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KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS IMMEDIATE EARLY GENE ACTIVITY

Vincent Lacoste [1], Cynthia de la Fuente [1], Fatah Kashanchi [1, 2] and Anne Pumfery [1]

[1]Department of Biochemistry and Molecular Biology, The George Washington University, Washington, District of Columbia 20037, USA, [2] The Institute for Genomic Research, Rockville, Maryland 20850, USA

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1. ABSTRACT

KSHV is the causative agent of three human proliferative disorders: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Herpesvirus gene expression and viral replication is a complex, tightly regulated process involving latent, immediate early, early, and late viral gene transcription. The immediate early genes generally code for transcriptional activators and are critical for initiating viral transcription. KSHV encodes for approximately nine immediate early gene products, including ORF50, K8, K9, K3, K5, ORF57, ORF29b, ORF45, and K4.2. This review will address the activities of these proteins and what roles they play in virus replication, evasion of the host immune response, and viral pathogenesis.

2. INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), is a member of the gamma-herpesvirinae sub-family and is the first known human rhadinovirus. Among the close homologues of KSHV, rhesus macaque rhadinovirus

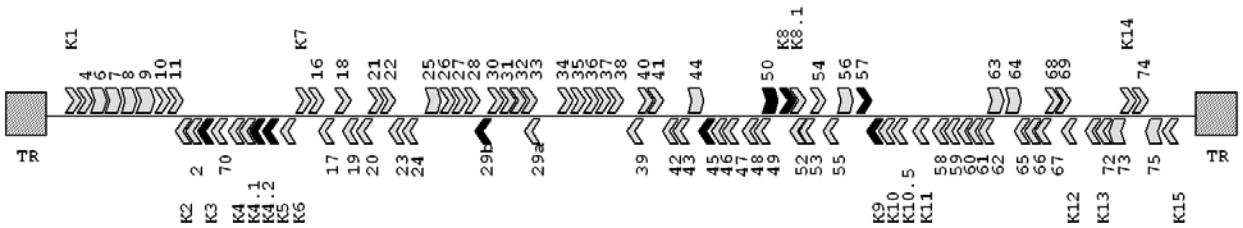

Figure 1. Schematic of the KSHV genome. Open reading frames and direction of transcription (arrows) are indicated within the unique region of KSHV. Terminal repeats (TR) are located at the 5' and 3' ends of the genome and indicated as hatched boxes. The immediate early genes K3, K4.2, K5, ORF29b, ORF45, ORF50, K8, ORF57, and K9 are indicated in black.

(RRV) (1), herpesvirus saimiri (HVS) (2), and Epstein-Barr virus (EBV) (3) are all associated with tumors in non-human primates and humans. KSHV infection is linked with three human proliferative disorders: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), initially named body cavity-based lymphoma (BCBL), and some forms of multicentric Castleman's disease (MCD). Support for an etiologic role of KSHV in these pathologic conditions is mostly based on epidemiological and molecular data and is especially strong for KS. KSHV sequences have been detected regularly in nearly 100% of KS lesions examined, whether in the setting of acquired immunodeficiency syndrome (AIDS) or in the classical and endemic forms of KS (4, 5). Geographic areas of high KSHV prevalence also have the highest incidence of KS, and an increase in anti-KSHV antibody titers precedes the outgrowth of KS in homosexual AIDS patients (4, 5).

Despite these associations and the accumulation of data on the function of individual viral gene products, the pathogenic role of KSHV in these diseases has not been conclusively elucidated. Immunohistochemistry and *in situ* hybridization studies have demonstrated that, in KS lesions, the virus is present in spindle cells, the presumed tumoral component of KS (6, 7). The presence of circular KSHV genomes in PEL cell lines and KS tissues is diagnostic of a latent herpesvirus infection (8, 9), and the expression of KSHV lytic genes is usually detected in only a small fraction of cells in KS, PEL and MCD lesions (5). These features are in agreement with a transforming role of latent KSHV, as is the case for EBV (10). Although the KSHV genome contains a number of potentially transforming genes, including cytokines, chemokines, and an interleukin 8 (IL-8) receptor homologue, many of these genes are part of the lytic program of gene expression and, therefore, are not expressed in the majority of infected tumor cells (5). However, since KS is often viewed as a reactive hyperplasia where dysregulated production of cytokines and angiogenic factors probably plays an important role, both latent and lytic modes of KSHV infection could be relevant to its pathogenesis (11, 12).

2.1. KSHV genome organization

KSHV was initially identified by representational differential analysis of Kaposi's sarcoma tissue, and sequence analysis revealed homology to EBV and HVS (13). Subsequent analysis determined that this virus had a typical herpesvirus genome (figure 1) with terminal repeats flanking a unique region (UR), seven blocks of conserved herpesvirus genes interspersed with genes unique to gamma herpesviruses or KSHV, and is present in an episomal form in latently infected cells (14-16). The UR is approximately 140 kbp in length with a G+C content of 54%. The terminal repeats are composed of one or more 801 bp direct repeat units with a G+C content of 85%. Sequence analysis indicated that the UR contained open reading frames (ORFs) with homology to genes from other herpesviruses including genes involved in viral DNA replication, nucleotide biosynthesis, viral transcriptional activators, and several conserved herpesvirus capsid and glycoprotein genes (16). The KSHV genome also contains a number of ORFs that are homologous to human genes, including cyclin (ORF72), G protein coupled receptor (ORF74), bcl2 (ORF16), and IL-6 (K2), among others. There are also quite a few genes that were initially determined to be unique to KSHV and were given a K designation (K1-K15) (16).

2.2. Nucleosomal structure of herpesviruses in latent infection

During latency, the viral genomes of herpesviruses are in an episomal structure and remain in the nucleus. The genomes are maintained by coordinated replication with cellular DNA replication (17). It has been determined that the genomes of several herpesviruses are in a nucleosomal structure during acute and latent infection, including EBV (18), Marek's disease virus (19), and herpes simplex virus type 1 (20, 21). The Latency-associated nuclear antigen (LANA/ORF73) is critical for replication and maintenance of the KSHV episome in latently infected cells (22, 23). LANA has been shown to associate with chromosomes and links viral DNA to chromosomes (23-26), which is mediated by LANA binding to the terminal repeat regions of KSHV (22, 27-30). Tethering of LANA and the KSHV genome to chromosomes is via binding to chromatin-associated proteins, including histone H1 (24), the methyl CpG binding protein MeCP2, or DEK (25). Recently, it was demonstrated that the ORF50 promoter of KSHV contains a single precisely positioned nucleosome (31). However, only 3 kb of the >150 kb genome was analyzed. As a nucleosomal or chromatin-like structure has been observed on several different herpesviruses including HSV, Marek's disease herpesvirus, and EBV (18-21), these results suggest that the KSHV genome most likely will be found in a similar structure in latently infected cells, which needs to be demonstrated in the future.

2.3. Role of chromatin in regulation of gene expression

Chromatin is composed of genomic DNA wrapped around a nucleosomal core composed of one

histone H3/H4 tetramer and two H2A/H2B dimers (32-35). The nucleosomal structure presents a significant barrier to transcription. DNA binding by transcription factors and transcription by RNA polymerase II can be subjected to nucleosomal repression at several stages, including formation of the pre-initiation complex, transcription initiation, and elongation. DNA binding can be facilitated by disruption of histone-DNA interactions via chemical modification of histone tails (36-39) or by chromatin remodeling complexes (40-42). The existence of various modifications of histone tails and differential binding of transcription factors and other activators to modified histones led to the 'histone code' hypothesis. This theory proposes that histone modifications are interdependent and culminate in specific histone 'landscapes', thereby providing entry sites for proteins responsible for chromatin organization and activation or repression of gene expression (36, 37).

