKV-infected rat cells (relative to the wild-type strain) thus leading to transformation.

Markowitz et al. [28] demonstrated that the regulatory elements of the archetype BKV strain WW were nearly quiescent when fused with a CAT gene and transfected into human embryonic lung fibroblasts. In contrast, regulatory elements from BKV(MM) and BKV(Dun), which have duplications and deletions

of the elements found in the archetype promoter, had 100-fold greater activity than BKV(WW). Interestingly, the junction of the rearranged elements created new AP-1 binding sites, suggesting that rearrangement conferred a growth advantage by promoting early transcription.

Small et al. [29] introduced the promotor and early region genes of BKV(Dun) into transgenic mice. One of the 3 mice (which contained >20 copies of the transgene), was abnormally proportioned and died at 2 weeks. The remaining 2 mice developed hepatocellular carcinomas and renal tumors. High levels of T protein were expressed in the liver tumor and interestingly in normal muscle as well. No amplification of the transgene was noted in the hepatocellular carcinoma. Offspring from 1 of these mice was later studied by Dalrymple and Beemon. In contrast to the parents, the offspring developed extremely hypertrophied thymus glands in addition to renal adenocarcinomas. Furthermore, the renal tumors had highly amplified transgenes with multiple rearrangements of the cellular DNA flanking the BKV transgene. T protein expression was found in the renal tumors as well as the enlarged thymuses. In total, these transgenic mice experiments demonstrated that the BKV(Dun) promoter had tissue-specific expression and moreover, that expression of the early gene product resulted in tumor formation with subsequent amplification of the transgene.

The significance of the heterogeneity of the regulatory region in both JCV and BKV remains controversial. It is theorized that normal individuals are infected with either archetype or type I/II virus which then establishes latency in either the urogenital tract or lymphoid system. During periods of immunosuppression, immunosurveillance of the virus is disrupted and it is hematogenously disseminated. It has been argued that changes in the regulatory elements might favor a lytic life cycle if the origin is left intact and the proper host nuclear factors are present for early transcription and replication. Alternatively, deletions in the origin of replication would result in a truncated life cycle in which only early genes are expressed (i.e. T protein). Lack of a lytic component would then predispose the host cell to a transforming event given the ongoing T protein expression.

Early Region Genes

As noted above, this region of the viral genome is the first segment to be transcribed in the viral life cycle coding for two proteins, large T and small t antigen. Start sites in the regulatory region direct transcription in a direction counterclockwise to the origin. Differential splicing of the early transcript results in two populations of mRNA, one of which codes for large T, the other for

small t protein. While the ORF for both proteins begins at the same nucleotide, the locations of the termination codons differ because of the splicing event. For instance, in JCV the termination signal for small t protein is at nucleotide 4495, while the one for large T is at nucleotide 2603. Consequently, these proteins share the same 5′ amino acid sequence, but differ at the 3′ portion. JCV, SV40 and BKV T proteins share extensive sequence homology, and consequently antigenic epitopes. This homology has allowed antibodies directed against SV40 T protein to also cross-react with JCV T protein.

JCV large T protein or antigen is a well-characterized viral oncogene, hence its name T(umor) protein. The oncogenic and immortalizing potential of JCV in a variety of cells (as will be described) is due to T antigen expression and presumed binding of cellular tumor suppressor gene products. As previously discussed, JCV T protein also binds to several sites at the origin and is an essential element in the initiation of DNA replication. Large T protein autoregulates early transcription as well as directs transcription of proteins in the late phase of the viral life cycle. Little is known about the role of small t protein in the JCV life cycle but it too is felt to be an oncogene. Gallia et al. [30] demonstrated that the single-stranded DNA binding protein Purα can physically associate with JCV T protein and attenuate its activity, blocking late transcription and viral replication. Conversely, JCV T protein is able to interact with the POU domain of the transcription factor Tst-1/Oct6/SCIP enhancing the activity of Tst-1/Oct6/SCIP [31]. Interestingly, this binding occurred in the amino-terminal portion of T protein in what is thought to be a J domain.

J domains consist of four α-helical segments present on heat-shock proteins which act as molecular chaperones. The J domains mediate the rate-limiting hydrolysis of ATP to ADP. It has been postulated that the J domain of T protein binds ATP-bound hsc70 while a second domain on T protein binds a target protein (e.g., retinoblastoma gene product). Energy derived from ATP hydrolysis leads to conformational changes of the bound protein. The altered target protein is then released. If the target protein were Rb, the conformational change might release E2F from the RB-E2F complex, facilitating E2F-dependent transcription of host genes. Harris et al. [32] in fact demonstrated that an intact BKV T antigen J domain was critical in inducing transcriptionally active E2F.

Late Region Genes

The late region codes for three structural proteins (VP1, VP2 and VP3) as well as a fourth protein termed the agnoprotein. These genes are transcribed clockwise from the origin of replication on the strand opposite that of the early gene. VP1 is the largest of the structural proteins (354 aa) and the most important

in viral adsorption. Antigenic cross-reactivity with SV40 has been difficult to demonstrate, suggesting that any shared epitopes are below the viral surface. The VP1 protein of JCV is able to cross-link sialic acid residues of erythrocytes, the basis of the hemagglutination assay. In contrast, SV40 does not hemagglutinate, again demonstrating a fundamental difference of the late gene products of these two viruses. While the VP1 gene is highly conserved among JCV isolates, Ault and Stoner [33] identified a series of point mutations in the 5′ portion of the VP1 and large T genes which allowed them to classify JCV isolates into two groups based on the mutant pattern. The ORFs for VP2 and VP3 lie in the 5′ portion of the late region and, based on sequence homology, it appears that VP3 is encoded by the carboxy-terminal sequences of VP2. Other than their sizes (VP2 is 344 aa, VP3 225 aa) little is known about these proteins in JCV. The ORF for the agnoprotein lies in the 5′ portion of the late region, codes for a putative 71 amino acid protein which, as its name states, has no known function.

Gene Expression

Cell-Specific Expression and Targeted Oncogenesis Is Dictated in Part by the JCV Promoter-Enhancer

With the elucidation of the complete nucleotide sequence of JCV and the establishment of the putative organization of the genome, studies were initiated to examine the activity of the designated promoter-enhancer region in different cell lines. The first such study was undertaken by Kenney et al. [34] who cloned the tandem 98-bp repeats of MAD1 into a CAT reporter plasmid. The first construct made had the proximal 98-bp repeat (closest to the DNA origin of replication) adjacent to the 5′ end of the CAT gene. Of the four cell lines (HeLa, human fetal glial, CV1 (green monkey kidney cell line) and human embryonic kidney cells) used in this study to measure enhancer activity, the highest activity was obtained with human fetal glial cells. Sevenfold less activity was seen in the CV1 cells with little to no activity obtained with the HeLa and embryonic kidney cells. When the regulatory region was cloned in the reverse orientation, the highest activity was again obtained with the fetal glial cells, followed by the CV1 cells. Similar experiments done in glial and nonglial cells of human and rodent lineage again found that transcriptional activity of the Mad1 regulatory region was highest in the glial cell lines [12, 35]. These initial studies demonstrated that the noncoding region between the early and late region could serve as a promoter-enhancer sequence and that the glial specific expression of JCV was determined by its regulatory region. Furthermore, glial expression of JCV was not species-dependent.

In addition to studying the promoter-enhancer activity of the prototype Mad1 regulatory region, the transcriptional activity of the regulatory region of JCV variants has been examined. Martin and Li [36] examined the enhancer activity of JCV MAD-1 (type I), MAD-8 (type II), and MAD-11 (type II) variants in HeLa cells, and two transformed human fetal glial cell lines, SVG and POJ. They found that in all three cell lines the type II promoters gave significantly higher activity than the Mad1 promoter and that this activity was higher in the glial cell lines. A study comparing the enhancer activity of type I and type II JCV variants in primary human fetal glial cells was reported by Frisque and White [37]. In contrast to the glial cell lines, in primary glial cells the type I Mad1 enhancer was more active than all the type II enhancers (HerI, Mad7d, Mad8 and Mad11) tested except for one (Mad11.3). As in prior studies, the activity of all JCV enhancers tested in human embryonic kidney cells was very low, while the enhancer from SV40 was much more active than the JCV enhancer in both human fetal glial and kidney cells. These studies demonstrated that, despite the promoter variability seen in JCV isolates, the glial specific expression remained unchanged.

A number of studies have demonstrated that if the JCV genome is altered sufficiently, the host cell expression can be expanded beyond glial cells. Miyamura et al. [38] infected human embryonal kidney cells with the MAD-1 strain of JCV, and unlike other groups, found that 30% of cells were positive for T protein 25 days postinfection. With serial passage, 90% of the cells became T-antigen-positive and cpe was prominent. Serial passage had apparently led to an adaptation of the virus, allowing for its growth in these cells. When this group later cloned the isolates adapted to human embryonal kidney cells they found that the regulatory region contained a triplicate promoter-enhancer sequence and origin of DNA replication [38]. Vacante et al. [39] constructed a chimeric JCV genome by inserting the 72- and 21-bp repeats of SV40 into the regulatory region of MAD-1. This chimera replicated well in human fetal glial cells, however, passage resulted in a large deletion of the chimeric regulatory region retaining the origin of replication, 78 bases of one JCV 98-bp repeat, 33-bp of one SV40 72-bp repeat and one complete 72-bp repeat. This adapted chimera demonstrated an extended species and host cell range, growing well in human fetal glial cells, human embryonic kidney, and rhesus monkey fetal and adult glial cells. Once again, this demonstrated that the host range was directly related to changes in the regulatory sequence.

To characterize-tissue specific expression of the JCV promoter in vivo, Small and colleagues [29, 40] produced a transgenic mouse which contained the early region of MAD-1, including the promoter-enhancer elements. Ten founder mice were produced, of which 5 survived to maturity. Four of these 5 developed widely metastatic adrenal neuroblastoma. JCV early region mRNA was easily

detected in these tumors, with little JCV mRNA expression in normal tissues, including the brain. Offspring of the founder mice which inherited the early region sequences were distinguished by a characteristic phenotype: vigorous shaking when moving and tonic seizures of 15–30 s duration, characteristics similar to the myelin-deficient strains of mice, *quaking and jimpy.* Histological examination of these offspring did in fact reveal a diffuse hypomyelination of the CNS, sparing the peripheral nervous system. The oligodendrocytes had enlarged, bizarre nuclei, with prominent macrophage phagocytosis of the myelin. In contrast to the founders, these offspring had abundant JCV T protein encoding mRNA (as seen by Northern hybridization) in the brain (and lung) with little expression in other organs. This expression was predominantly localized to the oligodendrocytes of the white matter using in situ hybridization [41]. Immunocytochemistry found decreased intensity staining of several constituents of myelin, including proteolipid protein, myelin-associated glycoprotein and myelin basic protein. They concluded that the in vivo data were very similar to those in vitro, i.e. the expression of the promoter element was restricted predominantly to the glial elements. Furthermore, the presence of JCV early gene products impaired the ability of oligodendrocytes to properly myelinate axons. A later paper by Beggs et al. [42] found that primary brain cultures from the transgenic mice contained many T-protein-positive astrocytes but surprisingly, T protein was not found in oligodendrocytes.

Feigenbaum et al. [43] constructed a transgene which consisted of the JCV regulatory region driving the SV40 T protein gene, instead of the JCV T protein. Transgenic mice with this chimera developed hypomyelination of the CNS and neuroblastomas, similar to the JCV construct made by Small's group [29, 40]. However, a transgenic mouse with the SV40 regulatory region driving the JCV T protein did not develop hypomyelination, but instead developed choroid plexus carcinomas. This again demonstrated that the regulatory region of the papovaviruses determines tissue expression.

Cis- *and* Trans-*Acting Factors*

Once it had been determined that the region between the 5′ end of the T antigen and agnoprotein coding sequence could serve as the promoter-enhancer for both early and late transcription, studies were carried out to identify the *cis- and trans*-acting factors responsible for expression and tissue specificity of the JCV genes. Khalili et al. [44] used a series of oligonucleotides which spanned the regulatory region, as DNA probes to detect specific nuclear binding proteins. Several areas within the regulatory region were found to bind nuclear proteins from HeLa and primary human fetal glial cells. Two areas were located at the 5′ and 3′ ends of each 98-bp repeat sequence. UV protein-DNA cross-linking studies showed that similar size proteins (M_r 82,000 and 78,000–80,000)

from both types of extracts recognized the 5′ and 3′ end, respectively, of the 98-bp repeat unit. In contrast, an oligonucleotide homologous to the central portion (nucl. 36–62) of the 98-bp repeat unit appeared to be recognized by different proteins from HeLa (M_r 85,000) and human fetal glial cells (M_r 45,000). In addition, it was reported that a protein of M_r 230,000 only from HeLa cells could bind to several areas within the 98-bp repeat unit. Tamura et al. [45] were able to detect several areas in the JCV regulatory region which bound nuclear proteins extracted from mouse brain by DNase I protection analysis. Two areas were within the 98-bp repeat units (nucl. 35–58 in the first repeat unit and the comparable region in the second repeat unit), and one just outside of the distal 98-bp repeat unit. All three protected sites contained a NF-1 recognition sequence. Using competitive binding assays with portions of the mouse myelin basic protein gene, BKV, and adenovirus type 2/5 promoter regions, which all have NF-1 binding sites, they were able to compete away the protection from DNase I digestion in the JCV promoter-enhancer region. In addition, a NF-1 preparation from HeLa cells was able to protect the NF-1 sites within the 98-bp repeat units but not the NF-1 site outside of the 98-bp repeat unit. This later NF-1 site was a weaker binding site than those within the 98-bp repeat units.

The identification of NF-1 and other nuclear protein binding sites in the JCV regulatory region was confirmed and extended by Amemiya et al. [46]. Nuclear proteins prepared from primary human fetal glial cells, SVGs, a glioma, and HeLa cells, all protected at least four sites from DNase I digestion. Three of the NF-1 sites (A, B, and C, relative to the DNA origin of replication) were the same as those reported by Tamura et al. [45] and the fourth site (D) was close to the translational start site of the agnoprotein on the late side of transcription. All the NF-1 sites were further confirmed with purified CCAAT (CTF)/NF-1 protein from HeLa cells. In addition to the NF-1 sites, activator protein (AP-l) or *c-Jun* binding sites were detected with both nuclear protein extracts and a purified *c-Jun* preparation. Interestingly, all the *c-Jun* or *c-Jun*-related protein binding sites in the JCV regulatory region were either adjacent to or overlapped the NF-1 binding sites. In fact, there appeared to be some interactions between the NF-1 and *c-Jun* proteins, since the binding of NF-1 at an NF-1 site was altered by the presence of *c-Jun* protein.

Transcriptional regulation of BKV bears a number of similarities to JCV. Chakraborty and Das [47] were the first to describe the location and nature of the transcription factors which bind to the BKV promoter. Using HeLa nuclear extract, they identified six domains in the regulatory region which bound transcription factors. Three domains bound NF-1, which also acted as a major activator of early transcription in vitro. Three other sites were identified which bound proteins with AP1- and SP1-like activity, however there in vitro transcriptional activity was less pronounced than that of NF-1. To study the functional

significance of these *cis*-acting elements, Ferguson and Subramani [48] made a series of constructs which contained different combinations of the six domains linked to a CAT gene. Using extracts from Vero cells, they found that the sites acted synergistically with one another and that certain combinations were clearly more synergistic than others. This suggested that certain rearrangements of the regulatory region during the viral life cycle could confer a growth advantage on the rearranged strain and might account for the changes seen in BKV when serially passaged in culture.

Other Trans-*Acting Factors*

Ahmed et al. [49] identified a protein of M_r 45,000 from calf brains that binds to an oligonucleotide (oligo B) from the central part of the JCV 98-bp repeat unit which contains the binding site for the NF-1 protein. However, it was not clear that this M_r 45,000 protein could be an NF-1-like protein given its ability to stimulate transcription of the JCV early promoter-enhancer but not that of myelin basic protein which also has an NF-1 binding site [50]. In another study, the oligo B domain was used to isolate a c-DNA clone from a human brainstem library which encoded a protein that could recognize the JCV oligo B domain [51]. The expression of this c-DNA clone, called glial factor-1 (GF_1), was greater in brain and kidney than in lung and spleen tissue. Co-transfection of a JCV early or JCV late promoter CAT-reporter plasmid with an expression plasmid carrying the GF_1 gene showed that the JCV late promoter was stimulated (6- to 10-fold) to a greater extent than the JCV early promoter (2- to 3-fold) in the presence of the GF_1 carrying expression plasmid. Analysis of the nucleotide sequence of GF_1 showed no homology to NF-1 or an NF-1-like protein.

The purine-rich region between the TATA sequence and the NF-1 binding site (termed the lytic control element) is believed to be important for both viral replication (as discussed above) and transcription. Several studies have reported the binding of nuclear protein(s) to this region, including a protein of M_r 53,000 present in both rat glioma and HeLa cell extracts [52], and a protein of M_r 56,000–60,000 from hamster fetal glial cells [53]. Tada and Khalili [54] isolated a single-stranded DNA binding protein from monkey brain, which interacted with the late-coding strand sequence 5′-AGGGAAGGGA-3′. This nuclear protein was called the lytic control element binding protein I. Mutations within the lytic control element decreased both binding of this protein and early mRNA transcription.

It has been reported that a protein called Tst-1, which is a member of the POU-domain family, had been identified in myelinating glial cells and found to stimulate JCV early and late transcription [55]. An overexpressed Tst-1 fusion protein bound to JCV sequences located at both the 5′ and 3′ ends of the 98-bp repeat unit (sites A and B respectively), with a greater affinity (2.5-fold)

for site A. Co-transfection of a Tst-1 expression plasmid and JCV early or late promoter reporter plasmids in a glioblastoma cell line (U138) showed that both promoters were stimulated 3- to 10-fold, although the expression of the early promoter construct was 10 times higher than that of the late promoter. Tst-1 also stimulated JCV Mad1 DNA replication, possibly as a result of the increased expression of T antigen. Because of the expression of Tst-1 in glial cells of the CNS, it was suggested that it too may be one of the cell-specific factors which restricts JCV infection to oligodendrocytes.

One of the hallmarks of the type II JCV variants is the presence of a 23-bp insertion between the TATA sequence and NF-1 binding sites in the first 98-bp repeat unit . The 23-bp insertion has a putative SP1 binding site, however, unlike the classical SP1 binding sequence found in SV40 (5′-GGGCGG-3′), the 23-bp insertion contains the sequence 5′-GGGAGG-3′. Henson [56] demonstrated that recombinant human SP1 protein could bind to an oligonucleotide containing the sequence of the JCV 23-bp insertion. It was shown by competitive binding assays that recombinant SP1 has a lower affinity for the JCV-23-bp insertion sequence than the classical SV40 SP1 sequence. In the same study, they showed by immunohistochemical staining that expression of SP1 in the brain appears to be localized to cells which can support JCV expression.

Oncogenesis

In vitro Transformation

From a mechanistic point of view, transformation of nonpermissive host cells by JCV and BKV is easily understood. Following infection of the target cells, early gene expression of T protein will occur in those cells which have transcription factors able to bind to the promoter-enhancer region and direct expression of T protein. The nonpermissive host will however not have the appropriate factors to promote viral DNA replication and block late gene expression as well. As a consequence, only the early gene products will be produced, tumor suppressor proteins p53 and Rb will be bound by T protein and the cell will become immortalized [57].

In vitro Transformation – JCV

Frisque et al. [58] demonstrated the transforming potential of four different isolates of JCV (Mad1, Mad2, Mad3 and Mad4) by infecting primary hamster brain cells in vitro. These cells had several characteristics of the transformed phenotype and expressed T protein, but neither viral structural protein nor recoverable virus was demonstrated. The cells were fibroblastic in appearance,

however, it was not clear whether they were fibroblasts or fibrous astrocytes. Howley et al. [59] cloned the MAD-1 genome into pBR322 and then transfected the recombinant into primary human amnion cells. Approximately 3–4 weeks post-transfection transformed foci were found in the cultures which expressed the viral T antigen. While full-length JCV DNA in a circular, episomal form could be identified, no cpe, HA activity or staining for the viral protein antigen could be detected in the cultures, suggesting that there was a block to late transcription and replication.

Two studies made hybrid viral genomes to determine which sequences within the viral genome contribute to the restricted transforming activity of JCV [60, 61]. Chimeras were constructed in which the JCV regulatory elements were removed and replaced with the regulatory elements of SV40 and BKV, leaving intact the early and late coding regions. These two hybrids transformed Rat2 fibroblasts and a baby hamster kidney cell line (BHK-21) more efficiently than the wild-type JCV genome. From this set of experiments it was concluded that inability of JCV regulatory sequences to adequately drive expression of T protein in cells of a nonpermissive host limit its transforming ability. A second set of experiments constructed hybrids in which the JCV regulatory region was kept intact, however the early region T protein gene was replaced with the T protein gene from SV40 or BKV. Surprisingly, these constructs were able to transform Rat2 and BHK-21 cells better than the JCV regulatory-BK/SV40 T protein chimeras. This demonstrated that the JCV transcriptional elements in the regulatory region are active in nonpermissive cells and, if the downstream oncogene is strong enough, apparently can lead to expression of adequate levels of an oncogene to result in transformation. Hence, both the regulatory region and the T protein of JCV restrict its ability to transform nonpermissive cells. It was also theorized that if the half-life of JCV T protein was shorter than that of SV40, that this too would contribute to weaker transforming ability. This was confirmed by a set of experiments by Lynch and Frisque [10] which demonstrated that the half-life of SV40 T protein is twice that of JCV protein.

Another group of experiments examined the different portions of JCV T protein to determine which portions had the greatest impact on transformation [60, 61]. Chimeric T proteins were constructed in which the wild type JCV T protein had sequences in both the carboxy- and amino-teminal portion substituted with sequences from BKV and SV40. It was concluded that the central and carboxy-terminal portions of JCV had reduced transforming ability, relative to BKV and SV40. As noted above, the downstream steps which will determine transformation by T protein are linked to the ability of the protein to bind the retinoblastoma gene product, Rb and the tumor suppressor p53. Given the poor ability of JCV to transform cells, it was not too surprising to find that wild-type JCV T protein poorly bound both RB and p53. In contrast, those JCV T protein

chimeras which had SV40 sequence in the central and carboxy-terminal regions (which also corresponds to the p53 binding domains) were able to bind and stabilize p53.

In total, the restricted transformation ability of JCV relative to BK and SV40 is due to: (1) limited transcriptional activity of the JCV regulatory elements in cells other than glia; (2) poor binding of p53 and Rb by JCV T protein and (3) short half-life of JCV T protein (relative to that of SV40).

In vitro Transformation – BKV

In 1975, Portolani et al. [62] demonstrated that primary hamster kidney cells could be transformed by BKV. The transformed cells produced BKV T antigen and BKV was rescued from these cells by fusion with permissive cells. Howley and Martin [63] in 1977 examined the DNA of three cloned lines of hamster kidney cells transformed by BKV and found 2.7–5.3 equivalents of viral DNA per diploid genome. In one cell line examined the entire viral genome was represented, consistent with the ability to rescue virus as demonstrated by Portolani. Takemoto and Martin [64] further demonstrated that transfection of BKV DNA was also adequate to transform hamster kidney cells with resultant T protein expression and the ability to rescue virus from the cells. This contrasted with a later study by ter Schegget et al. [65] who was unable to rescue virus from hamster tumor cells lines. The viral DNA in these cells was unintegrated and lacked part of the late region. Interestingly, in some cases the BKV DNA had acquired a TaqI site, suggesting that there had been recombination with host DNA, since the BKV genome lacks TaqI sites.

In 1981, Possati et al. [66] found that BKV-transformed hamster kidney cells at low passage showed high levels of T antigen expression, normal growth kinetics and low tumor-producing ability. In contrast, at higher passages T antigen expression declined, the growth properties approximated those of transformed cells and demonstrated high tumor-producing ability. In total, this suggested that cellular transformation by BKV is initiated, but not maintained, by the expression of genes regulating T antigen. Host factors clearly played a role in maintaining the transformed phenotype. Sabbioni et al. [67] extended this observation when they found multiple loci on human chromosome 11 control tumorigenicity of BKV. They found that transfer of a normal human chromosome 11 to BKV-transformed mouse cells suppressed the malignant phenotype. If however, the 11q13 segment was lost the phenotype could not be suppressed. Clearly, loss of host tumor suppressor genes as well as expression of T protein were required for the transformed phenotype.

BKV is able to transform cells of renal and CNS origin in a wide variety of species, including mouse, rat, hamster, rabbit and monkey. In contrast, transformation of human cells is very restricted. Human fetal brain cells are the only

primary cells that can be transformed by persistent viral infection. Theile and Grabowski [68] further demonstrated that JCV (and BKV) have mutagenic potential in a number of mammalian and human cell lines as well.

In vivo Oncogenicity

In vivo Oncogenicity of Human Polyomaviruses Caveats

Experimental oncogenicity in foreign hosts has to be interpreted carefully since they may not be an accurate reflection of human infection. Primary human infection with either BKV or JCV appears to be a subclinical event, probably respiratory in route, with a small inoculum presumably from aerosol. In contrast, experimental oncogenicity routinely uses routes of administration (intracerebral, intravenous) and inocula which are clearly artificial and not part of the natural history of polyoma infection. Moreover, the species-specific factors which govern viral replication will be absent in non-primate hosts. As such, the natural life cycle (including latency, replication and dissemination) of BKV and JCV are not thought to be approximated.

In vivo Oncogenicity – JCV

In 1973, Walker et al. [69] inoculated 1-day-old Syrian hamsters intracerebrally and subcutaneously with the JCV isolate now called MAD-1. Within 3 months, microscopic tumors were found in the CNS in the absence of demonstrable neurological signs. However, by 4–6 months post-infection the hamsters had tumors large enough to cause neurological deterioration. Of the animals inoculated, 83% developed tumors described as glioblastomas, medulloblastomas (a primitive neuroectodermal tumor (PNET)), unclassified primitive tumors and papillary ependymomas. The tumors were found throughout the parenchyma with some preference for the cerebellum and thalami. Virus was recovered from unpassaged explants of five tumors, confirming that JCV was the etiological agent.

Other investigators soon found that if the route of administration was changed, JCV was able to also induce extracranial tumors in the hamster. Intraocular inoculation [70] resulted in the development of retinoblastoma (a retinal PNET) in 20% of animals, and widely disseminated metastatic neuroblastoma in 30% of the hamsters. In addition to the route of administration, the viral isolate used was also a determinant in tumor induction [71]. If the MAD-2 isolate was inoculated intracerebrally, 95% of the hamsters developed cerebellar medulloblastomas and/or gliomas, similar to MAD-1. In contrast, the MAD-4 isolate induced pineocytomas in 10 of 22 hamsters as well as medulloblastomas in slightly less than half of the animals inoculated. Nagashima et al. [72], using

the Tokyo-1 isolate of JCV, were able to induce cerebellar medulloblastomas in 95% of the animals, and to a much lesser extent thalamic gliomas and ventricular ependymomas.

The remarkable ability of JCV to induce PNETs offered insight into the cellular origin of cerebellar medulloblastomas. It had been theorized that cerebellar PNETs in man originated from the vestigial cerebellar external granule cell layer. This was supported by Matsuda et al. [73] using Tokyo-1-induced medulloblastomas as a model. They followed the temporal and spatial patterns of JCV T protein mRNA using in situ hybridization. At times prior to the development of the medulloblastoma, cells migrating in the molecular layer and the internal granule cell layer hybridized to the JCV T protein probe. Since the nascent medulloblastomas originated in the internal granule cell layer, it suggested the following chain of events: JCV infected the cells of the external granule cell layer, which then migrated through the molecular layer to the internal granule cell layer to eventually become a medulloblastoma. Ressetar et al. [74] showed a similar chain of events using immunohistochemistry for JCV T antigen.

The oncogenic capability of JCV was tested in subhuman primates as well. London et al. [75] chose adult owl monkeys in their first set of experiments because they had little to no detectable antibody to any of the papovaviruses. The monkeys were inoculated intracerebrally, intravenously and subcutaneously with either JCV, BKV or SV40. None of the animals inoculated with BKV or SV40 developed tumors, however, 2 of 4 of the monkeys inoculated with JCV developed high-grade gliomas after 16–25 months. Interestingly, one of the gliomas had an area which appeared histologically to be a neuroblastoma. At autopsy, immunohistochemistry revealed T protein but not viral capsid proteins. Explants of the tumor grown in vitro also contained T protein, however, infectious virions were not isolated from the tumor either following serial passage in vitro or by direct co-cultivation of tumor extract with primary glial cells. Further characterization of these cells [76] found that JCV T protein was not complexed with the host p53 tumor suppressor protein and that the JCV genome was integrated into the cellular DNA at a limited number of sites [77]. This set of experiments provided the first demonstration of viral-induced brain tumours in owl monkeys.

Major et al. [78] attempted to develop a primate model of tumor formation with a shorter incubation period by inoculating 4 owl monkeys with a suspension of explanted JCV-induced owl monkey glioblastoma cells. Two years later, 1 of the monkeys developed a glioblastoma. The explanted tumor was successfully grown in culture and was found to be T protein positive and have JCV DNA (both integrated and episomal) similar to the grafted cells. However, unlike the donor cells, by passage 5 infectious JCV could be recovered from the cells. The JCV isolate from these cells had a 19-bp deletion in the second

98-bp repeat of the regulatory region, characteristic of the deletion found in the neuro-oncogenic MAD-4 strain. However, unlike MAD-4 and the donor cells, the T protein from this isolate (termed Owl-586) was able to form a stable complex with the cellular p53 protein of the explanted cells.

The ability of JCV to induce tumors of CNS origin in hamsters and primates and the rare co-association of PML with gliomas in some patients [79] suggested that JCV could be oncogenic in man. In 1978, Greenlee et al. [80] examined cell cultures established from 16 different types of CNS tumors, including glioblastomas, medulloblastomas and ependymomas, for the presence of T antigen using immunohistochemistry. None of the tumors examined stained with an antibody which recognizes a common epitope of T antigen from SV40, BKV and JCV. In 1987, Dorries et al. [81] examined 11 different types of CNS tumors, similar to those examined by Greenlee, for the presence of papovavirus DNA. None of the samples had DNA which hybridized to either a JCV- or SV40-specific probe, however, 11 of 24 specimens had detectable BKV DNA. Bergsagel et al. [82] examined 20 choroid plexus neoplasms and 11 ependymomas from pediatric patients for the presence of papovavirus T antigen gene sequence using PCR and a set of primers which recognize a sequence in the early region conserved among the viruses, followed by Southern hybridization with either JCV-, BKV- or SV40-specific probes. None of these tumors had detectable JCV or BKV DNA, although 10 of 20 choroid plexus tumors and 10 of 11 ependymomas generated amplification products which hybridized to the SV40 probe, suggesting that SV40 or an SV40-1ike virus was involved in the development of these childhood malignancies. In a separate study of human retinoblastomas (a retinal PNET), no JCV, BKV, papilloma or adenoviral DNA could be detected [83].

Krynska et al. [84] created a transgenic mouse which expressed JCV T protein driven by an archetype regulatory region. Unlike the results reported by Small, these transgenic animals had no dysmyelinating features. Rather, they developed PNETs of cerebellum and posterior fossa known as medulloblastomas. The PNETs expressed high levels of T protein which was associated with and stabilized p53. Moreover, 25–75% of the tumor nuclei were T protein positive by immunohistochemistry. This suggested that human medulloblastomas could likewise arise from JCV infection. Krynska et al. [85] examined 23 medulloblastomas and attempted to identify JCV genome using three different sets of PCR primers. Eleven of the 23 samples contained DNA sequences of the JCV genome that corresponded to all three amplified regions, 87% were positive for the N-terminal region of JCV T protein, 57% positive for the C-terminal region and 87% for the VP1 region. Moreover, immunohistochemistry identified T protein positive nuclei in 4/16 samples, ranging from 5 to 20% of all the cells seen on high-power field. VP1 staining was negative in 4 samples.

Interestingly, 5/23 samples contained SV40 DNA sequence in addition to JCV. Rencic et al. [86] also described an oligoastrocytoma in which expressed JCV DNA was detectable as was nuclear expression of T protein.

In vivo Oncogenicity – BKV

Like JCV, BKV is also oncogenic when inoculated into foreign hosts, particularly hamsters, mice and rats. Either intravenous or intracerebral inoculation of BKV into hamsters will result in a wide variety of tumors both within the neuraxis and extracranially. These tumor types include ependymoma, pinealoma, neuroblastoma, fibrosarcoma, osteosarcoma and pancreatic islet cell tumors [87–93]. Similarly, inoculation of BKV into either rats or mice results in gliomas, choroid plexus papillomas, fibrosacromas, osteosarcomas, liposarcomas and nephroblastoma [89, 90, 94]. Like JCV, there do appear to be some strains of BKV which are more oncogenic than others. Indeed, Gardner's initial isolate would appear to be the most oncogenic [95]. Changes in the regulatory region of BKV have been implicated as factors which increase early region expression, perhaps in a tissue-specific manner, thus resulting in targeted oncogenesis. One notable example is a deletion mutant of Gardner's original isolate which Watanabe et al. [93, 96] found could consistently result in insulinomas when inoculated in hamsters.

There are numerous reports describing the presence of BKV genomic sequences in a wide variety of human tumors. Fiore and DiMayorca [97] were the first to describe the presence of BKV DNA in human tumors using reassociation kinetics of ^{3}H-labeled BKV DNA with tumor DNA. They found evidence for BKV DNA in Wilms' tumor (a renal carcinoma), rhabdomyosarcoma (a muscle tumor) and spongioblastoma, a primary brain tumor. In 1983, Caputo et al. [26] found episomal BKV DNA in a pancreatic insulinoma from patient I.R. DNA extracted from the insulinoma was transfected into human embryonic fibroblasts with subsequent rescue of an isolate now called BKV-IR. BKV-IR was able to transform hamster kidney cells in vitro and had a deletion and insertion in the regulatory region setting it apart from wild-type BKV. This particular study was important in that BKV was known to produce pancreatic tumors in hamsters and now had a correlate in man. In a later study, Negrini et al. [98] also found episomal BKV DNA in Kaposi's sarcoma, Ewing and osteogenic sarcoma, glioma, meningioma and neuroblastoma. Again, BKV could be rescued from several of these tumors (ependymoma, meningioma, neuroblastoma, Kaposi's sarcoma and glioma) and had a regulatory region identical to BKV-IR. De Mattei et al. [99], using PCR amplification, were able to detect BKV genome in 85% of all primary brain tumors (100% of all astrocytomas, papillomas and spongioblastomas), 100% of all normal brain tissue, 78% of osteosarcomas, 38% of Ewing sarcomas and 71% of all normal peripheral lymphocytes.

