

MOLECULAR BIOLOGY OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

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1. ABSTRACT

Over the past twenty odd years, kaposi's sarcoma has launched from being a rare pathological curiosity to a significant public health concern. This massive increase in incidence, concurrent with the aids epidemic, has sparked a tremendous amount of interest in uncovering the etiopathogenesis of this disease. Due to its striking association with hiv infection, researchers have focused on identifying a possible infectious agent as the cause of this disease. Such an agent, termed kaposi's sarcoma-associated herpes virus (kshv) was identified in 1994. In the following years, virologists have employed all of their molecular tools to characterize this new agent and its role in the pathogenesis of human disease. Indeed, these efforts have been quite fruitful. It is now known that kshv is a gammaherpesvirus, which like its relatives establishes a latent form of infection that appears to be a prerequisite for expression of a disease phenotype. The relevant disease states and the basic virology are discussed.

2. HISTORY

In 1872, Moritz Kaposi, an Austrian-Hungarian dermatologist, published case reports of five elderly male patients with "idiopathic multiple pigmented sarcomas" (1). By 1891, another prominent dermatologist, Kobner had suggested that this condition be referred to as Kaposi's

sarcoma (KS) (2). Subsequent to this original documentation of the disease, Kaposi's sarcoma remained in relative obscurity until the dawn of the AIDS epidemic in the early 1980s. At this time, the US Centers for Disease Control and Prevention became aware of increased occurrence of two otherwise rare diseases in young gay men from New York and California: KS and *Pneumocystis carinii* pneumonia (PCP). These two conditions were eventually thought of as opportunistic and dependent upon the immunosuppression seen in patients with AIDS. Typically, in HIV-1 positive individuals, immunosuppression precedes clinical presentation of KS which often starts as described by Kaposi with a few skin lesions, but becomes disseminated affecting many organs systems including lung, liver, gastrointestinal tract, and spleen (3). KS today remains one of the most common AIDS-associated cancers and also represents one of the major contributors to morbidity and mortality in AIDS patients, largely due to the disseminative nature of KS associated with AIDS (AIDS-KS) (4, 5).

3. KAPOSI'S SARCOMA: CLINICAL VARIANTS

AIDS, however is not the only clinical setting where KS is commonly manifest. Four distinct clinical variants of KS have now been described; classic KS,

endemic KS, iatrogenic KS, and AIDS-associated KS (3). The form of KS that was originally described by Kaposi has now been termed classic KS. Classic KS most commonly presents in uniformly HIV negative elderly male patients of Southern European or Middle Eastern ancestry (6). Classic KS is a relatively indolent form of the disease which very rarely includes more than a few cutaneous nodules and almost never disseminates to internal organs. Another form of KS, endemic KS, is prevalent in equatorial, eastern and southern Africa (7). Unlike classic KS, endemic KS often presents in HIV-negative children and as a lymphadenopathy rather than skin lesions (8, 9). Endemic KS is also substantially more aggressive than classic KS; nearly all affected African children succumb to the disease (8). It should be noted that in the case of classic and endemic KS, disease is associated with neither HIV nor immunosuppression. Another form of the disease is iatrogenic or post-transplant KS (10, 11). In this case, patients who are undergoing immunosuppressive therapy, typically to prevent graft rejection after organ transplantation, develop KS approximately 6 months after initiating the immunosuppressive therapy (12). Interestingly, patients born in countries where classic KS is prevalent are at a higher risk of developing this condition, even if they migrated to a country, such as the US or the UK where classic KS is not highly prevalent (13). More similar in course to AIDS-associated KS, iatrogenic KS is fairly aggressive and may disseminate (14). Though withdrawal of immunosuppressive therapy often results in remission of iatrogenic KS, this is impractical as immunosuppressive therapy is required to prevent rejection of a vital organ (15). For these reasons, iatrogenic KS has become an increasing contributor to morbidity and mortality in iatrogenically immunosuppressed patients, notably among renal transplant recipients (16).

The most aggressive form of KS is the AIDS-associated variant. This form of KS is most commonly presented in gay and bisexual males, whereas AIDS patients with hemophilia and intravenous drug users only rarely develop KS, suggesting that the disease may be sexually transmitted (17-21). As previously mentioned, immunosuppression associated with AIDS often precedes the clinical appearance of the disease, which generally starts as a few cutaneous lesions, similar to classic KS. In sharp distinction to classic disease, however, AIDS-KS often rapidly progresses to include many overlapping cutaneous nodules. Additionally, AIDS KS often disseminates to vital internal organs, which too often results in the death of affected individuals. Patients with AIDS-KS therefore share a rather grim prognosis, whereby KS is often the precipitant of mortality in patients with advanced AIDS (22). Interestingly, however, in cases where affected patients' HIV load can be controlled with treatment such as highly active antiretroviral therapy (HAART), KS disease may also remit (23). Nonetheless, AIDS-KS is by far the most prevalent form of KS in North America and is still a significant public health concern (24). Regardless of the clinical variant, KS lesions share a distinct histopathology. The tumor cell or "spindle" cell makes up the bulk of the tumor and is thought to be derived from endothelial cells (25, 26). Additionally, an

inflammatory component is often present with infiltrating interstitial inflammatory cells. These components are interspersed with jagged and poorly organized vascular spaces which are sometimes lined by a recognisable endothelium and are often filled with extravasated erythrocytes (27). This feature gives rise to the characteristic grossly apparent purple color of KS lesions.

4. KAPOSI'S SARCOMA: VIRAL ETIOLOGY

Until recently, the etiology of KS remained obscure. Several lines of evidence suggested an infectious agent as being at least partially responsible. First, as previously mentioned, the two historical forms of KS, classic and endemic, both retain exquisite associations with a particular geography. This geography could represent regions where a particular undiscovered infectious agent might be indigenous. Another line of evidence that suggests an infectious etiology has to do with the relationship between KS and immunosuppression. An immunosuppressed patient would be unable to mount an appropriate immune response to the infectious agent, which would allow the agent to exert its pathogenic effects in an unchecked fashion. In this case, KS would result from an opportunistic infection, as is commonly seen in AIDS cases. Furthermore, since AIDS-KS appears to be associated with gay and bisexual men with AIDS, but not with intravenous drug users or hemophiliacs with AIDS, KS may, at least in these cases, be potentially sexually transmitted rather than transmitted through contaminated blood transfusions or needle sharing (28). Well before the AIDS epidemic, however, a viral etiology for KS was suspected. In 1972, an electron microscopy study revealed the presence of herpesvirus like particles in KS tumor cells (29-31). These particles were later attributed to the presence of cytomegalovirus (CMV) (31, 32). CMV however was not the only virus considered as a potential cause of KS. Human retroviruses, human papilloma viruses, and the human polyoma virus, BK, have all been detected, at least under some conditions, in KS lesions (33-35). These potential agents, however, have not been detected in all samples and therefore little data has accumulated that supports any of these as being important for the development of KS.