2.4. Herpesvirus gene expression

The use of chemical inhibitors has allowed for the temporal classification of herpesvirus genes (17). These inhibitors include cyclohexamide to inhibit protein synthesis and phosphonoacetic acid (PAA) or phosphonoformate (PFA) to inhibit viral DNA replication. Herpesvirus genes have been classified as latent, immediate-early (alpha or IE), early (beta or E), and late (gamma or L). Latency is characterized by the persistence of the viral genome without the production of viral progeny and viral gene expression is typically limited to a few genes. The IE genes are expressed soon after infection and do not require *de novo* viral protein synthesis. The IE genes generally encode transactivators which induce E gene expression. Therefore, the transcription of E genes is suppressed by cyclohexamide treatment. The E genes encode proteins that are involved in viral DNA replication and, in some cases, additional transactivators. Following E gene expression, viral DNA replication begins and L gene expression is activated. L genes are transcribed only after the initiation of viral DNA synthesis. Consequently, suppression of viral DNA replication by treatment with PAA or PFA inhibits L gene expression. For a more complete review of latent, early, and late herpesvirus gene expression the reader is referred to the following references (17, 43).

2.4.1. KSHV latent gene expression

Like other herpesviruses, KSHV persists in a latent form in the majority of infected cells as well as in tumor tissues (8). During latent infection, the KSHV genome replicates as extra chromosomal episomes in the absence of virion particles. Only a restricted number of viral genes are expressed during latency, including LANA (ORF73), v-FLIP (ORF71/K13), a group of short membrane-associated proteins named Kaposin A, B, and C (K12), and v-Cyclin (ORF72) (44, 45). LANA, which is required for maintenance and replication of the episomal genome (22, 23, 28), also binds to p53 and Rb, thereby inhibiting the activation of p53-dependent promoters (46) and inducing the activation of E2F-dependent genes (47). The viral FLICE inhibitory protein (v-FLIP) can block FAS-induced apoptosis and has been postulated to act as a tumor progression factor by interfering with apoptotic signals induced by virus-specific killer T cells (48). v-FLIP has also been proposed to contribute to the continuous NF-kappaB activation observed in PEL cells (49). A group of transcripts originating in the K12/Kaposin locus was reported to encode several proteins (50-52). Among these, Kaposin A can transform rodent fibroblasts (50, 53). v-Cyclin is a homologue of the cellular D-type cyclins (54) and has been shown to bind to several cellular cyclin dependent kinases (cdks) (55-59), phosphorylating a number of cellular substrates including Rb, histone H1, $p27^{kip}$, Orc1, Cdc6, as well as others *in vitro* (55, 56, 58-62). Although v-Cyclin was initially described as a D-type cyclin, it has a number of properties that make it distinct from these cellular cyclins, including extending the substrate specificity of cdks (56-58, 60, 61), pushing quiescent cells into the S phase (55, 59, 62), and a lack of inhibition by cellular cdk inhibitors (CKI) (58, 59, 61, 62). Although cellular cyclins have been demonstrated to be involved in human cancers, only recently has v-Cyclin been demonstrated to be an oncogene for KSHV (63).

2.4.2. The switch from latent to lytic gene expression

A recent study has suggested that NF-kappaB suppresses IE gene expression to mediate a latent infection in certain cell types (64). KSHV latency is established mainly in lymphocytes. Lymphocytes, due to the instability of IkappaB, contain constitutively high levels of NF-kappaB in the nucleus (65). Additionally, the latently expressed gene, v-FLIP, induces NF-kappaB activity in transfected cells (66, 67), suggesting that v-FLIP may be involved in the maintenance of the latent phenotype in infected cells. Co-transfection of the p65 subunit of NF-kappaB with a reporter containing the ORF50/Rta promoter inhibited transcriptional activation in a dose dependent manner (64). Furthermore, treatment of latently infected cells with a specific inhibitor of NF-kappaB induced lytic protein synthesis. Although the mechanism of NF-kappaB mediated suppression of IE gene expression is not known, unpublished reports indicate that the DNA binding activity of the p65 subunit of NF-kappaB is not required (64). It is not known what factor(s) induce KSHV lytic gene expression *in vivo*. A number of chemical inhibitors have been used to induce expression in latently infected cells *in vitro*, which will be discussed in more detail below.

2.4.3. KSHV lytic gene expression

It has been difficult to determine the transcription profile in KSHV infection, as a cell line suitable for primary KSHV infection has not been available until recently. Several laboratories have determined that human dermal microvascular endothelial cells could be infected with cell-free virus but the virus becomes latent soon after infection (68-71). Instead, latently infected PEL cell lines have been used extensively to study the transcription profile of KSHV genes. Renne *et al.* were the first to determine that KSHV lytic gene expression and replication can be induced by treating latently infected PEL cell lines with 12-O-tetradecanoylphorbol 13-acetate (TPA) (9). Viral gene expression and DNA replication can also be induced by treating latently infected cells with sodium butyrate (NaB) (72, 73). The difficulty with this model

system is that a global inducer, such as TPA or NaB, is needed to induce viral gene expression and it is not known how this mimics what happens *in vivo* following *de novo* infection. Recently, Gao *et al.* were able to infect primary human umbilical vein epithelial cells (HUVEC) with virions generated from a bacterial artificial chromosome (BAC) clone (74). Productive infection was demonstrated and the resulting virions could infect additional HUVEC cultures. This cell model system may prove extremely useful for the analysis of virus attachment and entry and immediate early gene expression.

Initial studies on the temporal regulation of KSHV gene expression utilized Northern analysis and cloned fragments of the KSHV genome. Sarid *et al.* (45) were the first group to globally analyze KSHV gene expression in a latently infected PEL cell line, BC-1, following induction with TPA. KSHV gene transcripts were classified based on their expression in uninduced cells or up-regulation following TPA treatment: class I (constitutive, strictly latent), class II (present at very low levels in uninduced cells but up-regulated with TPA treatment), and class III (present only after TPA induction). However, this classification does not facilitate the identification of the critical IE KSHV genes. Sun *et al.*, having initially demonstrated that ORF50 was an IE gene that can activate KSHV gene expression (75), determined the expression kinetics of a subset of genes using cyclohexamide or PAA pre-treatment followed by NaB induction (76). ORF50/Rta proved to be the only gene to be expressed as an IE gene. Expression was first detected at 4 hrs after induction and was not suppressed by cyclohexamide treatment. K8, K3, and K5 were expressed after ORF50, significant expression was detected at 8 hrs post-induction, and expression was inhibited by cyclohexamide. Transcripts identified as early included those involved in nucleotide biosynthesis, thymidylate synthase, and dihydrofolate reductase, as well as the pro-inflammatory cytokines, v-IL6, v-MIPI, and v-MIPII (76). However, Zhu *et al.*, using NaB and cyclohexamide treatment and isolation of cDNAs, identified four immediate early loci, (KIE-1-4), which encoded ORF50, ORF45, and K4.2 and several early genes (also known as delayed early), ORF57, K3, K5, and K8 (77), while Seaman *et al.* identified ORF50 as an IE and K8 as an E gene (78).