It was also identified in very high percentages of cell lines derived from glioblastomas, meningiomas, neuroblastomas, osteosarcomas and giant cell bone tumor. In contrast to Negrini's data, sequence analysis of 12 different samples, both normal and tumor tissue, revealed wild-type BKV regulatory sequence. Monini et al. [100, 101] also described the presence of BKV genome in a variety of urogenital tumors, including tumors of the bladder, prostate, kidney and ureter. Given that BKV persistently infects the urogenital tract, it was not surprising that BKV would be implicated in the pathogenesis of urogenital malignancies. Interestingly, sequence analysis of nine samples found the identical sequence of the regulatory region in all the samples. The regulatory region was notable for a novel combination of duplications and deletions which had previously not been reported. The authors named this new BKV strain URO1.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 28–43

The Bovine Papillomavirus Type-1 E5 Oncoprotein

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Many DNA tumor viruses induce the proliferation of their cellular hosts to establish a more suitable environment for viral replication, assembly, and propagation. Years of functional and structural analyses of viral transforming proteins have generated a wealth of knowledge regarding the cellular genes regulating the control of cell growth, gene expression, cellular differentiation and cancer progression. In many cases, viral transforming proteins either activate host proteins involved in inducing cell proliferation or neutralize tumor suppressor proteins involved in inhibiting cell cycle progression. The papillomaviruses are small (~8,000 base pairs) double-stranded DNA tumor viruses commonly associated with the benign proliferation of epithelial cells. For example, a subset of the human papillomaviruses (see chapter by Hubert and Laimins, this volume) is associated with cervical dysplasia, which occasionally progress to cervical carcinomas [1–3]. The bovine papillomavirus type 1 (BPV-1) belongs to a subclass of papillomaviruses that induces the formation of fibropapillomas in the skin of cattle. These tumors are benign proliferations consisting of both dermal fibroblasts and epidermal keratinocytes. BPV-1 has been a useful genetic model for studying papillomavirus-induced cellular transformation by virtue of its ability to efficiently transform monolayers of murine fibroblast cells growing in tissue culture [4]. For the past 20 years, this model system has been used as a basis for defining the specific viral and cellular genes, as well as the structural requirements of their gene products, that play an essential role for BPV-1-induced cellular transformation. This assay was used to identify the major BPV-1 gene product responsible for fibroblast transformation, a 44-amino-acid, highly hydrophobic protein, designated the E5 oncoprotein [5]. This chapter summarizes the genetic, functional and structural studies on the

E5 oncoprotein. Furthermore, it provides an overview of studies employed to identify E5 cellular targets and characterize the mechanism by which E5 induces cellular transformation.

Properties of the BPV E5 Oncoprotein

Although consisting of only 44 amino acids, the E5 protein induces the rapid and efficient transformation of mouse fibroblast cell lines, such as NIH 3T3 and C127, in the absence of other viral genes. The E5 protein shows relatively little homology to other known proteins. The first 30 amino acids of the protein are believed to constitute an α-helical transmembrane (TM) domain, and the carboxyl-terminal 14 amino acids are generally hydrophilic, including two cysteine residues that stabilize homodimer formation via disulfide bonds (fig. 1) [6–8]. To generate clues as to the function of the E5 protein, initial studies were focused on characterizing its subcellular distribution using an antiserum directed against the carboxyl-terminal 20 amino acids. Immunoelectron and immunofluorescence microscopy studies using this antiserum demonstrated that E5 exists as a type II TM protein that localizes predominantly to the Golgi apparatus with its C terminus facing the lumen [7] (fig. 2). E5 protein is detected at the plasma membrane only when vastly overexpressed in tissue culture using the baculovirus system. Further studies with this antiserum demonstrated that the E5 protein exists in transformed cells as a homodimer of 7 kilodalton (kDa) subunits [5].

Mutagenic Analysis of the E5 Oncoprotein

Early studies on the effects of amino-acid substitutions throughout the E5 protein led to the identification of only eight specific residues that are critical for cellular transformation of mouse fibroblast cell lines [6]. Of the eight residues identified, seven of these reside within the C-terminal, hydrophilic domain. Two of these include cysteine residues at positions 37 and 39 (Cys37 and Cys39) that were speculated to be responsible for homodimer formation by engaging in disulfide bonding. The eighth residue is a glutamine, the only nonhydrophobic residue in the TM middle third of the protein. Besides the glutamine residue, the replacement of amino acids in this region with other hydrophobic residues, including large stretches of apparently random hydrophobic residues, had little effect on transforming ability [9, 10]. However, replacement with charged residues appeared to destabilize the protein and resulted in transformation defects.

The ability of E5 to tolerate many mutations without dramatic affect on its focus-forming ability suggested that it did not possess intrinsic enzymatic ability,

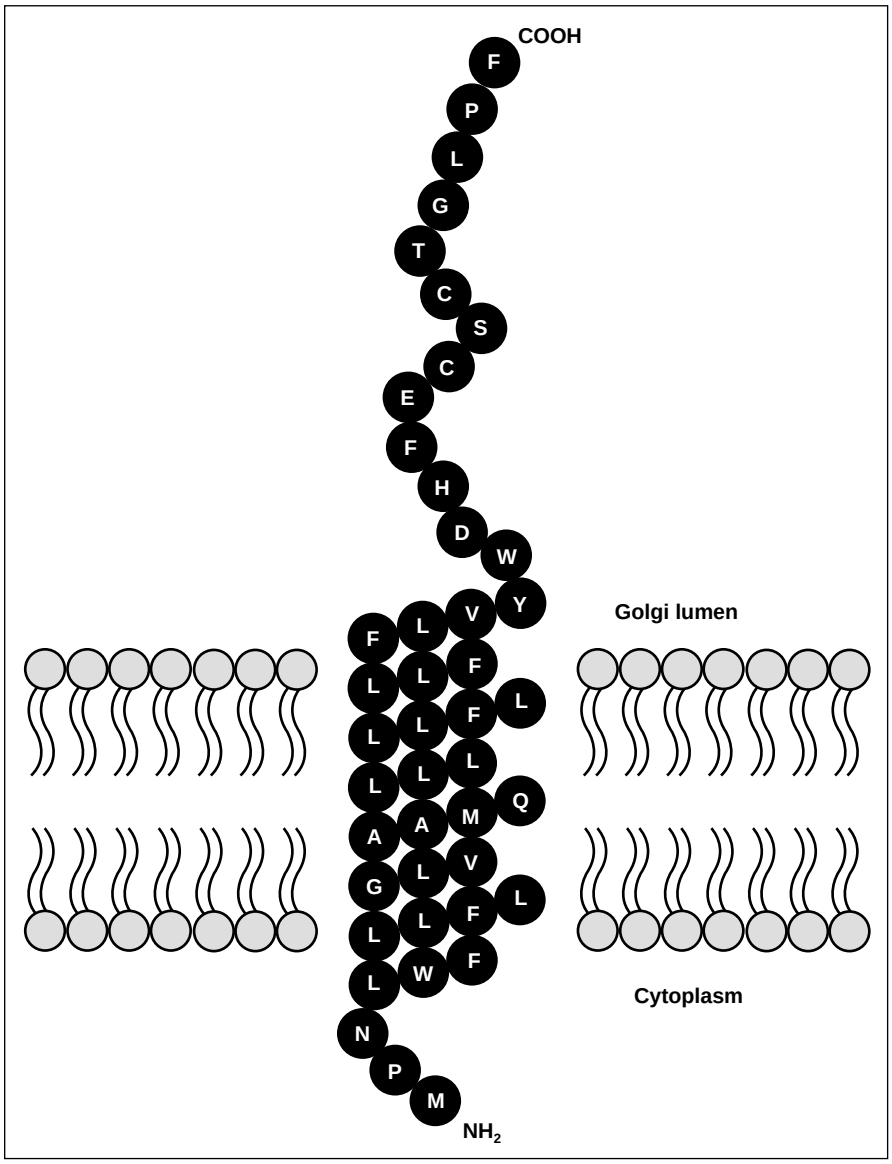

Fig. 1. Amino-acid sequence of the BPV-1 E5 protein. The E5 protein can be divided into two structurally distinct regions: the first 30 amino acids are generally hydrophobic and considered to be important for membrane insertion of the molecule while the 14 C-terminal amino acids are hydrophilic and contain two cysteine residues which contribute to E5 homodimerization. E5 is depicted here as a type II single-spanning membrane protein whose C-terminus extends into the Golgi lumen (see fig. 2).

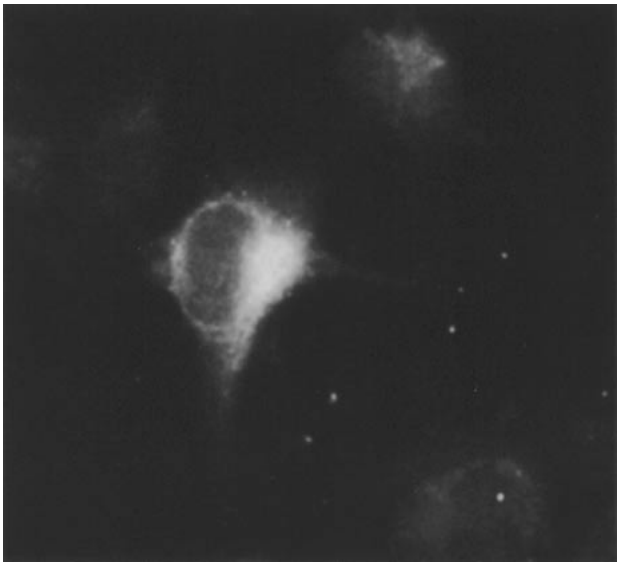

Fig. 2. The BPV-1 E5 oncoprotein localizes to the Golgi apparatus. Cos-1 cells were infected with a recombinant SV40, which expresses the E5 protein [43]. Seventy-two hours post-infection, the cells were fixed with formaldehyde, permeabilized with saponin, and reacted with a rabbit antiserum, which recognizes the carboxyl-terminus of E5. Indirect immunofluorescence microscopy using biotinylated goat anti-rabbit secondary antibodies and rhodamine-conjugated avidin revealed juxtanuclear Golgi staining only in cells infected with virus expressing the E5 protein.

but that it induced cellular transformation through interacting with or modulating the activity of cellular proteins that regulate cell growth. Those mutants that demonstrated transformation defects might thus correspond to E5 molecules that fail to properly interact with cellular targets and, as a consequence, fail to transform cells. The remainder of this chapter focuses on the identification of cellular targets of E5 and discusses the potential roles these interactions play in the transformation process.

Functional Interaction between the E5 and PDGF Receptor Proteins

Early biochemical studies by Martin et al. [11] indicated that E5 transforming activity of BPV-1 involves the activation of multiple protein-tyrosine kinase (PTK) growth factor receptors. They revealed that E5 cooperates with

the epidermal growth factor (EGF) and colony-stimulating factor-1 (CSF-1) receptors in transformation assays of NIH 3T3 cells. This increase in transformation was accompanied by an increase in receptor activity and half-life and a decrease in internalization of activated receptors. In addition, E5 transformation of mouse fibroblast cell lines was shown by DiMaio and colleagues [12] to involve the specific phosphorylation of the endogenous β-type receptor for the platelet-derived growth factor (PDGF). The specific activation of the PDGF-R was further supported by data demonstrating stable complex formation between E5 and the receptor [13, 14]. Further evidence suggested that E5-induced receptor activation and transforming activity involve the formation of physical complexes with two receptors: β-type PDGF-R and EGF-R [15]. Nilson and DiMaio [16] demonstrated that the E5 protein transforms a murine mammary epithelial cell line (NmuMG) only if these cells expressed β-PDGF-R as a result of transfection. Although these cells express readily detectable levels of endogenous EGF-R, E5 cellular transformation was not achieved without co-transfection with β-PDGF-R. These results were in apparent contradiction with earlier studies using rodent fibroblasts and a study demonstrating that E5 transforms epidermal keratinocytes which express EGF-R but not PDGF-R [17]. Consequently, it remained unclear whether the mitogenic and transforming activities of the E5 protein are a direct result of activation of a single growth factor receptor or whether E5 exhibits the ability to induce transformation through the activation of multiple PTK targets.

To further define the requirements for E5-mediated transformation and to identify which PTK receptors functionally interact with E5, we used a non-tumorigenic hematopoietic progenitor cell line, 32D, which is strictly dependent upon interleukin-3 (IL-3) for sustained proliferation in culture. 32D cells do not normally express PTK receptors for many growth factors (e.g., EGF, PDGF, CSF-1, Met, and Kit), thus allowing for the direct examination of the effects of E5 on individually expressed growth factor receptor genes. The expression of specific receptors and the addition of the appropriate ligand to the culture medium can substitute the IL-3 dependence for mitogenic signal transduction in these cells. For example, expression of EGF-R following transfection of the 32D cell line with an EGF-R expression vector conferred the ability to utilize EGF for the transduction of both a mitogenic and differentiation signal in these cells.

Using this system, we demonstrated that only the β-type PDGF-R conferred IL-3-independent growth and transformation of 32D cells when co-expressed with E5 [18]. Sustained IL-3-independent growth was accompanied by constitutive receptor tyrosine autophosphorylation and stable complex formation between E5 and the receptor. Despite considerable homology to the α-type PDGF-R, E5 was unable to cooperate or interact with this receptor to induce IL-3

independence, suggesting that cell proliferation induced by β-PDGF-R and E5 is highly specific. Furthermore, E5-induced growth factor-independent proliferation in a related cell system (Ba/F3 cells) required the expression of a catalytically active β-PDGF-R [19], indicating that the β-PDGF-R had to activate the receptor signaling cascade to deliver a mitogenic response.

Studies on the downstream effects of E5/PDGF-R co-expression in cells lends further credence to the hypothesis that the PDGF-R plays a central role in E5-mediated cell transformation. Activation of the PDGF-R signal transduction pathway is accompanied by a cascade of events, which begins with autophosphorylation of specific tyrosine residues on the receptor and binding of substrates via their SH-2 domains to these sites [20] (fig. 3). In E5 transformed cells, there was constitutive association between the receptor and phosphoinositol 3-kinase (PI3-K), phospholipase Cγ, and rasGTPase activating protein (GAP), SH-2 domain-containing cellular substrates that play important roles in response to PDGF stimulation of PDGF-R-expressing cells [19].

Interestingly, there exist certain E5 mutants that retained significant transforming ability yet exhibited a reduced level of binding to and activation of the β-PDGF-R [21, 22]. The existence of this class of E5 mutants that appear to lack correlation between PDGF-R activation and cell transformation indicates that there may be an additional pathway(s) by which E5 transforms cells. Schlegel and colleagues [23] recently demonstrated that this class of mutants elevated basal PI3-K activity in immunoprecipitates from NIH 3T3 cells despite failure to significantly activate the PDGF-R or the *ras*-dependent mitogen-activated protein kinase (MAPK) signal transduction pathway. Induction of PI3-K activity is presumed to result solely from the constitutive activation of receptor tyrosine kinases [24]. Therefore, Schlegel and colleagues proposed that under certain conditions, the E5 oncoprotein utilizes an additional signaling pathway for activating PI3-K and mediating cell transformation that is independent of PDGF-R activation (see below).

Specific Requirements for Functional E5/PDGF-R Interaction

To further analyze the molecular requirements for E5-mediated signal transduction through the β-PDGF-R and independent of other PTK receptors, the 32D-cell system was again employed. As described above, the 32D cells are strictly dependent on IL-3 for growth, thus permitting the selection in the absence of IL-3 for only those cells exerting a mitogenic stimulus through productive E5 interaction with exogenously expressed receptors. The α-PDGF-R, despite extensive amino-acid homology with the β-PDGF-R, does not interact with E5 and therefore provided an ideal partner for domain switching. Exchanging

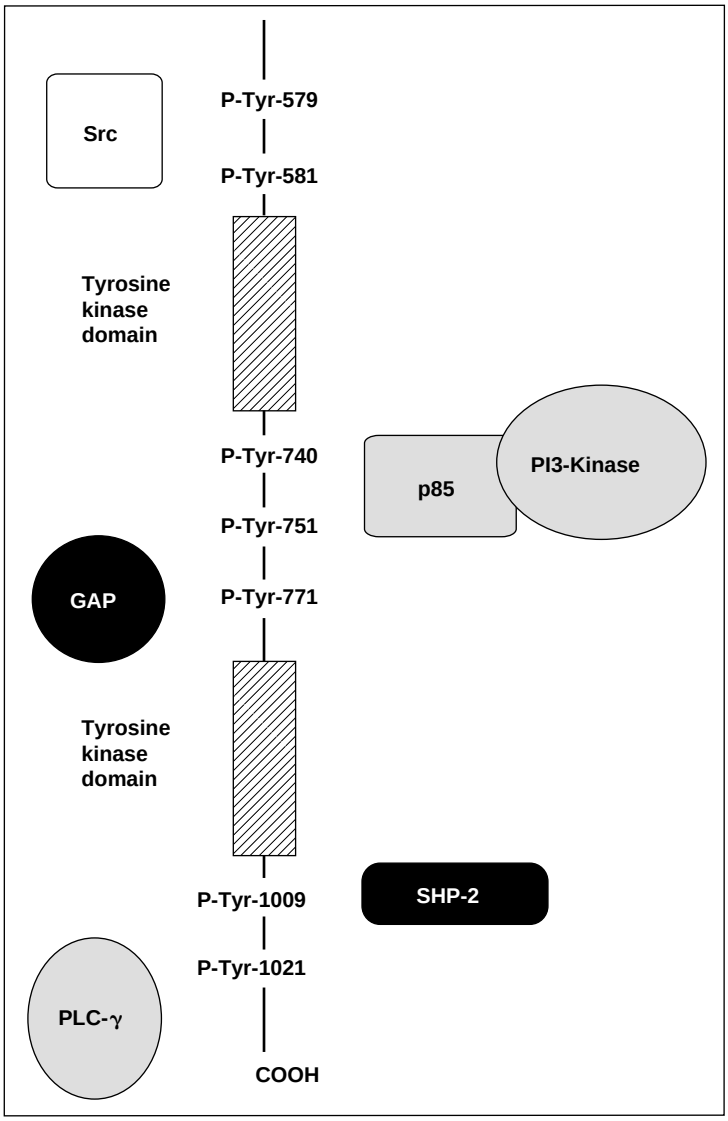

Fig. 3. Tyrosine kinase domain of the β-type PDGF receptor. The tyrosine kinase region (C-terminus) of the PDGF receptor resides in the cytoplasm of the cell. Growth-factor receptors dimerize upon binding of their appropriate ligand leading to activation of the kinase domains. Paired, active kinases specifically cross-phosphorylate tyrosine residues on the opposing receptor's C-terminus. These modified amino acids are targets for cell signaling molecules that bind specifically via their Src homology 2 (SH2) domains. Association of these accessory molecules to the receptor initiates a signal transduction cascade that results in mitogenesis.

extracellular domains critical for ligand-binding specificity [25, 26] had no effect on functional or physical interaction between E5 and receptor chimeras, demonstrating that the ligand-binding site of the β-PDGF-R is not involved in interaction with E5. Only those α/β chimeras containing a segment encompassing the juxtamembrane region on the extracellular side and the TM domain of the β-PDGF-R were able to cooperate with the E5 oncoprotein for induction of IL-3 independence in 32D cells [27]. These chimeras functioned like wild-type β-PDGF-R in that they were constitutively phosphorylated on tyrosine residues and bound to E5.

Further studies with a β-PDGF-R deletion mutant delineated the binding site to the TM, including a single lysine (Lys531) residue of the extracellular domain. It has been postulated that this lysine residue (positively charged) might participate in a charged interaction with an aspartic acid residue (negatively charged, Asp33) within the carboxyl-terminal juxtamembrane domain of E5 [28]. Interestingly, the α-PDGF-R encodes a glutamic acid residue (negatively charged) at the analogous position relative to the positively charged Lys531 in β-PDGF-R. Therefore, we hypothesized that the opposite charge in the α-receptor precludes E5 binding to this receptor species. To test the importance of the positive charge of the juxtamembrane residue of the receptor, the glutamic acid residue of a receptor was substituted with a lysine and tested for the induction of IL-3-independent growth in E5-expressing 32D cells. Although this mutant receptor induced transient proliferation in the absence of IL-3, no sustained growth was observed [D. Clarke and D. Goldstein, unpubl. data]. These results suggested that amino acids within the β-PDGF-R TM domain, in addition to the juxtamembrane Lys531, are required for productive E5/receptor interaction.

More recently, DiMaio and colleagues [29] speculated that there are at least two specific requirements for E5/β-PDGF-R interaction: (1) electrostatic interaction between opposed juxtamembrane residues of E5 (Asp33) and β-PDGF-R (Lys531), and (2) hydrogen bonding between the E5 Gln17 and a threonine residue within the center of the β-PDGF-R TM domain (position 545 of the human β-PDGF-R and 513 of the mouse β-PDGF-R). These residues are closely juxtaposed when the two proteins are aligned in an anti-parallel fashion. To test this hypothesis, they evaluated a series of E5 and β-PDGF-R mutants for their ability to cooperate in cell transformation assays in either the IL-3-based (Ba/F3) or C127 cell systems. These studies revealed a close correlation between the ability of E5 mutants to bind to and activate the PDGF-R and to transform cells [30]. Furthermore, all position 17 mutants unable to participate in hydrogen bonding were defective for complex formation, PDGF-R activation, and cell transformation. Lastly, a juxtamembrane negative charge on the E5 protein was required for all three activities. Although additional mutational

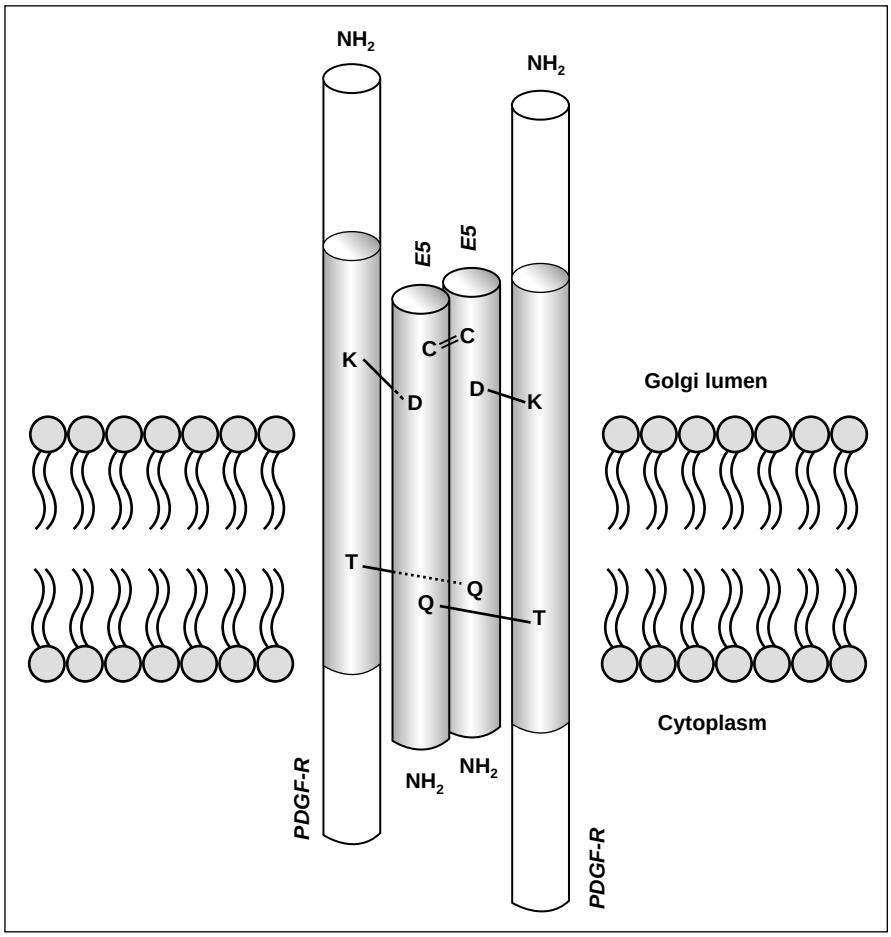

Fig. 4. A model for BPV-1 E5 and β-type PDGF receptor interaction. E5 and PDGF receptor TM domains (gray rods) are depicted in an anti-parallel orientation relative to each other. E5 homodimerization occurs through cysteine residue disulfide bonding and interactions between respective TM regions. Association between E5 and receptor occurs through non-covalent bonds within the respective TM and juxtamembrane domains. Molecular modeling has suggested that each receptor directly associates with each E5 within the complex [29].

analysis is required to determine if other specific residues participate in this interaction, the extent to which hydrophobic residues within the E5 TM domain can be substituted with minimal effect on transformation suggests that the identification of other key residues seems unlikely. Therefore, a model for the E5/PDGF-R interaction has been proposed: the E5 dimer, each with the binding

sites Gln17 and Asp33 (and probably other intervening residues) bring together two β-PDGF-R molecules by interacting with Thr545 and Lys531 of the receptor, thereby triggering receptor transphosphorylation [29] (fig. 4).

Based on alanine scanning mutagenesis, Schlegel and colleagues [21] identified three residues of the E5 TM domain, Gln17, Leu21 and Leu24, that are required for PDGF-R interaction. Interestingly, all three residues lie on the same face of the E5 α-helix, suggesting that they comprise part of the receptor-binding domain. Surprisingly, mutants containing alanine substitutions at Leu21 and Leu24 retained the ability to transform mouse fibroblast cell lines, despite their reduced ability to interact with the receptor. These results suggest that the association of E5 with PDGF-R may not be the sole determinant of E5 transforming activity. Supporting this hypothesis, recent studies on a cell line expressing one of these mutants revealed that tyrosine phosphorylation of EGF is elevated, suggesting a change in receptor specificity by these TM mutants [R. Schlegel, unpubl. data].

An Alternative Cellular Target of the E5 Oncoprotein

The 16-kDa subunit (16K) of the H^+-vacuolar ATPase proton pump (V-ATPase) [31] (also referred to as ductin [32]) was the first cellular protein identified as a target of the E5 oncoprotein. 16K is one of several subunits that make up the V-ATPase proton pump (fig. 5). Various cellular compartments, including the Golgi apparatus, endosomes, lysosomes and clathrin-coated vesicles, rely on the V-ATPase complex for pumping H^+ ions against a gradient. This ATP-dependent activity provides an acidic environment within these compartments that is essential for various cellular processes such as protein trafficking, protein sorting and endocytosis at the plasma membrane. 16K forms a homo-hexamer within the pump complex and is embedded in membranes. The remaining subunits of the pump use 16K as the foundation for the complex and are located on the cytoplasmic face of endomembrane compartments.

The association of E5 with 16K and the loss of transforming ability of certain TM mutants of E5 (substitutions at position 17) that no longer bound 16K led to the speculation that alterations in vacuolar H^+-ATPase activity by E5 contributed to cellular transformation [33]. An acidic internal endosome pH generated by vacuolar proton translocation is critical for dissociating ligand-receptor complexes as well as targeting these complexes for lysosomal degradation. Interference with the pump by E5/16K interaction might result in an increase in local pH, prolonged growth factor receptor/ligand interactions, and reshuttling of receptors to the cell surface. However, an increase in half-life or decrease in down-regulation was not observed for endogenously expressed PDGF receptors

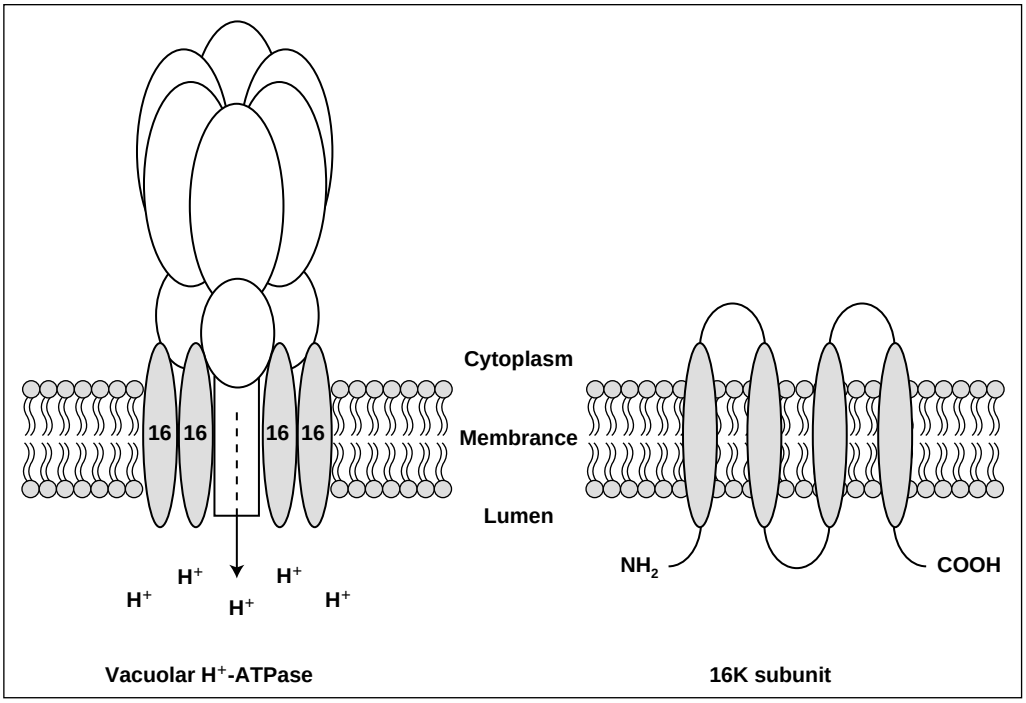

Fig. 5. The vacuolar H^+-ATPase (V-ATPase) and the 16K subunit**.** Left: The V-ATPase proton pump is shown as a membrane-bound complex of subunits that face the cytoplasm of compartments such as endosomes, lysosomes, clathrin-coated vesicles and the Golgi complex. 16K, which is in a homohexamer form within the complex, is the membrane-spanning, pore-forming component of the V-ATPase. Right: An individual 16K subunit. 16K has four hydrophobic, TM-spanning domains of which the fourth interacts with the BPV-1 E5 oncoprotein [40].

in mouse fibroblasts [34], suggesting that alterations in receptor processing through inhibition of proton pump activity may not be the primary mechanism by which BPV-1 E5 transforms cells.

The close correlation between E5 transformation and PDGF-R activation in fibroblasts suggested that the association between E5 and this receptor represents the primary mechanism for E5-mediated cell transformation. However, several studies suggested that alternative mechanisms might exist, such as E5 perturbation of V-ATPase activity. For example, BPV E5 is capable of transforming keratinocytes, which lack PDGF-R [17]. Moreover, several E5 mutants have been shown to either fail to transform cells, despite retaining the ability to induce PDGF-R autophosphorylation, or to retain transforming ability without

inducing significant PDGF-R autophosphorylation [22, 35]. Alterations of Golgi pH may thus provide an alternative mechanism to account for cell transformation in situations where E5 ability to activate the PDGF-R is compromised or in cells lacking this receptor. However, only until recently has it been possible to accurately measure intracellular pH in E5-transformed cells. Almost a decade after the first demonstration of E5 association with the 16K protein, Grinstein and colleagues [36] revealed using ratio imaging of Golgi-targeted, H^+-sensitive probes that the E5 oncoprotein impairs acidification of the lumen of the Golgi complex. This effect appears to be specific to the E5 protein and not merely a consequence of cellular transformation, since other oncoproteins (e.g., *sis* and *src*) failed to elicit this effect.

It is noteworthy that the HPV-16 E5 protein has also been reported to associate with the 16-kDa subunit of V-ATPase [37]. Furthermore, and in contrast to BPV E5-expressing fibroblasts, human keratinocytes expressing HPV E5 demonstrated higher levels of ligand-induced tyrosine phosphorylation of EGF receptors and an inhibition of EGF-R turnover [38]. This change in receptor processing is concomitant with the alkalinization of endosomes [39]. Therefore, these findings suggest a convergence of the biology of both HPV and BPV E5 proteins in that they both bind to 16K, inhibit the V-ATPase, and disrupt intracellular pH.

Comprehensive mutational analysis has been performed to characterize domains important for the BPV E5/16K interaction. Such studies revealed that the fourth TM domain of 16K is important for interaction with the hydrophobic domain of E5 [40]. More specifically, a glutamic acid residue within this domain of 16K (E143), which is known to be essential for the activity of the proton pump [41], is also critical for binding Gln17 of E5. Expression of two mutant forms of 16K (glutamic acid-to-arginine substitution at position 143, E143R, or deletion of the entire fourth TM domain) resulted in transformation of NIH 3T3 cells in a manner similar to E5 [40]. Most significantly, 16K E143R mutant induced a significant alkalinization of the Golgi complex, also resembling transformation-competent E5 molecules. Since the E143R mutant retains the ability to associate with wild-type 16K [40], this interaction may inhibit the activity of the pump. These genetic and biochemical findings indicate a potential role for 16K in E5 transformation.

Conclusions

The results described above provide strong evidence that the BPV-1 E5 oncoprotein transforms fibroblasts through specific activation of a growth factor receptor, the β-type PDGF-R. The mechanism by which the viral protein elicits

transforming ability through this PTK receptor represents a divergence from previously described mechanisms involving growth factors and their respective receptors. Whereas ligand interaction with PTK receptors involve the receptor extracellular domain, the E5 protein displays exquisite selectivity for the β-PDGF-R by forming both electrostatic and hydrogen bonds between very specific hydrophilic amino-acid residues either within the TM or juxtamembrane regions of each binding partner. However, the consequence of both mechanisms appears to be the same: juxtapositioning of two receptor molecules, autophosphorylation of specific tyrosine residues on their intracellular domains, recruitment of cytoplasmic signaling proteins to these residues, and initiation of a signal transduction cascade resulting in a mitogenic stimulus in the nucleus. However, an interesting distinction between ligands and E5 exists: ligands primarily activate receptors located at the cell surface, whereas the E5 protein activates intracellular, precursor forms of the receptor [19, 42] and does not traffic to the cell surface unless vastly overexpressed [7]. The activation of receptors at these discrete cellular compartments may lead to the recruitment of distinct subsets of signal transducing proteins, thereby eliciting different signaling effects on the cell. Our laboratory constructed a modified PDGF-R that fails to traffic to the cell surface, and thus cannot be activated by the PDGF-R ligand (PDGF BB [27, and unpubl. data]). Despite this apparent defect, this receptor cooperates with E5 to induce cellular transformation of mouse fibroblasts and IL-3-independent growth of 32D cells [D. Clarke and D. Goldstein, unpubl. data]. Reagents such as BPV-1 E5 and this modified receptor should be useful for further elucidating the downstream effects of activating receptors located within intracellular compartments, and determining whether this differs from activating receptors stationed at the cell surface.

Further studies are also required to determine whether E5 modulates growth regulatory pathways through alterations in V-ATPase activity. Alterations in the proton pump by E5 may result in pH changes within subcellular compartments, such as the Golgi apparatus or endosomes, and could lead to changes in receptor trafficking/processing or changes in signaling intermediates. Although binding to the V-ATPase may contribute to the transforming ability of E5, this association does not appear to be sufficient for transformation [22]. In addition to promoting transformation, Golgi alkalinization may also contribute to other aspects of the viral life cycle, such as interfering with antigenic presentation and evading immune recognition or virus assembly and release.

The growing body of evidence for the involvement of PTK receptors in papillomavirus-induced cellular transformation warrants further research in this area. Characterization of the viral/cellular protein complexes that are associated with receptor-activated, signal transduction pathways should help towards a better understanding of the complex mechanisms of papillomavirus transformation.

The data generated from these studies may also provide new insights into molecular and structural aspects of PTK receptors, receptor-mediated signal transduction, and the control of cell growth.

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The Action of E6 and E7 of Human Papillomaviruses in Cellular Immortalization and Transformation

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Background

Introduction

Papillomaviruses are small, nonenveloped DNA viruses that infect numerous vertebrate hosts, including humans. Viral infection targets epithelial cells and progeny virions are produced upon cellular differentiation. Infection by more than 70 human viral types identified to date [1] leads to benign hyperproliferative lesions or warts [2]. In addition, infection by a subset of human types (HPVs) is causally associated with the development of cancer. Such oncogenic HPVs include types 16, 18, 31, 33 and 35 and are referred to as 'high risk' to indicate the probability for malignant progression following infection [3].