Interestingly, HIV itself has been implicated as a potentially etiopathogenic factor in KS. Specifically, the HIV-1 Tat protein has been shown to promote angiogenesis and to induce expression of a number of cytokines known to be involved in the pathogenesis of KS (36, 37) (38, 39). Furthermore, overexpression of HIV-1 Tat in a transgenic mouse model resulted in the appearance of KS-like lesions, suggesting that Tat alone may promote the induction of KS (40, 41). This effect of Tat may be specific to the Tat sequence encoded by HIV-1, since the prevalence of AIDS-KS in Africa parallels that of HIV-1 infection but not that of HIV-2 infection (42). Along these lines, a recent study has shown that when the human Kaposi's sarcoma cell line was injected into HIV-1 Tat transgenic mice, tumor progression was substantially greater than that observed in control animals not expressing HIV-1 Tat (43). This suggests that Tat may at least have growth promoting

effects on Kaposi's sarcoma tumor cells, which might account for the particularly aggressive nature of AIDS-KS when compared to the other described clinical variants. This effect, however, may not be specific for Kaposi's sarcoma tumor cells. Indeed, AIDS patients are at a substantially greater risk for a large number of cancers, compared to the general population (44). Currently, the accumulated data suggests that HIV-1 may at least indirectly promote outgrowth of KS lesions through rendering infected individuals immunosuppressed.

In 1994, researchers at Columbia University employed a polymerase chain reaction based subtractive approach to compare DNA amplified from a KS lesion to that obtained from normal adjacent tissue from the same patient (45). This approach resulted in the identification of two DNA sequences present only in the KS but not in normal tissue. These sequences had significant homology to that of the herpesvirus family (45). Further analysis indicated that these sequences belonged to a novel member of the gammaherpesvirus family (46, 47). The virus was subsequently named Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8). Two years later, the now prototypical nucleotide sequence of this novel virus was published, which placed KSHV in the gammaherpesvirus subfamily (47). Based on the published sequence, the closest known relative to KSHV was herpesvirus saimiri (HVS), which is a member of the Rhadinovirus genus or the gamma-2 lineage of the gammaherpesvirus subfamily (48) (46). Interestingly, HVS retains the exquisite ability to growth transform human T-lymphocytes *in vitro*, and causes significant lymphoproliferative disease *in vivo* in new world primates other than its natural host, the squirrel monkey (49, 50). The most phylogenetically similar human virus is Epstein-Barr virus (EBV), which is a gamma-1-herpesvirus or Lymphocryptovirus (51). Like HVS, EBV appears to cause lymphoproliferative disease as well as a number of other solid cancers in its natural human host (51). That KSHV's closest relative viruses cause proliferative disorders in their natural hosts highlights the question of whether KSHV is indeed a tumor virus and does cause KS and/or other forms of human malignancy. Subsequently, several primate viruses related to KSHV have been identified. Among these are the recently sequenced Rhesus Rhadinovirus (RRV) and the retroperitoneal fibromatosis herpesvirus (RFHV) which are both highly colinear with KSHV, but appear to infect only Old World primates (52, 53) (54). Recently, gamma-2-herpesviruses that infect gorillas and chimpanzees have been discovered (55). A group of French researchers applied an immunofluorescence assay that detects both lytic and latent antigens encoded by KSHV to plasma from 25 wild born chimpanzees and 5 wild born gorillas from Gabon and Cameroon. Clear reactivity was detected in 20 out of 25 of the chimpanzees and two out of five gorillas, suggesting the presence of KSHV related agents that encodes antigens that are similar enough to cross react with antibodies directed against KSHV encoded antigens. The group of researchers followed up this screen with a nested PCR approach to amplify viral DNA sequences from peripheral blood mononuclear cells taken from four of the immunofluorescent-positive chimpanzees

and one immunofluorescent positive gorillas. Using degenerate primers to amplify DNA from the conserved herpesvirus DNA polymerase sequence, a 476 bp fragment was isolated and sequenced in each case (55). This sequence analysis revealed the presence of two new sequences detected in the chimpanzees, PanRHV1a and PanRHV1b, as well as a new sequence from the gorilla, GorRHV1. It is expected that these groups are currently working on cloning and sequencing the rest of the genomes of these new viruses, which will more clearly indicate the relatedness of these agents to KSHV. Nonetheless, phylogenetic analysis of the amplified polymerase DNA sequences places each of these viruses outside of the range of KSHV sequence variations in humans, their high degree of similarity suggests that they may represent KSHV's closest identified evolutionary relatives (55). Indeed, to date, KSHV remains the only identified gamma-2-herpesvirus or Rhadinovirus known to infect humans.

That such similar viruses appear common to lower primates suggests at least two possible mechanisms for the origin of KSHV (56). First, KSHV may be an "old" virus and may have coexisted and evolved with man since antiquity. Alternatively, KSHV may be the result of a zoonotic transmission of one of these related primate viruses at a time in the more recent evolutionary past. This appears to be the case for the two human retroviruses, HTLV-1 and HIV-1, which likely evolved after zoonotic transmission of STLV-1 and SIV from central African chimpanzees (57-59). If such an event is responsible for the presence of KSHV in humans, it likely occurred earlier than the zoonotic transmission of SIV, which likely took place in the last century. If KSHV was indeed responsible for the cases of KS described originally by Kaposi in the later 19th century, a zoonotic transmission of a related primate agent must have occurred earlier. Neither of these hypotheses completely explains the apparent geographic distribution of KS and KSHV infection, however. The origins of KSHV therefore await additional study. Nonetheless, the study of these highly related agents in lower primates suggests a potential animal model of Rhadinovirus-associated disease, which several groups are now exploring (52, 54, 55). Another potentially alluring avenue of study involves the identification of additional human Rhadinoviruses. This appears likely given the identified diversity and multiplicity of this virus subfamily in lower primates.