Two groups have recently developed microarrays for global analysis of KSHV gene expression (79, 80). Paulose-Murphy *et al.* classified genes based on their DTs (doubling time), the time at which expression is detected twice above background. The IE genes identified were ORF50, ORF57, K4.2, and K5. The expression kinetics of K8 was not determined, as this gene was not printed because the amplicons used could not differentiate between the various spliced products originating from this ORF. Jenner *et al.* isolated RNA at various times post-induction and used hierarchical clustering to group genes based on patterns of gene expression (80). The classification included primary lytic, secondary lytic, and tertiary lytic genes. Expression of the primary lytic genes was detected at 0-10 hrs, secondary genes at 10-24 hrs, and tertiary genes at 48-72 hrs post-induction. This should correspond to IE, E, and L genes as determined in other reports. However, a number of genes listed as primary lytic genes included genes involved in nucleotide metabolism and viral DNA replication and as such the primary lytic genes identified in this study do not strictly correspond to IE genes.

2.4.4. KSHV immediate early gene expression

These results and others (81, 82) suggest that there are several candidate IE genes in KSHV, including ORF50, K8, K9, K3, K5, ORF57, ORF29b, ORF45, and K4.2. Among the products of these genes, the Rta protein, which is encoded by ORF50, can activate the expression of several immediate early/early genes. This review will discuss the expression and function of these IE genes.

3. ORF50/RTA

In the case of EBV infection, the switch from the latent to the lytic cycle is mediated by the two immediate early proteins, BZLF1 and BRLF1, also known as Zta, Zebra, or Z and Rta, respectively (83, 84). The BZLF1 and BRLF1 genes encode transcription factors that activate the entire viral cascade of gene expression cooperatively, ultimately resulting in the production of viral particles. The KSHV genome possesses analogues to both proteins. The ORF50/Rta gene was initially identified due to its sequence similarities and positional analogies with its EBV and HVS analogues (75) and encodes multiple transcripts. The major transcript, consisting of five exons, is a 3.6 kb mRNA that encodes the entire Rta protein as well as the K8/K-bZIP protein (77, 81, 85). While ORF50 is singly spliced, K8 is multiply spliced. The ORF50 gene encodes a 691 amino-acid DNA-binding nuclear protein that can selectively transactivate KSHV or cellular promoters either by direct binding to the DNA or by interacting with different cellular as well as viral co-factors (86), which will be discussed further.

3.1. Regulation of expression of Rta

Epigenetic factors play an important role in regulating the state of KSHV infection in host cells. In the PEL cell lines, BCBL-1 and BC3, the ORF50 promoter has been shown to be heavily methylated and the chromatin surrounding the IE promoters to be in an unacetylated (inactive) form (87, 88). It has been further observed that the ORF50 promoter was unmethylated in most KS, PEL, and MCD patient samples compared to a latent asymptomatic KSHV carrier, demonstrating the importance of these epigenetic events in the progress and control of KSHV-associated diseases (87). Various agents that alter histone or DNA modifications can induce replication in latently infected PEL cells. These agents include NaB and trichostatine A (TSA), two chemicals known to inhibit the histone deacetylases (HDACs); TPA, which activates histone acetyltransferases (HAT); and 5-azacytidine, which reverses DNA methylation (31, 73, 87, 89). As observed by micrococcal nuclease digestion, histone acetylation induced either by NaB, TSA, or ectopic expression of CBP, led to nucleosomal remodeling and transcriptional activation of the ORF5O promoter (31). Sequence analysis of the ORF50 promoter has revealed the existence of numerous

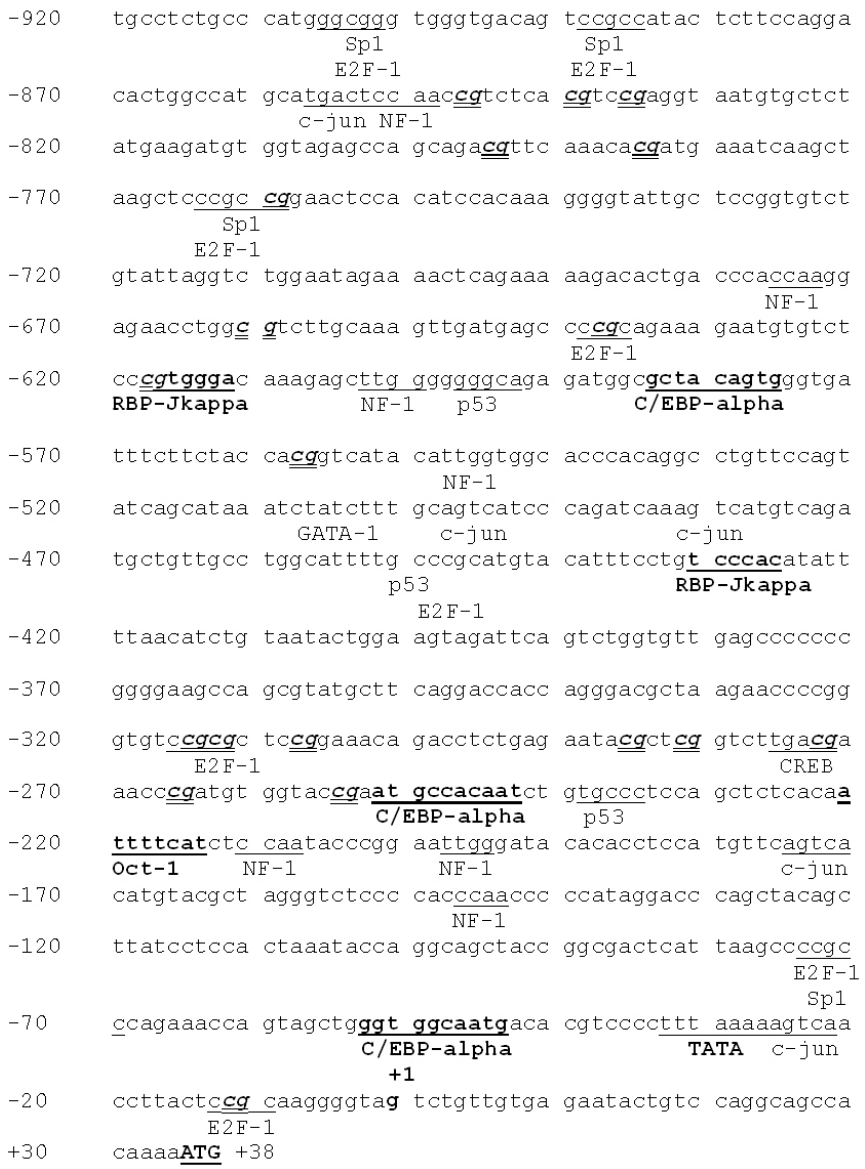

Figure 2. Nucleotide sequence of the 5'-flanking region of ORF50 and putative binding sites of transcription factors. A 955-bp fragment upstream of the ORF50 ATG initiating start codon is represented (from position 70640 to 71598 according to GenBank accession number U75698). The transcription start site is marked as +1 and indicated in boldface. Numbers refer to the number of nucleotide upstream (-) or downstream (+) of the transcription start site. The translational initiating ATG codon is shown in bold and underlined. The putative TATA box, -23 bp upstream of the transcription start site, and various transcription factor binding sites are indicated in bold and underlined. Transcription factor binding sites that have been verifed experimentally are in bold type. Binding sites identified by searching the Transfac database (90) using the Promo software http://www.lsi.upc.es/~alggen/recerca/promo_v2/ frame (-promo.html) are indicated in regular font. The CpG methylation sites are indicated in italic boldface and double underlined.