HPVs encode only 8–10 genes and most of the factors necessary for papillomavirus production, including those involved in viral transcription, translation, and DNA replication are provided by the host cell [reviewed in 4]. Production of viral progeny is coordinated by the action of the HPV oncoproteins E6 and E7 which modulate cell growth and differentiation, the replication and transcription factors E1 and E2, and the structural proteins L1 and L2. Cellular immortalization by HPVs is restricted to the high-risk types and is induced by the association of E6 and E7 with cellular proteins that regulate cell growth. The interactions of E7 with the retinoblastoma tumor suppressor protein (p105Rb) and E6 with the tumor suppressor p53 play central roles in these processes [4]. In the early stages of HPV infection, these protein–protein

associations are believed to alter the cellular environment to allow for the maintenance of viral genomes. Unfortunately, these same interactions also create a state of genomic instability which aids in cellular transformation. During the productive phase of the viral life cycle, HPV DNA replicates as a plasmid but is often found integrated into the cellular genome in high-grade lesions [5]. While integration does not appear to be essential for malignant progression it can contribute to dysregulated expression of the viral oncoproteins required for tumorigenesis. In this section ('Background'), we present a brief description of HPV biology and the mechanisms of cellular transformation. Below in the section on 'Biological Functions of the HPV E6 and E7 Oncoproteins', we describe the functions of the viral oncogenes E7 and E6 and their known effect on regulating cell growth.

HPV Classification, Pathogenesis and Cancer

Papillomaviruses (PVs) contain circular, double-stranded DNA genomes of about 8,000 base pairs in size. The capsids consist of virally encoded proteins which assemble with icosahedral symmetry into virions of about 55 nm in diameter [4]. PVs belong to the Papovaviridiae family, which includes polyomaviruses, and vacuolating viruses of primates and humans, such as simian virus (SV) 40, JC and BK virus [6]. PVs are named according to their host specificity, such as human (HPVs), bovine (BPVs), etc., and are numbered by viral genotype. The target cells of PV infection are basal keratinocytes, either cutaneus or mucosal, where the viral DNA establishes itself as a multicopy, nuclear plasmid. The production of progeny virions is restricted to terminally differentiated layers of the epithelium [reviewed in 5]. A small subgroup of PVs, the fibropapillomaviruses of ungulate animals, can also infect and replicate their DNA in fibroblasts as well as keratinocytes but no human viruses have been shown to exhibit such a dual tissue specificity [7].

The genomes of all known papillomaviruses are organized in a similar fashion and include common open reading frames (ORFs) [4]. A noncoding, upstream regulatory region (URR, also called the long control region or LCR) contains binding sites for transcription factors as well as an origin of DNA replication. Typically, PV genomes encode up to ten genes in overlapping ORFs which are transcribed unidirectionally and grouped into either 'early' or 'late' functions (fig. 1a). Early genes are expressed throughout the viral life cycle while late genes are only expressed during the productive phase in differentiated keratinocytes. Most HPV-encoded genes can be functionally classified into three groups: (1) the regulatory proteins E1 and E2 which control viral DNA replication and viral gene expression; (2) E6, E7 and E5, which control cellular growth by interacting with p105Rb, p53 and other cellular proteins, and (3) the structural virion components L1 and L2 [5].

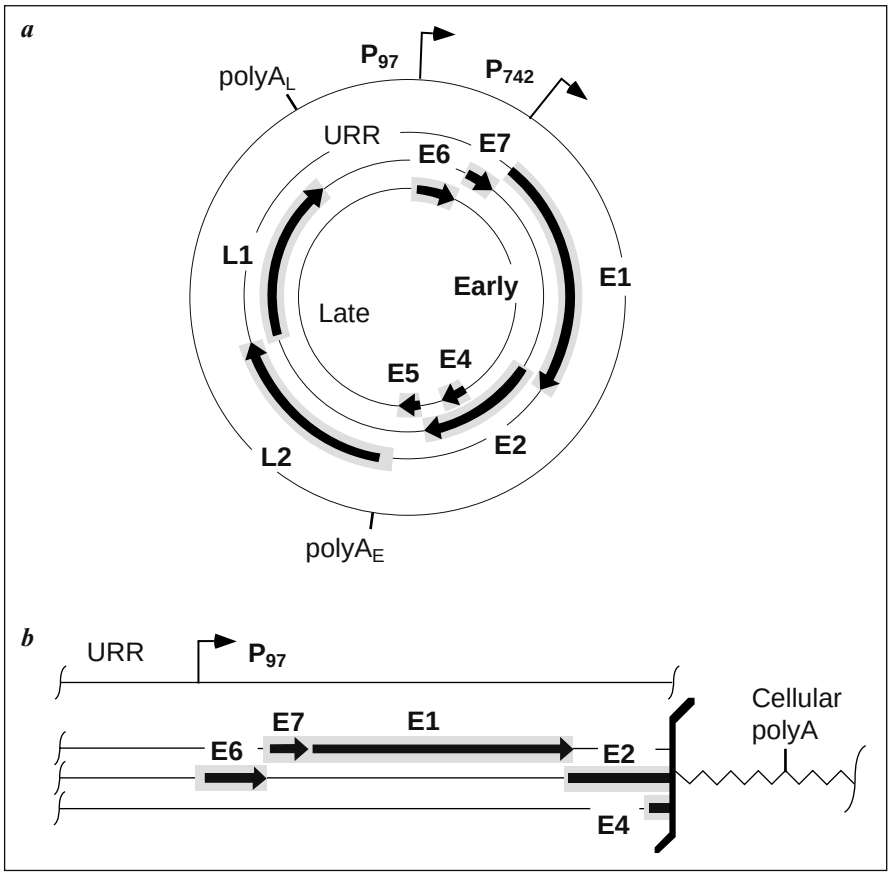

Fig. 1. Maps of the HPV31 genome and integrated HPV16 DNA. ***a*** The genetic organization of all known papillomaviruses is very similar. The circular genome of about 8,000 bp is divided into three distinct regions: early, late and upstream regulatory region (URR). These regions contain the viral genes (solid arrows within stippled arcs) in multiple, overlapping ORFs (stippled arcs). Early genes (E) are expressed throughout the viral life cycle, primarily from the major viral promoter P_{97} (angled arrow at the periphery). A differentiation-specific promoter P_{742} directs the expression of the late (L) genes during the productive phase of the life cycle only. The positions of the polyadenylation signals for early and late mRNAs are also indicated at the periphery. ***b*** Integration of HPV16 into the cellular genome often disrupts the E2 gene. Therefore, E6/E7-specific mRNA originating from the major viral promoter P_{97} contain a cellular 3′ end (zigzag line) which increases mRNA stability and contributes to high-level oncogene expression.

More than 70 distinct types of HPVs have been identified to date and all induce benign proliferative lesions. Furthermore, a subset of HPVs are associated with the development of malignancies. The most prevalent oncogenic, or high-risk, HPVs are types 16, 18, 31, 33 and 35 and all have a tropism for mucosal tissue in the anogenital tract [7]. HPV DNA can be detected in over 95% of all premalignant and malignant genital lesions, with HPV types 16 and 18 being the most prominent types [8]. Other common mucosal HPV types, such as types 6 and 11, induce benign genital or laryngeal papillomas but are rarely detected in cancers. Genital HPVs are sexually transmitted pathogens and a large segment of the population has been infected with these viruses. Specifically, over 60% of college age women in the USA acquire genital HPV infections in the first 2 years of college [9]. Most women will clear these infections within several months [10] but persistence of infection increases the risk for development of cervical cancer. The incidence of HPV-induced cervical cancer is particularly high in developing countries where diagnostic measures, such as the Papanicolaou (Pap) smear test, are not commonly available. Worldwide, cervical cancer is the second most common type of cancer among women [11]. Another group of oncogenic HPVs, such types 5, 8 and 20, have a tropism for the cutaneous epithelium and are associated with epidermodysplasia verruciformis (EV) as well as malignant squamous cell carcinomas. These malignancies are rare and require a genetic component to predispose a patient to tumorigenesis following HPV infection [2]. The remaining cutaneous HPVs, including the common types 1, 2 and 7, cause benign warts of the extremities.

HPVs infect dividing basal keratinocytes through microscopic lacerations of the skin or mucosa. While the molecular details of viral uptake are not completely understood, it is known that, within a few cell divisions after viral entry, the HPV plasmid DNA establishes itself in the nucleus of the infected host cells as a multicopy plasmid. During the ensuing persistent phase of infection the viral DNA is maintained at 20–50 copies per cell [5]. These cells can remain persistently infected for periods as long as several decades. HPV DNA is replicated through direct action of the E1 and E2 proteins while E6 and E7 induce proliferation of infected cells. The resulting cellular hyperproliferation leads to a thickened epithelium and forms the basis of the lesion. As infected keratinocytes leave the basal layer and begin differentiation they remain in the cell cycle and retain their nuclei. In contrast, normal keratinocytes stop dividing and become denucleated in the suprabasal layers of the epithelium. As part of the productive phase of the HPV life cycle, viral DNA synthesis continues in the postmitotic, suprabasal cells and plasmid copy numbers can be amplified to several thousand. Prevention of cell cycle exit, retention of the nucleus and viral DNA amplification require the continued expression of E6 and E7, as well as

E1 and E2 [12–14]. Concomitantly with DNA amplification, the structural HPV genes L1 and L2 are expressed. L1-containing capsomers form spontaneously and, together with L2, assemble into virions around one viral minichromosome. After assembly, HPV virions are disseminated to new host cells when virus-laden squames are shed from the surface of the epithelium [4].

Mechanisms of HPV Transformation and Tumorigenesis

The benign proliferative lesions induced by most HPV types usually regress spontaneously after a period due to activation of the host's immune response. The failure to mount an immune response against high-risk HPV infections allows a lesion to persist and predisposes an infected individual to develop neoplasia [7]. The malignant conversion of high-risk HPV-infected cells occurs at a much higher frequency than cells infected with low-risk HPVs. The increased risk to develop cancer is the direct result of the properties of the oncoproteins E6 and E7 of high-risk viruses. In addition to the action of the viral oncoproteins, tumor progression also requires other genetic changes in the expression of cellular genes. Such cellular mutations are acquired during the time between the initial infection and the onset of malignancy.

Insights into the molecular mechanisms of HPV transformation and tumorigenesis have been obtained from the analysis of tumor biopsy-derived cells, such as HeLa and Caski which contain HPV type 18 or 16 sequences, respectively. In these cells, the HPV DNA was found to be integrated into the cellular genome [15]. During the normal life cycle, cell growth is governed by the actions of the viral oncogenes and their expression is regulated through an E2-dependent control mechanism. The interactions of high-risk E6 and E7 with p53 and p105Rb, respectively, can bypass the cell cycle checkpoints in G1 and G2 resulting in genetic instability [16, 17]. These alterations can result in the integration of HPV DNA into the cellular genome (fig. 1b), disrupting either the E1 or E2 gene and abrogating E2 expression. Normally, E2 activates the major viral promoter (P_{97} in HPV16 and 31; fig. 1a) at low protein levels [18] but represses promoter activity at high levels [19–21]. In the absence of E2 protein, E6/E7 expression from P_{97} is no longer regulated and cells gain increased proliferative capacity [22].

A second effect of DNA integration is the alteration of mRNA structure for E6 and E7. Normally, these mRNAs are short-lived and the steady-state levels of oncoproteins, therefore, remain low. Integration of HPV DNA in E1 or E2 ORFs provides a new, cellular 3′-untranslated region (fig. 1b) for transcripts encoding E6/E7. As a result, the E6/E7 mRNA stability is increased which leads to increased levels of oncoproteins [23]. The disruption of E2-mediated repression of P_{97} and the increase of E6/E7 mRNA half-life are two major mechanisms that contribute to the maintenance of the tumorigenic state.

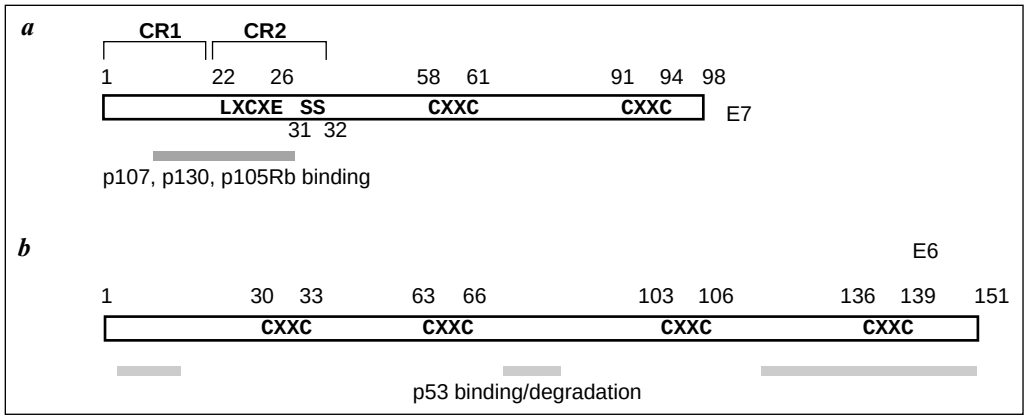

Fig. 2. Maps of the major oncoproteins of HPV16. ***a*** The E7 protein contains specific peptide domains: 'LXCXE' for interaction with p105Rb, 'SS' indicates the casein-kinase phosphorylation site, and 'CXXC' are zinc-finger domains of the protein. Large T antigen from SV40, E1A from adenovirus, and E7 from oncogenic HPVs share conserved regions (CR) which are involved in cellular transformation. The binding domains for the pocket proteins p107, p130 and p105Rb are indicated (stippled bar). ***b*** The E6 protein contains multiple CXXC Zinc-finger motifs. The protein domains involved in binding and degradation of p53 are indicated (stippled bars).

Biological Functions of the HPV E6 and E7 Oncoproteins

E7 Is a Major Regulator of Cell Growth

E7 proteins from the mucosal HPV types (high- and low-risk) are between 97 and 110 amino acids in length (fig. 2a). The C-terminus of E7 contains two repeated C-X-X-C motifs required for binding to zinc, while the N-terminus of E7 is comprised of two domains, termed conserved regions (CR) 1 and 2. CR1 and CR2 share extensive amino acid and functional similarity with E1A protein of adenovirus type 5 and the large T-antigen from SV40 [24, 25]. Both CR1 and CR2 are required for transformation of fibroblasts [26], immortalization of keratinocytes [26], as well as blocking TGFβ-mediated repression of cell growth [27]. Many of these properties of E7 are the result of specific protein-protein interactions with cellular regulatory proteins [reviewed in 28].

Numerous interaction partners of E7 from high-risk HPVs have been identified to date. These include the pocket proteins p105Rb [29], p107 [30] and p130 [30]; the cyclins A [30, 31] and E [32]; cyclin-dependent kinase inhibitors (CKI) p21 [33, 34] and p27 [35], histone H1 kinase [36], histone deacetylase (HDAC) 1 [37], members of the AP1 family of transcription factors [38], as well

as the interferon-stimulated gene factor ISGF3γ [39]. Binding of these cellular regulators to high-risk E7 has the following broad consequences: Enhanced entry into S-phase by indirect transcriptional activation of S-phase-specific genes and inactivation of the G1 and G2/mitotic spindle checkpoints. Other biological functions of high-risk E7 include the inhibition of cell cycle exit, retention of nuclei during differentiation, and induction of apoptosis. Finally, high-risk E7 also contributes to the abrogation of interferon-α-mediated signaling through the STAT1 pathway. The E7 proteins from high- and low-risk HPVs can be distinguished by differences in their binding affinities to pocket proteins. In addition, they associate with CKIs to regulate cell growth. The functional interactions of E7 with its cellular binding partners will be discussed further in the context of cellular growth regulation.

High-Risk HPV E7 Promotes Entry into S-Phase of the Cell Cycle

E7 can accelerate entry into S-phase by interacting with members of the Rb family of proteins, as well as histone deacetylase. One of the best characterized regulatory pathways for cell cycle progression is that involving p105Rb [reviewed in 40, 41]. In normal cells, p105Rb is hypophosphorylated during early G1-phase. Hypophosphorylated p105Rb associates with the transcriptional regulator E2F1 to inhibit E2F1's activation of S-phase-specific promoters, such as those for cyclin E, the proliferating cell nuclear antigen (PCNA), and ribonucleotide reductase. Upon exposure to a mitogenic signal, the cyclin D/cyclin-dependent kinase (cdk) 4 complex is activated and phosphorylates p105Rb resulting in the release of E2F1 and activation of S-phase-specific gene expression. The importance of p105Rb-mediated regulation of S-phase entry is underscored by the fact that in many human cancers the p105Rb-dependent pathway is functionally inactivated. This can occur through mutations in the Rb protein coding sequences, loss of the tumor suppressor p16 which regulates cdk4, and overexpression of cyclin D1. The binding of E7 to p105Rb, functionally equivalent to phosphorylation of p105Rb, leads to the release of E2F1, and abolishes the p105Rb-dependent control of S-phase entry [42]. Besides E2F1, the E2F family of transcription factors consists of at least four other members, some of which are regulated by binding to p107 while others associate with p130. Similarly, when E7 binds to p107 and p130, the corresponding E2F family member is dissociated from this complex to activate expression of S-phase-specific genes. Furthermore, binding of E7 to p105Rb also releases H1 deacetylase to relieve chromatin structure-mediated inhibition of transcription. When E2F factors dissociate from the pocket proteins, transcription of cyclin E is activated and cyclin E/cdk2 activity increases. In addition, cyclin E protein levels are also increased by E7 through a post-transcriptional mechanism [32, 43]. The abrogation of normal p105Rb function by high-risk E7 is increased further

by the ability of high-risk E7 to induce the degradation of the hypophosphorylated form of p105Rb through the ubiquitin pathway [44]. This destabilization of growth-inhibiting p105Rb is independent of the p53 status of the cell [45].

High-Risk HPV E7 Abrogates Cell Cycle Checkpoints

The mammalian cell cycle contains two major checkpoints that are induced by DNA-damaging agents and growth-inhibiting signals. The checkpoint in G1-phase has been well characterized to date, but the molecular details of the G2/mitotic spindle checkpoint are not well understood. E7 can overcome a G1-phase arrest by either binding to the CKIs p21 and p27 or through activation of E2F proteins [33–35, 46]. The p21-dependent G1 arrest is also invoked upon exposure of cells to DNA damage by genotoxic drugs or ionizing radiation to allow for DNA repair prior to S-phase. This checkpoint is controlled by cyclin D/cdk4, cyclin D/cdk6, and cyclin E/cdk2 protein complexes [reviewed in 47]. In the presence of the CKIs from the p21Waf1/p27Kip1/p57Kip2 or the p15/p16/p18 families, the cdk activities are downregulated and the regulatory pocket proteins p105Rb, p107 and p130 remain hypophosphorylated. Binding of high-risk E7 to p21 and p27 sequesters these CKI proteins and, as a result, the cyclin/cdk complexes become active and phosphorylate the pocket proteins. The E7 and p21 interaction also appears to stabilize p21 protein levels [45]. Furthermore, binding of E7 to p21 competes with the p21-PCNA interaction and thus removes a barrier for DNA replication [34]. E7 can also abrogate the G1-phase checkpoint independent of p21 by direct activation of E2F1 [46].

In addition to the G1-phase checkpoint, E7 can also act to bypass the mitotic spindle checkpoint [48, 49]. In the presence of compounds that inhibit the formation of the mitotic spindle, such as nocodazole, E7-expressing keratinocytes can undergo multiple rounds of DNA synthesis without proceeding through cell division. As a result, these cells accumulate polyploid genomes independent of their p53 status. The mouse double minute (Mdm) 2 oncoprotein and p105Rb have been implicated in the regulation of this checkpoint and Mdm2 levels are elevated in E7-expressing cells [49]. High-risk E7 also interacts with histone H1 kinase at the G2/M boundary of the cycle. This binding requires an intact CR2 and may modulate checkpoint control [36]. Thus, in addition to bypassing the G1 checkpoint, the interactions of E7 with p105Rb and p107 may also contribute to the abrogation of the G2/mitotic spindle checkpoint.

Role of E7 in Cellular Transcription, Differentiation, Apoptosis, and Interferon Response

As part of the normal differentiation program, keratinocytes exit the cell cycle as they leave the basal layer, migrate towards the top layer of the epithelium, and become denucleated. During this process, the morphology of the

keratinocytes is altered due to changes in cytokeratin expression. Keratinocytes which express high-risk E7 do not exit the cell cycle once they leave the basal cell layer, and retain a similar pattern of keratin expression that is similar to that of uninfected suprabasal cells [12, 50, 51]. Reactivation of DNA synthesis in these cells is thought to be facilitated by E7-mediated increases in cyclin E levels and cyclin E/cdk2 kinase activities. However, when both the CKI p21 and cyclin E are expressed at very high levels in suprabasal cells, no cellular or viral DNA synthesis takes place [14]. High-risk E7 has been found to interact with the members of the AP1 family of transcription factors which includes c-Jun, JunB, JunD and c-Fos [38]. Since p105Rb can also bind to these proteins [52] it is possible that the combined E7/p105Rb-mediated modulation of AP1-dependent transcription is also required for DNA synthesis to occur in suprabasal cells. The competence of differentiating keratinocytes to reinitiate DNA synthesis may in part be determined by which phase of the cell cycle the cells are in when they commit to terminal differentiation. Expression of E7 by itself in epithelial cells leads to apoptosis which can be reduced by coexpression of E6 from high-risk HPVs [53, 54]. This induction of apoptosis may occur in multiple steps whereby E7 expression first predisposes keratinocytes to cell death by increasing p53 and p21 protein levels. Such sensitized cells will then undergo apoptosis at a higher rate, either spontaneously or after treatment with tumor necrosis factor α [55].

Interferons (IFN) are cytokines that regulate cellular proliferation and modulate the immune response to viral infection [reviewed in 56]. The known IFN types α, β and γ use the Jak-STAT pathway of signal transduction to activate IFN-responsive genes [57]. IFNα signaling specifically involves a tetrameric protein complex composed of signal transducer and activator of transcription (STAT) proteins 1α and 1β, STAT2 and ISGF3γ that binds to IFN-stimulated response elements [58, 59]. The STAT proteins in this complex are collectively referred to as ISGFα proteins. In vitro studies have shown that E7 from high-risk HPVs binds to ISGF3γ and inhibits the formation of the tetrameric activator complex. IFNα signaling is thus abrogated without affecting the IFNγ pathway [39]. This bypass of the IFNα pathway may in part account for the poor success rate of IFNα therapy against high-risk HPV infections [60].

Properties of E7 from Low-Risk HPVs

Most of the transforming properties of high-risk E7 are dependent on the CR2 protein domain and this region differs most significantly between high-risk and low-risk E7 proteins [reviewed in 61]. In general, E7 from low-risk HPVs binds to p105Rb or related pocket proteins with reduced affinity compared to high-risk E7 [62]. The E7 proteins from low-risk HPVs are not able to immortalize cells since this property requires high affinity binding to Rb family members. However, low-risk E7 can overcome the p21- and p27-mediated G1-phase

checkpoint [63]. Therefore, the induction of efficient cellular proliferation observed with low-risk HPV infections may in part be controlled through the same molecular mechanisms that are utilized by high-risk HPVs.

E6 Is a Major Regulator of Cellular Proliferation

E6 proteins from the mucosal HPV types (high- and low-risk) are between 149 and 158 amino acids in length. Similar to E7, E6 proteins contain four repeated C-X-X-C motifs which are required for binding to zinc, interacting with cellular factors, and possibly for dimerization (fig. 2b). Alternative splicing of transcripts from high-risk HPVs could produce mRNAs encoding smaller E6 proteins (E6*I, E6*II, E6*III) which have been shown in vitro to modulate the activities of full-length E6 [64]. However, expression of these smaller E6 proteins in vivo has not been demonstrated conclusively. While E6 does not share sequence homology with proteins from other DNA tumor viruses, there is functional similarity among these factors. Like SV40 large T-antigen and E1B (55 kDa) from adenovirus, E6 can bind to the tumor suppressor protein p53. However, binding of E6 to p53 does not stabilize p53, as is the case with large T and E1B, but targets p53 for degradation [65].

Expression of high-risk E6 proteins results in the transformation of fibroblasts, immortalization of keratinocytes, and transcriptional modulation of heterologous promoters in transient assays. At present, the mechanisms of transcriptional regulation by E6 are not known and this activity of E6 does not correlate with its known transforming/immortalizing functions [reviewed in 66]. Numerous cellular proteins have been identified which bind to E6 and the biological functions of these interactions are well characterized. E6 can bind to the tumor suppressor p53 [67], the E6-associated protein (E6AP or ubiquitin ligase E3, [68]), the replication licensing factor multi-copy maintenance (Mcm) protein 7 [69, 70], the transcriptional coactivator CEBP/p300 [71, 72], the human homologue of the Drosophila disk large tumor suppressor protein (hDlg, [73, 74]), a putative GTPase-activating protein E6TP1 (E6-targeted protein 1 [75]), the calcium-binding protein ERC-55 (also termed E6-binding protein or E6BP [76]), the focal adhesion protein paxillin [77], the clathrin-adaptor AP-1 [78], the interferon regulatory factor (IRF) 3 [79], and the cytoplasmic tyrosine kinase Tyk2 [80]. Binding of these cellular regulators to E6 has the following broad consequences: E6/E6AP-induced degradation of p53 and inactivation of the G1 cell cycle checkpoint, abrogation of the G2/mitotic spindle checkpoint, induction of telomerase during cellular immortalization, as well as E6-mediated prevention of apoptosis and resistance to differentiation. Additional cellular proteins can interact with E6 and are implicated in p53-independent mechanisms of

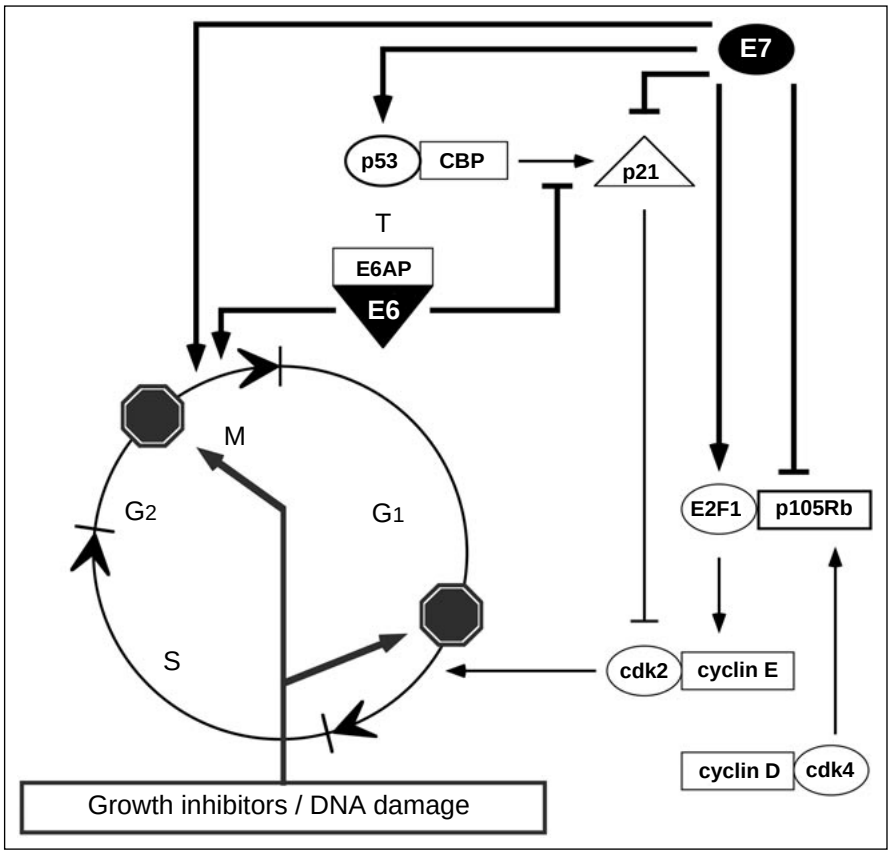

Fig. 3. Dysregulation of the cell cycle by the HPV oncoproteins. This schematic shows the major signaling mechanisms by which E6 and E7 can overcome the checkpoints of the cell cycle, induced by DNA damage and growth inhibitors. By dissociating E2F from the p105Rb complex, cyclin E expression is activated, a requirement for progression through the G1 arrest. E6 together with E6AP cause degradation of p53 which diminishes expression of the cyclin-dependent kinase inhibitor p21. The cyclin E/cdk2 complex is thus activated and the G1 checkpoint is bypassed. Through mechanisms that have yet to be established, E6 and E7 can also overcome the G2/mitotic spindle checkpoint.

transformation. E6 can also modulate IFN signaling by two distinct mechanisms. Finally, E6 proteins from low-risk and high-risk HPVs have some common cellular binding partners but differ in their ability to immortalize or transform cells. The functional interactions of E6 with its cellular binding partners will be discussed further in the context of cellular growth regulation (fig. 3).

High-Risk E6-Dependent Degradation of p53 and Abrogation of the G1 Cell Cycle Checkpoint

p53 is a key regulator of cell cycle progression and acts to ensure the genomic integrity of proliferating cells. p53 is the most commonly mutated gene in human cancers which demonstrates that p53 plays a central role in tumorigenesis [81]. Upon DNA damage resulting from exposure to chemicals or ionizing radiation, p53 levels are increased by a post-transcriptional mechanism. This increase activates transcription of the CKI p21 [82, 83]. At high protein levels, p21 associates with the G1-phase cyclin/cdk complexes and inhibits kinase activity leading to cell cycle arrest in G1. Activation of this checkpoint allows for the repair of damaged DNA prior to entry into S-phase.

High-risk E6 protein first binds to the cellular ubiquitin ligase, E6AP, and then recruits p53 into this complex [84]. After binding, p53 is polyubiquitinated which results in its rapid degradation by cellular proteases [reviewed in 85]. In the absence of p53, there is no increase in expression of the CKI p21 following DNA damage and the p21-dependent G1 checkpoint is inactivated. A second pathway of p53 inactivation by high-risk E6 involves binding of E6 to the coactivator CEBP/p300 which inhibits the expression of p53- and NFκB-responsive genes [71, 72]. Inactivation of p53 leads to the accumulation of cellular mutations over time which may predispose individuals for the development of cancer.

High-Risk E6-Dependent Abrogation of the G2/Mitotic Spindle Checkpoint of the Cell Cycle

In addition to the loss of the G1 checkpoint, E6-expressing cells can also bypass the G2 checkpoint [86]. The G2 checkpoint controls the end of S-phase and insures the proper replication of cellular origins once-per-cell cycle. Loss of this checkpoint leads to re-replication of DNA and can cause chromosomal translocations. E6-expressing cells accumulate chromosomal abnormalities over time which may lead to the loss of G2/mitotic spindle checkpoint control [87]. Abrogation of the G2 checkpoint, together with the shortening of telomeres (discussed below), leads to chromosomal instability [88]. Similar to the effect of E7, E6-expressing keratinocytes treated with the mitotic spindle inhibitor nocodazole arrest in M-phase and undergo multiple rounds of DNA synthesis [49]. In contrast to E7-expressing cells, however, the Mdm2 protein levels are low in E6-positive cells, indicating that E6 likely abrogates this checkpoint through other mechanisms. A prime candidate is p53 which has been implicated in the regulation of the G2/mitotic spindle checkpoint by several studies [89–91].

High-risk E6 has also been shown to associate with the human hMcm protein 7, a component of the DNA replication licensing system [69]. Together with other members of the Mcm protein family, Mcm7 ensures that cellular DNA

replicates only once-per-cell cycle [92]. The interaction of E6 with Mcm7 leads to degradation of Mcm7 which, in part, is mediated by E6AP [70]. Therefore, the loss of Mcm7-mediated control of DNA replication may also contribute to the abrogation of the G2/mitotic spindle checkpoint.

High-Risk E6 Proteins Induce Telomerase Activity during Cellular Immortalization

Telomeres consist of 4–15 kilobases of a repeated TTAGGG motif located at the ends of cellular chromosomes and are important for the replication of the chromosomal termini [reviewed in 93, 94]. During normal cell growth, the number of telomeric repeats is reduced upon each cell division and this loss eventually leads to senescence [95]. Telomeres of stem cells are maintained through the action of the cellular enzyme telomerase which consists of four subunits, three of which are constitutively expressed in cells. hTert, the fourth catalytic subunit, is not expressed in normal adult cells but is activated in most cancers. Keratinocytes expressing E6 exhibit activated hTert expression in a subset of infected cells prior to immortalization. Upon crisis, only keratinocytes with activated telomerase survive and become immortalized. The induction of telomerase by high-risk HPVs depends only on the expression of E6 and is independent of the status of p53 in cells [96]. Thus E6 contributes to cellular immortalization also in a p53-independent manner, activating telomerase activity possibly through a c-Myc-dependent pathway [reviewed in 97].

High-Risk E6 Inhibits Cellular Apoptosis and Differentiation

Expression of E7 alone induces programmed cell death or apoptosis through a p53-dependent mechanism which is similar to the induction of apoptosis by the E1A oncogene of adenovirus. Like the adenoviral E1B proteins (19 and 55 kDa) which have antiapoptotic functions, E6 of high-risk HPVs can also abrogate apoptosis through p53-dependent and -independent pathways [98, 99]. E6 abrogates p53-dependent apoptosis through E6AP-mediated degradation of p53 as described above. Prevention of p53-independent apoptosis by E6 involves Bak, a member of the Bcl-2 family of apoptosis modulators. The Bcl-2 family consists of the proapoptotic members Bak and Bax [100], and the antiapoptotic Bcl-2 and Bcl-XL whose viral homologue is E1B-19 kDa [101]. High-risk E6 and E6AP can form a complex with Bak, which induces its ubiquitination and subsequent degradation [102]. The antiapoptotic effect of E6 also protects cells against induction of cell death by tumor necrosis factor independent of p53 [103].

Apoptosis and terminal differentiation of suprabasal epithelial cells share several features, such as denucleation and expression of transglutaminases to form cornified envelopes [104]. Consistent with its antiapoptotic effects,

E6 expression in keratinocytes can inhibit keratinocyte differentiation which is induced by culturing cells in low serum/high calcium conditions. E6 reduces the incidence of apoptosis in differentiating keratinocytes by diminishing p53 levels, elevating Bcl-2, and suppressing Bax levels. However, E6 does not inhibit the expression of differentiation-specific proteins or overcome the growth arrest imposed by differentiation [105]. In general, the ability of high-risk E6 to inhibit differentiation correlates with its ability to degrade p53 in vitro [reviewed in 66].

p53-Independent Mechanisms of Transformation Depend on the Binding of E6 to Additional Cellular Proteins

Besides E6AP and p53, other cellular proteins have been identified that can interact with high-risk E6 and may play a role in E6-mediated transformation. These include hDlg and E6TP1, paxillin, ERC-55, and AP-1. Binding of the human homologue of the Drosophila disk large tumor suppressor protein (hDlg) to high-risk E6 proteins is required for the transformation of fibroblasts [73]. The binding of E6 to hDlg leads to proteasome-mediated degradation of hDlg [106]. Since hDlg can also bind to the adenomatous polyposis coli (APC) proteins whose genetic locus is mutated in colon cancers, the E6-hDlg interaction may affect HPV-induced tumor progression [reviewed in 66]. Similarly, E6TP1 protein can bind to high-risk E6 and is degraded by E6AP-mediated ubiquitination. E6TP1 is a putative GTPase-activating protein and its chromosomal locus is affected by loss of heterozygosity in human tumors. Since E6TP1 degradation occurs only with immortalization-competent E6 proteins, this functional interaction may affect HPV-mediated transformation [75].