5. KSHV SEROEPIDEMIOLOGY

Since the discovery of the KSHV, much has been done to characterize the prevalence of this agent in the various human populations through seroepidemiological survey. Since assays employed in such studies have not been standardized, significant variations in detected prevalence for a particular group of individuals has been reported (60). Nonetheless, these data generally suggest that, in contrast to other human herpesviruses such as EBV, infection by KSHV is not ubiquitous throughout the general human population. These data also lend support for a central role for KSHV in the development as the seroprevalence of KSHV appears to mirror the risk of developing KS. In areas highly endemic for KS, a range of

seroprevalence of KSHV in the general population has been reported from 32-100%, which might account for the high general risk of KS in these areas (17, 61, 62). Indeed, in some areas of Central Africa, KS is one of the most common cancers in the general population (7). In southern regions of Italy, including Sicily and Sardinia, where rates of classic KS are relatively high, the seroprevalence of KSHV is also quite high (63, 64). Several studies have now reported that, in this region, incidence rates for classic KS range from 1-3/100,000, (65, 66) with estimated KSHV seroprevalence ranging between 10-35% (63, 64, 67). These data however do not present a perfect correlation between the presence of KSHV and the risk of KS as, for example, one study of seroprevalence in Egypt noted a relatively high prevalence of anti-KSHV antibodies in the absence of significant rates of KS (68).

The seroprevalence reported for the general western population is significantly lower than that reported for highly endemic areas. Several groups have reported that the actual seroprevalence in this population ranges from 5-10%, which may account for the relatively low risk of KS for the general western world (18-20, 62, 69, 70). Consistent with the fact that KS does not present in western children, KSHV antibodies have been reported as being absent or detected only rarely in western children (71). The seroprevalence of KSHV in patients with KS, as expected, has been reported to be significantly higher than that detected in the general population. This is again consistent with a role for KSHV in the etiopathogenesis of KS. Antibodies against KSHV-encoded antigens have been detected in 70-100% of all individuals with KS, regardless of the clinical variant (19). It should be noted that detection of KSHV antibodies is somewhat lower in patients with AIDS KS than that detected from patients with classic KS, although this could be due to the aforementioned variation in testing procedures and results (19). Furthermore, since KSHV DNA is uniformly detected in KS lesions (45, 72-82), seropositivity may be a less sensitive measure of determining prevalence of the virus in various human populations. In this way, the aforementioned KSHV prevalence may underestimate the actual prevalence which might be detected by means more sensitive than serologic assays.

20-70% of homosexual HIV-positive, but clinically KS negative patients generally have detectable anti-KSHV antibodies in their serum (18, 21, 83). This correlates with the observed high risk of KS in this group as compared to that of HIV-positive hemophiliacs, who have a low risk of developing KS and also a concordantly low KSHV seroprevalence (18, 19, 62, 83). In fact, the KSHV seropositivity among HIV-positive and negative hemophiliacs is similar to that of the general population (18, 19, 62, 83). This data further supports not only the notion that KSHV is important for the pathogenesis of KS, but that KSHV is likely a sexually transmitted herpesvirus. The fact that hemophiliacs do not display a higher risk for KS than the general population suggests that KSHV is not parenterally transmitted like HIV, Hepatitis viruses B and C (HBV and HCV), and other pathogenic viruses. In non-endemic areas KSHV therefore appears to be primarily

transmitted via male homosexual sex, since data is lacking to support other modes of transmission, including heterosexual sex. In endemic areas, however, the modes of KSHV transmission appear to be somewhat different. Due to the fact that endemic KS often presents in African children with documented seropositivity for KSHV, sexual transmission seems unlikely as the primary mode of KSHV transmission, at least among children in this region (84-86). In these cases, transmission of KSHV appears to take place prior to puberty, but generally after the age of two (87-90). This argues against the possibility of maternal transmission, such as that has been observed for HIV transmission through breast feeding. KSHV does however, appear to be transmitted among families (84, 86, 91). KSHV has been detected in saliva samples which suggests that, as is the case for other human herpesviruses, that spread by saliva could be responsible for some transmission of KSHV (92, 93). If KSHV is indeed spread by saliva it appears that this may be a relatively inefficient mode of transmission, as salivary transmission of other herpesviruses appears to account for their near ubiquity, whereas KSHV appears to prevail only in particular subsets of the general population. The precise mode of this transmission remains somewhat obscure and awaits further study. Taken together, these data suggest that KSHV is likely transmitted through male homosexual sex, but also that other more complex means of transmission are likely among patient populations other than homosexual and bisexual men.

Seroconversion and seropositivity do appear to predict the risk of developing KS in a particular patient population (94, 95). Among HIV positive homosexual men, having anti-KSHV antibodies or seroconverting to KSHV seropositivity strongly predicts the likelihood of developing KS lesions (96-98). In a serologic study of 593 men with a KSHV seropositivity rate of 37.6%, the ten year probability of developing KS was calculated to be 49.6%, indicating a correlation between prior evidence of KSHV infection and the development of clinical KS (97). This correlation is also manifested in the study of renal transplant patients, who are at a documented risk of KS, likely due to treatment with immunosuppressive drugs. A recent study of renal transplant recipients found that, within 4 years, among 39 patients who seroconverted to KSHV seropositivity after transplantation, 2 went on to develop clinical KS, whereas of the 181 seronegative kidney recipients, none developed KS (12). This data suggests that KSHV infection precedes the development of KS and is likely a prerequisite for subsequent development of the signs and symptoms associated with this human malignancy.

6. KSHV AND CAUSALITY OF KS

In terms of the direct evidence for KSHV as being causal in the pathogenesis of KS, the following five summary observations should be noted:

KSHV viral DNA is detectable in virtually every biopsied sample of KS tissue, regardless of the clinical variant. Similar PCR-based methods rarely, if at all, detect KSHV DNA in connective tissue tumors other than KS (45, 72-82).

Detection of KSHV DNA in the peripheral blood of HIV-positive individuals accurately predicts who might subsequently develop KS disease (99).

Populations historically at a greater risk for KS also display a similarly greater KSHV seroprevalence. Additionally, a higher anti-KSHV titer also correlates with an increased risk of developing KS. In this way prevalence of the virus correlates with the incidence of all clinical variants of KS.

In advanced KS lesions, latent KSHV gene products are detectable in nearly all of the neoplastic spindle cells (100, 101). This indicates a correlation between the presence and expression of KSHV and the development of the neoplastic state on even the cellular level.

In vitro infection by KSHV of some cells including endothelial cells results in the development of features similar to those observed among spindle cells in KS lesions (102, 103).