transcription factor binding sites (figure 2). Binding to and control of the ORF50 promoter by RBP-Jkappa, Oct-1, and C/EBPalpha will be discussed further below. Potential binding sites were identified by searching the Transfac database (90) using the Promo software (http://www.lsi.upc.es/~alggen/recerca/promo_v2/frame-omo.html). Conserved binding recognition sites for c-jun, NF-1, p53, E2F-1, GATA-1, and SP1 were identified in the ORF50 promoter. c-Jun is a component of the AP-1 complex, which regulates multiple genes involved in cell proliferation, differentiation, and apoptosis (91). Furthermore, AP-1 is induced by TPA (92). NF-1 expression is low to undetectable in B cells but highly expressed in fibroblasts and adherent cells (93) and activates a number of viral promoters (94-97). p53 expression and activity is induced by a number of stressors which results in either growth arrest or apoptosis (98). The transactivating function of p53 is suppressed by Rta and K-bZIP (described below). E2F-1 is a transcription factor which is involved in gene activation during cell cycle

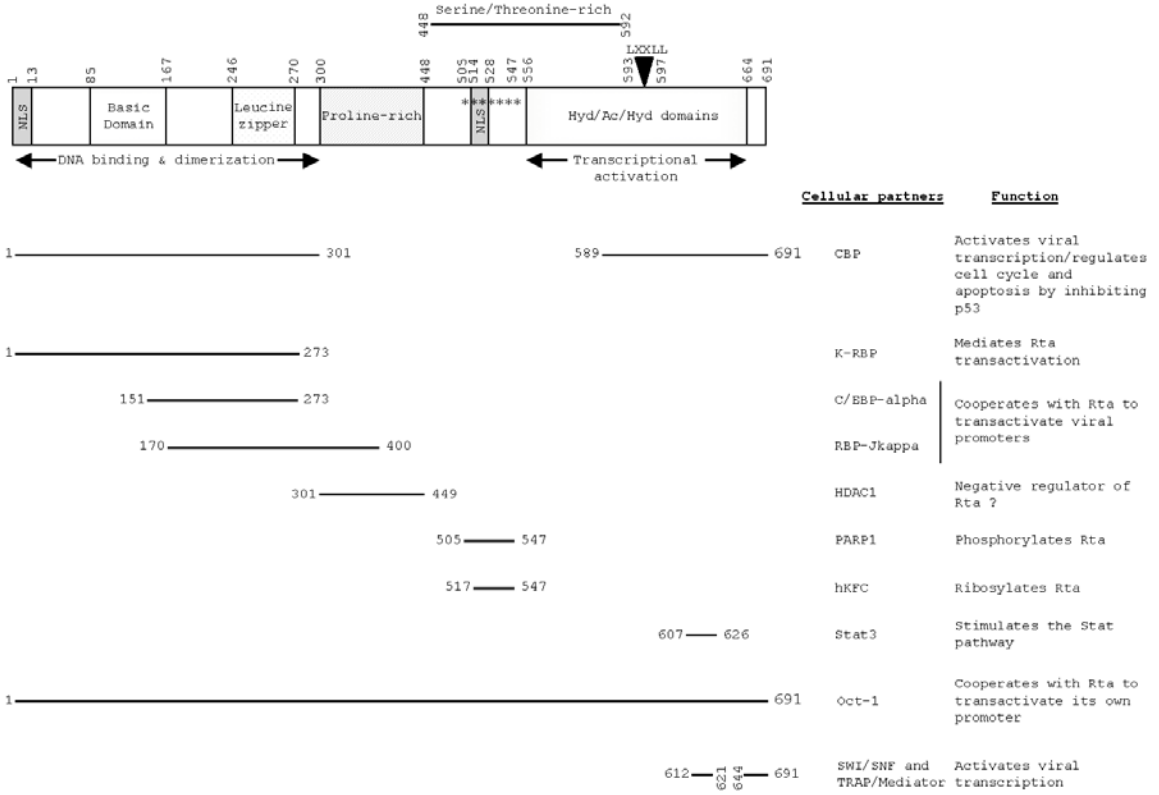

Figure 3. Domain structure of Rta. Rta contains a number of important protein domains. Amino acid positions are indicated in numbers and represent approximate domain boundaries. The N-terminal nuclear localization signal (NLS), basic domain, and leucine zipper are involved in DNA binding and dimerization. The C-terminal hydrophobic/acidic/hydrophobic (Hyd/Ac/Hyd) domain is involved in transcriptional activation. The LXXLL motif is required for interaction with CBP. Proline-rich and serine-threonine-rich regions are indicated. * identifies potential ribosylation and phosphorylation sites mediated by PARP1 and Ste20-like kinase hKFC, respectively. Regions that bind to various cellular partners are indicated below along with their various functions.

progression and whose activity is controlled by binding to Rb (99). GATA-1 is essential for erythroid maturation (100) and whose expression is down-regulated during monocyte differentiation (101). However, cellular gene expression is altered in KSHV infected cells (102, 103) and GATA-1 expression may be induced to allow for Rta expression. SP1 binds to GC-rich elements, which are widely distributed in housekeeping and tissue-specific genes (104). Interestingly, SP1 is required for the latent expression of the EBV C promoter (105) and cooperates with NF-1 to suppress expression of the EBV Zta (homologue of KSHV K-bZIP) promoter (106). On the other hand, the NF-1 and SP1 sites overlap in the EBV Zta promoter (106) yet do not overlap in the ORF50 promoter. It is not known, however, how these transcription factors control the expression of the Rta promoter. Furthermore, in addition to the auto-regulation of its own promoter, the expression of Rta is controlled by an additional strategy implying post-translational modifications of the encoded protein. The activation and activity of Rta will be discussed in further detail below.

3.2. The Rta protein and its post-translational modifications

While the 691 amino acid Rta protein was predicted to have a molecular mass of 73.7 kDa, the expressed protein was shown by western blotting to be about 110 kDa (85). Figure 3 illustrates the various domains of Rta as well as regions involved in binding to cellular proteins. The Rta protein consists of a N-terminal basic DNA binding domain flanked by two nuclear localization signals (NLS), a central dimerization domain, and a C-terminal acidic activation domain. Amino acids 300 to 592 of Rta also contain proline-rich and serine/threonine rich regions. Lukac *et al.* have generated a transdominant negative protein by deleting the C-terminal activation domain (aa 531 to 691), which maintained its DNA-binding activity but ablated spontaneous reactivation from latency and suppressed viral reactivation induced by all known stimuli (85). These results demonstrate that Rta plays an essential role in KSHV lytic replication. Recently, Gwack and co-workers have shown that the Rta protein is post-translationally modified by the cellular poly(ADP-ribose) polymerase 1 (PARP1) and Ste20-like kinase hKFC (human kinase from chicken), which functions as a mitogen-activated protein (MAP) kinase kinase kinase kinase (107). These two proteins transfer poly(ADP-ribose) and phosphate groups, respectively, to the internal serine/threonine-rich region of Rta. These modifications repress the recruitment of Rta to lytic viral promoters regulated by Rta, inhibiting its capacity to induce lytic viral replication.