Paxillin is involved in the organization of actin filaments and attachment of cells to the extracellular matrix through focal adhesion proteins. E6 from BPV1 can bind to paxillin and disrupt the actin cytoskeleton. High-risk E6 has also been shown to associate with paxillin but its role in tumorigenesis remains to be established [77]. Currently, the biological function of the interaction of E6 with ERC-55 (E6BP [76]), a calcium-binding protein of the endoplasmic reticulum, is not known. Similarly, the significance of E6 binding to the clathrin adaptor AP-1 [78] which is involved in intracellular trafficking is unclear. The contribution of these interactions to HPV-mediated transformation remains to be established.

E6 Modulates Cellular IFN Signaling

Similar to E7, high-risk E6 protein can bind to cellular proteins involved in the IFN response. Two mechanisms have been identified that implicate E6 in the downregulation of IFNβ expression as well as abrogating IFNα-mediated Jak-STAT signaling. IFNα and IFNβ expression is transcriptionally regulated

by the IRFs which consists of IRF1, IRF2, IRF3, IRF4, IRF7, the IFN consensus sequence binding protein (ICSBP) and ISGF3γ (discussed above with E7). IRF3 expression is normally activated in response to viral infection [107]. High-risk E6 can bind to IRF3 and lead to diminished expression of IFNβ [79]. Furthermore, IFNα-mediated signaling is also affected by E6. Normally, transcription of IFN-responsive genes is activated by the ISGF3 complex which consists of ISGF3α and ISGF3γ proteins. Upon IFNα/β treatment, the Stat-1 and -2 (ISGF3α) proteins are phosphorylated by the cytoplasmic Jak1 and Tyk2 tyrosine protein kinases, translocate to the nucleus, and form the tetrameric ISGF3 activator complex. High-risk E6 can bind to the Tyk2 kinase, diminish its autophosphorylation, as well as phosphorylation of ISGF3α. As a result, the IFNα-mediated response is abrogated without affecting IFNγ-mediated signaling [80]. The interactions of E6 with proteins involved in the IFNα and IFNβ signaling cascades thus contribute to the downmodulation of the cellular antiviral response.

Properties of E6 from Low-Risk HPVs

Like the E6 proteins of high-risk HPVs, low-risk E6 can activate transcription form heterologous promoters such as the Adenovirus E2 promoter. As with the high-risk E6 proteins, transcriptional activation by low-risk E6 proteins does not correlate with cellular immortalization or tumorigenesis suggesting that this property is not essential for transforming ability [reviewed in 66]. Low-risk E6 proteins do not bind to p53 with high affinity or degrade p53 through E6AP, but they can associate with E6AP. This presents a quandary and indicates that the low-risk E6-E6AP complex may have different biological functions than the high-risk E6-containing complex. In contrast to high-risk HPVs, infection by low-risk HPVs leads to extensive proliferation of HPV-positive cells and the mechanisms of low-risk E6-dependent growth control appear to be p53 independent. Low-risk E6 proteins can induce low levels of telomerase but are unable to immortalize human keratinocytes [96]. Low-risk E6 does not interact with either ERC-55 [76] or paxillin [77], and binds only with low affinity to Mcm7 [69]. These findings suggest that other mechanisms may be responsible for the action of the low-risk E6 proteins.

Summary and Outlook

E6 and E7 expression occurs throughout the normal viral life cycle of high-risk HPVs and leads to abrogation of cell cycle checkpoints, a reduction of the rate of cellular apoptosis, and a delay in cellular differentiation. The net result of these combined effects of E6 and E7 is an expansion of HPV-infected

keratinocytes. The loss of key regulators of the cell cycle and the concomitant increase in cellular proliferation, therefore, adversely affects the genetic integrity of HPV-infected cells. Under these conditions, high-risk HPV-infected cells can become tumorigenic after additional mutational changes have occurred. In a similar manner, both oncoproteins also contribute to the downregulation of the IFN-mediated antiviral response of HPV-infected cells. Further studies are necessary to identify the mechanisms by which high-risk E6 proteins can activate telomerase and how low-risk E6 proteins regulate cell growth.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 64–87

HSV-2 Transformation: A Multistep Process Mediated by Distinct Mutagenic DNA Sequences and Viral Genes Includes Activation of the Ras/MEK/MAPK Mitogenic Pathway

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Herpes simplex virus type 2 (HSV-2) is a sexually transmitted human virus that is responsible for most cases of genital disease. During pregnancy, infection is associated with spontaneous abortion, prematurity and congenital neonatal herpes. HSV-2 has also been associated with an increased risk of infection with human immunodeficiency virus (HIV) and increased severity of HIV disease [1]. Despite educational efforts directed at containing the HIV epidemic, the prevalence of HSV-2 infection has increased significantly during the last decade [2]. Several reports of severe hyperproliferative lesions caused by HSV-2 in patients co-infected with other agents, acyclovir-resistant virus, or HIV-induced immunosuppression [3, 4] have stimulated renewed interest in the ability of HSV-2 to cause cellular proliferation. Here we review our present understanding of HSV-2-induced cellular proliferation and neoplastic transformation with particular emphasis on the mechanisms involved in these processes and their relationship to the virus life cycle.

HSV-2 DNA Codes for Multiple Transforming Functions

The HSV genome consists of approximately 150 kb of double-stranded linear DNA. There are two unique stretches, a long (U_L) and a short (U_S) stretch which are flanked by inverted repeats. 15 kb of DNA sequences represent inverted

repeats of terminal regions inserted between the U_L and U_S domains. Genes are contained both within unique and repeat sequences. There are at least 84 different open reading frames (ORFs), only 38 of which are required for the ability of HSV type 1 (HSV-1) to replicate in cell culture. Transcripts from genome domains not known to specify proteins and ORFs that are read antisense to others on the HSV genome were also described [reviewed in 5], suggesting that there is ample genetic information to code for functions that cause cellular alterations characteristic of tumor cells, globally known as transformation.

HSV-2 transcription patterns are basically similar to those described for HSV-1. Viral genes are classified into three major kinetic classes on the basis of the time and regulation of their synthesis during productive infection. The immediate early (IE), or α genes are transcribed as early as 2 h postinfection (p.i.) and in the absence of de novo protein synthesis. Expression of IE genes is initiated by the interaction of the virion tegument protein VP16 with the *oct-1* cellular transcription factor at the octamer/TAATGAARAT sequence in the IE gene promoters. The synthesis of the next class of HSV genes, designated delayed early (DE) or β, begins at 4–7 h p.i. It consists primarily of enzymes involved in DNA synthesis, and is followed by the third class (late or γ), that primarily consists of structural proteins. The expression of the β and γ kinetic classes requires competent IE gene expression, especially a functional ICP4 protein [reviewed in 5, 6].

Ribonucleotide reductase (RR) reduces ribonucleotides to deoxyribonucleotides, thereby providing precursors for DNA synthesis. It consists of two subunits. The large subunit (R1) is a 140-kDa protein specified by a 5.0-kb mRNA which is encoded by U_L39. The small subunit (R2) has a molecular weight of 38 kDa and it is encoded by a 1.2-kb transcript the 3′-end of which is shared with the R1 transcript [7]. R2 is synthesized with classic DE kinetics and it determines the kinetics of the RR enzymatic activity [6]. By contrast, R1 is synthesized with IE kinetics. Synthesis begins at 2 h p.i. (maximal at 4–8 h p.i.) and in the absence of de novo protein synthesis [8, 9]. The R1 promoter has an octamer/TAATGAARAT sequence that responds to activation by the VP16/*oct-1* complex [10–12]. As will be discussed later, IE regulation is probably required for the protein kinase (PK) function of the R1 protein encompassed within its one-third amino-terminal domain [13–17].

In the 13 years following the original 1973 report that HSV-2, the lytic activity of which was inactivated by UV exposure, causes morphological transformation of rodent cells [18], many reports confirmed and extended these findings. Transformation of human and other mammalian cells was described, using various HSV-2 strains and inactivation procedures. Some studies used the focus formation assay which takes advantage of the altered morphology of tumor cells and their decreased serum requirement for growth. However, these properties do

not always reflect a constitutively increased rate of proliferation, and lines established from the foci were not always oncogenic. Other studies measured anchorage-independent growth which takes advantage of the ability of tumor cells to proliferate in the absence of cell adhesion, and these lines were oncogenic in animals. The results of the transformation studies varied widely, with some systems exhibiting some, but not all the transformation-associated properties and losing the viral DNA upon successive passage, while others retained and expressed viral DNA and acquired oncogenic potential [18–45]. In vivo tumorigenicity studies indicated that inactivated HSV-2 and viral DNA caused preinvasive and invasive tumors when applied to the mouse cervix, and preimmunization of the animals with HSV-2 prevented tumor development [46–50]. Nonetheless, the apparently discrepant transformation data caused some investigators to question the ability of HSV-2 to cause malignant transformation. The problem was further compounded by the finding that HSV-1, which is 47–50% homologous to HSV-2, does not cause malignant transformation.

The first interpretation of these apparently contradictory findings was provided by Manak et al. [51]. These investigators transformed cells with HSV-2 the DNA of which was modified by bromodeoxyuridine substitution thereby allowing for the inactivation of lytic functions (by UV-mediated fragmentation) at various times p.i. They showed that functions expressed during the first 2 h p.i. which primarily consist of IE proteins, caused focus formation, but virtually all foci failed to acquire anchorage-independent growth and neoplastic potential. The majority of the foci did not survive passage in culture and this was associated with the loss of the viral DNA. By contrast, cells transformed by HSV-2 functions that were expressed at 3–6 h p.i. (when R1 synthesis was maximal), acquired unlimited proliferative potential. These cells survived in vitro passage, they evidenced anchorage-independent growth and tumorigenic potential and they retained viral DNA sequences. These findings were the first indication that the HSV-2 genome codes for multiple transforming functions which are differentially selected under various experimental conditions.

HSV-2 DNA Has Two Transforming Regions

Original studies designed to identify HSV-2 transforming functions used rodent and human cells transfected with viral DNA fragments and various transformation assays (focus formation and/or anchorage-independent growth). They identified two separate transforming regions located at an adjacent position in the U_L domain of the viral genome (fig. 1a). One of these, also known as mtrII, is contained within the BglII-N fragment (at map position 0.58–0.62) which also encodes R2 [52–56]. The other transforming region, also known

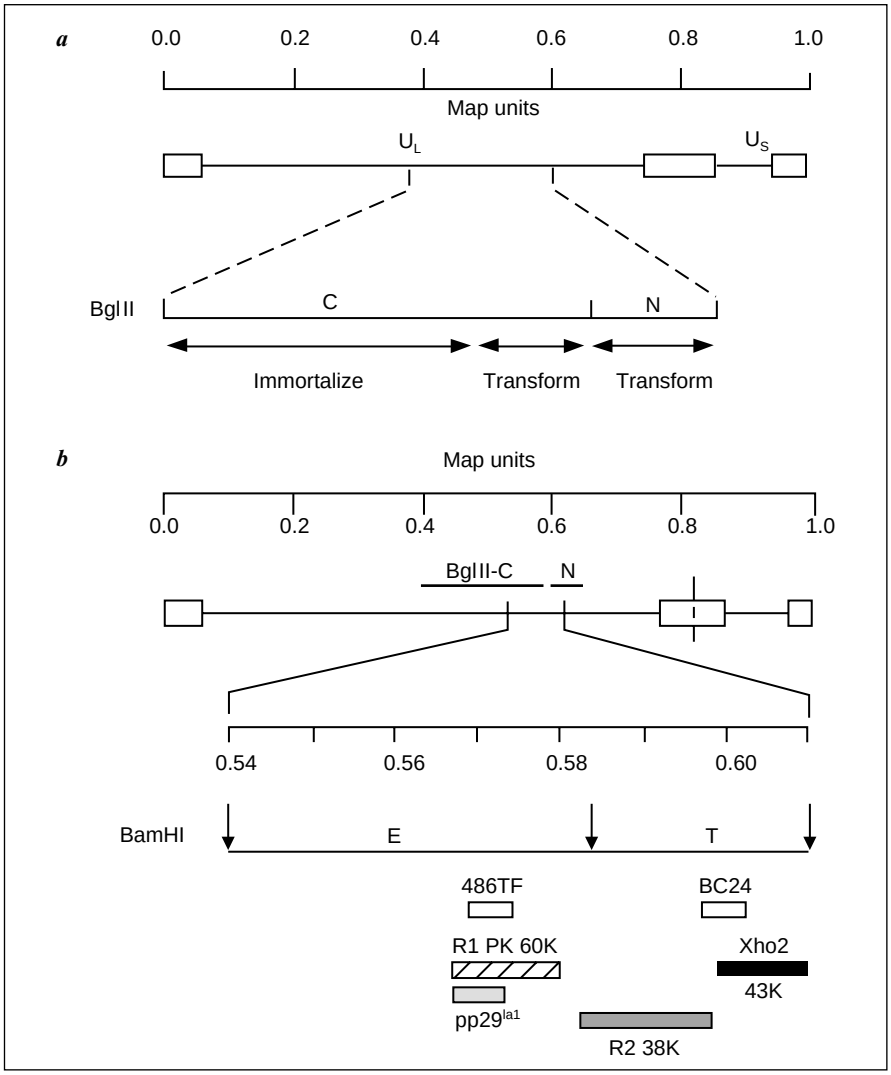

Fig. 1. Physical map of the location of HSV-2-transforming genes. ***a*** Genome organization showing the regions BglII-C (also known as mtrIII) and BglII-N (also known as mtrII). BglII-C DNA contains an immortalizing and a transforming region. ***b*** BamHI fragmentation of the transforming regions, identifies the E fragment that codes for the large (R1) subunit of ribonucleotide reductase (RR) and the T fragment that codes for the small (R2) subunit of RR. The minimal transforming fragment within BamHI-E is 486TF. The minimal transforming fragment within BamHI-T is BC24. Also identified are the 60-kDa R1 PK oncoprotein and the minimal PK catalytic core $pp29^{la1}$, the 38-kDa R2 protein and the putative 43-kDa protein in the transforming Xho2 fragment within BglII-N.

as mtrIII, is contained within the BglII-C fragment (at map position 0.54–0.58) which also encodes R1 [57–60]. Because the 3′ ends of the R1 and R2 transcripts are co-linear [7], it was speculated that RR activity is required for transformation [61]. However, this is unlikely since both RR subunits are required for enzymatic activity and they are not encompassed within either one of the transforming DNA fragments.

Transformation by BglII-N DNA Includes a Mutagenic Component

BglII-N DNA caused focus formation of immortalized rodent cells [52–56], but it did not impart long-term proliferative potential when transfected into normal diploid cells [58]. When retained, DNA sequences were present in low copy number and they were lost upon passage and/or in tumor derivatives [52–56]. Focus formation was unrelated to R2 expression, since the presence and expression of the R2 DNA did not correlate with acquisition of the transformed phenotype [62]. However, several studies documented activation of cellular genes including heat-shock proteins, phospholipase, cyclooxygenase, serum amyloid A protein and a 90-kDa protein, the function of which is still unclear [63–67]. By analogy to cells transformed by mutagenic chemical carcinogens, DNA from cells transformed by BglII-N was hypomethylated [68]. Loss of BglII-N DNA from the transformed cells was attributed to 'repressor' sequences [69]. BglII-N includes a region that codes for interferon resistance [70], but its relationship to the putative 'repressor' sequences is unclear.

Galloway et al. [71] reported that the focus forming potential of BglII-N localizes in a 737-bp fragment that does not lie within coding sequences, designated BC24 (fig. 1b). BC24 contains a stem-loop structure bounded by direct repeats which include a region resembling an insertion sequence (IS). The stem-loop structure resembles the P elements of Drososphila but it does not code for a transposition factor. It does not conform to the classical description of an insertion sequence and its structure is unstable because of base mismatches. It was proposed that BC24 acts as a mutagen, the so-called 'hit-and-run' hypothesis [72]. Transformation could also result from the disruption of genes that regulate cell proliferation or through gene rearrangement resulting from mutation due to BC24 insertion into area(s) of cell homology [73]. The presence in BglII-N transformed cells of extrachrosomal DNA that contained the selectable neo^r marker, BglII-N and cellular sequences and had a 1,000-fold higher transformation efficiency than BglII N [74], is consistent with such an interpretation. Also, cellular sequences homologous to BC24 were recently identified by polymerase chain reaction (PCR) assays in some (but not other) human populations [75] and they could be a putative site for BC24 integration. However,

computer-assisted analyses indicated that IS and mutagenic sequences, other transposable elements and areas of homology to cellular DNA sequences are common to many regions of the viral genome, including those that fail to cause focus formation [76], and mutagenic potential was also localized to a component of the incoming HSV-2 virion or viral DNA [77].

Reports of BglII-N-mediated transformation independent from mutation at the hrpt locus [78] were followed by the finding that human keratinocytes immortalized by human papillomavirus (HPV) types 16 or 18 are converted to a neoplastic phenotype by transfection with an expression vector for the Xho2 fragment within BglII-N (fig. 1b). The cells retained and transcribed Xho2 DNA and caused invasive and noninvasive indolent cystic squamous carcinomas when injected into severely immunocompromised (SCID) mice [79]. As previously reported for BglII-N [58], the Xho2 sequences did not transform normal diploid cells. They contained an ORF that encodes a putative 42- to 43-kDa protein with 66% homology to the HSV-1 UL43 protein with multiple hydrophobic stretches characteristic of proteins which create channels in the plasma membrane [79]. However, the mechanism of transformation by the Xho2 DNA fragment, the role of transcription and the importance of immunosuppression in tumorigenicity, are still unclear. Inasmuch as transformation by the Xho2 fragment was only seen in HPV 16/18 immortalized cells, BglII-N appears to contain distinct transforming regions with cell- and/or species-specific activities.

Transformation by BglII-C DNA Is a Multistep Process Consisting of Immortalization and Neoplastic Transformation

BglII-C DNA caused multistep neoplastic transformation of primary (diploid) cells (fig. 1a). The left-hand region immortalized primary rodent cells [57–60], but not human fibroblasts [80], suggesting that the requirements for immortalization are species- and/or cell type-specific. The right-hand region, represented by the BamHI-E (fig. 1b) and more specifically by the SalI/HpaI (fig. 2a) or PstI-C subfragments caused neoplastic transformation of rodent and human cells immortalized by various means [57–60, 81, 82], indicating that the tumorigenic function is not similarly restricted.

The mechanism of immortalization is unknown. By analogy to the prototype DNA tumor viruses, immortalization may involve disarming of tumor suppressor genes, such as p53 or the retinoblastoma protein family which includes pRb, p107 and p130. Indeed, functional inactivation of the pRb pathway results in deregulated activity of the E2F transcription factors in a cell cycle-dependent manner. This, in turn, regulates the expression of several growth-promoting genes, thereby contributing to immortalization. For example, expression of two

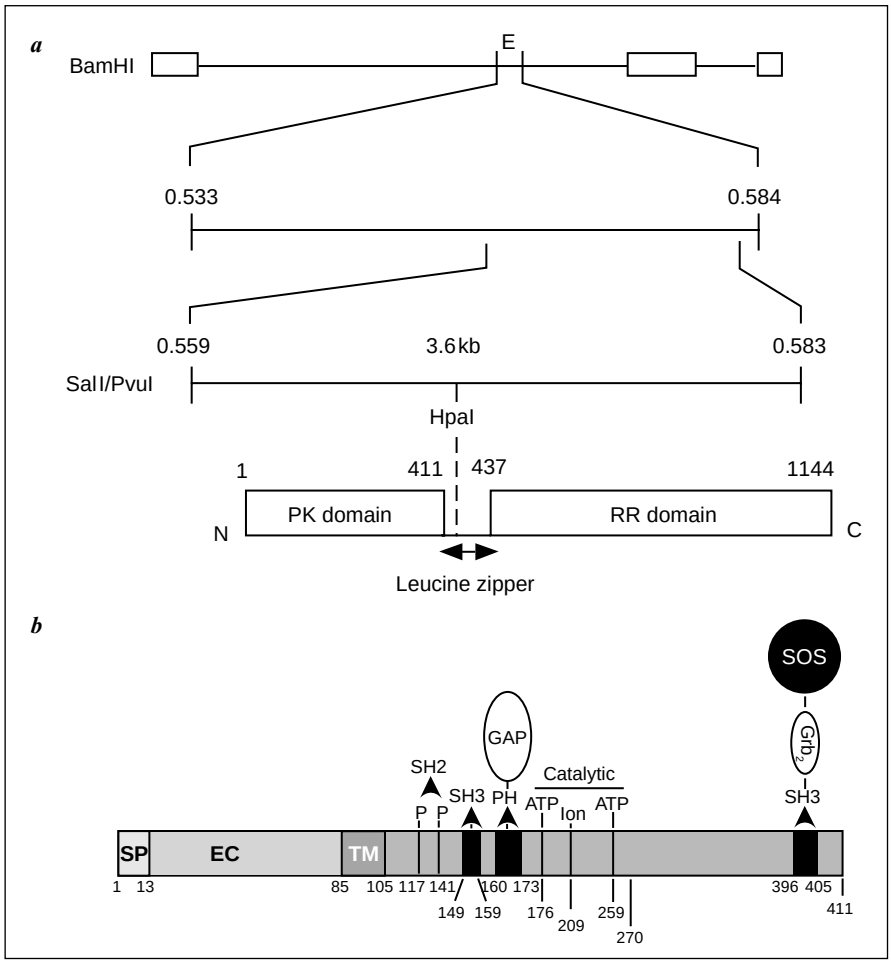

Fig. 2. Physical map of the location of the R1 PK oncogene and schematic representation of the oncoprotein. ***a*** SalI/PvuI fragmentation of the transforming BamHI-E fragment defines the R1 coding region. The unique HpaI site represents the 3′ end of the known transforming region. It cuts the R1 gene after the codon for residue 417 within the leucine zipper-like region which provides protein flexibility. ***b*** Schematic representation of the expanded PK domain includes the signal peptide (SP, aa 1–13), extracellular domain (EC, aa 14–85), transmembrane domain (TM, aa 86–105) and catalytic core located at residues 106–270 and encompassed within pp29[la1]. The catalytic core contains two ATP-binding sites (Lys^{176} and Lys^{259}) and the ion-binding site (Glu^{209}). The major Ras-GAP-binding site is a WD40-like motif at position 160–173 which binds the Ras-GAP PH domain. Residues $pThr^{117}$ and $pThr^{141}$ bind the Ras-GAP N-SH2 motif in vitro. The major Grb_2-hSOS-binding site is the proline-rich, SH3-binding motif at position 396–405. The minor site is the SH3-binding motif at position 149–159.

E2F-1 targets, phosphatase CDC25A and cyclin E, is sufficient to induce entry into S phase in quiescent fibroblasts [83]. HSV causes rapid and large increases in cell cycle-regulated free E2F and S phase p107/E2F DNA-binding activities resulting in increased c-myc promoter activity [84]. HSV-2 selectively activates cdk2, that is involved in G1 to S phase transition [85], and a factor present in crude extracts and supernatants of HSV-2-infected cells induces cellular proliferation [86]. However, the relationship of these functions to immortalization is unknown.

Of the genes known to be contained within the immortalizing HSV-2 DNA fragment, only UL31 and UL34 are not involved in virion morphogenesis and code for phosphoproteins the size of which is similar to that of viral proteins in immortalized cells. Like prototype immortalizing genes [87], the UL31 protein partitions with the nuclear matrix [88] which is involved in the regulation of mRNA maturation and transport, and in gene expression [89]. The unphosphorylated UL34 protein complexes with cellular phosphoproteins, and it may function to activate a cellular PK or inactivate a cellular phosphatase [90]. The HSV-1 UL34 protein was also shown to bind the intermediate chain of the dynein complex [91], a microtubule-based motor involved in several cellular functions including cell division [92].

Transforming BglII-C Sequences Code for the R1 PK Oncoprotein

The transforming DNA sequences within BglII-C were first localized to the BamHI-E fragment that codes for R1. However, the entire gene is not required for transformation since the activity was delimited to fragments SaI/HpaI or PstI-C [17] that only code for the amino-terminal one-third domain of the R1 protein (fig. 1b). This domain is unique to the HSV R1 proteins and it can be dissociated from the remaining two-thirds (RR domain) by various means, including intracellular proteolysis [13]. It has serine-threonine (ser-thr)-specific PK activity that is not required for nucleotide reduction (PK domain) [13–17] (fig. 2a). A HSV-2 mutant deleted in the PK domain (ICP10ΔPK) was not transforming [93], indicating that HSV-2-transforming activity localizes within the R1 PK domain. The PK and RR domains are connected by a leucine zipper-like motif which provides structural flexibility [94] (fig. 2a).

The HSV R1 PKs differ from most eukaryotic PKs in that they function with fewer conserved catalytic motifs. The enzymatic activity of the HSV-2 R1 PK is localized to a catalytic core located within the first 270 amino acids, as evidenced by the 29-kDa phosphothreonine (pThr)-specific R1 PK mutant, pp29^{la1} [17, 95] (fig. 1b). The catalytic core is preceded by a transmembrane domain (TM) which is followed by a basic residue that anchors the TM into the plasma membrane [96] (fig. 2b). Genetic and biochemical analyses of the HSV-2

R1 PK protein (also known as ICP10 PK) indicated that the conserved catalytic motif I is not required for kinase activity [96], as also reported for several other PKs [97]. Replacement of the invariant Lys residue in catalytic motif II (Lys^{176}) caused a significant decrease in kinase activity (K_m = 1.2 and 6.6 μM for R1 PK and Lys^{176} mutant respectively), but a similar reduction (K_m = 9.4 μM) was achieved by replacement of an adjacent Lys residue (Lys^{259}), indicating that both Lys residues are required for PK activity. Both residues bind ATP, as evidenced by FSBA-binding and competition studies. A null phenotype was only achieved when both Lys residues were mutated [98]. Together with the Glu residue in catalytic motif III (Glu^{209}), they form the core catalytic function for the HSV-2 R1 PK (fig. 2b). Glu^{209} replacement severely compromised both MnATP- and MgATP-dependent PK activity, suggesting that the ion pair which is presumably formed between the two charged residues (Lys and Glu) provides a docking site for either MnATP or MgATP [98]. Other ser-thr PKs which are expressed at high levels, also use several Lys residues to bind ATP [99].

The TM domain of the HSV-2 R1 PK is required for kinase activity as evidenced by the finding that a TM-deleted mutant (p139™) is PK negative [96]. This, presumably, reflects a stringent requirement for presentation of the MnATP/MgATP docking site which is only achieved through relative structural rigidity imparted by protein anchorage to the plasma membrane. Myristylation also insures that the R1 PK protein is associated with the plasma membrane [14]. Indeed, the HSV-2 R1 PK is located on the cell surface, and like all growth factor receptors, it is internalized by receptor-mediated endocytosis [100, 101]. Phylogenetic analyses [101] indicated that it belongs to a subfamily of growth factor receptor ser-thr PKs that includes the HSV-1 R1 PK and FAST, a PK that is activated during Fas-mediated apoptosis [102].

HSV-2 R1 PK Activates the Ras/MEK/MAPK Mitogenic Pathway

Signaling pathways, the ultimate targets of which are nuclear transcription factors, determine the cell's ability to respond to external stimuli. Transduced signals can be interpreted as mitogenic/proliferative, differentiating or apoptotic, depending on the cell type and the nature and duration of the stimulus. The mitogenic Ras/MEK/MAPK pathway is initiated by growth factor-mediated activation of cognate receptors on the cell surface. The growth factor receptor binds a protein complex consisting of an adaptor protein (viz. Grb_2) and a guanine nucleotide-releasing factor (viz. hSOS), thereby bringing the latter in the vicinity of the membrane-bound G protein Ras. hSOS promotes the conversion of the inactive GDP-bound Ras to the active, GTP-bound state. In turn, Ras coordinates the activation of a cascade of ser-thr PKs that begins with Raf and

is followed by MAP kinase 1 and 2 (MEK1/2) and mitogen-activated protein kinase (MAPK1/2) and culminates in the expression of c-Fos [103], which is important for promoting cell cycle progression into S phase [104]. The GTPase-activating protein Ras-GAP, a major negative regulator of Ras activity, acts to enhance the weak intrinsic GTPase activity of the Ras protein, thereby accelerating the hydrolysis rate of bound GTP to GDP [105]. Ras-GAP inactivation by phosphorylation on ser-thr residues has been implicated in Ras activation [106]. The specificity of the signal transduction is determined by protein domains such as SH2, SH3 and PH that bind unique motifs in target proteins for recruitment into signaling complexes [107].

Comparison of human cells neoplastically transformed by HSV-2 R1 PK DNA to nontransformed cells that are stably transfected with the PK-negative mutant p139™ (which is deleted in the TM domain) indicated that both proteins are stably expressed, but only R1 PK is localized on the cell surface and functions as an activated growth factor receptor that stimulates the Ras/MEK/MAPK mitogenic pathway [100, 101, 108]. In transformed cells, the R1 PK oncoprotein bound the Grb_2-hSOS complex, thereby bringing it in the vicinity of Ras and causing Ras to adopt an active, GTP-bound state [108]. Mutational analyses indicated that binding occurs at proline-rich SH3 binding modules in R1 PK [98]. The major binding site is a class II SH3 binding motif (^{396}LPPVPPNAYT^{405}) with basic residues (His^{408} and/or Arg^{410}) at the carboxy-terminus which is located at position 396–405. Binding (albeit at a 20-fold lower level) also occurred at another class II motif (^{149}AVPPPPPPPFPWGH^{159}) which is located at position 149–159 (fig. 2b). In peptide competition assays Grb_2 was found to bind R1 PK at its carboxy-terminal SH3, with an affinity similar to that with which it bound hSOS at its amino-terminal SH3 [98]. p139™ did not bind the Grb_2-hSOS complex, suggesting that both SH3-binding sites are masked [108]. Presumably, binding sites are properly exposed only when protein flexibility is reduced through anchorage into the plasma membrane (fig. 3). However, it is still unclear whether: (a) the minor Grb_2-hSOS-binding motif is functional in transformed cells, and (b) pp29^{la1}, which is deleted in the major Grb_2-hSOS-binding motif (fig. 2b), has transforming activity.

Ras-GAP was also complexed to R1 PK in transformed cells and it was phosphorylated on ser-thr residues. Accordingly, its GTPase activity was significantly decreased [108]. In vitro binding assays with Ras-GAP fusion proteins and peptide competition studies [9], demonstrated that binding occurs at Ras-GAP N-SH2 and PH modules. Binding of the Ras-GAP PH fusion protein was phosphorylation-independent (seen with dephosphorylated oncoprotein and PK-negative mutants) and it involved a WD40-like sequence located at position 160–173 in the oncoprotein (fig. 2b). Binding of the N-SH2 Ras-GAP fusion protein was phosphorylation-dependent (not seen with dephosphorylated

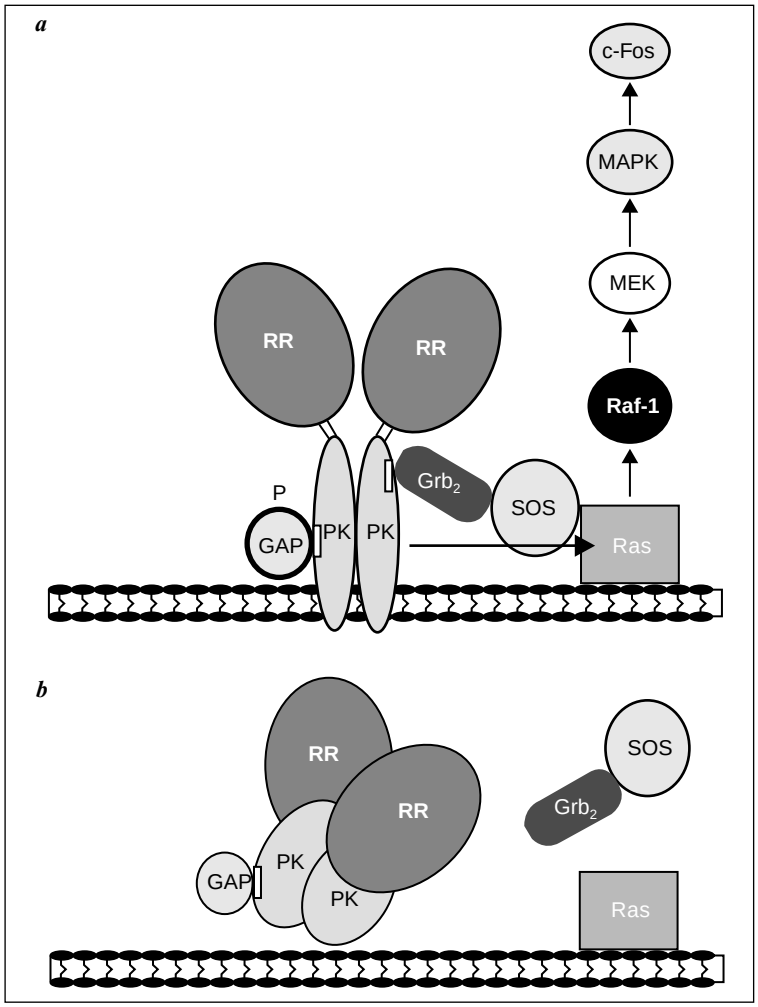

Fig. 3. Schematic representation of R1 PK-mediated activation of the Ras/MEK/MAPK mitogenic pathway. ***a*** The R1 protein consists of the PK and RR domains connected by the flexible leucine zipper. The PK domain is anchored to the plasma membrane where it behaves as a growth factor receptor. The relatively rigid configuration imparted by plasma membrane anchorage allows for the proper presentation of the ion-ATP docking site and the site for Grb_2-hSOS binding. hSOS binding brings it in the vicinity of the membrane-bound Ras. R1 PK also binds and phosphorylates Ras-GAP, thereby causing its inactivation. The combined effect is activation of Ras and the kinase cascade that culminates in increased c-Fos expression/stability. ***b*** p139™ which is TM deleted is not anchored to the plasma membrane and, therefore, assumes a flexible configuration that masks the ion-ATP-and Grb_2-hSOS-binding sites. Ras-GAP is bound, but it is not phosphorylated (i.e. it is active). The outcome is the failure to activate the Ras/MEK/MAPK mitogenic pathway.

oncoprotein and PK-negative mutants) and it involved phosphothreonine residues $pThr^{117}$ and $pThr^{141}$, consistent with previous reports that SH2 binding can occur at pThr residues [109]. Binding at both Ras-GAP sites could stabilize the interaction and/or improve Ras-GAP presentation to the adjacent PK catalytic core. However, we conclude that in transformed cells, it occurs at the PH domain, because Ras-GAP is bound by p139™ which does not have PK activity (fig. 3b). The combined effect of hSOS binding and Ras-GAP inactivation in human cells transformed by the R1 PK oncoprotein, was a significant increase in the levels of activated Ras, phosphorylation (and thereby activation) of Raf, MEK1/2 and MAPK, and increased expression of c-fos (fig. 3a). These changes were not seen in nontransformed cells stably transfected with p139™, which did not bind Grb_2-hSOS and did not phosphorylate the bound Ras-GAP, thereby failing to decrease its GTPase activity (fig. 3b) [100, 101, 108].