Taken together, these data indicate that KSHV is central to the etiopathogenesis of KS, however, as previously suggested, KS may require factors in addition to KSHV to achieve its pathogenic potential. In this way, KS may be seen as a complex multifactorial disease, which, nonetheless is often due to infection by KSHV.

7. KSHV AND OTHER HUMAN DISEASES

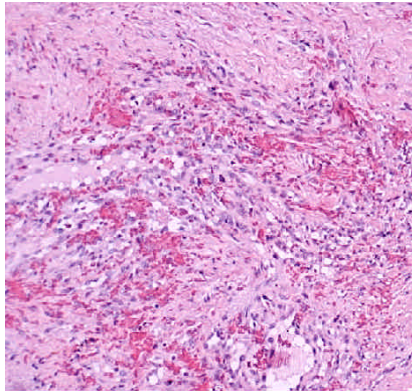
In the several years since the original identification of KSHV from KS lesions by Chang and colleagues, the virus has been implicated in a startling number of diseases and conditions. Epithelial skin tumors following solid-organ transplantation, pemphigus vulgaris/foleyaeus, sarcoidosis, and angiolymphoid hyperplasia have all been reported as being associated with the presence of KSHV, however none of these findings have been confirmed by several other investigating groups (104-107). Since these results have not been reported subsequently by other independent groups and are largely based upon PCR analysis for detection of KSHV DNA, which can be fraught with false positivity, it is difficult to conclude that KSHV plays a significant role in any of these diseases.

Other groups have suggested a role for KSHV in some forms of B-cell gammopathy. One group has detected KSHV in cultured bone marrow dendritic cells from patients with multiple myeloma (108), a disease that involves clonal expansion and proliferation of a mature B-lymphocyte which results in hypersecretion of a monoclonal antibody. Another group has detected KSHV in bone marrow biopsies of patients with multiple myeloma and a proportion of patients with monoclonal gammopathy of unknown significance (MGUS), a related, but less aggressive disorder (109). Conflicting data has now emerged regarding the reproducibility of these results. Some groups have confirmed these observations (110, 111) while others have been unable to do so either by PCR or

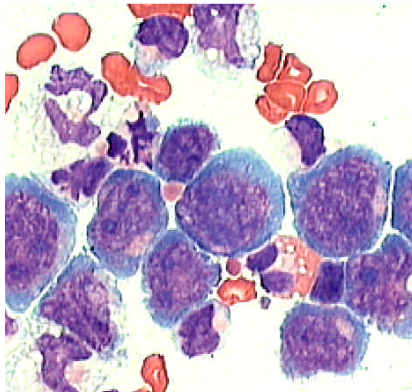
serologic assay (112-116). A potential role for KSHV in these disorders therefore remains quite controversial. Substantial further investigation will be required for determining what, if any, contribution KSHV makes to the pathogenesis of these diseases.

In spite of the sometimes confusing literature surrounding potentially KSHV-associated diseases, two diseases in addition to KS have emerged as being likely to be caused by KSHV (Figure 1). The first of these, body-cavity-based-lymphoma (BCBL), or more commonly, primary effusion lymphoma (PEL) is a rare malignant non-Hodgkin's lymphoma that generally presents as a serous pleural or pericardial effusion or ascites without a detectable mass or peripheral lymph node involvement (117). Alternately, PEL can present as a solid mass in the lymph nodes, lung, or gastrointestinal tract (117-119). PEL typically presents in patients also infected with HIV-1, however, PEL in HIV-negative patients has been described (120). Furthermore, PEL cells are often, but not always, coinfecting by EBV and KSHV, since EBV negative, KSHV positive PEL has also been described, primarily in HIV-negative patients (120, 121). Interestingly, the EBV commonly present in PEL cells may often represent the recombination of two different subtypes (types 1 and 2) of EBV which may therefore retain unique biologic properties (122, 123). Histologically, PEL appears to lie between large-cell immunoblastic lymphoma and anaplastic large-cell lymphoma (124-126). Most PEL cells appear to express CD45, CD30 and CD38, but typically lack other B-lymphocyte associated cell surface markers except CD138/syndecan-1, which is associated with the late stages of B-cell differentiation (127). These observations along with the finding that PEL cells lack expression of BCL-6, a marker specific to germinal center B-cells, define PEL cells as preterminally differentiated, post germinal center B-cells (128). Most PEL cells have undergone immunoglobulin gene rearrangement but notably lack c-myc rearrangement and activation of other known proto-oncogenes (117, 119, 129). PEL often presents in concert with KS, and, like KS, is also associated with HIV-positive homosexual males (119, 130). PEL is thought to be uniformly associated with KSHV, and the presence of KSHV in PEL is used diagnostically to distinguish PEL from other rare lymphomas, since KSHV is almost never detected in other forms of malignant non-Hodgkin's lymphoma (131). Southern blot analysis has shown that PEL cells harbor KSHV genomes at a copy number (50-150/cell) substantially greater than that observed in KS spindle cells (132). Several cell lines have been established from PEL samples which maintain infection by KSHV and are readily propagated in culture (121, 128, 132-134). For these reasons, PEL cell lines have been important for studying the basic KSHV virology and the pathogenesis of associated disease.

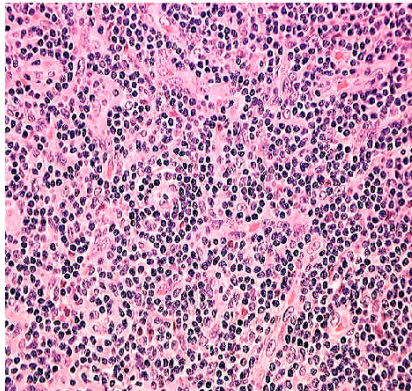
The other disease, in addition to KS that appears to be associated with infection by KSHV is multicentric Castleman's disease (MCD). MCD is a systemic variant of Castleman's disease (CD) which is a polyclonal lymphoproliferative disease, which has long been thought to be mediated by overexpression of interleukin (IL)-6



Kaposi's Sarcoma



Pleural Effusion Lymphoma



Multicentric Castleman's Disease

Figure 1. Histopathology of KSHV associated diseases. Kaposi's sarcoma (KS) shown on the left (adapted from the Erlanger image database) is characterized by the presence of disorganized vascular lumens containing red blood cells (RBCs) made contains endothelially derived "spindle cells". Pleural Effusion Lymphomas (PELs) is shown in the center of the montage (adapted from the University of Virginia School of Medicine Department of Pathology) is characterized by the abundance of large pleomorphic lymphocytes with basophilic cytoplasm in the effusion. Multicentric Castleman's Disease (MCD) is shown on the right (adapted from the University of Pittsburgh School of Medicine Department of Pathology) is characterized by the display of follicular hyperplasia of the lymph node with plasm cell proliferation and hyaline vascular alterations.