3.3. Direct DNA binding to specific sequences

Rta has been reported to directly transactivate the promoters of numerous downstream KSHV genes in the KSHV lytic cycle, including those for K12/Kaposin, ORF6/single-stranded DNA binding protein, ORF57/Mta, K14/vOX2, ORF74/vGPCR, K2/vIL6, vMIP-I, K8/K-bZIP, K9/vIRF-1, K1, thymidine kinase, and the polyadenylated nuclear (PAN) RNA also known as T1.1 or nut-1, as well as its own promoter (75-77, 81, 85, 108-124). Initial studies have shown that Rta binds to several viral promoters in a sequence-specific fashion, but does not seem to recognize the same motif in all responsive promoters suggesting both direct and indirect mechanisms for Rta transactivation (109, 119, 125). Rta-responsive elements (RRE) from some of the viral promoters have been defined by transient transfections of Rta and mutational analysis. These studies have defined a RRE within the PAN promoter for which Rta demonstrated a very strong binding affinity (119, 120). A sequence search of the KSHV genome revealed that the promoter of K12, also responsive to Rta activation, shares a 16-bp motif with the PAN promoter, but required additional bases on the 5' end of the core element for optimal Rta-dependent expression (126). Other groups have shown that the RRE of ORF57 also confers direct binding affinity to Rta (109) and that the K8 promoter possesses a highly homologous ORF57 RRE (117). These studies have revealed a 16-bp homology between the 5' sequences preceding the start of ORF57 and K8, which were not homologous to the 16-bp motif shared by the K12 and PAN promoters. Finally, another RRE has been identified in the vIL-6 promoter, presenting a 26-bp binding element for Rta, with no obvious similarity to the PAN, K8, K12, and ORF57 RREs.

Recently, two different groups have focused on characterization of the RRE within the K8 promoter (124, 127) and identified two different elements. Liao *et al.* identified a 47-bp sequence, RtaRE1, as a minimal Rta response element. Sequence comparison of this RtaRE1 with other known RREs of the PAN, K12, and ORF57 promoters revealed a pattern of multiple A/T triplets spaced with a periodicity of 10 or 20 bp. Furthermore, a second RRE of 60-bp has been identified in the K8 promoter, named RtaRE2, downstream of the 47-bp motif and upstream of the TATA box (127). Substitutions of the in-phase, but not out-of-phase, A/T trinucleotides of the RtaRE1 with G/C bases greatly diminished Rta responsiveness and binding. It was further shown that an optimal Rta response was obtained with phased A/T trinucleotides with an arbitrary 7-nucleotide spacer of high G/C content. Liao *et al.*, using *E. coli* derived Rta, demonstrated that Rta binds to the K8 RRE as an oligomer suggesting that the subunits of the oligomer might make multiple contacts with a tandem array of phased A/T trinucleotides. At the same time, another study by Seaman and Quinlivan also identified two different Rta responsive elements in the K8 promoter (124). They observed that a minimal 12-bp element contained the major response region required for Rta activation in lymphoid cells and that this element resided within a palindrome named PAL2. Sequence analysis of this motif indicated some homology between PAL2 and a previously identified palindrome response element in the K8 promoter, PAL1. The last 24-bp of RtaRE1 identified by Liao *et al.* corresponded to the 24-bp 5' sequence of the K8 PAL2 and the last 35-bp of RtaRE2 corresponded to the entire K8 PAL1 identified by Seaman and Quinlivan (124, 127).

These data demonstrate some structural and functional variability in the Rta response elements and that Rta utilizes multiple mechanisms to maintain a tight control over KSHV lytic replication. As will be detailed below, Rta binds directly to the palindromic sequence shared by the ORF57 and K8 promoters but transactivation of the ORF57 promoter, enhanced by Rta binding to this sequence, also requires a direct interaction with the RBP-Jkappa protein, a sequence specific transcription factor that is the key target of the Notch signaling pathway (122). Furthermore, *in vitro* experiments demonstrated that the N-terminal 272 aa DNA binding domain of Rta, which is sufficient for binding *in vitro* to these two promoters, bound also to the heterologous element shared by two other promoters, PAN and K12. Nevertheless, fusion of this N-terminal domain to a heterologous activation domain is not able to transactivate the K12 promoter *in vivo*. Activation requires a larger N-terminal domain of Rta (from aa 1 to 490), strongly suggesting that this region probably interacts with other cellular DNA binding factors.

3.4. Rta cellular partners and transcriptional activation

Ectopic expression of Rta is sufficient to disrupt viral latency and activate lytic replication. Although the detailed mechanisms of Rta-mediated transcriptional activation are still not fully characterized, numerous cellular proteins, including Oct1, Stat3, C/EBP-alpha, the novel cellular protein MGC2663, RBP-Jkappa, as well as the CREB-binding protein, CBP, and certain subunits of the SWI/SNF and TRAP/Mediator complexes have been found to interact and synergize with Rta (116, 122, 125, 128, 129). The locations of these interactions on the Rta protein are illustrated in figure 3 and will be discussed in more detail herein.

3.4.1. Oct-1

Rta has been shown to activate its own promoter (78, 113, 123). Using a series of 5' deletion mutants of a 950-bp fragment upstream of the ORF50 coding region, Sakakibara *et al.* found that the region spanning from –259 to –163 was required for Rta transactivation (125). Furthermore, an intact octamer element located approximately 200-bp upstream (from position –227 to –208) of the ORF50 transcription start site (figure 2) was critical for Rta responsiveness. Octamer-binding protein 1 (Oct-1) bound to this element as demonstrated by electrophoretic mobility shift assay (EMSA). The Oct-1 protein is a member of the POU family and specifically interacts with the octamer-binding sequence 5' ATGCAAAT 3' (130). Mutational analysis of the octamer-binding sequence strongly impaired Rta responsiveness and expression of Oct-1 enhanced the transactivation of the reporter plasmid containing the minimum wild-type promoter sequence with the octamer element. Although disruption of the octamer-binding sequence in the ORF50 promoter did not disrupt transactivation completely, these

results demonstrate that auto-activation of ORF50 requires an interaction with Oct-1 (125), but also suggest that another cis element might be present in this promoter.

3.4.2. KSHV Rta binding protein

In addition to Oct-1, it is likely that other cellular proteins participate in Rta function. Using the yeast two-hybrid system with an EBV-transformed B-cell cDNA library and an Rta C-terminal mutant as a bait, Wang *et al.* identified a human cellular protein able to interact with Rta (116). This protein, referred to as MGC2663 by GenBank, whose cellular function is still unknown, is 554 aa in size and displays a typical structure of members of the KRAB-zinc finger protein family. Amino acid sequence analysis showed that it contains a N-terminal KRAB domain (for Krueppel-associated box) and a C-terminal zinc-finger domain with up to 11 well-conserved C2H2-type zinc finger motifs. MGC2663 expression has been detected in several different primate cell lines and does not seem to be regulated by Rta in a PEL cell line (BC3) following TPA treatment. MGC2663 synergized with Rta to activate transcription of different viral promoters, including ORF57, K8, K6, and ORF50, regardless of whether they were expressed early or late after infection. Over expression of MGC2663 further enhanced Rta transactivation of these promoters in transfection assays. However, MGC2663 has no effect on general transcription. Wang *et al.* also demonstrated, by an *in vitro* pull-down assay as well as by co-immunoprecipitation, that these two proteins interact *in vitro* and *in vivo*. Due to these Rta binding features, they renamed MGC2663 as K-RBP for KSHV Rta binding protein. The N-terminal 273 aa of Rta (figure 3) bound to K-RBP while the potential C-terminal zinc finger domain of K-RBP bound to Rta. Interestingly, the C-terminal activation domain of Rta was not implicated in this interaction. Although it is still not clear whether K-RBP can directly and specifically interact with DNA, nor whether it can play a role in transcriptional regulation in the absence of Rta, this study suggests that K-RBP plays a significant role in mediating Rta transactivation *in vivo*.