Oncoprotein Expression and Ras/MEK/MAPK Activation are Required for Maintenance of the Transformed/Tumorigenic Phenotype

Human cells transformed by R1 PK caused poorly differentiated invasive adenocarcinomas when injected into nude mice and the tumor cells were positive for R1 PK oncoprotein expression. The presence and expression of the transforming DNA sequences are required for the maintenance of the transformed phenotype and for tumor formation in animals, as evidenced by the inhibition of cellular DNA synthesis and cell proliferation with an antisense oligonucleotide that is complementary to the oncoprotein translation initiation site and inhibits its synthesis. Tumor formation was also inhibited by treatment of the animals with the oncoprotein-specific antisense oligonucleotide, indicating that its expression is required for tumor growth [110]. Activation of the Ras/MEK/MAPK pathway is required for the maintenance of the transformed phenotype because the pathway was not activated in nontransformed cells stably transfected with p139™ [100, 101, 108]. Conversely, a R1 PK mutant deleted in both Ras-GAP-binding sites but retaining PK activity, had a significantly increased level of activated Ras and its transforming potential was higher than that of the wild-type (wt) R1 PK oncoprotein. This was evidenced by a significant increase in anchorage-independent growth (cloning efficiency = 0.8–1.4% and 4.4–8.6% for wt and mutant respectively), and colony size (200–300 μm and $\geq$1,500 μm for wt and mutant respectively) (fig. 4) and a shorter time required for colony formation (14–22 and 7 days for wt and mutant respectively).

The following additional conclusions are implicit in these observations. First, membrane anchorage of the R1 PK oncoprotein is a stringent requirement

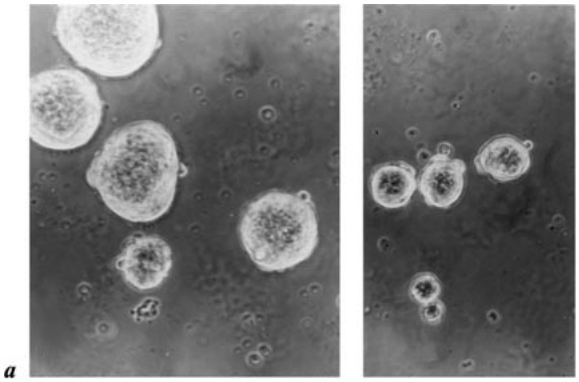

Fig. 4. Anchorage-independent growth of immortalized human cells (293) transformed with the R1 PK mutant deleted in the Ras-GAP-binding sites evidences increased transforming activity as shown here by the significantly larger size of the colonies (***a***) relative to those of cells transformed by the wt R1 PK oncoprotein (***b***).

for PK activity and Grb_2-hSOS binding. Presumably this is due to the increased structural rigidity of the anchored protein which results in appropriate presentation of the ion-ATP and major Grb_2-hSOS-binding sites. Similar conclusions were independently reached for the PK activity of the v-fms oncogene [111]. By contrast, the requirement for binding of Ras-GAP is significantly less stringent, as evidenced by its interaction with the TM-deleted mutant p139™. Taken at face value, the data imply that: (a) the minor SH3-binding site in R1 PK is essentially nonfunctional in transformed cells, and (b) the ability of the R1 PK oncoprotein to bind Ras-GAP is a dominant property that exerts a level of Ras down-regulation even when Ras-GAP is phosphorylated. However, inasmuch as we did not exclude the possibility that structural alterations resulting from the deletion of the Ras-GAP-binding site increased Grb_2-hSOS binding, the relative contribution of hSOS vs. Ras-GAP towards Ras activation in R1 PK-transformed cells is still unclear. Because the Ras/MEK/MAPK pathway is activated by the tyrosine-specific, but not ser-thr-specific growth factor receptors, the second conclusion implicit in these observations is that R1 PK functionally bridges these two families of growth factor receptors. Indeed, epidermal growth factor (EGF) which functions via a tyrosine PK receptor (EGFR) uses a similar strategy to activate the Ras/MEK/MAPK pathway and cause cellular transformation in cells transfected with a chimera consisting of the EGFR ligand-binding domain and the R1 PK oncoprotein [112].

It should be mentioned that DNA fragmentation studies indicated that a 486-bp PstI/SalI fragment designated 486TF, which does not contain PK activity

(fig. 1b), also causes transformation of Rat-2 cells and alters the growth of human fibroblasts or keratinocytes immortalized by HPV 16. 486TF caused rearrangement of HPV 18 DNA sequences in immortalized keratinocytes and chromosome changes in HPV 16 immortalized human cell lines, and the HSV-2 DNA sequences were retained in the transformed cells [80, 82, 113]. In transient transfection assays, 486TF was shown to be a complex transcriptional regulatory element composed of two distinct promoters, a transcriptional silencer and a distal trans-activating domain [114, 115]. 486TF contains repetitive DNA sequences which can be folded into relatively stable hairpin structures [82, 115]. Nuclear proteins, newly expressed in transformed cells, specifically bind to sequences within these structures [115]. It has been suggested that stem-loop structures in 486TF mediate transformation by facilitating recombination or altering expression of growth regulatory genes consequent to its integration. However, the efficiency of transformation by the PstI-C fragment of HSV-2 DNA, which encompasses 486TF but has R1 PK activity, was significantly higher [116].

Taken in toto, the data indicate that both transforming HSV-2 DNA fragments (BglII-N and SalI/HpaI) contain mutagenic sequences (BC24 and 486TF respectively) which are likely to function by direct alteration of regulatory cellular genes. The transforming DNA fragments also code for viral proteins (R1 PK oncoprotein and Xho2 ORF) (fig. 1b) that function via specific pathways, such as the mitogenic Ras/MEK/MAPK pathway in the case of R1 PK (fig. 3). The finding that a HSV-2 mutant deleted in R1 PK (ICP10ΔPK) fails to activate the Ras/MEK/MAPK pathway [9] and does not have transforming activity [93] indicates that pathway activation is a major mechanism responsible for the transforming activity of HSV-2. However, this does not exclude transformation by other mechanisms (including BC24 or 487TF-mediated mutagenesis), under other conditions.

HSV-1 R1 DNA Codes for a Distinct PK and Does Not Have Transforming Activity

Inasmuch as the HSV-2 and HSV-1 genomes are 50% homologous overall, the finding that HSV-1 R1 PK DNA does not have transforming activity [52] was originally puzzling. However, subsequent studies indicated that the level of homology within sequences that encode the R1 PK catalytic core is relatively low (38% as compared to 93% in the RR encoding domain) and there are a number of insertions and deletions between both sequences [117]. The differences are particularly evident at sites that are functionally relevant. For example, the HSV-1 R1 PK protein lacks a conserved catalytic motif II [13] and its ATP-binding site is downstream of amino acid 350, outside of the catalytic core [118]. The protein also lacks the basic amino acid residue which anchors the TM into the plasma

membrane and, therefore, it does not localize on the cell surface [119]. These properties are incompatible with robust PK activity, and are consistent with the original report by Chung et al. [13] that HSV-1 R1 does not have PK activity, as well as subsequent reports that its activity is low [16] or only autophosphorylating [15]. In direct comparison of the autophosphorylation activities of native R1 PK proteins, we found that the K_m of the HSV-1 R1 PK was 10-fold lower than that of the HSV-2 R1 PK [Lee and Aurelian, unpubl. data]. These properties presumably account for the failure of the HSV-1 R1 PK to activate the Ras/MEK/MAPK mitogenic pathway [120, 121] and cause cellular transformation. The findings suggest that the two viruses developed distinct regulatory constraints which were probably related/dictated by their regional specificity.

R1 PK Is Required for HSV-2 Growth

Because it is unlikely that viral DNA sequences evolved in order to cause cellular transformation, an important question is the role of the R1 PK oncoprotein in the HSV-2 life cycle. Viruses take advantage of signaling pathways for their growth. For example, vaccinia virus encodes a protein that mimics EGF in terms of its ability to stimulate cognate receptors [122]. SV40 small T antigen binds protein phosphatase 2A, thereby preventing it from dephosphorylating MEK and MAPK2 and prolonging their activated state [123] and a coxsackievirus protein (Sam68) binds Ras-GAP and inactivates it, thereby activating Ras [124]. Inasmuch as R2 synthesis requires a functional ICP4 and it is regulated with the classic DE kinetics also evidenced by the RR enzymatic activity, the IE-type regulation of R1 [10–12] is likely required for its PK activity. Also likely to be required for the PK activity is the regulation of R1 by AP-1 transcription factors, specifically c-Fos [10, 11] that is important for promoting cell cycle progression into S phase [104]. R1 is the only HSV-2 protein the expression of which is known to depend on AP-1 transcription factors.

Our recent studies of a HSV-2 mutant deleted in the R1 PK oncoprotein (ICP10ΔPK) [9, 16, 125] indicate that HSV-2 uses a strategy similar to that of coxsackievirus in order to activate Ras/MEK/MAPK and this activation is required for IE gene expression and timely onset of virus growth [9]. Thus, immunoprecipitation/immunoblotting studies indicated that Ras-GAP was complexed to R1 PK as early as 2 h p.i. and it was phosphorylated. Maximal levels of phosphorylated Ras-GAP were seen at the time of maximal R1 PK synthesis (4–8 h p.i.) and this corresponded to maximal reduction in GTPase activity and conversely, maximal levels of activated Ras. Similar kinetics were seen for MAPK activation (by phosphorylation) and c-Fos expression. The stability of the c-Fos protein was also increased, presumably due to phosphorylation on

C-terminal ser-thr residues [126]. MAPK activation and increased c-Fos expression/stability were not seen when infection was in the presence of a MEK-specific inhibitor (PD98059), suggesting that they are due to Ras/MEK activation. Increased c-Fos expression/stabilization provided a positive feedback loop for R1 PK expression as evidenced both by promoter activation and gel shift mobility studies [9]. Ras-GAP was not bound/phosphorylated by the R1 protein deleted in the PK domain (p95) which is expressed by the ICP10ΔPK virus. Consequently, Ras and MAPK were not activated and c-fos levels were not increased. Onset of virus growth was similarly delayed (14 as compared to 2 h p.i.) in cells infected with HSV-2 in the presence of PD98059 and in those infected with ICP10ΔPK, suggesting that activation of the Ras/MEK/MAPK pathway by R1 PK is required for timely onset of virus growth [9]. Because the onset of IE protein synthesis, most notably ICP4, is also delayed in ICP10ΔPK-infected cells [125], activation of Ras/MEK/MAPK may also be required for timely expression of HSV-2 IE genes. However, a function which is not inhibited by PD98059 (MEK-independent) is ultimately induced and it provides the cellular environment conducive to virus growth. The identity of this compensatory function and the mechanism responsible for its induction are presently unknown. Potential candidates are other Ras effector pathways [104] and cellular genes functionally similar to the R1 PK protein, such as H11 (discussed below).

Is Ras/MEK/MAPK activation involved in virus pathogenesis? Presumably, pathway activation is responsible for the HSV-2-induced hyperproliferative lesions recently described in immunosuppressed and other patients [3, 4]. Because AP-1 transcription factors are induced by stimuli which cause reactivation of latent virus [127] and R1 is the only known viral gene that responds to AP-1 [10, 11], it is likely that reactivation-inducing stimuli induce R1 expression. This, in turn, results in the activation of the Ras/MEK/MAPK mitogenic pathway providing a positive feedback amplification loop for R1 expression, timely expression of viral IE genes and RR activity which is required for DNA synthesis in nonreplicating (neuronal) cells. The outcome is initiation of the lytic cascade and the production of infectious virus. Because activated Ras has antiapoptotic activity in neurons [128], its activation by R1 PK may also be required for latency establishment. Consistent with these interpretations, ICP10ΔPK is impaired in latency reactivation and establishment [93, 130].

A Cellular Protein (H11) Functionally Similar to R1 PK Is Required for Melanoma Cell Growth

Inasmuch as the PK domain is uniquely present in HSV R1 proteins, the possibility must be considered that it originated from an ancestrally captured

cellular gene. Implicit in this interpretation is the conclusion that the upstream (5′) recombination site is within the promoter of the ancestral HSV-2 R1 gene and the C-terminus (3′) recombination site, at the junction of the PK and RR domains of the chimeric protein, presumably occurred within the promoter region of the ancestral HSV-2 R1. The presence of enhancer core and functional promoter elements at the PK and RR junction is consistent with this interpretation [114]. Cellular proteins that cross-react with antibodies to the HSV-2 R1 PK protein were recently identified in HeLa and melanoma cells and a cDNA (designated H11) was cloned from these cells using antibodies to the R1 PK protein. H11 cDNA codes for a 25-kDa protein that is 30% identical to R1 PK, but the homology is scattered and its biological significance, particularly as relates to the evolution of R1 PK, is unclear [129]. Notwithstanding, the H11 protein is functionally similar to the HSV-2 R1 PK (has ser-thr PK activity and induces anchorage-independent growth of immortalized human cells), suggesting that it (and/or other potentially similar proteins) may provide the complementary function that allows the virus to grow when activation of the Ras/MEK/MAPK pathway is prevented [9, 125]. H11 RNA and phosphoprotein levels were significantly increased (47- to 100-fold) in at least some melanoma cells as compared to normal melanocytes and antisense oligonucleotides that inhibit H11 expression-inhibited melanoma cell growth, indicating that H11 is required for cell growth. The role of H11 in melanoma is still unclear. However, given its functional similarity to HSV-2 R1 PK, it is tempting to propose that it may be involved in melanocyte transformation, for example by inducing entry into the S phase through activation of growth-associated transcription factors [129].

Recent studies indicate that H11 is a previously unrecognized member of the small stress protein superfamily. Its expression in normal cells is required during the cell division to differentiation transition [Aurelian et al., in preparation].

Summary and Conclusions

The recent increase in the rates of infection with HSV-2 and the finding that it causes hyperproliferative lesions in immunosuppressed subjects and in those infected with acyclovir-resistant virus, have renewed interest in the virus-transforming potential. The ability of HSV-2 (the lytic functions of which were inactivated) to cause neoplastic transformation was unequivocally established by numerous in vitro and in vivo studies. This review emphasizes the complexity of the transformation process which is mediated by multiple viral genes and DNA sequences and ranges from cellular immortalization to neoplastic transformation. Immortalization was seen in rodent cells, but its mechanism is still unclear. By contrast, HSV-2 contains at least two mutagenic DNA

sequences (BC24 and 486TF) and codes for at least two functions (R1 PK oncoprotein and Xho2 ORF) that convert immortalized human cells to a neoplastic phenotype. The R1 PK oncoprotein, which was studied in more detail, is a growth factor receptor ser-thr PK that binds secondary messenger proteins known to interact with growth factor receptor tyrosine kinases (viz. Ras-GAP and the Grb_2-hSOS complex) to activate the Ras/MEK/MAPK mitogenic pathway. Gene expression and pathway activation are required for maintenance of the transformed phenotype and tumorigenic potential. Activation of the Ras/MEK/MAPK pathway appears to be the major mechanism of virus-mediated transformation, since a HSV-2 mutant deleted in R1 PK was not transforming. This is to be expected, since activation of the mitogenic pathway is involved in the virus life cycle. Indeed, pathway activation is a very early event during productive infection which is regulated with IE-type kinetics and is required for the expression of regulatory IE genes (such as ICP4) and timely onset of virus growth. Expression of later viral genes which inhibit cellular macromolecular syntheses (lytic functions) is likely responsible for counteracting the establishment of constitutive cellular proliferation. If such genes are not expressed or are not functional, HSV-2 infection may cause hyperproliferative conditions. The HSV-1 counterpart of R1 PK is structurally and functionally distinct, it does not activate the mitogenic pathway and does not have transforming potential. Thus, small sequence differences at critical sites can result in major biological differences between two proteins that share an otherwise good level of homology. This cautions against facile conclusions about protein function based solely on sequence homology. H11, a recently identified cellular gene the expression of which appears to be required for melanoma cell growth, is similar to the HSV-2 R1 PK oncoprotein. Although the exact relationship of R1 PK to H11 is still unknown, the data suggest that R1 PK may have originated from the small stress protein superfamily and functions to initiate virus replication upon exposure of latently infected ganglia to reactivation inducing stress stimuli. Implicit in this interpretation is the conclusion that the transforming potential of R1 PK results from deregulation of a function that is required for the control of latency.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 88–106

Transforming Genes of Human Cytomegalovirus and Human Herpesvirus 6

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Overview

This chapter reviews the transforming genes of the β-herpesviruses, human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6). Both viruses have been implicated in the etiology of several human cancers. HCMV has been associated with cervical carcinoma [70, 71, 81] and adenocarcinomas of the prostate [9, 87] and colon [35, 41]. In vitro transformation studies have identified three HCMV morphologic transforming regions (mtr), i.e., mtrI, mtrII and mtrIII. While all of them can transform rodent cells in culture, only mtrII (UL111A) is retained and expressed in both transformed and tumor-derived cells. The transforming and tumorigenic activities of mtrII have been localized to a 79 amino acid (aa) open reading frame (ORF) designated as the mtrII oncogene. In studies to understand mechanism of mtrII transformation, the mtrII protein was shown to bind to the tumor suppressor protein p53 and to inhibit its ability to transactivate a p53-responsive promoter [76].

Other studies have shown that the HCMV immediate early (IE) genes, IE72 (IE1; UL123) and IE86 (IE2; UL122) may also be involved in transformation. Shen et al. [91] reported that IE72 and IE86 can cooperate with the adenovirus E1A gene to transform rodent cells in vitro. Moreover, the IE86 protein has been shown to interact with cell cycle regulatory proteins such as p53 and Rb [12, 95].

HHV-6, considered a possible cofactor in AIDS progression, has also been linked to several lymphoproliferative diseases. In vitro studies have identified three transforming fragments, i.e., *Sal*I-L, ZVB70 and ZVH14. Of these, only

*Sal*I-L (DR7) is retained in transformed and tumor-derived cells. The transforming and tumorigenic activities of *Sal*I-L have been localized to a 357 aa ORF-1 protein. The ORF-1 protein was expressed in transformed cells and like the HCMV mtrII, bound to p53 and inhibited its ability to transactivate a p53-responsive promoter.

Thus, the transforming genes mtrII and ORF-1 of HCMV and HHV-6 respectively, share a common mechanism of transformation and like the transforming genes of other DNA tumor viruses such as SV40, human adenovirus 5 and human papillomavirus types 16 and 18, bind to and inhibit the function(s) of one or both of the tumor suppressor proteins p53 and Rb. Future investigation into specific pathways involved will certainly clarify the role of HCMV mtrII and HHV-6 ORF-1 oncogenes in human malignancy.

Human Cytomegalovirus

Introduction

HCMV is a ubiquitous β-herpesvirus, that generally causes asymptomatic infections in normal immunocompetent individuals and severe, life-threatening disease in immunocompromised individuals such as AIDS patients and organ transplant recipients [80, 97]. HCMV can be transmitted from the mother to the fetus and cause severe birth defects [22, 96]. Following primary symptomatic or asymptomatic infection, HCMV has the ability to persist in the host and cause severe disease upon reactivation.

Because of the ubiquitous distribution of HCMV and high seroconversion rates, an etiological association between HCMV infection and human cancer has been difficult to establish. However, evidence based on virologic, epidemiologic and molecular studies has demonstrated the presence of viral DNA or antigens in tumor tissues. Seroepidemiologic studies linking HCMV to cervical cancer have yielded conflicting results [30, 34, 78, 102]. However, HCMV DNA has been detected in many cervical cancer biopsies and their derived cell lines [28, 39, 40, 73]. The presence of HCMV DNA and nuclear antigens has also been reported in prostatic carcinoma cells [17] and adenocarcinomas of the colon [21]. These observations have led to the development of experimental systems to identify and characterize the transforming DNA fragments of HCMV.

Transformation of Mammalian Cells in vitro

Initial studies using in vitro transformation assays demonstrated the oncogenic potential of HCMV. Both infectious and UV-inactivated virus transformed a variety of rodent and human cells in vitro [2, 10, 31, 40]. Moreover, transformed cells expressed HCMV-specific antigens and were tumorigenic in nude mice [32].

Several investigators have reported that HCMV infection can modulate a number of cellular properties often associated with the malignant phenotype (for review, see Cinatl et al. [19]). Transcriptional activation of cellular proto-oncogenes such as *fos*, *jun* and *myc* has been observed following HCMV infection with both laboratory strains and clinical isolates [5–8]. Increase in the levels of several cell cycle regulatory proteins such as cyclins, p53 and phosphorylated Rb has also been reported [13, 14, 49]. Zhu et al. [121] have shown that HCMV infection blocked the induction of apoptosis, or programmed cell death and that this block was mediated by the viral IE regulatory genes.

The ability of HCMV to induce tumorigenic transformation of a variety of mammalian cells as well as to modulate the expression of various proteins involved in cell cycle regulation and apoptosis, provided a rationale for studying specific viral genes and their role in cellular transformation.

Morphological Transforming Regions of HCMV

Transformation of rodent cells with either intact or UV-irradiated HCMV DNA suggested the presence of a transforming gene. Restriction fragments of HCMV DNA were tested for their ability to convert normal cells to a transformed phenotype, to grow in soft agar, as well as to induce tumors in mice. Using these criteria, three distinct genomic fragments capable of transforming rodent cells in vitro were identified. These fragments were designated as morphological transforming regions (mtr)I (5.0 kb), mtrII (3.0 kb) and mtrIII (2.1 kb) [20, 24, 77, 107] (fig. 1).

Subsequent studies showed that mtrI identified in strain AD169, and mtrIII identified in strain Towne, were not retained in the transformed cells. This led to the proposal that mtrI and mtrIII transformed cells by a 'hit-and-run' hypothesis of insertional mutagenesis. In contrast, mtrII identified in strain Towne was consistently retained in both the transformed and tumor-derived cells suggesting a role for the viral gene sequence in transformation. In other studies, cooperation between mtrII and mtrIII led to a 7-fold increase in transformation frequency compared to rodent cells transfected with either alone [47]. The mechanism involved in enhanced tumorigenicity by mtrII and mtrIII has not been elucidated.

The MtrII Oncogene

Localization of mtrII Activity to a 79 Amino Acid ORF (UL111A). The transforming activity of the original 3.0-kb mtrII fragment was localized to a smaller 980-bp fragment [83]. Nucleotide sequence of the 980-bp fragment revealed ORFs of 79, 83 and 34 aa respectively. The 5′ terminus of the ORFs contained regulatory elements that included CAAT boxes, Sp1-binding sites, and TACAAA and ATA transcriptional initiation signals which suggested a promoter region. This region also contained the heptanucleotide sequence

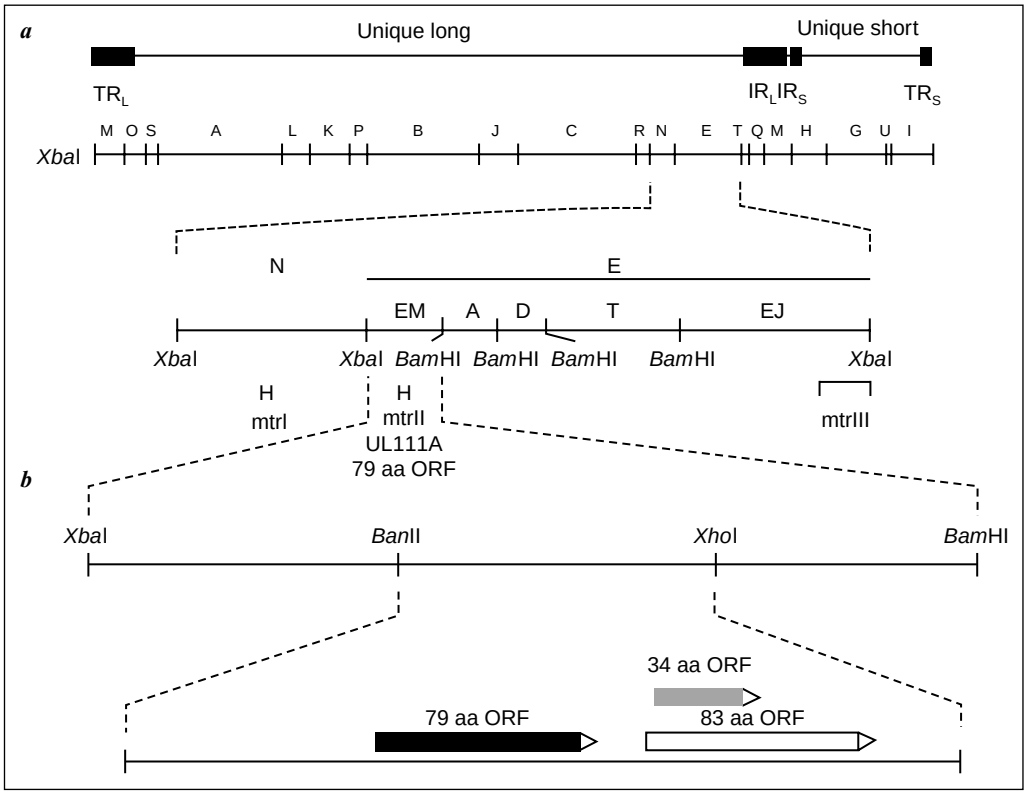

Fig. 1. ***a*** Restriction map of HCMV strain Towne showing the location of mtrI, mtrII and mtrIII. MtrI has been identified in strain AD169. MtrII, also designated as UL111A, contains the 79 aa ORF. MtrII and mtrIII were identified in strain Towne. Adapted from figure 1 of Muralidhar et al. [76] with permission of the authors and publisher. ***b*** Within the *Xba*I/*Bam*HI EM fragment of strain Towne is the *Ban*II/*Xho*I (mtrII 980) fragment with its ORFs. The filled, open and gray rectangles represent the 79, 83 and 34 aa ORFs, respectively.

GGTGA/GTC with similarity to the simian virus (SV40) enhancer core consensus sequence. That this was a functional promoter sequence was established by cloning the putative promoter sequences upstream of a chloramphenicol acetyltransferase (CAT) reporter gene [44, 84]. Furthermore, a major 410 base (b) mRNA transcript encoding the 83 and 34 aa ORFs and a minor 720 b mRNA transcript encoding the 79 aa ORF were detected in HCMV-infected cells at 24 h post-infection [83]. However, of the three ORFs, only the 79 and 34 aa ORFs contained motifs with homology to the Kozak consensus translational initiation sequence.

Analysis of the transforming activity of the colinear mtrII regions in HCMV strains AD169, Towne and Tanaka provided a clue to the role of the 79 aa ORF in HCMV-induced transformation. While the mtrII region of strains AD169 and Towne exhibited similar levels of transforming activity, mtrll from strain Tanaka showed a 75% reduction in transforming activity [46]. Sequence analysis revealed a frameshift mutation in the 79 aa ORF in the strain Tanaka, which was proposed to be responsible for the lower levels of transforming activity.

Direct evidence for the role of the 79 aa ORF in transformation was reported by Thompson et al. [106]. Plasmid pCHCmtrII, containing the 79 aa ORF expressed from the HCMV IE promoter, was shown to transform NIH 3T3 cells. Cell lines established from the transformed foci expressed mtrII mRNA and were tumorigenic in immunodeficient athymic nude mice. Similar stable cell lines were developed after transfection with pCHCmtrII-TTL_{24} or pCHCmtrII-TTL_{49} which expressed mutant mtrII that terminated translation after aa 24 or 49, respectively. MtrII-TTL_{24} cells failed to produce tumors indicating that the N-terminus alone was not sufficient for tumorigenesis. However, the mtrII-TTL_{49} cell line produced tumors, but with less efficiency than wild-type mtrII. Thus, the transforming ability of the 79 aa ORF (mtrII) in the absence of the 34 and 83 aa ORFs was demonstrated.

Mechanism of Transformation by mtrII

Binding of mtrII Protein to p53 and Inhibition of p53-Activated Transcription. DNA tumor virus oncoproteins such as the SV40 T antigen, adenovirus E1A and E1B, and HPV-16 and -18 E6 and E7 commonly bind to the cellular tumor suppressor proteins, p53 and/or Rb [23, 88, 89, 116, 117] and inactivate the check points in the cell cycle, resulting in uncontrolled cellular growth and division. The interaction of the mtrII protein with p53 was analyzed by Muralidhar et al. [76] in order to determine the mechanism of transformation.

Binding of mtrII protein to p53 was demonstrated by co-immunoprecipitation assays both in vitro and in vivo in cell extracts of mtrI-transformed rodent cells. In the in vivo studies, mtrII protein co-immunoprecipitated with p53 when an anti-p53 antibody was used. The in vitro studies using ^{35}[S]-labeled mtrII and p53 proteins and their deletion mutants, also established the interacting domains of the two proteins. The mtrII-binding domain was mapped to the N-terminal region of p53, aa 1–106, with a critical region from aa 1–44. Interestingly, this is the domain of p53 which is required for binding to transcription factors such as the TATA-binding protein (TBP) and its associated factors (TAFs) [60]. Importantly, this is also the domain that is targeted by the negative regulator of p53, viz., mdm2 [59], and other DNA tumor virus oncoproteins such as adenovirus E1B 55 kD protein [59]. Conversely, using C-terminal truncated peptides of mtrII, the p53-binding domain on mtrII protein was determined as aa 1–49.

The functional consequence of the mtrII/p53 interaction was analyzed by examining the effects of mtrII protein on the transactivation of the reporter CAT gene by p53. MtrII inhibited p53-activated transcription both in transiently transfected cells as well as in stably transformed cells. When a reporter construct containing a CAT gene driven by a p53-responsive promoter (p53G5BCAT) was co-transfected with pCMV/p53 which expressed p53 protein, CAT activity increased over 70-fold. When increasing amounts of the mtrII construct, pCHCmtrII, were co-transfected, a dose-dependent decrease in CAT activity was observed. The above result indicated that binding of mtrII to p53 can inhibit the transactivation function of p53.

When similar experiments were carried out with mtrII TTL mutants, inhibition of p53-activated CAT activity was observed in the presence of pCHC mtrII-TTL_{49} but not pCHC mtrII-TTL_{24}. This result correlated with the ability of the mutants to bind to p53 in vitro. While full-length and TTL_{49}-truncated mtrII proteins bound to p53, TTL_{24} did not. Importantly, both wild-type and mtrII-TTL_{49} transformed rodent cells were tumorigenic in nude mice, whereas mtrII-TTL_{24} cells were not [106]. The concordance of these results indicates a causal relationship between mtrII binding to p53, mtrII inhibition of p53 function, and mtrII tumorigenic activity.

Some viral oncoproteins such as the SV40 T antigen have not only been shown to bind to and functionally inactivate p53, but also stabilize the levels of p53 in transformed cells by making them inaccessible to the ubiquitin degradation pathway [88, 89]. Interestingly, the steady-state level of p53 protein in mtrII-transformed NIH 3T3 cells was 10- to 20-fold greater than in parental cells or cells transfected with vector plasmid [76]. This stabilization of p53 was not due to an increase in the rate of transcription and translation of p53 but due to a decrease in the rate of its degradation as determined by the p53 half-life studies. In spite of the high steady-state levels of p53, the level of CAT activity in transformed cells was only 25–30% of that observed in control cells after transfection with p53G5BCAT. Therefore, despite the high steady-state levels, p53 was functionally inactive in mtrII-transformed cells. Elevated p53 levels have also been observed in HCMV-infected human embryonic lung [75] and smooth muscle cells [95]. In light of the above interactions between mtrII and p53, mtrII could contribute to the elevated levels of p53 observed in HCMV-infected cells.

The HCMV mtrII oncogene has many features of the small DNA tumor virus oncogenes. In fact, mtrII is not only expressed in transformed cells, but it can also bind to p53 in vivo and inhibit p53-activated transcription. These similarities characterize mtrII as a human herpesvirus oncogene, at least in as far as its ability to transform rodent cells in culture. Further studies are required to determine the pathways involved in mtrII transformation. Binding of p53 by the mtrII oncoprotein may affect p53 function(s) in the cell.

Wild-type p53 is a pleiotropic gene exhibiting both growth and transformation suppressor activities that result in a G_1 arrest in the cell cycle [56]. P53 is activated in response to DNA damage, stress and hypoxia, and has been shown to transactivate a number of genes involved in growth arrest, apoptosis, and DNA repair pathways [25, 54, 74]. Disruption of p53 function is a characteristic of several human cancers [37, 66].

The functional consequences of mtrII binding to p53 remain to be elucidated. In addition, the expression of the mtrII-transforming gene in human tumors needs to be investigated. Identification of mtrII as a transforming gene will make this search more specific and will hopefully lead to the identification of relevant pathways involved in transformation in vitro and oncogenicity in vivo.

The HCMV Immediate Early (IE) Genes

The HCMV IE gene locus encodes several proteins because of differential splicing of the primary transcript [99–101]. Of these, the two major proteins IE72 (IE1; UL123) and IE86 (IE2; UL122) are the best characterized. Several studies have demonstrated that the IE genes are the major regulators of viral early and late gene transcription. Moreover, IE86 is a strong transactivator of both heterologous viral and host genes [79, 119]. Although these IE proteins have not been demonstrated to be transforming in vitro, they have been shown to interact with p53 and Rb. Shen et al. [91] have shown that IE72 and IE86 cooperate with the adenovirus E1A oncoprotein to transform baby rat kidney cells. In addition, the transformed cells exhibit mutations in cellular genes such as p53, suggesting the mutagenic potential of the IE proteins. However, the expression of the IE proteins was transient, and neither IE DNA nor IE proteins were detected in the transformed cells. Based on these observations, the investigators proposed that the IE genes mediate a 'hit-and-run' mechanism of transformation, by inducing mutations in cellular genes. Zhu et al. [121] have shown that the IE proteins can inhibit the induction of apoptosis by either tumor necrosis factor α (TNF-α) or the adenovirus E1A protein and could therefore, presumably promote the replication and persistence of the virus.

Binding of HCMV IE86 Protein to p53

Several studies have investigated the effect of IE86 protein expression on the levels and functional status of p53. Speir et al. [95] have proposed that the induction of p53 following HCMV infection plays a role in coronary artery restenosis, characterized by hyperproliferation of smooth muscle cells. Latent HCMV has been detected in arterial walls of patients suffering from atherosclerosis [36, 72, 93]. The authors found that a majority of the restenotic lesions that exhibited immuno-detectable p53 were also positive for HCMV DNA

(as determined by PCR). Furthermore, upon culturing smooth muscle cells from restenotic lesions, 4 out of 7 cultures were immuno-positive for both p53 and IE86. As a result, Speir et al. [95] have proposed that activation of latent HCMV infection by physical injury during angioplasty may lead to elevated levels of functionally inactivated p53. The authors demonstrated binding of IE86 and p53 expressed in the baculovirus system by co-immunoprecipitation. In addition, they showed that IE86 abrogated p53-mediated transactivation of a CAT reporter plasmid containing p53-responsive elements, when all three constructs were co-transfected into human smooth muscle cells. These data suggested that HCMV infection of smooth muscle cells lead to enhanced cell proliferation through the inactivation of p53 function by IE86.

To determine whether the interaction of IE proteins with p53 abrogated G_1 checkpoint function, Bonin and McDougall [12] established stable clonal human foreskin fibroblast cell lines expressing IE72 or IE86. IE86 protein, but not IE72, was co-immunoprecipitated with p53 from cell lysates demonstrating the binding of IE86 to p53 in these cells. However, upon treatment of cells with a DNA-damaging agent such as actinomycin D, no difference was observed in the percentage of cells in G_1/S phase in the parental and IE72 and IE86 expressing cells as determined by fluorescence flow cytometry. Furthermore, no differences were found in levels of p53, p21 (CIP1/WAF1) and mdm2 proteins and the phosphorylation status of Rb after actinomycin D treatment of IE expressing cells. These data demonstrated that although IE86 bound to p53, it failed to abrogate the p53-mediated G_1 arrest induced by DNA damage.