(135-137). CD is rare, but has, like KS, been increasing in incidence in parallel with the AIDS epidemic of the last approximately twenty years (138). CD is further classified as two distinct histopathologic variants. The more common hyaline-vascular type often presents as a solitary mass in the upper thorax or retroperitoneum and is usually cured by surgical resection (138, 139). The rarer form of CD, the plasma cell variant often presents with generalized lymphadenopathy, immune abnormalities and B symptoms, such as fevers and night sweats (138, 139). Like CD, MCD is often diagnosed in the setting of concurrent infection by HIV-1. KSHV can be detected in most of HIV-positive cases of MCD and in approximately 40% of HIV-negative MCD cases (139). Interestingly, KSHV-positive MCD is almost always is of the plasma cell type with KSHV uniformly infecting these cells, which have been referred to as plasmablasts (139, 140). These cells appear to be specific to KSHV-positive MCD as they are absent in KSHV-negative MCD (140). KSHV-positive MCD has a poorer prognosis than KSHV-negative, probably due to the tendency of the KSHV infected plasmablasts to progress to a frank plasmablastic lymphoma, which is also KSHV-positive (140). This suggests that KSHV-positive plasmablastic lymphoma may represent an evolution of the less aggressive KSHV-positive MCD (140).

Although KSHV is less frequently associated with MCD in HIV-negative patients compared to HIV-positive patients, the HIV-negative patients with KSHV-positive MCD tend to have experience a more severe clinical progression (141). In these patients, the typical symptoms associated with MCD are frequently complicated by autoimmune hemolytic anemias and polyclonal gammopathies (141). In distinction to PEL cells, cells in MCD have not been shown to be coinfecting with EBV. Unlike the other diseases associated with KSHV, a significant amount of KSHV RNA associated with lytic infection can be detected in MCD (142). This suggests that KSHV's role in the pathogenesis of MCD depends, at least in part, on some degree of lytic infection. In this way, KSHV gene products may drive proliferation in MCD, potentially through paracrine mechanisms. Consistent with this idea, immunohistochemical methods have detected significant amounts of vIL-6, the cellular homolog of which, as previously mentioned, has long been thought to contribute to CD pathogenesis (141, 143).

These data indicate that KSHV is playing a central, and probably causal role in several human tumors including all forms of KS, PEL, and MCD-derived plasmablastic lymphoma, and may therefore tentatively be considered a new oncogenic virus. Indeed, KSHV has now been shown to maintain some transforming capacity *in vitro* (102, 103, 144, 145). Several groups have now infected different sources of primary human endothelial cells with KSHV (102, 103, 144, 145). Though different experimental conditions applied in each case, each group observed changes consistent with growth transformation in cells that became latently infected by KSHV. Additionally, each group observed that the endothelial cells experimentally infected with KSHV developed a spindle-cell like morphology similar to that observed in KS lesions

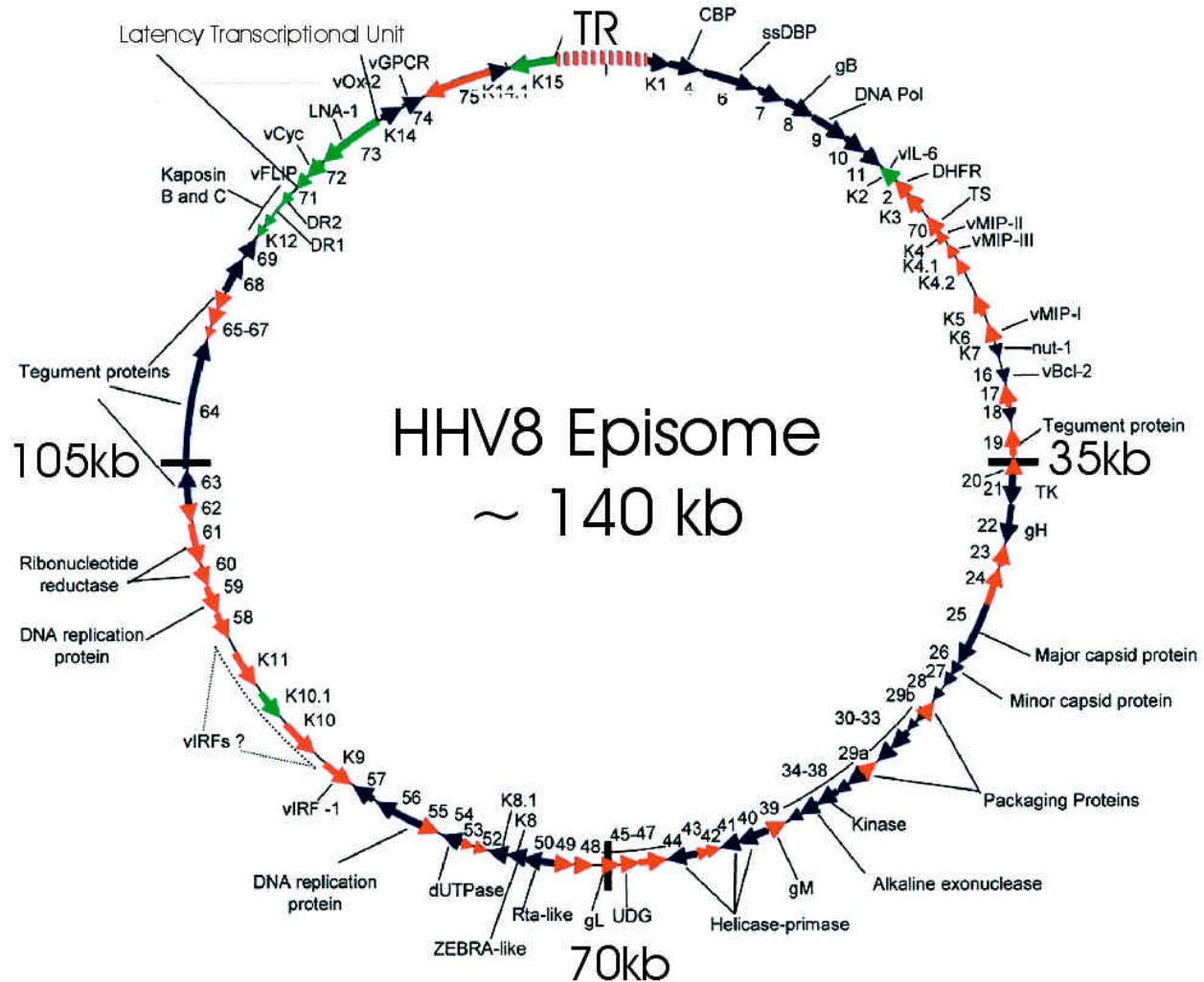


Figure 2. Schematic showing the HHV-8 genome. Human cells latently infected with HHV-8 harbor multiple copies of the circularized genomes. As depicted above the circular episome represents a fusion of the terminal repeats at each end of the linear genome. The episome is approximately 140 kilobase pairs in length and contains open reading frames which code for viral proteins which mediate latent infection as well as modulate cellular processes (adapted from Sharp and Boshoff, 2000. *J. Virol.* 74(2): 97-104).