3.4.3. RBP-Jkappa

Liang *et al.* discovered that Rta can directly interact with the cellular protein recombination sequence signal-binding protein-Jkappa (RBP-Jkappa) also known as CBF-1 (for Cp-binding factor 1) or CSL, which belongs to the CSL/CBF-1 family (122). RBP-Jkappa is a sequence specific transcription factor, recognizing the 5' GTGGGAA 3' sequence, a key target of the Notch signaling pathway. RBP-Jkappa is normally thought to repress transcription by recruiting co-repressor complexes to responsive promoters (131, 132). RBP-Jkappa and Rta interaction was confirmed by co-immunoprecipitation in transfected 293T cells, as well as in the KSHV-infected PEL cell line BCBL-1 (122). By using an RBP-Jkappa null cell line, the specificity of the Rta and RBP-Jkappa interaction was further confirmed. Two contiguous but separate regions of RBP-Jkappa have been shown to bind to Rta, the N-terminal domain and the central repressor domain. The central repressor domain overlaps with the binding site for the Notch effector. The smallest region of Rta capable of binding RBP-Jkappa is between aa 170 and 400 (figure 3), which contains a leucine-rich repeat region (from aa 246 to 270). To determine whether the RBP-Jkappa recognition sequence can serve as an Rta-responsive element, three copies of the RBP-Jkappa binding site were cloned in tandem upstream of a TATA box driving basal expression of a luciferase reporter. Co-expression of Rta with a wild-type RBP-Jkappa in the human endothelial cell line SLK derived from a KS tumor strongly induced luciferase activity compared to a mutated RBP-Jkappa reporter, containing a mutation known to ablate RBP-Jkappa binding *in vitro*. Furthermore, transfection of the wild type RBP-Jkappa luciferase reporter with Rta in a mouse embryo fibroblast cell line derived from RBP-Jkappa$^{-/-}$ mice resulted in very low luciferase expression, demonstrating that RBP-Jkappa is involved in the Rta response and that Rta cannot activate an RBP-Jkappa site directly. RBP-Jkappa consensus sites within the promoter regions of different viral genes, including PAN, ORF57, SSB, TK, and ORF50 (figure 2), were identified. These promoters have already been identified as Rta responsive. Liang *et al.* further demonstrated that these promoters were induced by Rta and that mutations of the RBP-Jkappa recognition site in these promoters strongly impaired Rta responsiveness (122). This study demonstrated that the interaction between these two proteins targets Rta to RBP-Jkappa recognition sequences via the C-terminal activation domain of Rta and results in reversal of the intrinsic repressive activity of RBP-Jkappa.

Because many KSHV lytic genes contain RBP-Jkappa binding sites, RBP-Jkappa-mediated repression may be central to the establishment of latency following *de novo* infection (133). Liang and Ganem tested this hypothesis by examining KSHV infection of the murine fibroblast cell lines OT11 (RBP-Jkappa$^{-/-}$) and OT13 (RBP-Jkappa$^{+/+}$) (133). Following infection, OT11 and OT13 expressed LANA in a comparable number of cells with a characteristic punctate nuclear pattern. Furthermore, the viral genome was present as an episome with comparable efficiency in both cell lines, demonstrating that KSHV latency was efficiently established in these cells. To explore the impact of RBP-Jkappa loss on lytic infection, both cell lines bearing latent KSHV genomes were infected with an adenovirus vector constitutively expressing Rta. Rta expression efficiently induced lytic cycle gene expression in wild type cells but not in RBP-Jkappa null cells. These results were also confirmed by Gardella gel analysis demonstrating that there was no accumulation of progeny linear viral DNA in the null cells as compared to the wild type cells. Finally, there was no release of progeny virus in the absence of RBP-Jkappa. These results demonstrate that Rta-mediated redirection of RBP-Jkappa is critical for KSHV lytic reactivation.

3.4.4. Stat3

Stat proteins (for Signal Transducers and Activators of Transcription) are a family of cytoplasmic signal transducers that are activated by cytokines or growth factors (134). These proteins have an amino-terminal protein/protein interaction domain, a DNA interaction domain, a SH2 domain, and a single tyrosine phosphorylation site. Tyrosine phosphorylation stabilizes the association between two Stat monomers through interaction

with a phosphotyrosine and a SH2 domain (135, 136). Activated Stat dimers then translocate to the nucleus where they can bind to their cognate DNA response elements to activate gene expression. It has been shown that a number of cellular oncoproteins can activate specific Stats (137, 138). Furthermore, several tumor viruses, such as human T-cell lymphotropic virus type 1 (HTLV-1), HVS, and EBV, are associated with Stat activation (139-141). Gwack *et al.* have shown that Rta activates transcription of Stat3-driven reporter genes without mediating tyrosine phosphorylation (129). Rta and Stat3 were found to associate *in vivo* and this interaction induced dimerization and nuclear localization of Stat3. Physical association between Rta and Stat3 required the carboxy-terminal transactivation domain of Rta (figure 3) and multiple regions of Stat3, which were not fully identified but may include the N-terminal protein/protein interaction domain, the DNA binding c-jun interaction domain, as well as the SH2 phosphotyrosine recognition activation domain. Rta also activates the expression of cellular IL-6 (108) as well as v-IL6 of KSHV (108). As v-IL6 has transforming activities through the Stat pathway, Gwack *et al.* proposed that Rta and v-IL6 might cooperate to stimulate the Stat pathway and demonstrated that Rta contributes to the modulation of cell growth (129). Furthermore, it remains possible that Rta associates with other proteins of the Stat family.

3.4.5. CBP/HDAC

Cyclic AMP (cAMP)-responsive element binding protein (CREB)-binding protein (CBP) is a transcriptional co-activator possessing an intrinsic histone acetyltransferase activity (HAT) that contributes directly to the acetylation of histones (142, 143). Histone deacetylases (HDACs), however, are known as repressors of transcription. HDACs reverse the acetylation process and induce the formation of tightly packaged nucleosomes, which are inaccessible to transcription factors (144). Rta interacts with CBP to activate Rta-mediated viral transcription (128). Rta bound to the C/H3 and the C-terminal transcriptional activation domains of CBP, while CBP bound to the amino-terminal basic and the carboxyl-terminal transactivation domains of Rta (figure 3). The C-terminal domain contains a LXXLL motif, which constitutes a CBP-interacting motif in several proteins (145, 146). Interestingly, the basic domain and the LXXLL motif of Rta interacted independently with CBP and these two domains were necessary for Rta transcriptional activation. Furthermore, the adenovirus E1A protein, which also binds to the C/H3 domain of CBP, repressed the transcriptional activation activity of Rta. However, the cellular protein c-Jun, another CBP-binding protein, bound to the activation domain of Rta and stimulated Rta-mediated viral transcription.

Rta also interacts with HDAC-1 (128). The HDAC-1-interacting domain of Rta was shown to be a central proline-rich sequence (figure 3). A model for the regulation of Rta transcriptional activation has been proposed: CBP functions as a positive regulator of the Rta transactivator, while HDAC-1 acts as a negative regulator, suggesting that both HAT and HDAC activities may play important roles in controling latent and lytic cycles of KSHV. Others have also shown that ectopic expression of CBP stimulated plasmid-based ORF50 transcription in a histone acetyl transferase (HAT) manner, further confirming that CBP recruitment to the ORF50 and other promoters can be an initiating event for transcription and viral reactivation (31).

Because p53 uses CBP as a transcription co-factor and binds to the carboxyl-terminal transactivation domain of CBP (147, 148), whether or not Rta could inhibit transcriptional activation by p53 was analyzed (128). By sequestering CBP, Rta prevented the interaction of CBP with p53 and repressed the transcriptional activation function of p53 and p53-induced apoptosis. Furthermore, the inhibitory effect of Rta on the transcriptional activity of p53 was reversed by the addition of CBP. Rta mutants, which were defective for interaction with CBP, lost the inhibitory effect on p53. These results suggest that Rta not only acts as a transcriptional activator of KSHV but also as a regulator of the cell cycle and apoptosis to sustain viral persistence. CBP may also be a key molecule in the interaction of Rta with transcriptional factors.