Binding of HCMV IE86 Protein to Rb

A number of viral oncoproteins such as SV40 T antigen and adenovirus E1A have been shown to bind to the tumor suppressor protein Rb [3, 38, 42, 117]. Likewise, Sommer et al. [92] demonstrated the binding of HCMV IE86 to Rb using both wild-type and deletion mutants of IE86 expressed in bacteria as glutathione S-transferase (GST)-IE86 fusion proteins. Binding of GST-IE86 fusion protein to in vitro transcribed/translated ^{35}[S]-labeled Rb was assessed by immobilization onto glutathione-coated beads. The authors showed that IE86 bound to wild-type Rb and mapped three internal domains of IE86, i.e., aa 85–135, 136–290 and 291–364, that could independently bind to Rb.

Another study by Fortunato et al. [29] determined the domains of Rb required for binding to IE86. More than one IE86 binding domain was found within Rb, i.e., the C-terminal domains from aa 768 to 926 and the Rb A/B pocket from aa 379 to 776. Most other proteins such as SV40 T and adenovirus E1A that bind Rb require the A/B pocket region, or the C-terminal end or both [104, 112, 113].

In summary, HCMV IE86 binds to both Rb and p53. However, there is no evidence that IE86 interferes with Rb or p53 cell cycle regulation thus leading

to an extended life span or transformation (as in the case of SV40 T antigen). Further studies are necessary to determine if the IE genes play a role in HCMV transformation.

Human Herpesvirus 6

Overview

HHV-6 is a T-cell tropic virus originally isolated from AIDS patients with lymphoproliferative disorders [51, 86] and later detected in other human lymphoid malignancies [1, 64, 65, 103]. HHV-6 infection has also been linked to a number of other diseases such as exanthem subitum in children [118], meningoencephalitis [45], infectious mononucleosis [4, 98], autoimmune disorders [55], chronic fatigue syndrome [15], pneumonitis [16], and multiple sclerosis [18].

HHV-6 has been proposed to be a co-factor in AIDS progression because co-infection of CD4+ human T cells with both HIV-1 and HHV-6 results in accelerated cytopathic effects [63]. HHV-6 may also play a role in the reactivation of latent HIV-1 as well as in the up-regulation of HIV-1 expression as has been observed by Ensoli et al. [27]. Additionally, Lusso et al. [62] have demonstrated that HHV-6 infection of CD8+ human T cells induced CD4 expression rendering these cells susceptible to HIV-1 infection. Therefore, HHV-6 may augment AIDS progression either by increasing HIV-1 viral production or by increasing the population of HIV-1-susceptible cells.

HHV-6 DNA sequences have been identified in various human cancers including African Burkitt's lymphoma, Hodgkin's lymphoma and EBV-negative B-cell lymphoma [26, 48, 50, 110]. In fact, integration of the HHV-6 genome into the 17p13 region of chromosome 17 has been demonstrated in peripheral blood mononuclear cells isolated from individuals with Hodgkin's disease and non-Hodgkin's lymphoma [61, 109]. Whether the integration of HHV-6 contributed to the etiology of the above lymphomas remains to be determined. These observations have encouraged researchers to identify HHV-6-transforming gene(s) and to evaluate the potential role of HHV-6 as an oncogenic virus.

Identification of HHV-6 Transforming Fragments

Transformation of NIH 3T3 cells by either total genomic HHV-6A DNA or non-overlapping subfragments, ZVH14 (8.7 kbp) and ZVB70 (21 kbp) (fig. 2) was first demonstrated by Razzaque [82]. The transformed cells exhibited anchorage-independent growth in agarose and tumorigenicity when injected into athymic nude mice. Upon analysis, no ZVH14 DNA was detected by Southern blotting in either genomic DNA or ZVH14-transformed cells or their tumor-derived lines. In contrast, G418-selected cell lines contained intact or

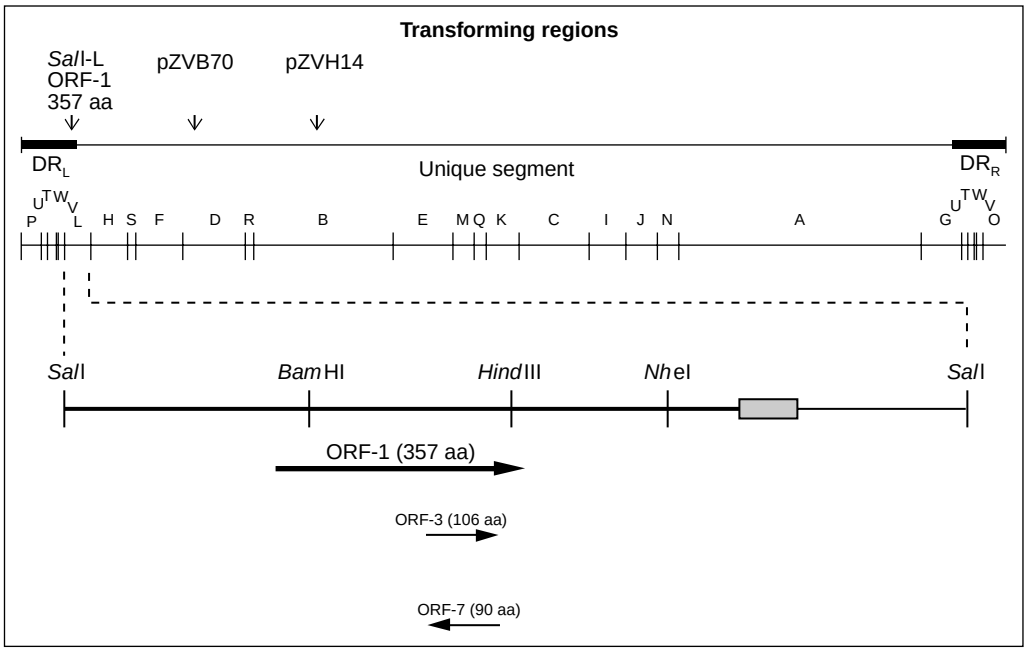

Fig. 2. A schematic map of HHV-6 strain U1102 showing the unique segment and the left and right direct repeats regions (DR_L and DR_R, respectively). Shown above are the three transforming fragments *Sal*I-L, pZVB70 and pZVH14. Shown below is the SalI restriction map. The *Sal*I-L is enlarged to depict the positions of the ORF-1, ORF-3 and ORF-7. The direction of the arrow indicates the direction of the ORF.

rearranged ZVH14 sequences. Since the ZVH14-transforming region was not retained in the transformed cells except under selective pressure, its role in the maintenance of the transformed phenotype was questioned. In another study, Thompson et al. [105] tested five fragments of HHV-6 strain U1102 for transformation of NIH 3T3 cells. Of these, only the 3.9 kbp *Sal*I-L fragment caused transformation (fig. 2). The number of transformed foci observed for *Sal*I-L was similar to that obtained with the HCMV mtrII oncogene. The *Sal*I-L-derived transformed cell lines exhibited anchorage-independent growth in agarose and produced tumors with a latency period of under 2 weeks in athymic nude mice. Southern blot analysis revealed *Sal*I-L DNA sequences in the tumor-derived cell lines suggesting that they are required for the maintenance of the transformed phenotype.

Identification and Characterization of the ORF-1-Transforming Gene

Localization of Sal*I-L-Transforming Activity to ORF-1 (DR7).* Sequence analysis of *Sal*I-L revealed seven ORFs of greater than 75 amino acids. To

determine the transforming regions within *Sal*I-L, Kashanchi et al. [52] subcloned six subfragments and tested them for transformation of NIH 3T3 cells. The results showed that only the *Sal*I-HindIII subfragment-containing ORFs 1, 3 and 7 (fig. 2) induced morphological transformation. The individual ORFs 1, 3 and 7 were then subcloned in a mammalian expression vector and tested for their ability to induce focal transformation. Only ORF-1 was found to induce transformed foci above background levels. Moreover, ORF-1-transformed cell lines were tumorigenic in athymic nude mice. To demonstrate that ORF-1 translation was required for transformation, ORF-1 was constructed with a translation termination linker (TTL) inserted after residue 172 (TTL_{172}). The location of the TTL was upstream of ORF-3, leaving ORF-3 intact. Cell lines transfected with wild-type ORF-1 exhibited transformation while those transfected with the TTL_{172} mutant did not. Expression of wild-type and mutant ORF-1 proteins in these cell lines was confirmed by Western blot analysis using rabbit polyclonal antibody raised against purified bacterially expressed ORF-1 protein. When wild-type ORF-1 and TTL_{172} cell lines were tested for tumorigenicity, only wild-type ORF-1 cells produced fibrosarcomas in nude mice, while TTL_{172} mutant cells did not. Furthermore, ORF-1 protein was detected in tumor tissue by Western blot analysis, suggesting that the expression of ORF-1 protein was required for tumorigenesis. In other studies, ORF-1 was expressed in HHV-6-infected human T cells with both mRNA and protein detected at 18–48 h post-infection, suggesting that ORF-1 is an HHV-6 early gene [52].

Association of ORF-1 with Human Malignancies. With the in vitro identification of the ORF-1 oncogene, studies were performed to determine if ORF-1 was detected in human malignancies [52]. Glioblastomas and pathologic lymph nodes from patients with angioimmunoblastic lymphadenopathy, angioimmunoblastic lymphadenopathy-like lymphoma, Hodgkin's disease (HD), and both B- and T-cell lineage, non-Hodgkin's lymphoma (NHL) were examined by PCR. ORF-1 sequences were rarely detected in most of these malignancies. However, ORF-1 sequences were found in 5 out of 12 lymph nodes of angioimmunoblastic lymphadenopathy, while nonmalignant lymph nodes and normal brain tissue specimens were negative. Thus, ORF-1 sequences, which exhibited oncogenic properties in vitro, were retained at variable frequency in various human tumor tissues. These observations, while not proof per se, are a necessary prerequisite for establishing the role of ORF-1 in human malignancies.

The Interaction between ORF-1 and p53 Proteins. The expression of the ORF-1 protein in transformed cells and tumor tissues suggested a maintenance function for ORF-1. Because binding to p53 is characteristic of several viral oncoproteins, including HCMV mtrII previously described in this chapter [43, 58], ORF-1 was also tested for binding to p53. In GST pull-down assays, ORF-1 was observed to bind specifically to GST/p53. Furthermore, anti-ORF-1 serum

co-immunoprecipitated the p53/ORF-1 complex. Co-immunoprecipitation experiments performed with truncated p53 proteins demonstrated that the ORF-1-binding domain of p53 was between aa 28 and 187 with a critical region between aa 107 and 187. This is the sequence-specific DNA-binding domain of p53 to which other viral oncoproteins such as and SV40 T antigen binds.

The ability of ORF-1 to affect p53-activated transcription was examined using a p53-responsive reporter construct (p53G5BCAT). Wild-type ORF-1 cells transfected with p53G5BCAT exhibited 7- to 8-fold reduction in CAT activity as compared to parental NIH 3T3 cells expressing the ORF-1-TTL_{172} mutant. Thus, ORF-1 specifically suppressed p53-activated transcription. The binding of ORF-1 to p53 may alter the ability of p53 to regulate cellular genes important for growth control.

Conclusion

Human herpesviruses have been investigated for over the past several decades as possible oncogenic agents. Studies cited in this review demonstrate that HCMV and HHV-6 contain in vitro transforming genes which are retained in both transformed and tumor-derived cell lines. These genes may be in part responsible for the tumorigenic phenotype observed in some human cancers. In the case of HCMV, the mtrII oncoprotein was expressed in transformed and tumor-derived cell lines. Furthermore, mtrII protein bound to p53 and inhibited p53-activated transcription. In the case of HHV-6, the transforming activity was localized to ORF-1. Like mtrII, ORF-1 also bound to p53 and inhibited p53-activated transcription. These activities coupled with the detection of ORF-1 in malignant tissues may indicate a role of ORF-1 in human cancer. Both the HCMV and HHV-6 oncoproteins exhibit the same characteristics as the oncoproteins of several DNA tumor viruses such as SV40 and polyomavirus T antigens [116], adenovirus E1B [117] and HPV 16 E6 [23] because of their ability to bind to and inactivate host tumor suppressor proteins such as p53. This binding may uncouple normal growth control processes and lead to cellular transformation. However, mtrII and ORF-1 differed in the domains of p53 they bound. While mtrII bound to the N-terminal transactivation domain of p53, ORF-1 bound primarily to the central sequence-specific DNA-binding domain (fig. 3). As a result, the mechanism by which they inhibit p53 function may differ. Although the HCMV mtrII and IE86 as well as the HHV-6 ORF-1 proteins have been shown to bind to and inhibit p53-activated transcription, the effects on specific p53-mediated pathways such as apoptosis, stress-induced G1 and G_2 growth arrest, and DNA repair have not yet been characterized.

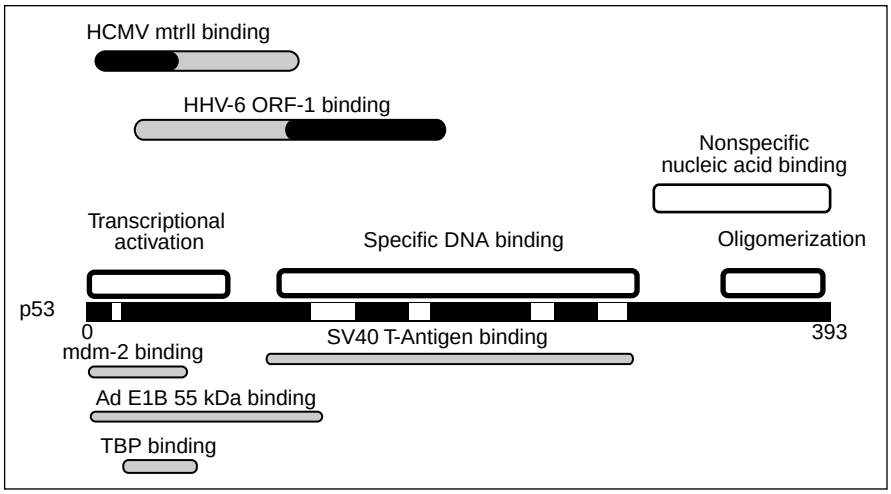

Fig. 3. Map of the human p53 protein showing the locations of the transcriptional activation, specific DNA binding, oligomerization and the nonspecific DNA-binding domains. Depicted above are the HCMV mtrII and HHV-6 ORF-1-binding domains, with the critical regions for binding indicated in black. Shown below are the locations where other viral oncoproteins such as the SV40 T antigen and adenovirus E1B (55 kDa) bind to p53, as well as cellular proteins, mdm-2 and TATA-binding protein (TBP).

The acquisition of a fully malignant phenotype by normal cells is thought to require several mutations/dysfunctions in a number of cellular genes/proteins. Inhibition of tumor suppressor functions as discussed above could be one mechanism by which herpesviruses modulate the malignant potential of cells. In the case of HCMV infection, a number of other mechanisms such as stimulation of growth factors or proto-oncogenes and inhibition of cellular apoptotic pathways, may also play a role in altering cell growth [5–8, 11, 13, 49, 121].

In recent years, it has become increasingly clear that viral evasion of host immune responses plays an important role, not only in disease, but also in transformation and tumor development. In this regard, a number of viruses and viral oncogenes have been shown to modulate the expression of major histocompatibility complex (MHC) genes [69]. Loss of MHC antigen expression in infected or transformed cells may serve as a mechanism for survival and escape from the host immune system. Further analysis of the above mechanism(s) of transformation by herpesvirus oncogenes together with their detection in human cancers will provide insights into the multistep process of malignant transformation.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 107–119

Transforming Genes of Human Herpesvirus 8

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Overview

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV) is etiologically associated with all epidemiologic forms of Kaposi's sarcoma (KS), i.e., Mediterranean classic, African endemic, post-transplant or iatrogenic, and the AIDS-associated [8, 43, 57]. Moreover, HHV-8 infection is associated with AIDS-associated B-cell malignancies, i.e., primary effusion lymphoma (PEL), also called body cavity-based lymphoma (BCBL) and multicentric Castleman's disease (MCD) [9, 60]. The linkage of HHV-8 with the above malignancies is based on the detection of HHV-8 DNA sequences in malignant tissue biopsies as well as seroepidemiological studies correlating conversion to HHV-8 seropositivity with the onset of malignancy [24, 31, 36, 41, 59]. The pattern of HHV-8 infection in KS, PEL and MCD can be distinguished by cellular tropism and by differences in viral gene expression [61]. While KS is a vascular tumor of possible endothelial origin, PEL and MCD are both B-cell malignancies [22, 26, 50].

The genomic DNA sequence has indicated that HHV-8 is a member of the rhadinovirus subgroup of herpesviruses [44]. The genome exhibits significant homology to herpesvirus saimiri (HVS), a rhadinovirus prototype New World primate tumor virus and to the Epstein-Barr virus (EBV), a human γ-herpesvirus linked to Burkitt's lymphoma and nasopharyngeal carcinoma [54]. However, HHV-8 lacks homologs of the transforming genes of either HVS or EBV. Like other rhadinoviruses, HHV-8 contains numerous homologs of cellular genes

that are involved in nucleotide metabolism, regulation of cell cycle and apoptosis, signaling proteins, cytokines, chemokines, as well as modulators of the immune response. Additionally, HHV-8 contains unique genes designated K1–K15, which lack homology to other herpesvirus or cellular genes. This chapter reviews the roles of several HHV-8 genes including K1, K9, K12, ORF 72 and ORF 74 that are involved in transformation of rodent cells in vitro and are thus candidates for being involved in oncogenicity in vivo.

Genome Structure and Organization

The HHV-8 genomic sequence has been obtained from both cosmid and phage genomic libraries of the PEL cell line, BC-1 [49, 54], as well as from a KS lesion [44, 47]. Its genome structure is similar to that of HVS and consists of a 140.5-kb long unique region (LUR), flanked on either side by a variable number (30–50) of 801-bp tandem repeats [54]. The tandem repeats are a conserved feature of herpesviruses, serve as the site for circularization of the linear genome during its transition to the latent plasmid genome, and contain conserved packaging and cleavage signals for viral DNA at the end of the replication cycle. The LUR contains 75 known open reading frames (ORFs), (ORFs1–75) with similarity to those of HVS [54], in addition to unique ORFs, K1–K15. Recently, four additional unique ORFs have been identified and assigned decimal numbers K4.1, K4.2, K8.1 and K10.1. Other genes including the major capsid protein (ORF25), minor capsid protein (ORF26), DNA polymerase (ORF9), thymidine kinase (ORF21), thymidylate synthase (ORF70) and dihydrofolate reductase (DHFR; ORF2) are all conserved in both HHV-8 and HVS. However, the last two genes are located at different positions on the genomes [4, 49]. The extensive homology between HHV-8 and HVS indicates an evolutionary relationship between these viruses. However, the reported transforming genes of HVS, i.e., saimiri transforming protein (STP) and tyrosine kinase-interacting protein (TIP) are not present in HHV-8 [4, 54].

Identification of Transforming Genes

Since HHV-8 lacked homologs of the transforming genes of either HVS or EBV, several unique K genes have been tested for their ability to transform tissue culture cells and to promote cell proliferation in vitro. Among the unique HHV-8 genes, K1, K9 and K12 (kaposin), have been shown to induce tumorigenic transformation of rodent cells [23, 35, 45]. ORF K1 induced T-cell lymphomas in common marmosets when substituted for the STP gene of HVS [35].

Table 1. HHV-8 genes involved in in vitro transformation

Viral gene	Cellular homolog	Expression in KS lesions	Expression in PEL	In vitro transformation	Possible functions
K1	–	–	Lytic	Yes	Promote cellular growth
K9	IRF	–	Latent/lytic	Yes	Immune regulation; promote cellular growth
K12 (kaposin)	–	+++	Latent/lytic	Yes	Promote cellular growth
ORF 72	Cyclin D	+	Latent	Yes	Promote cellular growth
ORF 74	GPCR	+	Lytic	Yes	Promote cellular growth

+ = Expression detected; +++ = abundant expression detected; − = not expressed.

ORF K9 (v-IRF) which exhibits some homology to the negative regulator of the interferon signal transduction pathway, IRF-2, also transformed rodent cells [23]. Moreover, ORF K12 (kaposin) transformed rodent cells in culture which upon inoculation into nude mice, induced tumors [45]. Interestingly, the transcript encoding kaposin is the most abundant latency- associated transcript detected in all stages and in nearly every KS biopsy as well as PEL [62, 74]. In addition, ORF 72, a homolog of cyclin D, and ORF 74, a homolog of GPCR, have also been demonstrated to promote cellular growth [5]. Furthermore, ORF 74 has been shown to induce tumorigenic transformation of rodent cells as well as to activate pathways promoting angiogenesis [6]. The above-mentioned transforming genes, their expression pattern in KS and PEL, and their proposed functions are summarized in table 1. Of these, ORF K12, ORF 72 (v-cyclin D), and ORF 74 (v-GPCR) are clustered along with other genes such as the latency-associated nuclear antigen, ORF 73 (LANA), ORF K13 (v-FLIP) and ORF K14 (v-OX-2) in a 13-kb region at the right end of the LUR designated as the 'oncogenic cluster'(fig. 1) [7, 70].

ORF K1

ORF K1 encodes a membrane glycoprotein with a cysteine-rich extracellular domain which exhibits high variability in sequence. This variability is the basis for the classification of HHV-8 into four major subtypes, A, B, C and D, as well as into multiple variants or clades in different human populations [76].

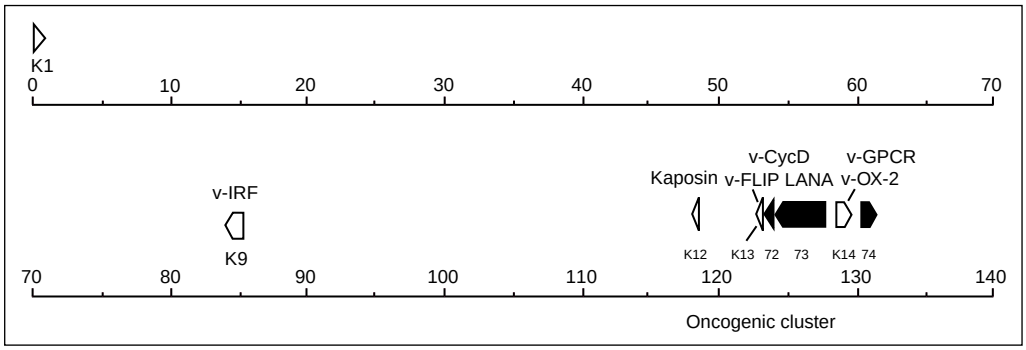

Fig. 1. Map of the HHV-8 long unique region (LUR) showing the locations of the in vitro transforming genes of HHV-8. The solid arrows indicate ORFs conserved in HVS and open arrows indicate the unique HHV-8 ORFs. Arrows indicate the direction of transcription. The designated ORF numbers are shown below the arrows. V-IRF = Viral interferon regulatory factor; v-FLIP = viral FLICE inhibitory protein; v-Cyc D = viral cyclin D; LANA = latent nuclear antigen; v-OX-2 = viral NCAM-like adhesion protein; v-GPCR = viral G protein-coupled receptor. Also indicated is a 13-kb region at the right end of the LUR designated as the 'Oncogenic cluster'.

ORF K1 is the positional equivalent of the transforming gene of HVS, STP, but does not exhibit any sequence homology. Lee et al. [35] demonstrated that transfection of K1 into rodent fibroblasts induced morphologic changes and focus formation indicative of transformation. Furthermore, an HVS chimera containing the K1 gene in place of STP not only immortalized primary T lymphocytes to IL-2-independent growth but also induced T lymphomas in common marmosets. The cytoplasmic domain of K1 contains a functional immunoreceptor tyrosine-based activation motif (ITAM), which when fused to the human CD8α polypeptide induced cellular tyrosine phosphorylation and intracellular calcium mobilization in B cells upon stimulation with anti-CD8 antibody [34]. However, unlike other ITAM-based signal transduction pathways, K1 signaling occurred constitutively, in the absence of extracellular ligands. These studies suggest that K1 can deregulate signal transduction pathways in HHV-8-infected cells. However, expression of K1 has been observed only upon TPA treatment of PEL-derived cells [56], and has thus far not been demonstrated in KS lesions (table 1).

ORF K9 (Viral-Interferon Regulatory Factor; v-IRF)

ORF K9 has low sequence homology to the IRF family of proteins involved in the interferon signal transduction and regulation [42]. IRFs are either positive regulators (transcriptional activators), for example, IRF-1, or negative regulators (repressors), for example, IRF-2, induced through the

Jak/STAT signal transduction pathway [67]. The v-IRF appears to act as a negative regulator of interferon signaling, analogous to IRF-2 [38, 75]. Transfection of v-IRF into cell lines such as 293, HeLa and microvascular endothelial cells inhibited interferon-mediated induction of reporter plasmids containing an interferon-stimulated response element (ISRE). The mechanism of this inhibition is currently not understood because the direct binding of v-IRF to ISRE or to members of the interferon signal cascade has not been demonstrated [23]. Stable transfection of v-IRF into NIH3T3 cells resulted in the development of transformed cells which were tumorigenic in nude mice [23]. V-IRF transformed cells exhibited down-regulation of the cyclin-dependent kinase (CDK) inhibitor, p21/WAF1, which may contribute to cell cycle deregulation. Thus v-IRF may function to evade interferon-mediated host cell responses as well as to contribute to transformation. Expression of v-IRF has not been detected in KS spindle cells [23]. However, v-IRF is expressed and induced in PEL cells [42] (table 1).

ORF K12 (Kaposin)

ORF K12 encodes a small 60 amino acid, highly hydrophobic protein called kaposin [74]. It is translated from a 0.7-kb mRNA (T0.7) which is abundantly expressed in PEL cell lines (latently infected with HHV-8) as well as in the spindle cells of KS lesions at all stages of tumor progression [53, 56, 63, 65]. It has been proposed that in most HHV-8 isolates from KS and PEL, kaposin is encoded by a larger 2.3-kb transcript [55]. Nevertheless, the kaposin encoding mRNA has been consistently detected in both KS and PEL as an abundant latency-associated transcript by in situ hybridization using the T0.7 sequence as a probe (table 1). It continues to be routinely used as a marker for HHV-8 latency [62–64].

The kaposin gene has been shown by our laboratory to induce tumorigenic transformation of Rat-3 cells when expressed either from its endogenous promoter or from a heterologous promoter [45]. Kaposin-induced tumors were high-grade, highly-vascular, undifferentiated sarcomas. Tumor-derived cells expressed kaposin protein in the cytoplasm, where it colocalized with the 58-kD Golgi membrane protein as determined by indirect immunofluorescence [46]. Kaposin protein was also detected in the cytoplasm of PEL-derived cell lines, BCBL-1 and KS-1. Western blot analysis of PEL cell lines BC-1, BC-3, BCBL-1 and KS-1 revealed multiple bands ranging from 16 to 40 kD. Several of these bands were also detected in rodent cells expressing the 60 amino acid ORF K12, suggesting that kaposin may be post-translationally modified by glycosylation and/or phosphorylation. Preliminary studies have indicated that kaposin may be involved in the activation of serine-threonine kinases such as PKC, leading to cell proliferation [46]. The expression pattern of kaposin in KS and PEL and its ability to induce tumorigenic transformation in vitro support its role as a candidate oncogene.

Sadler et al. [55] have described a complex translational pattern for kaposin in HHV-8-infected cells, with several proteins initiating at non-AUG codons such as CUG and GUG within the direct repeat region upstream of ORF K12. Two kaposin proteins of 38 and 54 kD contain the coding sequences of ORF K12. Others include a 48-kD protein and two 32-kD proteins in alternative reading frames. The role of these alternatively initiated proteins in in vitro transformation is currently being investigated.

ORF 72 (V-Cyclin D)

ORF 72 (v-cyclin D) exhibits limited amino acid identity with the human cyclin D2 [10]. It has been reported to interact with CDK6 and phosphorylate Rb in vitro, much like the cellular cyclin D [25]. In addition, v-cyclin D/CDK6 can also phosphorylate histone H1, suggesting that it may be important at other stages of the cell cycle as well. The functionality of v-cyclin D has been demonstrated by its cotransfection with a plasmid encoding Rb (pRb) into an osteosarcoma cell line, SAOS, which has a homozygous deletion for both Rb and p53 tumor suppressor genes [11]. Expression of v-cyclin D in these cells overcame Rb-mediated senescence and induced cell proliferation. ORF 72 (v-cyclin D) also stimulated progression from G1 to S in quiescent mouse fibroblasts [66]. Furthermore, Duro et al. [15] showed that v-cyclin D activated cellular cyclin A which is normally expressed at very low levels in the presence of antiproliferative signals such as serum starvation, loss of cell adhesion and DNA damage [27, 58]. ORF 72-induced activation of cyclin A under such conditions has also been observed for the adenovirus E1A and human papillomavirus-16 E7 oncogenes [72, 73]. The ability of v-cyclin D to activate cyclin A expression may also contribute to the transformed phenotype of HHV-8-infected cells. Interestingly, v-cyclin D has evolved an advantage over its cellular counterpart in that it is not inhibited by the CDK inhibitors p16INK4A, p21WAF1/CIP1 and p27KIP1 [66]. Recently, Yao and Browning [71] reported that v-cyclin D lacks a PEST (proline, glutamate, serine, threonine) sequence which targets cellular cyclins for ubiquitination, and is therefore able to escape ubiquitin-mediated degradation. ORF 72 (v-cyclin D) is expressed in latently infected spindle cells in KS lesions, as well as in PEL (table 1) [14, 56, 64], a finding possibly important for maintaining the proliferative nature of transformed cells.

ORF 74 (V-GPCR)

ORF 74 (v-GPCR) exhibits high-sequence homology to the CXC family chemokine receptor, IL-8R [10, 28]. Transient transfection studies in Cos-1 (SV40 transformed African green monkey kidney) cells revealed that ORF 74 functions as a bona fide IL-8R by binding the ligand IL-8 with high affinity, and activating the same signal-transduction pathway as IL-8, i.e., the phosphoinositide-inositol

triphosphate-protein kinase C pathway leading to the activation of AP-1, a transcription factor involved in cell proliferation and survival [5]. However, unlike its cellular counterpart, ORF 74 activates the signal transduction pathway constitutively in an agonist-independent manner [5]. Cellular GPCRs that are constitutively active due to mutations can transform cells and have been implicated in the pathogenesis of several human tumors [3, 13, 30]. Another feature that distinguishes v-GPCR from its cellular counterpart is its ability to bind to a wide range of CXC and CC chemokines and therefore activate other signal transduction pathways. Transfection of v-GPCR into the normal rat kidney fibroblast cell line, NRK-49F, resulted in enhanced proliferation [5]. Bais et al. [6] have shown that v-GPCR transformed NIH3T3 cells (mouse fibroblasts) induced highly vascularized tumors in nude mice. Interestingly, they also showed that v-GPCR induced a switch to an angiogenic phenotype in these cells. In addition, conditioned medium obtained from v-GPCR transformed NIH3T3 cells was able to induce vascular endothelial cell growth and angiogenesis in vitro. Induction of angiogenesis was mediated by activation of vascular endothelial cell growth factor receptor (VEGF-R) resulting in signaling through the JNK/SAP kinase and p38MAP kinase signal transduction pathways, which are commonly activated by inflammatory cytokines and stress. This indicated that v-GPCR can utilize the protein kinase pathways linked to cellular proliferation and possibly transformation as well as to pathways linked to induction of angiogenesis, which may be relevant to the pathogenesis of KS.

V-GPCR is expressed in PEL-derived cells upon induction with TPA, indicating that it is a lytic gene (table 1) [56]. In KS biopsies, expression of v-GPCR has been detected by RT-PCR [10, 62, 64]. Since KS lesions contain both latently and lytically infected cells, it is difficult to determine the pattern of v-GPCR expression and its role in the proliferation of HHV-8 latently infected spindle tumor cells.

In addition to the genes discussed above, other HHV-8-encoded genes with cellular growth-promoting potential have been reported. One gene of particular interest is ORF 73 that encodes LANA. LANA is an immunodominant antigen that exhibits distinct nuclear staining with a characteristic stippling pattern in an indirect immunofluorescence assay (IFA). LANA is routinely used in serological testing of patient sera for HHV-8 positivity [32, 52]. LANA is analogous to the EBV-encoded nuclear antigens (EBNAs) which also show similar staining patterns with IFA [31, 32, 52]. LANA is similar to the EBNAs in its hydrophilicity (38% charged residues), proline-rich sequence, extensive repetitive domain, and leucine zipper motif [54]. The EBNAs have been shown to be required for B cell transformation by EBV [33]. Whether LANA plays an analogous role in cellular transformation by HHV-8 is not currently known. Preliminary data failed to demonstrate the transformation of rodent cells by LANA, a system in which kaposin (ORF K12) was routinely positive [Muralidhar et al., unpubl. results].

Recently, Flore et al. [21] demonstrated that HHV-8 infection of primary endothelial cells (with a finite life span) resulted in long-term proliferation of the cells accompanied by the acquisition of telomerase activity and anchorage-independent growth, both of which are characteristics of transformed cells. However, only a small subset of the cells retained HHV-8 sequences as determined by PCR, Southern blotting, in situ hybridization and expressed latent antigens as determined by IFA. Upon treatment with TPA, about 10% of the latently infected cells expressed lytic viral antigens. The observation that only a small percentage of endothelial cells in culture were positive for HHV-8 indicated that other paracrine factors may be involved.

Role of Cytokines and Other Autocrine and Paracrine Factors

Evidence in vitro suggests that autocrine and paracrine factors as well as inflammatory and angiogenic cytokines may play a role in the pathogenesis of KS, both AIDS-associated and non-AIDS-associated, as well as PEL and MCD. AIDS-KS cells have been shown to constitutively express high levels of IL-6, basic fibroblast growth factor (bFGF), IL-1β, vascular endothelial cell growth factor (VEGF), oncostatin M and γ-interferon (IFN-γ) [16]. In addition, moderate levels of the granulocyte-monocyte colony-stimulating factor (GM-CSF), transforming growth factor β (TGFβ), platelet-derived growth factor-A and -B (PDGF-A and -B) and vascular permeability factor (VPF) are also expressed. Many of these cytokines including bFGF, IL-6, IFN-γ and IL-1β, have been shown to exhibit growth-promoting activities in addition to promoting neovascularization (angiogenesis) [19]. IL-1 can also stimulate endothelial cells, smooth muscle cells and fibroblasts to produce both GM-CSF and IL-6, which are multifunctional cytokines capable of influencing the vascular, immune and hematopoietic systems [12]. In addition to expressing the above cytokines and growth factors, AIDS-KS cells have also been shown to respond to them, thus creating autocrine and paracrine loops that help to promote their growth.