in vivo. Furthermore, these infected cultures appeared to maintain a small subset of cells undergoing lytic infection, again similar to that observed in KS lesions. While many researchers are addressing potential mechanisms of KSHV-mediated oncogenesis, much work remains to be done to clarify and better characterize the molecular details of how this new agent may promote growth transformation *in vivo* and subsequent human disease.

8. KSHV: GENOME STRUCTURE AND BASIC VIROLOGY

Regardless of its role in human disease, the identification and sequencing of KSHV as a novel herpesvirus has enticed a host of scientists in the field of virology. As mentioned, KSHV is the first example of a human Rhadinovirus. As such, the KSHV genome bears a characteristic structure (Figure 2), consisting of an

approximately 140.5 kb central coding region, or long unique region (LUR), flanked on either side by multiple copies of an approximately 800 bp highly GC-rich (~80%) terminal repeat (TR) sequence (47). The KSHV LUR includes at least 85 open reading frames (orfs), which have been broken into two categories depending upon homology to genes encoded by the related primate gamma-2-herpesvirus, herpesvirus saimiri (HVS) (47). Genes bearing homology to known HVS genes are numbered with the orf prefix, while genes that appear specific to KSHV are numbered with a K prefix. The first unique gene located at the far left end of the LUR, adjacent to the TR, K1 appears to be highly variant in sequence (146). Hayward and colleagues analyzed the K1 sequence from numerous KS lesions and PEL cell lines and proposed four variant KSHV subtypes, A, B, C, and D, based on clustering of K1 sequence variation from isolate to isolate (146). The KSHV open reading frames are also classified according to their

sensitivity to chemical induction by butyrate or phorbol esters in PEL cell lines (147). Class I genes are constitutively expressed and independent of chemical induction. Class II genes are detectable at a low level in the absence of stimulation, however, expression increases significantly upon induction. Class III genes are dependent upon induction; they are undetectable in the absence of stimulation. Class I genes are generally thought to be associated with a latent infection, while class III genes are thought to be associated with lytic infection (147). Class II genes are not restricted to either category and may be expressed during latency at a low level compared to that observed during lytic infection.

Like related herpesviruses, it is likely that following primary lytic infection, KSHV establishes a latent or persistent infection (148). This latent virus may retain the potential to be reactivated under certain circumstances, such as immunosuppression. While both KS and MCD lesions have some cells that appear to be undergoing lytic infection, the vast majority of the cells in these lesions and all PEL cells are latently infected by KSHV (142). This suggests that at least the maintenance of the neoplastic or hyperplastic state in these conditions is likely to be mediated by gene products expressed during latency. This appears to be the case for both HVS and EBV, the best characterized relatives of KSHV which have been associated with neoplastic and hyperplastic conditions in their respective natural hosts. For these reasons, any effort to identify the molecular mechanisms by which KSHV may cause disease, should, at least in part, be focused on latent infection and the viral gene products expressed therein.

Again similar to other described gammaherpesviruses, latent infection by KSHV appears to be characterized by the maintenance of the viral DNA as an extrachromosomal plasmid or episome (149). These episomes are presumed to be formed by joining of the two TR sequences through homologous recombination or other mechanism of ligation. As has been observed for EBV, KSHV episomes appear to be maintained at a stable copy number through cycles of host cell division, suggesting that KSHV episomes are replicated only once per S-phase of the cell cycle in a manner similar to that for host chromosomes (149, 150). Though several gene products have been shown to promote oncogenesis, such as the viral G-protein coupled receptor (vGCR) encoded by orf 74, the aforementioned K1 membrane protein, and the viral homologs of the interferon regulatory factors (vIRF), they are expressed during lytic and not the latent infection which is associated with the neoplastic state *in vivo* (151, 152) (153-155). Presently, researchers have turned their attention to viral genes ubiquitously expressed during latency in an effort to determine potential mechanisms whereby latent infection by KSHV may result in human cancers.

Several gene expression studies have revealed that in spite of the high number of coding sequences on the KSHV genome, only a very restricted set of genes are expressed during latent infection (156-158). The most abundantly expressed latent transcripts are encoded around

the KSHV K12 orf. K12 encodes a small membrane protein, kaposin A (152, 156). Kaposin A has been shown to retain some oncogenic potential, however significant further investigation will be required to verify expression of the kaposin protein in latently infected tumor cells as well as precisely determine the means by which it might exert its oncogenic potential in the context of latent infection (152). The function and expression of additional protein products from this K12 orf, kaposins B and C have yet to be characterized (159).

Another latent membrane protein from the far right end of the genome adjacent to the TR sequence is encoded by the K15 orf (160). K15 bears some domain similarity to both latent membrane protein (LMP) 1 and LMP2, encoded by EBV. Like the well characterized EBV oncogene, LMP1, K15 has a putative TRAF binding domain, which, might also stimulate signalling events which contribute to KSHV-associated oncogenesis (160). Like the kaposins, K15's precise function remains obscure and awaits further experimentation to determine what contribution it makes to latency and oncogenesis by KSHV. In addition to these expressed genes, KSHV also expresses a cluster of three latent genes from a common promoter located around 128 kb from the left hand TR (161). These three genes include orfs 71, 72, and 73, which encode a viral flice inhibitory protein (vFLIP), a viral D-type cyclin (vCyc), and the latency associated nuclear antigen (LANA) (161). Two transcripts are detected from this promoter, the first encoding all three proteins, whereas, the second encodes only the vCyc and vFLIP proteins (161). The vFLIP functions analogously to cellular FLIP proteins in that it interferes with apoptosis signalled by the TNF family of receptors (162). This function may be critical for preventing CTL-induced apoptosis which might otherwise be induced as result of the viral infection. The vCyc likely plays a significant role in dysregulation of the cell cycle in latently infected cells by forming active kinase complexes with CDK6 to phosphorylate the retinoblastoma (Rb) protein in a manner similar to that of the cellular D-type cyclins (163, 164). Interestingly though, the vCyc is resistant to inhibition by the cellular CDK inhibitors, p16, p27, and p21 (165). Furthermore, the KSHV vCyc/CDK6 kinase complex also phosphorylates and inactivates p27, which is known to be important for the negative regulation of the cyclin E/CDK2 (166). In this way, the vCyc appears to activate two of the key pathways required for G1/S-phase progression. In spite of these data strongly implicating a role for the vCyc in growth transformation, overexpression of the vCyc by itself does not cause malignant change or growth transformation.