3.4.6. C/EBP-alpha

CCAAT/enhancer binding protein alpha (C/EBP-alpha) was the first identified member of the leucine zipper (bZIP) family of transcription factors including C/EBP-beta, CHOP-10, c-jun, c-fos, ATF, CREB, and Zta of EBV (149). C/EBP-alpha can positively regulate its own promoter and has been shown to control differentiation and inhibit cell proliferation by G_1 cell cycle arrest (150-152). In PEL cell lines, C/EBP-alpha expression has been shown to be induced either during lytic-cycle induction by TPA or by direct introduction of exogenous K8, leading to host cell cycle arrest at G_1, which will be discussed below (153, 154). As previously mentioned, Rta has been shown to be a transcriptional activator of the K8 promoter through an RRE containing a 16-bp consensus motif. Recently, Wang *et al.* identified two potential consensus C/EBP-alpha binding sites in the K8 promoter (155). One of these two sites, which overlap the 16-bp consensus motif in the RRE, had a high affinity for C/EBP-alpha. C/EBP-alpha strongly transactivated the K8 promoter both alone and in combination with Rta or with Rta and K8. Furthermore, introduction of exogenous C/EBP-alpha in BCBL-1 latently infected cells induced K8 expression. By site-directed mutational analysis of the K8 promoter, deletion of the strongest C/EBP-alpha binding site impaired the responsiveness to C/EBP-alpha but also to Rta as this site overlaps the RRE. *In vitro* GST pull-down assays and *in vivo* co-immunoprecipitation experiments demonstrated that C/EBP-alpha and Rta physically interacted, which required the N-terminal DNA binding domain of Rta (figure 3). Furthermore, deletion of a 17 aa basic motif of Rta abolished not only its capability to bind C/EBP-alpha but also its DNA binding activity resulting in the loss of Rta transactivation and C/EBP-alpha cooperativity. Finally, chromatin immunoprecipitation (ChIP) showed that these proteins associate specifically with the K8 promoter *in vivo* and that removal of C/EBP-alpha resulted in the reduction of Rta binding. From these data, Wang *et al.* proposed a model in which Rta activation of the K8 promoter is

partially mediated by C/EBP-alpha: C/EBP-alpha first stably binds to the core motif of the crucial binding site, then Rta physically interacts with DNA-bound C/EBP-alpha and interacts secondarily with the adjacent RRE motif to maximize the level of expression of the K8 promoter (155). Therefore, the strong activation of the K8 promoter by Rta seems to be attributable to the unique juxtaposition of a C/EBP-alpha binding site next to an Rta-specific motif.

As a C/EBP-alpha site was found in the RRE of a KSHV promoter, C/EBP-alpha might also play a role in activating KSHV immediate early gene expression. Wang *et al.* demonstrated that C/EBP-alpha also activated the ORF50 promoter and that addition of Rta resulted in additional activation (156). By using EMSA and deletion analysis, three C/EBP-alpha binding sites were identified (figure 2) that mediated cooperative transactivation of the proximal 914-bp ORF50 promoter by C/EBP-alpha and Rta. Direct introduction of exogenous C/EBP-alpha into KSHV-infected cells led to induction of Rta transcription. Furthermore, expression of exogenous Rta also induced expression of endogenous C/EBP-alpha. Interaction of both C/EBP-alpha and Rta with the ORF50 promoter was then confirmed *in vivo* in the TPA-induced PEL cell line JSC1 by ChIP. Finally, analysis of other Rta-responsive viral promoters revealed that PAN and ORF57 promoters also possess C/EBP-alpha binding sites. Both promoters were responsive to C/EBP-alpha, demonstrated C/EBP-alpha and Rta cooperativity, and were bound by C/EBP-alpha and Rta *in vivo*. These data suggest that C/EBP-alpha and its interaction with Rta play important roles in the control of viral gene expression in the early stages of the lytic cycle.

3.4.7. SWI/SNF and TRAP/mediator subunits

The association of transcription factors, recruitment of co-activator complexes, and the targeting of gene promoters is a sequential process resulting in transcriptional activation (157). The SWI/SNF complex is a chromatin remodeling complex that uses the energy of ATP hydrolysis to alter the accessibility of promoter regions embedded in nucleosomal structures (158). The complex has been associated with both transcriptional activation and repression, as it may favor recruitment to the DNA of both activators and repressors (159, 160). Using affinity purification and mass spectrometric analysis, Gwack *et al.* found that Rta directly interacted with different subunits of the SWI/SNF chromatin remodeling complexes (BAF155, BAF170 and Brg1), some of the thyroid hormone receptor-associated protein (TRAP)/Mediator subunits (TRAP37, TRAP55 and TRAP100), and CBP (88). The interaction of Rta with SWI/SNF and TRAP/Mediator complexes were confirmed *in vitro* by GST pull-down assays. Using a system in which Rta was expressed under the control of an inducible promoter (82), they demonstrated that these complexes directly bound to Rta *in vivo* and were specifically recruited by Rta to its dependent promoters, ORF50, ORF57, PAN, K9 and K14, playing a critical role in transcriptional activation of these KSHV genes (88). Rta recruited CBP, SWI/SNF, and the TRAP/Mediator co-activator to these viral promoters through interaction with a short acidic sequence in the carboxyl region of Rta (figure 3), demonstrating that the recruitment of these complexes is essential for Rta-dependent viral gene expression.

3.5. Rta as an inducer of apoptosis

Recently, Nishimura *et al.* showed using an Rta-inducible system that Rta was able to induce apoptosis through a caspase-dependent pathway in KSHV-uninfected cells (BJAB) but not in KSHV-infected cells (BCBL-1) (161). Their results suggest that induction of apoptosis by Rta may be opposed by one of the many genes encoded by KSHV that have an anti-apoptotic function such as v-Bcl-2, v-FLIP, v-IRF, or K7 (162-166).

3.6. Summary

KSHV ORF50 is an immediate early gene, which is regulated at the transcriptional level by promoter methylation and by its encoded protein Rta. Rta is a potent transcriptional activator capable of inducing the entire virus lytic cycle to completion in KSHV-infected PEL cell lines. Rta activates the expression of numerous viral genes through direct binding to specific sequences and/or interaction with various cellular transcriptional factors as well as the SWI/SNF chromatin remodeling and TRAP/Mediator complexes. Its transcriptional activity is regulated by phosphorylation and ribosylation, which have been shown to repress the recruitment of Rta to viral promoters. However, whether these modifications affect the binding capability of Rta to its cellular protein partners is not yet known. Furthermore, as most of the protein-protein interactions of Rta with transcriptional factors have been demonstrated in uninfected cells, their biological relevance in KSHV-infected cells remain to be evaluated. Nevertheless, the results obtained to date demonstrate that the molecular mechanisms that underlie Rta-mediated transcriptional activation require a large number of transcriptional co-factors and that their actions ultimately direct well-controlled viral gene expression and thereby viral lytic reactivation.