The highly aggressive nature of HIV-associated KS has led to the suggestion that HIV-encoded proteins enhance the pathogenesis and progression of KS. The HIV-1 Tat protein has been implicated in the pathogenesis of AIDS-KS, either directly by the activation of HHV-8 replication, or indirectly by the activation of cytokines [20, 29]. Tat protein is released by infected cells and can act extracellularly in promoting the growth of AIDS-KS cells in vitro. The Tat protein contains a basic domain rich in arginine and lysine similar to that of other potent angiogenic proteins such as bFGF and VEGF-A [1]. Tat has also been shown to bind and activate the VEGF-A tyrosine kinase receptor, Flk-1/KDR, which is highly expressed in AIDS-KS cells [2]. Furthermore, Tat-induced angiogenesis

can be inhibited by agents that block the Flk-1/KDR receptor [18]. Ensoli et al. [17] have demonstrated that Tat acts in synergy with bFGF in inducing KS-like lesions in mice. The growth-promoting and angiogenic activities of Tat may play a role in the aggressive nature of KS lesions seen in AIDS-associated KS.

The importance of autocrine and paracrine growth factors in non-AIDS-associated KS has also been documented. Various growth factors and cytokines including bFGF, IL-6, PDGF-B, VEGF and oncostatin M were shown to serve as modulators of KS cell proliferation in vitro [19, 37, 39, 40, 68]. KS-derived cells were shown to express a functional bFGF receptor, flg [37], and PDGF-A and -B receptors [69]. Constitutive expression and activation of growth factors and their specific receptors could facilitate the mechanism by which viral oncogenes and oncoproteins create an autocrine loop leading to self-sustained aberrant growth.

Summary and Conclusion

HHV-8-encoded genes that can transform rodent cells in culture have been described (table 1). In particular, the K1, K9 (v-IRF), K12 (kaposin) and ORF 74 (v-GPCR) have been shown to induce tumorigenic transformation in mice. ORF 72 (V-cyclin D) has been shown to induce cell proliferation of rat fibroblasts. Although these studies indicate the oncogenic potential of HHV-8, many questions remain as to the mechanism of transformation. The recent demonstration of transformation of primary endothelial cells by HHV-8 infection may provide a system to study the interplay between viral gene expression, cytokines as well as other autocrine and paracrine growth factors. Since KS, PEL and MCD show distinct biology and patterns of viral gene expression, differences in the mechanism of transformation will probably exist for each malignancy and therefore must be addressed in the context of each disease.

Rapid advances in HHV-8 research have begun to provide insights into viral tumorigenesis. The oncogenic potential of several viral genes have been determined, and their role in human malignancies must now be dissected. The hunt for the KS oncogene(s) together with its (their) interaction(s) with other viral and cellular targets will hopefully lead to the eradication of the HHV-8-associated malignancies.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 120–139

Transformation by the Epstein-Barr Virus

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Epstein-Barr virus (EBV) is the prototype member of the human herpesvirus subfamily, γ-herpesviridae [1]. The distinguishing feature of this group of herpesviruses is their ability to establish latent infection in lymphoid cells and induce cellular proliferation. In vitro, EBV can infect primary B lymphocytes and usually establishes a nonpermissive infection that results in cellular immortalization with continuous cellular proliferation and the establishment of permanent cell lines. Transformation of lymphocytes is the primary experimental system for the study of EBV transformation and has been used to genetically determine which viral proteins are essential for transformation [1]. These studies have revealed that establishment of latent infection and transformation of lymphocytes is a complex process that involves the carefully regulated expression of at least nine viral proteins. Six of the proteins are predominantly located in nucleus and are called EBV nuclear antigens or EBNAs. The EBNAs carefully regulate expression of themselves and the EBV latent membrane proteins 1 and 2 (LMP) [1, 2]. The EBNAs also affect expression of specific cellular genes. LMP1 and LMP2 are integral membrane proteins that can apparently activate or affect cellular signaling cascades [3, 4].

To understand the molecular and biochemical properties of EBV proteins, the individual viral genes have been expressed in different cell types to determine their individual effects on cellular expression and growth control. LMP1 was initially shown to transform established rodent fibroblast cell lines. Subsequent studies have revealed transforming properties in rodent fibroblasts of additional viral proteins, including proteins associated with viral replication.

Molecular biologic techniques have also identified specific interactions between viral and cellular proteins. These approaches have identified some of the key molecular functions of the viral proteins and how they affect cellular and viral growth control.

This chapter will review the viral proteins that are essential for transformation of lymphocytes and the expression of EBV proteins in specific cancers. The molecular and biochemical properties of these EBV proteins and their effects on cellular growth regulation will be presented.

EBV and Cancer

EBV is a ubiquitous infectious agent and infects greater than 90% of the world's population [5]. However, EBV is closely linked to the development of several human cancers [6]. Some of the cancers develop with high incidence in specific populations or in particular geographic regions [7]. These differences in incidence suggest that genetic and environmental components contribute to the development of cancer.

Lymphomas Associated with Immunosuppression

The ability of EBV to cause cancer is most evident in the development of B-cell lymphomas in patients who are immunosuppressed. EBV lymphoproliferative disease can develop in patients with congenital immune deficiency, during post-transplant immunosuppression, and in AIDS [6]. In post-transplant lymphoma (PTL) and in some examples of acquired immunodeficiency (AIDS) lymphoma, the tumor cells express all of the EBV genes that are also expressed in latently infected transformed lymphoid cell lines transformed in vitro [8]. These include the EBV nuclear antigens, EBNA1, EBNA2, EBNA-LP, EBNA3A, 3B and 3C, LMP1, and LMP2. This state of latent infection and viral expression is called type 3 latency [1].

The EBNA3 proteins are the main targets for cytotoxic lymphocytes and post-transplant lymphomas tumors remain susceptible to CTL killing. Reduction in immunosuppression can induce regression of these proliferations and adoptive immunotherapy using EBV-specific CTLs that have been amplified in vitro has also been successful [9–12].

Burkitt's Lymphoma (BL)

BL is an unusual childhood malignancy that develops with high incidence in equatorial Africa in an area marked by high malarial infection [13]. The endemic form of BL consistently contains EBV while BL tumors that develop with lower incidence in other areas are associated with EBV in 10–20% of cases

[14, 15]. Increased incidence of BL has also been described in patients with AIDS [16]. Endemic and sporadic cases of BL are marked by specific chromosomal translocations involving the myc oncogene and the regulatory sequences for the immunoglobulin genes [17]. It is likely that the translocation and resulting deregulated expression of c-myc is essential for the malignant growth of BL. It is believed that only a single viral protein, EBNA1, is consistently expressed in BL. This limited state of expression is called type 1 latency [18].

Nasopharyngeal Carcinoma (NPC)

NPC is a malignancy which develops in the squamous epithelium in the posterior nasopharynx [19]. The disease is the major cause of death from cancer in Southern China and also develops at high incidence in Mediterranean Africa and in Eskimo populations. EBV is consistently detected in NPC and present in all cells [20, 21]. Epidemiologic investigations to identify the factors that contribute to the extraordinary incidence in endemic areas suggest that the disease results from a combination of environmental, genetic and virologic factors [7]. Tumor-promoting chemicals such as phorbol esters and nitrosamines have been identified in Chinese salted fish and in food products in other areas with elevated incidence [22]. Several recent studies have identified areas of loss of heterozygosity on several chromosomes [23, 24]. These areas may contain a genetic susceptibility locus or critical cellular genes that are affected by the mutagenic environmental factors.

Within the tumor, EBV DNA is clonal, suggesting that the tumor represents a proliferation of a single EBV-infected cell [25]. Early stages in malignancy such as dysplasia, or carcinoma in situ are extraordinarily rare, and in most cases are detected concomitantly with invasive carcinoma. Examples of early lesions that were studied all contained clonal EBV indicating that they are a clonal proliferation of latently infected cells [26]. The rarity of these premalignant lesions and the detection of dysplasia, concomitant with invasive cancer, suggests that NPC develops from a single EBV-infected cell and that this proliferation rapidly progresses to malignancy. However, the monoclonality of NPC suggests that, in addition to EBV, other genetic changes occur that contribute to the development of cancer.

In NPC and in most of the cancers linked to EBV, EBNA1, LMP1 and LMP2 are expressed in the absence of expression of the EBNA2 and EBNA3 proteins that activate and regulate viral expression in transformed lymphocytes. This state of infection is termed type 2 latency [1]. In addition, a family of highly spliced mRNAs transcribed through the BamHI A fragment, that potentially encode additional proteins, are also expressed at high levels in NPC [27–30].

Other EBV-Associated Malignancies

Multiple other types of cancer have also been linked to EBV including Hodgkin's lymphoma, T-cell lymphoma, gastric carcinoma, leiomyomas and leiomyosarcomas [6]. In all examples, identification of the EBV termini revealed that the infection was nonpermissive and clonal with regard to EBV. [31, 32]. These additional tumor types usually manifest type 1 or type 2 latency. The consistent expression of specific viral proteins suggests that they are critical factors to the deregulated malignant growth.

Biology of EBV Infection

EBV Expression in Lymphocyte Transformation

Infection of primary human lymphocytes with EBV in vitro consistently results in the establishment of continuously proliferating lymphoid cell lines [33]. This infection is usually nonpermissive and is considered 'latent' [1]. When the virus enters the cell, the linear viral DNA circularizes to form an extrachromosomal plasmid or episome that is then amplified by some unknown mechanism to multiple copies [34–36]. With each cell division, the viral genome is replicated by the host DNA polymerase and then evenly partitioned to daughter cells.

Cellular transformation is dependent upon the establishment of a latent infection and carefully regulated viral expression. In transformed cell lines, multiple viral genes are expressed. These genes include three integral membrane proteins, latent membrane proteins 1, 2A and 2B (LMP), six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C and EBNA-LP), and two small, noncoding nuclear RNAs (EBERs). This state of infection is termed type 3 latency [6]. After infection, the first mRNA that is transcribed is bicistronic and can encode EBNA-LP and EBNA2, which are the first proteins detected [37]. Expression of these two proteins activates promoters for the mRNAs that encode the other EBNAs and the LMPs [38]. Genetic analyses have identified the genes that are essential for B-lymphocyte transformation and the potential functions and biochemical properties for some of these proteins have been determined [1].

Epstein-Barr Virus Nuclear Antigen 1 – EBNA1

The EBNA1 is expressed in all transformed lymphoid cell lines and EBV-associated tumors. This protein binds to the origin of replication for the plasmid form of the viral genome (ori-p) and associates with host chromosomes [39, 40]. This property enables the viral episome to segregate with the host chromosomes during mitosis. This is the single viral gene product that must be expressed to enable the viral DNA to be transmitted to the daughter cells of an activated, dividing B cell.

EBNA1 recognizes a palindromic sequence and binds at three sites in the EBV genome [41]. Two of the sites comprise ori-p and the third site, within the BamHI Q fragment, is immediately 3′ to the promoter for EBNA1 that is used in type 1 and type 2 latency in BL and NPC [42]. Binding of EBNA1 to this site negatively regulates its own expression [43, 44]. EBNA1 is also thought to activate a transcriptional enhancer element within ori-p [45]. EBNA1 also may activate specific cellular enhancers and affect cellular gene expression and has been shown to activate expression of the lymphoid recombinase RAG genes [45, 46]. This activation might promote chromosomal rearrangement and translocations characteristic of BL and could possibly also facilitate viral integration [17].

One study indicated that transgenic mice that contained EBNA1 under the control of the immunoglobulin heavy chain promoter have an increased incidence of lymphoma [47]. This suggests that EBNA1 may have oncogenic properties and may contribute to the growth of EBV-transformed cells and cancers. It is possible that EBNA1 activates expression of critical cellular genes and affect cellular growth control in addition to its essential role in maintenance of the viral genome.

Interestingly, this protein is rarely recognized by cytotoxic lymphocytes [48]. One mechanism for this inhibition is that EBNA1 is not processed by the proteosome in the classical pathway for presentation in class I MHC molecules [49]. The EBNA1 protein contains a simple repeat of glycine and alanine that is homologous to some cellular proteins [50]. It has been demonstrated that transfer of this repeat element to a heterologous protein can sequester that protein from processing and presentation within MHC class I molecules [51]. This unique property would enable an EBV-infected lymphocyte that only expressed EBNA1 to escape immune recognition in vivo.

Epstein-Barr Virus Nuclear Antigen 2 – EBNA2

EBNA 2 was the first protein to be shown to be essential for growth transformation of lymphocytes [52]. Early studies of EBV transformation determined that the HR1 strain of virus was unable to transform lymphocytes and renaturation kinetic analyses and restriction enzyme mapping identified a specific deletion within the EBV HR1 genome. This region was subsequently shown to encode the EBNA2 protein and marker rescue studies proved that reintroduction of the EBNA2 gene restored transformation ability. [Cohen, 1989 #9027].

Marker rescue of lymphocyte transformation has been exploited to genetically manipulate EBV [1]. Transfection of EBNA2 coding sequences and induction of viral replication in the HR1 cell line will produce both transforming and nontransforming virus and only virus that has had EBNA2 coding sequences recombined into the genome will be able to transform lymphocytes. Co-transfection of a selectable marker or altered viral gene will produce virus of

which approximately 10% is a double recombinant with the selectable marker or second gene incorporated into a second site via homologous recombination [53]. The transforming ability of recombinant viruses that harbor mutations in specific genes can be assessed by capturing the recombinant genomes into EBV-negative B-cell lines [54]. These cell lines can then be induced to produce the recombinant virus that can then be tested for transforming ability. Using this approach, the EBNA2, 3A, 3C and LMP1 proteins have been shown to be essential for B-cell transformation while EBNA3B, LMP2A and B, and the small, nonpolyadenylated RNAs, the EBERs, are not essential [1].

EBNA2 is the major regulator of viral transcription and activates expression of LMP1 and LMP2, and the major promoter, Cp, that initiates transcription of the EBNA mRNAs [2, 55–57]. EBNA2 also regulates expression of the B-cell activation marker, CD23, and the EBV receptor, CD21 [58, 59]. Transactivation of gene expression by EBNA2 is mediated, in part, through the interaction of EBNA2 with the DNA binding protein, RBP-Jκ (CBF1) [60, 61]. RBP-Jκ is a component of the Notch signaling pathway and is activated through its interaction with the intracellular cytoplasmic domain of activated Notch [62]. The interaction of EBNA2 or Notch with RBP-Jκ converts it from a repressor of transcription to an activator. Thus EBNA2 seems to be a functional homolog of activated Notch in EBV-infected cells. Expression of constitutively activated Notch can substitute for EBNA2 and activate LMP1 expression [63]. Notch is an important factor in development and has been implicated in the development of cancer [64]. Chromosomal translocations detected in human T-cell leukemias result in expression of truncated, activated forms of Notch and expression of similar forms of Notch are also transforming in murine T cells [65, 66].

EBNA2 has an acidic transactivation domain at the carboxy-terminus that interacts with the basal transcription machinery, through TFIIB, TFIIH and TAF40 [67]. EBNA2 also interacts with the human homolog of the SNF2/SWI complex, which is involved in nucleosome structuring [68]. The EBNA-LP protein cooperates with EBNA2 to greatly increase transactivation of responsive viral promoters [69, 70].

Surprisingly, two types of EBNA2 have been identified, encoded by quite divergent sequences. The type of EBNA2 gene, EBNA2A or 2B, has been used to define two types of EBV, EBV1 or 2 [52, 71, 72]. The EBNA3 genes, EBN3A, 3B, and 3C, are also encoded by divergent sequences that usually co-segregate with the EBNA2 type [73]. Therefore, the sequence of the EBNA2 and EBNA3 genes define the type 1 and type 2 of EBV. Type 2 EBV is less efficient in B-lymphocyte transformation, however, the two EBV types do not seem to differ in their pathogenic properties and both have been identified in all types of diseases and cancers associated with EBV [74]. Genetic studies have

shown that the efficiency of transformation is dependent on the EBNA2 type. Thus, recombinant type 2 EBV-containing type 1 EBNA2 and type2 EBNA3 proteins is as efficient in transformation as wild-type EBV [75].

EBNA-LP

EBNA-LP can be encoded by a bicistronic mRNA that also encodes EBNA2 [37]. There are two alternate splicing patterns for the mRNA, one of which forms the initiating methionine for EBNA-LP. EBNA-LP has been reported to bind p53 and Rb but does not seem to affect their function [76]. In concert with EBNA2, EBNA-LP can induce cyclin D2 and can greatly enhance EBNA2-mediated transactivation of LMP1 expression [77]. Transgenic mice expressing EBNA-LP die of heart failure without evidence of tumor development [78].

The EBNA3 Proteins, 3A, 3B and 3C

The EBNA3 proteins are encoded by three genes tandemly placed in the EBV genome. The proteins are similar and are each encoded by short 5′ and long 3′ exons and all contain repetitive elements encoding different repeating polypeptides. All three bind RBP-Jκ, the same cellular DNA binding protein that binds EBNA2 [79, 80]. These proteins may affect the binding of EBNA2 to RBP-Jκ and modulate EBNA2 effects on transactivation of viral gene expression [81]. The EBNA3 proteins also likely affect cellular gene expression, independently of EBNA2. EBNA3C has been shown to induce expression of the EBV receptor, CD21 [59]. EBNA3C can act as a repressor and activator of transcription [82]. In addition to its interaction with RBP-Jκ, EBNA3C interacts with histone deacetylases and prothymosin α [83, 84]. These interactions likely facilitate its effects on transcription

EBNA3C has transforming properties in rodent fibroblasts when expressed in combination with activated ras [85]. EBNA3C also affects accumulation of the cyclin-dependent kinase inhibitor, p27, possibly affecting the retinoblastoma tumor suppressor pathway [86].

In summary, all of the EBNA proteins seem to function in the maintenance and coordinated regulation of expression of the viral genome in establishing a latent, transforming infection. Individually, the proteins are not transforming, although they may also contribute to growth transformation.

The EBV Oncogene – Latent Membrane Protein 1 (LMP1)

LMP1 is considered the EBV oncogene as it has transforming ability in rodent fibroblasts, transforming Rat-1 cells in vitro to anchorage-independent growth and tumorgenicity in nude mice [87]. Expression of LMP1 at levels comparable to infected lymphocytes also alters the phenotype of lymphoid cells

inducing expression of B-cell activation antigens, adhesion molecules, transferrin receptor and sensitivity to TGF-β [88]. LMP1 also has significant effects on epithelial cell growth and inhibits differentiation and induces morphologic transformation of some cell lines [89–91]. LMP1 induces expression of the epidermal growth factor receptor (EGFR) in epithelial cells and EGFR is expressed at high levels in NPC [92]. The induction of EGFR expression in NPC may be an important contributing factor to transformation in this epithelial tumor. LMP1 also induces expression of CD40 and secretion of IL-6 in epithelial cells and decreases expression of cytokeratins and E-cadherin [93–95].

LMP1 has been shown to inhibit apoptosis in B lymphocytes which may be due to its induction of expression of the bcl-2 oncogene. In epithelial cells, LMP1 specifically inhibits p53-mediated apoptosis and this inhibition is due to LMP1 transactivation of expression an antiapoptotic factor called A20 [96]. A20 is also induced by tumor necrosis factor (TNF) and then blocks apoptosis induced by TNF [97]. This protection from p53-mediated apoptosis may be responsible for the lack of p53 mutations in EBV-associated cancers that express LMP1.

LMP1 is an integral membrane protein with a complex molecular structure containing a cytoplasmic amino-terminus, six transmembrane domains, and a long cytoplasmic carboxy-terminal portion (fig. 1). The molecule is processed after being placed in the membrane with a specific cleavage site and phosphorylation on serine and threonine. The carboxy-terminal tail of LMP1 has an 11 amino acid (aa) repeat element and a region of 10 aa that is deleted in some strains of EBV [98]. Several studies have shown that there are consistent sequence variations in the LMP1 gene that can be used to distinguish strains [99, 100]. Many studies have analyzed the presence of the 10 aa deletion in LMP1 and it has been suggested that this deletion is linked to pathogenesis [101]. This deletion is present in an EBV strain that is prevalent in China and it has been shown that this deletion increases the transformation ability of LMP1 in rodent fibroblasts [102, 103]. Other studies have revealed additional differences in the properties of the different forms of LMP1. Two studies showed that LMP1 variants isolated from NPC samples had increased ability to activate NFκB [104, 105]. Interestingly, this ability mapped to the amino-terminus and transmembrane regions of the LMP1 variants and was not due to the presence of the 10 aa deletion.

Two domains within the carboxy-terminal tail of LMP1 have been identified, CTAR1 and CTAR2, which both can activate the NFκB transcription factor (fig. 1) [106]. The membrane proximal domain, CTAR1, interacts with the cellular molecules that mediate signals from the TNF family of receptors including CD40 [3]. These molecules, entitled TRAFs, form heteromeric complexes that transduce signals that depending on the receptor may activate NFκB, induce cellular growth, or induce apoptosis. TRAF1, 2, and 3 assemble on the TRAF interacting domain in CTAR1, while the TRAF adaptor proteins, TRADD

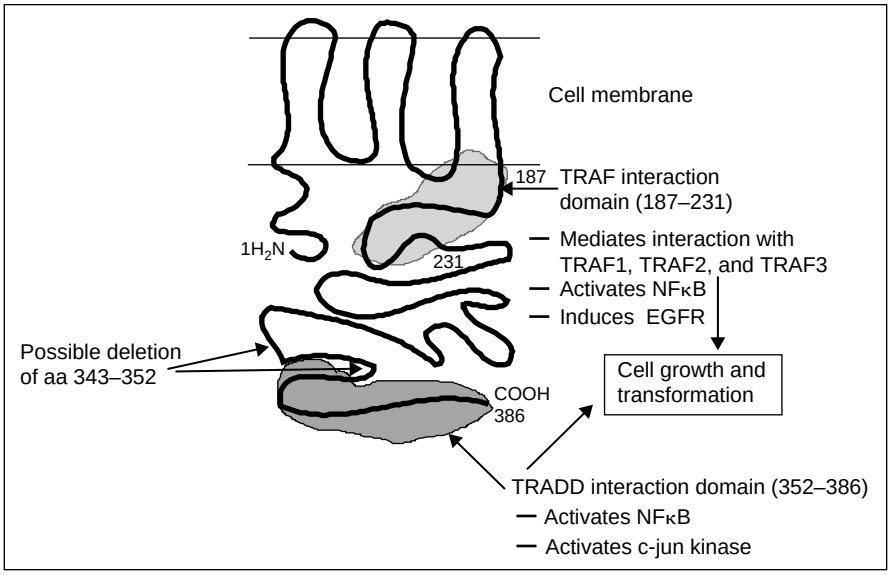

Fig. 1. LMP1 structure and functional domains. The LMP1 protein consists of an amino terminal cytoplasmic domain, 6 transmembrane domains, and a carboxy terminal domain. The TRAF interaction domain (CTAR1), located between amino acids 187 and 231, is essential for transformation and activates NFκB and EGFR expression. The TRADD interaction domain (CTAR2), located between amino acids 352 and 386, is the major NFκB activation domain and activates c-jun kinase. Several strains of EBV have a deletion of aa 343–352.

and RIP, bind to CTAR2 [107]. LMP1 apparently acts as a constitutively activated member of the TNF receptor family and constitutively activates NFκB. Although both CTAR1 and CTAR2 can activate NFκB, CTAR2 is the major activator in transient reporter assays [108]. Analysis of the forms of NFκB activated by LMP1 indicated that CTAR1 and CTAR2 activate different heterodimeric forms of NFκB [109]. CTAR2 has also been shown to activate the c-jun amino-terminal kinase, resulting in activation of the AP1 transcription factor [110].

Using deletion derivatives of LMP1, it has genetically been shown that LMP1 containing the TRAF interacting domain, CTAR1, but lacking the TRADD interacting domain, CTAR2, is sufficient for induction of EGFR expression [111]. As CTAR2 is the major activator of NFκB in transient expression assays, this was the first identification of an effect due to TRAF activation that was distinct from NFκB activation. LMP1 induction of expression of TRAF1 and EBI3 is also mediated only by CTAR1 suggesting that an additional pathway, in addition to NFκB, governs expression of some cellular genes [112]. However,

expression of a constitutively activated form of IκB, the inhibitor of NFκB expression, does significantly reduce but not eliminate EGFR expression [109]. CD40, another member of the TNFR family, also interacts with TRAFs 1, 2 and 3 and overexpression of CD40 also can induce EGFR expression, supporting the role of TRAF signaling in specifically inducing EGFR expression [109].

Genetic analysis of recombinant EBV expressing deleted forms of LMP1 revealed that CTAR1 but not CTAR2 was required for transformation [113]. However, transformation efficiency with this virus was reduced and the transformed lymphocytes required fibroblast feeder layers or seeding at high density for continued growth [113]. Thus, CTAR2 is not absolutely essential but is an important contributing factor in transformation.

LMP1 Transgenic Mice

Two studies have analyzed the effects of LMP1 expression in transgenic mice. In one study LMP1 was expressed under the control of the polyoma early promoter and enhancer resulting in mice with epidermal hyperplasia and altered keratin gene expression. Replacement of the polyoma enhancer with immunoglobulin heavy chain enhancer resulted in embryonic lethality with epidermal hyperplasia. Many studies have revealed toxicity associated with high levels of LMP1 expression and the high level of lethality in transgenic mice may reflect this toxicity [114].

Another study established transgenic mice with LMP1 expressed under the control of the heavy chain immunoglobulin promoter/enhancer [115]. In three lineages, lymphoma developed with increased incidence. The incidence increased significantly with age with lymphomas developing in 50% of transgenic mice over 18 months. LMP1 expression was detected at high levels in the lymphoma tissues but was present at only trace levels in normal spleens. Immunoglobulin heavy chain gene rearrangement indicated monoclonality or oligoclonality in all lymphomas. These data revealed that LMP1, without expression of other EBV genes, can be oncogenic in vivo and suggested that LMP1 is likely a major contributing factor to the development of EBV-associated lymphomas. However, the extended time to tumor development and the monoclonality of the tumors suggest that other genetic changes must occur that complement the growth-stimulating effects of LMP1. These changes may activate pathways that are affected by other viral proteins during transformation of lymphocytes. Similar genetic changes may also contribute to the development of EBV-associated cancers such as NPC.

Latent Membrane Protein 2 – LMP2

The LMP2 proteins are encoded by highly spliced mRNAs that contain exons located at both ends of the linear EBV genome [116, 117]. Therefore, LMP2 can be only transcribed across the fused termini of the episome or from

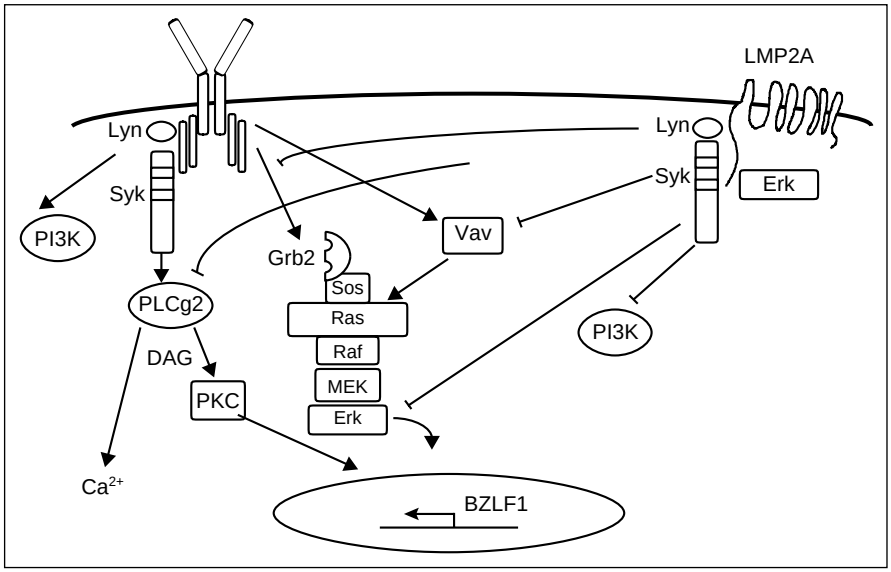

Fig. 2. LMP2A effects on signal transduction from the B cell receptor. Activation of the B-cell receptor leads to phosphorylation of the lyn and syk tyrosine kinases and the activation of the PI3 kinase, PLCgamma2, protein kinase C (PKC), and MAPK pathways. Activation of PKC or MAPK activate expression of the EBV replication activator, BZLF1, leading to EBV reactivation and replication. LMP2A blocks phosphorylation of lyn and syk, activation of the downstream pathways, and blocks EBV reactivation.

rare integration events of two tandem copies. The two forms of LMP2 (LMP2A and 2B) differ in that only LMP2A has a 119 aa cytoplasmic N-terminus that is critical for its function in B lymphocytes [118]. The proteins are both very hydrophobic and contain twelve transmembrane domains.

In lymphocytes, LMP2A is constitutively phosphorylated on tyrosine and the amino-terminus contains nine tyrosine residues [4]. Two of the tyrosines are within an immunoreceptor-tyrosine-based activation motif (ITAM). ITAMs are found in the B- and T-cell receptors where they are phosphorylated by src family kinases and bind syk and ZAP70. In B cells, activation of syk activates multiple signal transduction pathways including activation of the ras/mitogen-activated protein kinase (MAPK) pathway, release of intracellular Ca^{2+}, and activation of PI3kinase (fig. 2). In cells expressing LMP2A, the lyn and syk kinases are believed to be sequestered and possibly degraded by association with LMP2A [119, 120]. This sequestration blocks signaling from the B-cell receptor and keeps the lymphocyte in an inactivated state. In the absence of LMP2A, B-cell receptor engagement leads to activation of trancription factors

that activate expression of the EBV replicative cascade. Therefore, expression of LMP2A helps maintain a latent infection in B lymphocytes.

In epithelial cells, LMP2A becomes phosphorylated on tyrosines upon adhesion to extracellular matrix (ECM) proteins that are ligands for integrin receptors [121]. The phosphorylation is not mediated by the src family kinases and may be linked to activation of the csk kinase, a kinase that negatively regulates src. Epithelial cell interactions with the ECM regulate epithelial cell behavior. In squamous epithelium, integrins are expressed in the proliferating, basal layer in contact with the basement membrane and integrin ligands [122]. As cells move from the basal layer to form the upper epithelium, integrin signaling is downregulated, the cells cease to proliferate, and begin to differentiate. Some epithelial cell lines will mimic this process in organotypic raft cultures where they will differentiate and become enucleated [123]. In organotypic raft cultures with the HaCat epithelial cell line expressing LMP2A, differentiation was inhibited and the cells continued to proliferate [124]. The LMP2A expressing HaCat cells also formed colonies in soft agar indicating that they continued to proliferate without attachment to matrix and these cells formed aggressive tumors in nude mice. Analysis of signaling pathways revealed that some of the known targets of integrin signaling, such as the MAPK pathway and focal adhesion kinase, were not activated in the tumors (fig. 3). However, PI3kinase seemed to be constitutively activated as evidenced by activation of the Akt kinase. Thus in epithelial cells, LMP2A is also transforming, possibly due to activation of Akt.

The EBV Encoded Noncoding RNAs – EBERs

The most abundant RNAs in EBV-infected cells are small nuclear RNAs transcribed by RNA polymerase III [125, 126]. These RNAs, called EBERs, are present at approximately 105 copies/cell but are not necessary for lymphocyte transformation [127]. However, they are expressed in all of the malignancies associated with EBV and presumably contribute in some way to the maintenance of latency in vivo. Interestingly, expression of the EBER RNAs seems to be downregulated during differentiation. Thus, examples of NPC that have differing degrees of differentiation lack EBER expression in differentiated areas [21]. The EBER RNAs are also not detected in the permissive EBV infection, hairy leukoplakia, and are downregulated during viral replication [128, 129].

Derivatives of the EBV-positive BL cell line, Akata, can be isolated that have lost the EBV genome. The EBV-negative Akata cells do not grow in soft agar and cannot form tumors in nude mice. Reinfection with EBV restores these properties [130]. The viral protein(s) that are responsible for the tumorigenic phenotype have not been identified, however, it has been demonstrated that expression of the EBER RNAs partially restores growth transformation [131].

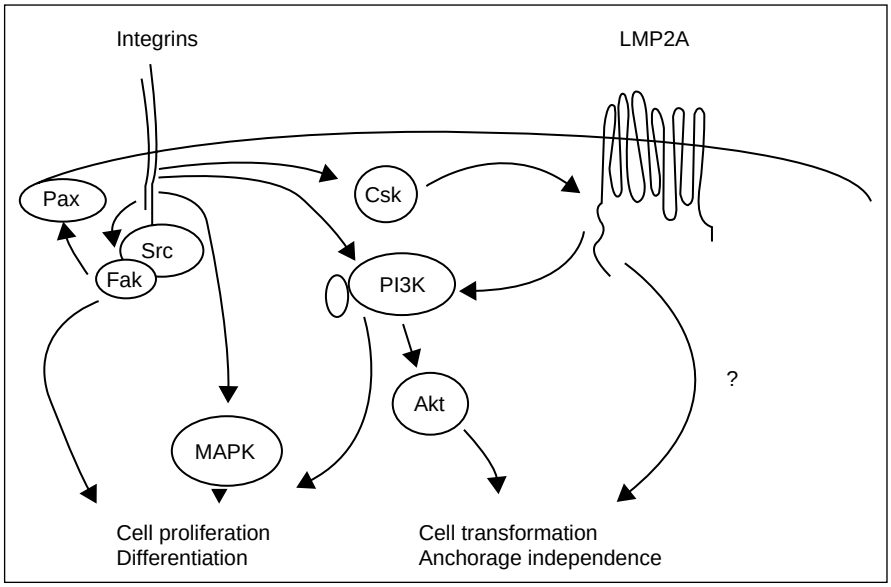

Fig. 3. LMP2A signaling in epithelial cells. Integrin-mediated signaling activates src kinases, focal adhesion kinase (Fak), the csk tyrosine kinase, paxillin phosphorylation, and activation of PI3 kinase. In epithelial cells, LMP2A is phosphorylated by csk and LMP2A constitutively activates PI3 kinase and the Akt kinase in the absence of integrin signaling. This partial substitution of integrin signaling by LMP2A leads to cell transformation and anchorage-independent growth of the HaCat keratinocyte cell line.

Conclusions and Speculations

A significant aspect of most of the cancers associated with EBV is that multiple viral proteins are expressed that are probably essential for the abnormal and deregulated growth. As EBV infection is a major contributing factor to the development of these cancers, a successful viral vaccine that eliminated viral infection should greatly reduce the incidence of most of the EBV-associated cancers. However, it will be difficult to develop a vaccine that will produce sufficient mucosal immunity to completely neutralize EBV. It might also be possible to directly target critical signaling pathways that are activated by EBV-transforming proteins. Inhibitors of NFκB, EGFR and PI3kinase are available that might block EBV transformation. Alternatively, the molecular interactions between viral and cellular proteins that are responsible for activation of these signaling pathways could be targeted using molecular therapies specifically

designed to block the interactions. The continued study of the effects of EBV proteins on cellular gene expression and growth regulation in model systems is likely to uncover new potential molecular targets and provide experimental systems to evaluate inhibitors of EBV transformation.

Acknowledgments

I wish to thank Drs. Frank Scholle and Shuichi Kusano for the diagrams used in this document and for assistance in manuscript preparation.