9. LATENCY-ASSOCIATED NUCLEAR ANTIGEN

The third latent gene encoded in this cluster of genes is LANA, encoded by the viral orf 73 (167-169). Of all the genes that are associated with latent infection by KSHV, LANA is the most ubiquitously expressed protein. Indeed, detection of LANA appears to be a common denominator in latent KSHV gene expression studies. As such, serological surveys of antibodies directed against LANA have served as sensitive markers of latent infection

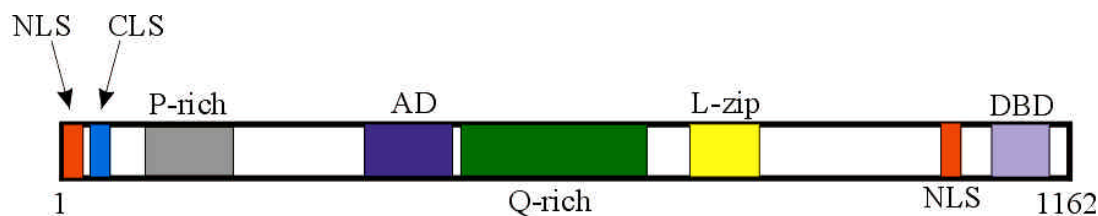


Figure 3. Domain architecture of the KSHV LANA protein. As shown above LANA is a 1162 amino acid protein (reference strain BC-1). Identified domains include an N-terminal proline rich domain, central acidic repeat domains, acidic domain (AD) and glutamine rich domain (Q-rich). Immediately C-terminal to the central repeat is a putative leucine zipper (L-zip). LANA appears to have nuclear localization signals (NLS) in both the amino and carboxy terminal regions. A chromosome localization signal (CLS) has also been mapped to the amino terminus. A DNA binding domain (DBD) has been mapped to the distal carboxy terminus.

by KSHV since shortly after the discovery of the virus in 1994. For these reasons, LANA remains uniquely poised to play a central role in the maintenance of latent infection by KSHV as well as in induction and maintenance of the hyperplastic and neoplastic states seen in KSHV-associated human diseases.

In the few years since its identification, several functions have been assigned to the 222-234 kDa LANA protein (Figure 3). LANA is a large 1162 amino acid domain protein with several identified domains. Two nuclear localization signals (NLS) have been mapped to the N- and C-termini, respectively. The N-terminus contains a Proline rich (P-rich) domain, while the central portion contains a highly repetitive acidic region, reminiscent of some transcriptional activators. Although, the central repeat is highly acidic throughout, the amino acid sequence varies somewhat. The proximal portion of the repeat, labeled acidic domain (AD) consists primarily of DEED repeats, while the glutamine rich domain (Q-rich) is comprised primarily of DEQQQ repeats. Variation in the size of this repeat gives rise to some variation in the size of the LANA protein encoded by different KSHV isolates. Immediately C-terminal to this acidic region, lies a hydrophobic zipper repeat region that is presumed to form a leucine zipper. With the exception of an identified nuclear localization signal, the LANA C-terminus does not bear any significant homology to any known protein or domain structure. Additionally, the sequence that mediates chromosomal localization (CLS) has also been mapped to the N-terminus. The fact that LANA is one of the few viral proteins ubiquitously expressed in cells latently infected by KSHV, has suggested that it might exert a function or functions critical for the maintenance of the latent phenotype. This has clearly been brought to bear as LANA has now been implicated in several processes relevant to viral latency. First, LANA has been shown to be critical for the stable maintenance of KSHV-derived cosmids (170). Additionally, LANA has been shown to tether KSHV cosmids to host chromosomes during mitosis, presumably to ensure efficient segregation of newly synthesized viral episomes to newly formed daughter cells during mitosis (171). Another recent publication identifies a binding sequence within the TR and shows that the TR sequence likely serves as an origin of replication as TR-containing plasmids are efficiently maintained in cells expressing LANA for at least a month after transfection (172). Additional work by our group has recently shown that

LANA binds to multiple distinct sites at the left end of the genome through its unique amino terminus (173). Taken together these data suggest a model of episome maintenance in which LANA binds to multiple sites clustered in and around the multimerized TR. This interaction likely mediates localization of episomes via a tethering mechanism to the host chromosomes and may facilitate episome replication through recruitment of host replication factors (Figure 4).

10. COMPARISON TO EBV

Nonetheless, these data suggest that the KSHV replicon bears at least some similarity to that of the EBV. Only one viral protein, Epstein-Barr nuclear antigen 1 (EBNA1) is required in-trans for maintenance and stable propagation of genomic episomes through many rounds of cell division (174). This protein, along with the 1.8 kb of EBV DNA known as oriP, represents the basic viral machinery by which latent EBV episomes are propagated in infected cells (175-177). Interestingly, though EBNA1 appears to be necessary for latent episome replication, it does not appear to interact with replication-associated enzymes such as a helicase or ATPase, that would be consistent with a direct role in replication (178, 179). Indeed, latent EBV episomes have been shown to be replicated by the cellular DNA polymerase during S phase (180). Though some controversy exists regarding the timing and precise activity of this protein, EBNA1 is thought to exert its effects by direct binding to specific viral DNA elements including oriP (174, 181). Specifically, oriP contains two sets of sites to which EBNA1 binds with high affinity (181). The dyad symmetry (DS) element contains 4 EBNA1 consensus sites (TAGCATATGCTA) and is the functional replicator at which bidirectional replication is initiated (174, 182, 183). The family of repeats (FR) contains 21 repeats of a 30 bp sequence that contains the EBNA1 consensus site (174, 183). The FR functions to prevent loss of viral plasmids in actively dividing cells likely by tethering plasmids via EBNA1 to cellular metaphase chromosomes as they segregate during karyokinesis and also serves as an enhancer of replication (184-186). Interestingly, the N-terminus of the EBNA1 protein has recently been shown to be responsible for the chromosomal localization of the protein (185, 187, 188). The presence of this chromosomal localization domain was also shown to be necessary for the promotion of efficient episomal replication by EBNA1 (188). Furthermore, by