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Rosenthal LJ (ed): Mechanisms of DNA Tumor Virus Transformation.
Monogr Virol. Basel, Karger, 2001, vol 23, pp 140–158

Oncogenic Transformation of T Cells by *Herpesvirus saimiri*

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Classification, Genomic Structure and Subgroups of *Herpesvirus saimiri*

Gamma or lymphotropic herpesviruses are implicated as causative agents of malignant diseases. They have been further classified to gamma-1 and gamma 2-subgroups. Epstein-Barr virus (EBV) represents the gamma-1 subgroup. The recently discovered Kaposi's sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus-8 (HHV-8), and the simian *H. saimiri* (HVS), belong to the gamma-2 subgroup, also referred to as the genus *Rhadinovirus*. HVS is a ubiquitous agent of squirrel monkeys (*Saimiri sciureus*). The virus can be reproducibly isolated from the peripheral blood of apparently healthy animals [58] by co-cultivation of T cells with owl monkey kidney cells. Among the human herpesviruses, KSHV is the closest relative of HVS. Many of the HVS open reading frames (ORFs) are colinear with those of KSHV as revealed by DNA sequencing [3]. Both viruses encode several genes with significant homology to host sequences and these cellular homologues will be discussed later in detail. The architecture of both viral genomes is similar as they encode a large number of terminal tandem repeats.

As shown in figure 1, the genome of HVS consists of about 113 kb of unique sequences (called L-DNA, about 35% G + C content) flanked by tandem repeats (H-DNA, about 71% G + C) [7]. H-DNA contains consensus packaging and cleavage sites allowing generation and packaging of full-length genomes. Recent data also indicate that the H-DNA plays a role in replication of latent episomal genomes [unpubl. data]. At least 75 ORFs, which are likely to be

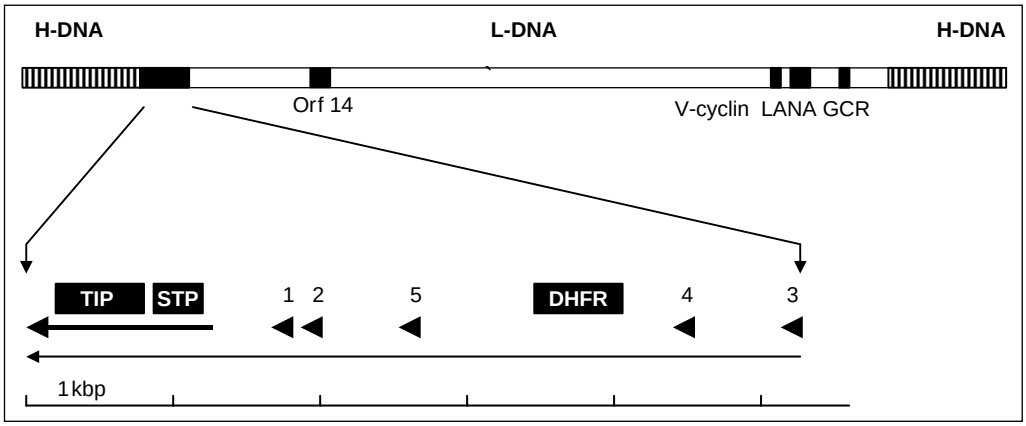

Fig. 1. Genomic structure of HVS and map positions of genes with proven or potential roles in immortalization of T cells and oncogenesis. The unique L-DNA region is about 113 kb and is flanked by repetitive H-DNA. The number of terminal H-DNA repeat units is variable. Approximate map positions of genes with possible roles in oncogenic transformation are depicted by solid rectangles. The leftmost region is shown enlarged depicting ORFs TIP, STP and DHFR. Long arrows represent mRNA. Short arrowheads indicate small non-coding U-type nuclear RNAs (HSURs).

expressed as proteins, are encoded by the L-DNA of HVS [3]. The genome of HVS encodes a set of genes commonly described in all herpesviruses including immediate-early transactivator genes, several enzymes involved in viral DNA metabolism, structural proteins such as capsid components, and various glycoproteins [3]. These common herpesvirus genes function during the lytic replication cycle.

Similar to other herpesviruses, HVS strains can be readily distinguished by restriction enzyme cleavage site polymorphism [17]. However, the left end 2 kb 'oncogenic' region is much more variable than the rest of the genome [56]. On the basis of DNA hybridization experiments, the various isolates of HVS have been classified into DNA groups A, B and C [56] (group C was formerly called group non-A, non-B) [56]. Comparison of DNA sequences confirmed this finding and no significant homology was found among groups within the leftmost 2.5 kb of L-DNA [6, 31]. To explain the lack of homology among groups in the left end 2 kb L-DNA region, it has been hypothesized that different cellular sequences have been acquired by these strains and this issue will be discussed later.

The recent nomenclature of virus strains now reflect the virus group. For example, a strain formerly called 'HVS strain 11' is referred to as HVS A11.

Oncogenic Potential and Immortalization of T Cells in vitro by HVS

The most intriguing feature of HVS is its ability to induce acute T-cell lymphomas and/or lymphoid leukemias. These experimental malignancies develop within a few weeks after inoculation of the virus into New World monkeys, marmosets, and New Zealand White rabbits [13, 55, 59]. Most T lymphocytes transformed in vivo or in vitro by HVS are CD8+ [5, 53, 64, 66] although some CD4 and double positive cell lines have been reported [5]. HVS-transformed T cells are strongly cytotoxic against various tumor cell targets [5, 35, 53], thus, resemble lymphokine-activated killer (LAK) cells which have been under intense investigation. However, unlike uninfected LAK cells, which require interleukin-2 (IL-2) for induction of cytotoxic activity, HVS-immortalized T cells are strongly cytotoxic regardless of IL-2.

Although HVS-associated malignancies are restricted to T cells, a much wider range of cell types can be persistently infected by HVS [1, 70] suggesting that T-cell specificity of tumors is not determined by a T-cell-specific cellular receptor but HVS encodes genes responsible for the unique immortalization of T cells.

Tumor-derived cells isolated from HVS-infected animals and T cells infected and immortalized in vitro display characteristics of malignant lymphocytes. Unlike uninfected lymphocytes, HVS-immortalized or transformed T cells can be cultured in media without IL-2 and cell growth is sustained practically indefinitely. These cultures are also morphologically distinct from normal T cells in the peripheral blood and immortalized cells are typically enlarged resembling lymphoblasts and typically grow in large clumps. Immortalized T cells contain circular episomal viral genomes suggesting an active role of the virus and its genes in the immortalization process and transformed cells are oncogenic in syngeneic animals [2, 5, 19, 23, 40, 53, 78]. Another easily measurable consequence of immortalization of T cells by HVS is abrogation of the requirement for IL-2 for growth. Sudden removal of IL-2 followed by incubation in IL-2-free medium induces apoptosis of normal T-cell cultures [22], however, such treatment has no effect on the viability of the HVS-infected cultures [53].

Not only DNA variability but also the transforming ability of strains correlates with subgroups. Group C strains appear to be the most potent oncogenic/transforming agents, group A strains rank second, and B strains rank last in this respect. Viruses of groups A and C can efficiently and reproducibly immortalize common marmoset peripheral blood lymphocytes (PBL) in vitro [19, 78]. Group B strains can immortalize PBL only at a low frequency and group B immortalized cells require IL-2 for optimal growth [78]. Tumor formation in

rabbits also correlates with DNA grouping. New Zealand White rabbit experiments showed that a group C strain 484–77 is highly oncogenic in New Zealand White rabbits, however group A or B viruses are not oncogenic in these rabbits [55].

Immortalization of human cells has been also reported by using group C strains 484–77 and 487–77; group A or B strains were negative in these experiments [5, 53]. These T lymphocyte cultures can be propagated without stimulation by lectins or antigen for a prolonged period of time [5, 53]. However, the long-term maintenance of HVS-immortalized human T cells in tissue culture is a much more difficult task than that of immortalized marmoset cells and special conditions and media are required [5, 53]. Fickenscher and Fleckenstein developed a special growth medium for the transformation of human T cells which contains 45% RPMI 1640, 45% GC medium, 10% fetal calf serum, glutamine, and 40 U/ml IL-2 (Boehringer Mannheim). GC medium is available from Vitromex GmbH, Dr. F. Zimmermann, Adilgestr. 33, D–94474 Vilshofen, Germany. AIM-V medium (readily available from Gibco-BRL Inc) supplemented with 10% fetal calf serum, 100 U/ml recombinant IL-2 and antibiotics was also found suitable for the immortalization and long-term maintenance of HVS-infected human T cells [53].

HVS Encodes Several Genes of Cellular Origin

DNA sequencing and sequence comparison revealed that most genes from the leftmost 7-kb L-DNA sequence of HVS genome and several ORFs encoded by other regions of the genome show significant homology with cellular genes [3, 30, 31, 46, 79]. Some of these viral homologues such as the seven U-type small RNAs (HSURs), the saimiri-transforming protein (STP), the tyrosine kinase-interacting protein (TIP) are involved in oncogenicity and/or T-cell activation by the virus. These transforming HVS genes will be discussed later in greater detail. The second group of genes include ECRF3, ORF15 and CCHP, which are probably important in survival of virus-infected cells against host immune attack. A third group of genes of cellular origin are DHFR and TS which may function in nucleotide metabolism.

These data leave little doubt that HVS is a transducing virus, similar to acutely transforming retroviruses. The transduced genes of HVS have no introns, thus, most likely have been transduced from mRNA transcribed to cDNA by an unknown reverse transcriptase. The virus encodes no known reverse transcriptase and one can only speculate that perhaps endogenous or exogenous squirrel monkey retrovirus reverse transcriptases are responsible for the generation and insertion of these cellular sequences into the HVS genome.

Features of Latent Genomes in Transformed T Cells

In latent/persistent infection, which occurs in T lymphocytes, the viral genome is a circular episome. Tumor tissues and cell lines established from tumors or by in vitro immortalization carry multiple copies of the viral DNA in covalently closed circular form [5, 24, 28, 53, 68, 78]. No evidence is available that HVS would integrate into the host genome.

Although viral episomes in transformed T cells are very stable, large spontaneous deletions in the middle of the L-DNA are commonly found in tumor cell lines maintained in tissue culture [15, 24, 40]. In contrast, the leftmost and the rightmost approximately 15-kb L-DNA sequences do not suffer of such deletions, suggesting that the middle of the genome is not essential for the maintenance of the transformed state, and perhaps, sequences relevant to immortalization and maintenance of the circular episome are located in the left and right H-L DNA junctions.

Most of the viral genes encoded by HVS are inactive in immortalized T cells and only a limited number of gene products can be detected. Extensive methylation of the episomal HVS DNA at C-G residues in immortalized T cells has been described which is thought to correlate with the lack of gene expression in mammalian cells [16, 18, 81]. On the other hand, a few unmethylated sites of the left end region have been also reported in tumor cells [16] suggesting that some selected genes at the left end of L-DNA are expressed.

Deletion Analysis and Gene Expression in the Left End of the Viral Genome Implicates Two ORFs in Oncogenic Transformation

Several studies with deletion mutants revealed that the left end genomic segment is important for oncogenic transformation [12, 19, 43, 53, 62]. Large deletions of the left end sequences of the L-DNA results in loss of immortalization of T cells and oncogenicity by HVS, and transformation-deficient deletion mutants of all three virus subgroups have been described. A common feature of these mutants is that they are entirely competent for lytic replication.

These data strongly suggests that protein products corresponding to the ORFs are involved in the process of immortalization of T cells. Deletion of the STP ORF in strain 11 of group A correlated with loss of oncogenic and transforming potential of the virus [12, 19, 43, 62]. Deletion mapping of a group C strain showed the importance of two ORFs, STP and TIP [53]. STP is a collagen-like oncoprotein and TIP is an IL-11-like protein; these genes and their role in transformation are further discussed below.

To localize the region of the genome conferring the highly oncogenic phenotype to strain 484–77 (group C), strain B-C recombinants have been constructed. Two recombinants consisting of strain B virus DNA, in which the left end 9 kb of unique DNA is replaced by group C virus DNA, were oncogenic in rabbits [55]. These experiments showed that the left end 9-kb DNA of the group C strain contains gene(s) relevant to the transforming and oncogenic potential.

Consistent with data on these deletion mutants, gene expression has been demonstrated in the left end oncogenic region of the group C strain 484–77. A 1.2-kb polyadenylated virus-specific RNA is transcribed from the leftmost region of L-DNA in lymphocytes transformed by the highly oncogenic group C strain 484–77 [30, 31]. The 1.2-kb transcript codes for the two ORFs [31]. Next we will discuss experiments implicating two proteins and small RNAs encoded by the left end region in the transformation process.

Oncoprotein Product STP Transforms Rodent Cells and Interacts with Cellular Proteins Ras, Src and TRAF in Transformed T Cells

The first ORF of the 1.2-kb mRNA is termed either as SCOL (for saimiri collagen) [31] for strain 484–77, or STP (for saimiri-transforming protein) [6]. We will continue to use the term STP in this review because these proteins can transform rodent cells.

The STP protein of subgroup C is composed of three domains: an acidic amino-terminus, a central collagen-like region and a hydrophobic carboxy end. STP is expressed in all virus-induced tumor cells and in vitro immortalized T cells tested [37, 54]. The subcellular localization of STP is not entirely clear although all investigators agree that STP is associated with membranes of tumor cells [31]. It is clear that the highest amount of this protein is in the cytoplasm [37, 54]. STP may associate with the Golgi apparatus [37] and/or is expressed on the outer surface of tumor cells [54].

Several studies using different approaches proved that STP is a viral oncogene. STP is expressed in tumor-bearing animals as revealed by antibody responses [54]. Jung and Desrosiers [37] demonstrated that collagen-like sequences of strain 488–77 overexpressed by a retrovirus can transform rat kidney cells and these transformed cells were oncogenic in nude mice. These investigators also found that the corresponding ORF from group A strain 11 also induced tumors in nude mice. Experiments with transgenic mice also provided strong evidence for the role of STP in transformation; a strain of mice carrying the STP transgene developed various tumors [60]. Paradoxically, no T-cell tumors were observed in these transgenic mice [60].

STP-488 has been shown to bind to and activate ras [36]. Mutations in STP that interfere with its ability to bind ras abrogate the transforming activity of STP [36]. The level of activated ras is also elevated in STP C488-transformed cells [36]. Furthermore, mitogen-activated protein kinase (map kinase), which acts downstream of ras, is constitutively activated transformed cells [36]. AP-1 transcription factor also acts downstream of ras, and its activity is upregulated in transformed cells [32].

By replacing STP with v-ras, an activated form of ras, Guo et al. [32] showed that the transforming activity of STP involved the ras signaling pathway. Recombinant virus expressing v-ras is able to transform common marmoset T lymphocytes as efficiently as wild-type virus [32]. Recombinant virus in which STP was replaced with normal cellular ras (c-ras) is also able to transform common marmoset T lymphocytes, but with much lower efficiency [32]. Furthermore, these recombinant viruses are able to induce lymphomas in common marmosets, but the onset of disease is delayed in v-ras recombinants compared to wild-type. Disease onset is delayed even further in animals infected with c-ras recombinants [32]. Although lymphomas caused by these recombinant viruses were similar in pathology to those caused by wild-type virus, they differed in the subpopulations of T lymphocytes that were transformed [32]. Cells transformed by recombinant virus were predominantly CD4+CD8+, whereas cells transformed by wild-type virus are predominantly CD8+ [32]. In addition, T cells transformed by v-ras recombinant virus express much higher levels of ras than cells transformed by either wild-type virus or c-ras recombinant virus [32]. Therefore, the role of ras in HVS-mediated cell transformation is not entirely clear.

STP A11 has been shown to bind src and to be phosphorylated by it [45]. By comparing the sequences of STP from different subgroup A isolates, a highly conserved motif, EExxYEAV/I, was discovered [45]. This sequence is similar to the SH-2 binding domain of src family kinases, which is EExxYEEV/I [73]. By mutational analysis it was shown that the tyrosine residue in this motif is essential for src binding [45]. Once STP A11 is phosphorylated by src, it is then able to bind the nonreceptor tyrosine kinases lck, which is T-cell-specific, and fyn, which is highly expressed in T cells [45].

TNF receptor-associated factors (TRAFs) interact with the cytoplasmic portion of TNF receptors during TNF-mediated signal transduction [67]. Two TNF receptors, CD40 and CD30, have a common motif, PxQxT/S that has been shown to interact with TRAFs [25]. LMP1 of EBV, which has been shown to interact with TRAF2, also contains this sequence [20]. This sequence is also found in STP A11, and a similar sequence, PxExT is found in STP C488. By mutational analysis it was shown that these sequences are essential for TRAF binding [44].

TRAF2 has been implicated in the activation of NFκB through interaction with LMP1 of the Epstein-Barr virus [20, 72] NFκB is also upregulated in cells transformed by wild-type HVS strain C [44]. However, NFκB activation is not induced in STP C488 mutants that are unable to bind TRAFs [44]. These mutants are unable to transform primary human lymphocytes, suggesting a critical role for the interaction of STP C488 with TRAFs in cell transformation. Wild-type STP-A11 does not induce NFκB activation. However, both wild-type STP and mutants in which STP is unable to bind TRAFs are able to transform marmoset primary T lymphocytes and cause fatal lymphoproliferative disease in marmosets as efficiently as wild-type HVS [44]. This suggests that different mechanisms of cell transformation mediated by STP are utilized by these strains.

The origin of the collagen-like domain in the STP sequence is uncertain. The repeat is perhaps the result of a recent amplification of an 18-bp sequence by the virus; this recent amplification of the collagen 18-bp unit is supported by the fact that the 18-bp sequence is perfectly repeated 9 times [31]. Alternatively, the repeats may be amplified by the host cell and then transduced by the virus. Interestingly, some collagen exons are 54-nucleotide in length, like in the chicken collagen gene, where most of the 50 exons contain 54 nucleotides. In HVS the 162-nucleotide sequence appears to be the result of exactly 3 reiterations of a 54-nucleotide unit [31]. The absence of splicing of the viral collagen-like domain may refer to a reverse transcription step prior to integration into the viral genome.

Expression of TIP and Interaction with Lck and STATs in Transformed T Cells

Figure 2 illustrates schematically that the TIP-484 protein is expressed in HVS-immortalized T cells and associated with the outer cell membrane; most of the protein is localized on the cytoplasmic side of the plasma membrane [47]. TIP-484 and TIP-488 forms a very stable complex with Lck as proved by several different methods [6, 49–51]. Association of TIP-484 with Lck also markedly and constitutively increases tyrosine kinase activity of Lck [48]. This activation also takes place in HVS-transformed cells in vivo.

There are two conflicting papers in the literature regarding the effect of TIP-488 (a different C strain) on Lck. Jung et al. [38] reported a downregulation of tyrosine phosphorylation in cells expressing TIP-488, while Wiese et al. [80] observed activation of Lck by TIP-488. More studies are required to understand this paradox.

A small subfragment of TIP-484, designated as the Lck-binding domain (LBD), is sufficient for maximal Lck activation [48]. Two regions within the

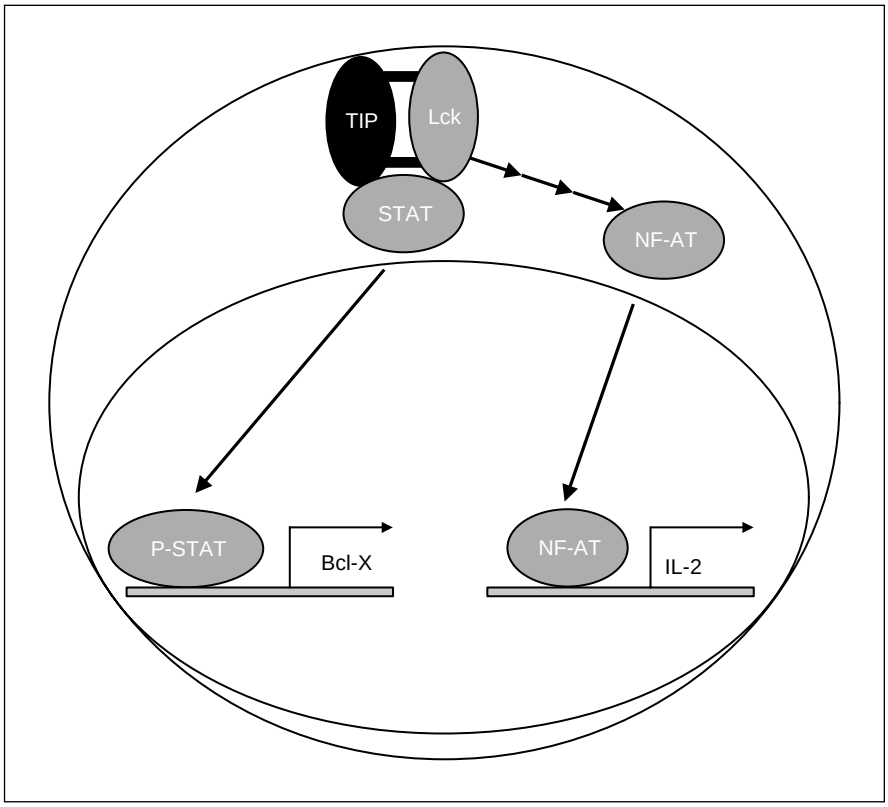

Fig. 2. Proposed mode of action of TIP. Two interaction domains of the membrane protein TIP tightly binds two regions of the nonreceptor tyrosine kinase Lck [50, 53]. Lck is constitutively activated and the TIP-Lck complex binds and activates STAT3 transcription factors. The phosphorylated STAT3 is translocated into the nucleus. Bcl-X has been shown to be activated by STAT3 although this has not been described with TIP. NF-AT is also activated by TIP but the mechanisms involved in activation of NF-AT and the IL-2 gene are unknown.

LBD are involved in binding and activation of Lck [51] A region containing proline residues 132–141 binds to the SH3 domain of the Lck protein. The other Lck-binding region 104–113 of TIP binds the catalytic domain of Lck. Simultaneous binding of both regions to the Lck protein is required for maximal activation [51].

Activation of Lck by TIP-484 correlates with assembly and phosphorylation of a membrane-associated complex that includes not only TIP-484 and Lck, but two transcription factors STAT1 and STAT3 [50]. Signal transducers and activators of transcription (STATs) are transcription factors responsible for

transducing signals from a variety of cytokine cell surface receptors, including IL-2, IL-10 and interferon [for review, see 27, 34]. STATs are inactive in the cytoplasm until a ligand-induced activation of cell surface receptors occurs. This leads to phosphorylation followed by dimerization of STATs on tyrosine residues. Phosphorylation of STATs can occur by a receptor-associated member of the Janus kinase family (Jak-1, -2, -3 or Tyk2). Phosphorylated, dimerized STATs then translocate to the nucleus where they directly activate transcription [27, 34].

Phosphorylation of STAT3 protein is also increased by the presence of TIP-484 and Lck [50]. These unique events lead to activation, constitutive upregulation and translocation of STAT transcription factors to the nucleus [50]. The LBD that is sufficient for Lck activation is also sufficient for STAT activation [50, 51]. While these experiments show that STATs are activated by HVS, it is unknown whether STATs are essential for oncogenic transformation.

HVS-immortalized T cells produce IL-2 in an autocrine manner (discussed below) and express high-affinity IL-2 receptor [10, 14, 57]. STP and TIP are essential for IL-2 gene expression as suggested by deletion mutational analysis [10]. Cyclosporin, an inhibitor of IL-2 gene expression and NF-AT transcription factors, blocks growth of HVS-transformed T cells [10]. Consistent with these data, TIP activates transcription of a construct containing NF-AT transcription factor sites [unpubl. data]. These data suggest that STP and TIP genes of HVS cooperate to induce the IL-2 cascade but the details are unknown.

Viral Small RNAs in Transformed Cells and Identification of Cellular Proteins Which Bind Both Viral AUUUA Repeats and the 3′ End of Unstable mRNAs of Lymphokines

Four viral nonpolyadenylated HSURs are expressed in tumor cells transformed by strain 484–77 [30]. Similar transcripts (seven small RNAs) have been described in marmoset cell lines transformed by strain 11 [46, 61] (group A). Although the functions of these HSURs have not been elucidated yet, some data suggest involvement of these transcripts in mRNA stabilization. Sequence comparison between group A and C strains showed that the small RNAs 1 and 2 encode conserved AUUUA repeats [6, 31]. Interestingly, the same AUUUA repeats occur at the 3′ noncoding regions of growth factor, lymphokine and proto-oncogene mRNAs; these sequence motifs are involved in rapid mRNA degradation and several trans-acting factors have been shown to complex with this 3′ noncoding mRNA sequence. Removal of the AU-rich region confers greater stability to mRNA produced from transfected constructs [69]. Similarly, addition of a short DNA segment containing AUUUA to stable mRNA destabilized mRNA [69].

As shown by in vitro binding assays, the HSUR AU-rich repeats form specific complexes with a 32-kD and a 70-kD cellular RNA-binding protein. The 32-kD factor has been implicated in mRNA destabilization and binds HSURs of both group A and group C strains [29, 31, 63]. It has been proposed that HSURs and lymphokine and oncogene mRNA sequences compete for the 32-kD AUUUA-specific binding protein [29, 63], and as a consequence of such competition, the 32-kD destabilizing factors could be titrated out by HSURs which would allow mRNA to stabilize.

A lectin-inducible AUUUA-specific novel 70-kD binding factor has been also identified (termed AUBF70) which binds both the 3′ noncoding region of IL-4 mRNA as well as the AUUUA repeats of a viral small RNA [9, 29]. HSURs and the IL-4 sequence compete for the 70-kD AUUUA-specific binding protein [29]. However, it is more likely that AUBF70 is not a destabilizing factor but a stabilizing positive regulator and HSURs facilitate its transport from the cytoplasm to the nucleus [9]. According to this hypothesis, AUBF70 forms a complex with HSURs in the cytoplasm and the complex would be transported into the nucleus. In the nucleus AUBF70 then binds oncogene and/or lymphokine mRNAs which have higher affinity for 70 kD; binding would result in mRNA stabilization. This hypothesis is supported by the following data: (a) U-RNAs are known to be exported to the cytoplasm where they complex with proteins before returning to the nucleus [52, 82]; (b) HSUR1 and HSUR2 contain AUUUA repeats and are detectable in both the nucleus and cytoplasm [9, 29]; (c) AUBF70 is inducible by mitogens in T cells [9]; (d) IL-4 mRNA 3′ end exhibits 10-fold higher affinity for binding than HSUR indicating that the IL-4 sequence could easily take up proteins from the HSUR complex [29]. However, further experiments are required to unravel the role of HSURs in mRNA stabilization.

Secretion of Lymphokines and Expression of Their Receptors

The relatively autonomous growth of malignant cells has been known for many years; it has been suggested that cells could become malignant by the endogenous production of polypeptide growth factors acting on their producer cells via external receptors. This mechanism is termed ‘autocrine secretion’ [33, 75]. Long-term growth of activated uninfected T cells can be supported by several lymphokines including IL-2 or IL-4 [71, 74]. Therefore, constitutive upregulation of these growth factors can result in uncontrolled growth of T cells. For example, the long terminal repeat of gibbon ape leukemia virus is integrated into the IL-2 gene of MLA 144 cells; MLA 144 cells secrete high levels of IL-2 [8].

HVS-transformed lymphocytes cannot survive at low cell density, and a T-cell growth-promoting activity was found in conditioned media suggesting

that autocrine secretion of growth factor(s) could be involved [10]. Attempts to demonstrate constitutive high-level expression of IL-2 by HVS-transformed cells have failed although small amounts of IL-2 mRNA can be detected [10, 14]. Studies have also demonstrated that high-affinity receptors for IL-2 are present in both in vitro immortalized cells and tumor cells [57]. The growth of these cells is strongly inhibited by a monoclonal antibody to the receptor of IL-2 suggesting that IL-2 may be involved in autocrine secretion and proliferation of HVS-transformed cells [10, 14, 57].

Deletion analysis experiments suggest a possible role for STP and TIP in the induction of lymphokines. IL-2-independent growth of HVS-infected cells requires both the STP and TIP sequences as revealed by analysis of deletion mutant-infected T cells [53]. To examine the effect of HVS deletions on lymphokine gene expression, IL-2 and IL-4 mRNA levels were compared in T cells infected with a series of deletion mutants by polymerase chain reaction-based assays [10]. IL-2 and IL-4 mRNA expression was readily observed in wild-type-infected samples, however, STP and TIP mutant-infected cells did not express detectable cytokine mRNAs [10].

The role of IL-2 and IL-4 in the process of oncogenic transformation in the HVS model is still subject of debate. One plausible explanation is that these lymphokines, especially IL-2, may act as autocrine growth factors. IL-4 is, however, not only a growth factor but it selectively stimulates development of Th2 T-cell subsets [76]. It is proposed that IL-4 secretion in tumor-bearing animals could shift the balance of immune responses from Th1 subsets representing cellular T-cell responses towards Th2 humoral immunity in HVS-infected animals [10]. Some of the cell lines that secrete IL-4 (1670 and 70N2) have been isolated from monkey tumors. If IL-4 is secreted by virus-transformed tumor cells in vivo, it would cause a shift in T-cell subsets which could help tumor cells to escape from elimination by Th1-mediated cellular immune responses.

Recently, a homologue of IL-10, designated as AK 155, has been identified in HVS-immortalized T cells [41]. This novel cytokine is specifically induced in HVS-immortalized T cells and is expressed in normal human T cells at a much lower level [41]. More studies are required to understand whether AK 155 plays a role in HVS-specific transformation.

The Latency-Associated Nuclear Antigen Homologue (LANA), v-Cyclin and Orf 14 Superantigen

KSHV encodes a nuclear protein designated as latency-associated nuclear antigen (LANA) that is expressed in all primary effusion lymphoma (PEL) cells, suggesting a possible function in episomal maintenance. Ballestas et al. [4] and

Cotter and Robertson [11] reported that the LANA protein co-localizes with viral episomes. Ballestas et al. [4] also showed that uninfected B cells expressing LANA support episomal replication of a cosmid derived from the left end of the genome. In vitro and in vivo studies indicate that LANA binds to a specific sequence in the terminal repeats anchoring the viral genome to chromosomal structures [manuscripts submitted].

The KSHV LANA protein appears to be a multifunctional protein since it was also shown to bind the tumor suppressor protein p53 [26]. Loss of p53 function is implicated in various human tumors and viral oncogenesis. KSHV LANA also inhibits p53 transcriptional activity and inhibits apoptosis induced by p53 [26].

HVS encodes a positional homologue of LANA orf 73 [3]. Alignment of the KSHV and HVS LANA shows a high level of conservation of the central glutamate-rich domain and several potential phosphorylation sites. HVS RNA corresponding to the LANA ORF is expressed in stringently latent cell lines [unpubl. results].

Important questions include whether p53 binding is essential for oncogenesis and whether the HVS LANA homologue has similar dual functions as proposed for the KSHV protein.

HVS also encodes a protein, v-cyclin, that is homologous to cellular type D cyclins [39, 65]. V-cyclin, when complexed to cdk6, strongly induces phosphorylation of Rb in vitro, and has a much higher kinase-inducing activity than cyclin D1/cdk6 [65]. Furthermore, v-cyclin/cdk6 complexes are resistant to inhibition by the cdk inhibitors $p21^{Cip1}$, $p27^{Kip1}$ and $p16^{Ink4a}$ [77]. These properties of v-cyclin may therefore contribute to the oncogenicity of HVS by deregulation of the cell cycle.

The role of the orf14 HVS 'superantigen' in T-cell immortalization is somewhat controversial. While Knappe et al. [42] reported that orf 14 deletion mutants could immortalize T cells, Duboise et al. [21] found orf 14 essential for IL-2-independent growth. These groups used slightly different assays, which can explain this paradox.

Conclusions: Possible Mechanisms of Transformation

Oncogenic transformation by HVS is a distinctly unique phenomenon. The T-cell-specific human retroviruses HTLV-I and HTLV-II integrate in the host genome, however, there is no evidence for integration of the HVS DNA into the cellular genome. Since the disease caused by HVS is extremely acute and tumors are polyclonal, it is also unlikely that integration of viral enhancer sequences adjacent to a cellular proto-oncogene would be the mechanism of

Table 1. List of HVS genes with proven or likely roles in immortalization

Gene	Proven role in immortalization	Mode of action	References
Saimiri-transforming protein (STP)	Yes (in vivo, in vitro)	Activates ras, TRAF, Src	31, 32, 36, 37, 44, 45, 53
Tyrosine kinase interacting protein (TIP)	Yes (in vivo, in vitro)	Binds, activates Lck, STAT 3, IL-2, NF-AT?	6, 31, 38, 47–51, 80
HSURNAs	Yes (only in animals)	mRNA stabilization	9, 29, 30, 61, 63
v-Cyclin	Not tested	Cell cycle regulation	39, 65, 77
G-coupled receptor	Not tested	Signaling	65
Orf 14	Yes/no (contradiction)	Superantigen	21, 42
LANA (orf 73)	Not tested	p53 binding?	3

transformation by this virus. Although EBV and HVS are probably evolved from a common ancestor, their target cell specificity is quite different. In addition, the various genes involved in transformation by EBV and HVS are entirely unrelated which further supports the idea that HVS is a unique transforming agent.

Table 1 summarizes our present knowledge on the various viral genes that have been proven to be or are likely to be involved in immortalization. Studies on the STP gene established that it is a viral oncogene, however it is very clear that this collagen-like protein is not the only factor responsible for growth transformation. Since deletion of STP cannot completely eliminate IL-2 independence [10], other sequences in the viral genome must be also involved. Deletion of TIP resulted in a phenotype similar to the STP mutant (10). These data suggest that the two proteins cooperate in T-cell stimulation and this is achieved through stimulation of various signalling pathways. Their regulation is also tightly coordinated as encoded by a bicistronic mRNA. It is tempting to speculate that TIP represents the T-cell-specific component of the transformation process since its target the Lck protein is available abundantly in T cells only.

Small RNAs are also likely candidates as co-factors in the process of transformation through their AUUUA motifs. In addition, preliminary experiments with deletion mutants also suggest that the two small RNAs with AUUUA repeats are required for oncogenicity in rabbits [M. Medveczky, P. Geck and P. Medveczky, unpubl. data]. Since detailed studies regarding the vast majority of the viral genome have not been published yet, it is possible that several genes outside of the well-studied left end of HVS genome are also involved in transformation of T cells. Some likely oncogene candidates are listed in table 1.

It is also worth emphasizing another unique feature, i.e. that oncogenes acquired by HVS from the cellular genome are highly variable. The viral collagen-like STP and the TIP protein are only found in subgroup C of HVS and are both absent in group A and B strains. It appears, therefore, that the leftmost area, where these genes are located, is a 'hot spot' to acquire host DNA sequences. It is also clear, however, that even if these genes are of cellular origin, they are significantly diverged from their cellular ancestor. Discovery of viral oncogenes in retroviruses and identification of their cellular counterparts (proto-oncogenes) represented a historic breakthrough in cancer research. It is possible that cellular homologues of these HVS genes are also involved in regulation of growth of normal cells or could be activated in human cancers. Future studies are required to identify putative cellular homologues of STP, TIP, HSUR and several other HVS genes which may have evolved from cellular counterparts.

Application of HVS as an immortalizing agent is also a useful research tool for various immunological studies, and among many possibilities, cloning CD8 lymphocytes is one of the important tasks that can be achieved by this method. Infection of human T cells with HVS also provides a model which could facilitate studies on growth regulation of human cytotoxic T cells; CD8 cells are important effector cells in immune responses against infectious diseases and cancer.

It still remains a hypothesis that aberrant regulation of lymphokines and/or their receptors are involved in immortalization and tumorigenicity by HVS. The details of molecular mechanisms by which viral gene products of HVS activate T cells is not yet fully understood. Information learned in this model should provide us with some unique information regarding T-cell activation and lymphomagenesis which should help us to understand how human lymphomas develop. This can contribute to improved diagnostic and therapeutic approaches.

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Subject Index