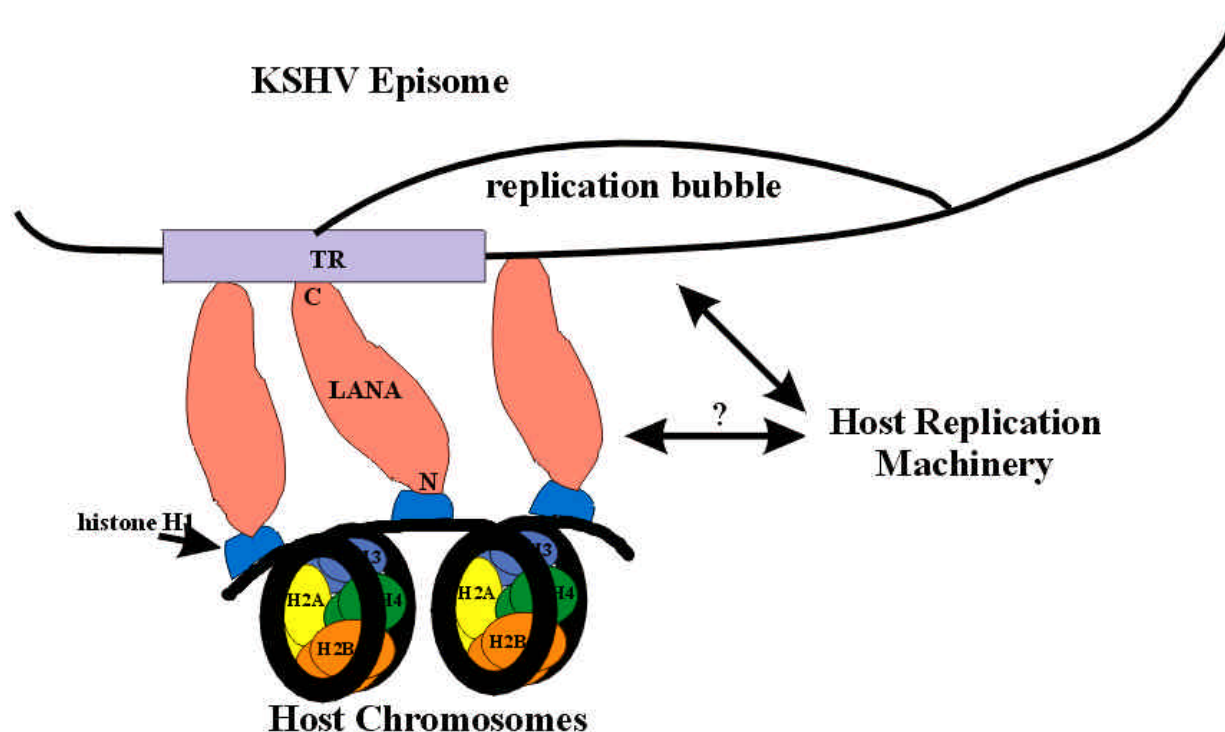


Figure 4. Model of mechanism of latent HHV-8 episome maintenance. In this model, LANA tethers KSHV episomes to the host genetic material, by interacting with DNA binding sites embedded within or adjacent to the terminal repeats, as well as simultaneously with chromosomal proteins including histone H1. this appears to be critical for replication of viral episomes and may involve recruitment of the cellular replicative machinery to the episomal replication bubble.

replacing this domain with either HMG-1 or histone H1 (two known chromosome binding proteins), the resultant EBNA1 fusion promoted replication with efficiency equivalent to wild-type EBNA1 (188). These data clearly indicate that the chromosomal localization function of EBNA1 is critical for its function with regards to episome replication and maintenance, in a way that appears to be similar to the chromosomal localization function of the LANA protein, however, much more experimentation will be required to further compare and contrast latent replication of KSHV and EBV episomes as well as further define the minimal replicator of KSHV.

11. REGULATION OF TRANSCRIPTION BY LANA

Long term maintenance of latent episomes however is not the only aspect of LANA function. Additional data has suggested that LANA functions as a regulator of transcription in a variety of settings. Recent work has shown that LANA can protect cells from apoptosis by negatively regulating p53-dependent transcriptional events through direct interaction with the p53 protein (189). LANA has also been shown to interact with pRb and thereby derepress E2F-dependent transcriptional events (190). In targeting these two key tumor suppressor pathways, LANA is also likely to play a significant role in malignant transformation of latently infected cells. Consistent with KSHV's controversial role as a tumor virus we have shown that LANA has the capacity to transactivate the reverse transcriptase subunit of the human telomerase holoenzyme (191). This is important,

as a robust debate exists in the literature regarding KSHV's ability to transform cells similar to some closely related gammaherpesviruses. Since activation of telomerase is thought of as a critical step in the process of cellular transformation (192), this data lends additional mechanistic support for a role for KSHV as a tumor virus.

Several other groups have shown that LANA maintains the capacity to repress transcription in a number of other settings. One group has shown that LANA interacts with the mSin3/HDAC1 corepressor complex to repress transcription (193). By interacting with a histone deacetylase, LANA may therefore mediate the post-translational modification of histone tails specifically at promoters to which it is recruited. Another group, Choe and coworkers have reported that LANA interacts with and inhibits the ATF4/CREB2 transcription factor (194), which is a key regulator activator of transcription which interacts with several members of the basal transcriptional machinery, such as TFIIB, and TBP (195). Wilson and colleagues have shown, by a series of GAL4 DBD-LANA fusion studies, that both the N and C terminal domains of LANA maintain a repressive capacity (196). Another, more comprehensive study of LANA's effects on promoters containing classical transcription factor binding sites has shown that LANA can activate promoters containing ATF, AP-1, and Sp1 binding sequences (197). Additionally, LANA has also been shown to activate transcription from its own promoter (197, 198).

Taken together, the current evidence supports a multifunctional, complex role for this 222-234 kDa protein in the promotion of latent infection and perhaps oncogenesis by KSHV. These functions are likely to be required for the full expression of KSHV-associated diseases including, at least, KS, PEL, and MCD.

12. ACKNOWLEDGMENTS

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