4. K8/K-bZIP

K8, also known as replication-associated protein (RAP) or K-bZIP, was originally identified as a 719-bp ORF predicted to encode a 239 amino acid protein with no amino acid sequence identity to known cellular or viral proteins (16). Transcriptional analysis of the ORF50/K8 region, however, indicated that K8 was actually expressed as a spliced transcript and exhibited weak identity to the EBV Zta protein (78). Despite the weak identity with Zta, K8 is believed to be the evolutionary equivalent to Zta based on its co-linear genome localization and similar 3' splicing patterns producing a protein with a leucine zipper motif. The EBV Zta protein, also known as BZLF1, Zebra, or EB1, along with the EBV Rta protein, activates the EBV lytic cycle (167-171), suggesting that K-bZIP may also cooperate with the KSHV Rta protein to induce the KSHV lytic cycle. Further characterization of the K8 ORF revealed that there were three coding exons (172, 173). Exons I and II contain basic regions and exon III contains a prototypic ZIP (leucine zipper) domain (173). The leucine

zipper is a heptad of leucine repeats that form a coiled-coil of alpha-helices involved in dimerization (174, 175). The basic domain of exon II and the ZIP domain of exon III could form a bZIP domain. The DNA contact residues that are conserved in c-Jun, c-Fos, and Zta, however, are not well conserved in K-bZIP (173), and, to date, sequence-specific DNA binding by K-bZIP has not been demonstrated in viral promoters. The K-bZIP protein has been shown to form homodimers, which is mediated by the bZIP domain (172, 173). K-bZIP, though, does not bind to other known bZIP proteins such as Jun, Fos, and C/EBP (172). The Hayward laboratory, however, later demonstrated binding of K-bZIP to C/EBP-alpha (154, 156). The nuclear localization signal (NLS) of K-bZIP has been determined and in a chimeric protein can localize GFP to the nucleus. Furthermore, a wild-type K-bZIP can heterodimerize with a mutant K-bZIP, with the NLS mutated to neutral residues, and drive the nuclear localization of the mutant protein (176). The NLS of K-bZIP is unique, though, as it is composed of a single basic sequence whereas the NLS of other bZIP proteins is composed of two basic clusters. K-bZIP has been shown to have a number of functions including inducing cell cycle arrest, binding to the KSHV lytic origin of replication, and regulating KSHV gene expression with Rta, which will be discussed herein.

4.1. Phosphorylation by cyclin dependent kinases (Cdk)

The EBV Zta protein is phosphorylated *in vivo* on serine 186 (S186) in its basic domain, which is mediated by protein kinase C (177). Phosphorylation of Zta *in vitro* inhibited its DNA binding activity; however phosphorylation of Zta *in vivo* was shown to be required for TPA-induced increases in DNA binding. Earlier results had demonstrated that mutation of S186 to alanine suppressed the ability of Zta to initate the EBV lytic cycle but was able to activate Zta-responsive promoters (178, 179). These incompatible results have yet to be reconciled but suggest that phosphorylation of the Zta transactivator may control its activity. Orthophosphate labeling of BCBL-1 cells demonstrated that K-bZIP was phosphorylated on threonine 111 and serine 167, which were located in Cdk recognition motifs. *In vitro* kinase assays demonstrated that Cdk1/Cyclin B, Cdk2/Cyclin A, and Cdk2/Cyclin E mediated phosphorylation of K-bZIP (180). Although the KSHV v-Cyclin can bind to a number of cdks to phosphorylate cellular substrates (54-56, 58, 60, 62, 181, 182), it is not known if v-Cyclin/cdks can phosphorylate K-bZIP. As K-bZIP has not been shown to be able to induce lytic gene expression, Polson *et al.* determined if phosphorylation of K-bZIP inhibited its ability to reactivate latent KSHV by transfecting phosphorylation site mutants into BCBL-1 cells. Neither the wild-type K-bZIP, nor the phosphorylation site mutants, were able to induce reactivation (180). These results indicate that the inability of K-bZIP to induce reactivation is not due to inhibitory phosphorylation of the protein. The EBV Zta protein is not only involved in transactivation of the EBV genome, it is also an origin binding protein which is required for lytic replication (183, 184). The EBV Zta protein has also been shown to associate with helicase-primase proteins and may be involved in the formation of the EBV replication complex (185). Although K-bZIP is not able to induce lytic gene expression, it could play other roles in the KSHV life cycle, including acting as a sensor of host cell cycle progression (based on its phosphorylation status) or be involved in the formation of a KSHV replication complex (discussed below).

4.2. Cell cycle arrest

Many viruses, including the herpesviruses, arrest the host cell cycle during lytic infection (186). Cell cycle arrest allows for the shift of nucleotide stores and cellular machineries from cellular transcription and DNA replication to viral gene expression and replication. Treatment of KSHV+ PEL cell lines with TPA results in a G_1 arrest (153, 187). Following induction of viral gene expression, K-bZIP mRNA levels increased within 12 hrs, which was followed by an increase in p21/cip-1 and p27 levels (187) as well as C/EBP-alpha expression (154). Furthermore, transfection of a K-bZIP expression vector into HeLa cells induced C/EBP-alpha and p21/cip-1 expression (153). K-bZIP binds to C/EBP-alpha and p21/cip-1 *in vivo* and *in vitro*, and stabilizes these proteins, increasing their half-lives 10-fold (154). C/EBP-alpha, p21/cip-1, and p27 are all involved in regulation of the cell cycle. p21/cip-1 and p27 are inhibitors of cyclin dependent kinases (188) while C/EBP-alpha regulates the cell cycle by inducing the expression of p21/cip-1 (151, 189), inhibiting E2F transcription (190), and directly inhibiting cdk2 and cdk4 activity (191, 192). These results indicate that K-bZIP mediates a G_1 arrest during the KSHV life cycle by inducing the expression of and stabilizing the key cell cycle proteins p21/cip-1 and C/EBP-alpha.

K-bZIP also regulates the cell cycle through Cdk2 (187). Immunoprecipitation of K-bZIP from KSHV+ cells precipitated Cdk2 with Cyclins A and E. The interaction of K-bZIP with Cdk2 required the basic region of K-bZIP and inhibited Cdk2 phosphorylation of histone H1. The basic region of K-bZIP contains two RXL motifs (187). RXL motifs mediate interaction of inhibitors and substrates of cdks to the cyclin subunit (193), suggesting that K-bZIP binds to cyclins as well as Cdk2. Interestingly, the two RXL sites are preceded by the sequence S/TPXK/R, which are motifs shown by Polson *et al.* to be phosphorylated by Cdk2 (180). Transfection of 293 cells with K-bZIP resulted in growth arrest of transfected cells and barely detectable levels of Cdk2 activity. Transfection of K-bZIP into BCBL-1 cells likewise resulted in growth arrest, which was augmented by TPA treatment. Growth arrest was also accompanied by a decrease in Cdk2 activity and an increase in p21/cip-1 and p27 levels (187).

These results indicate that K-bZIP can mediate cell cycle arrest during KSHV lytic replication by induction and stabilization of p21/cip-1 and C/EBP-alpha proteins. p21/cip-1 inhibits G_0 progression by blocking cyclin/cdk activity (194) and by preventing PCNA from activating DNA polymerase delta which is essential for cellular DNA replication (195). Induction and stabilization of C/EBP-alpha would result in further expression of p21/cip-1, inhibition of E2F transcription, and inhibition of Cdk2 and Cdk4. Furthermore, K-bZIP down-regulates Cdk2 activity.

Cdk2 associates with Cyclin E and regulates progression of the cell cycle from the G_1 to the S phase (196). Cdk2 also associates with Cyclin A, which is required during the S phase to regulate DNA replication (196, 197). Therefore, inhibition of Cdk2 activity by K-bZIP would further mediate cell cycle arrest at the G_1/S restriction point.

4.3. Lytic replication

KSHV has six genes with various levels of homology to the equivalent EBV, HSV, and human cytomegalovirus replication proteins (16). They are ORF9 (polymerase), ORF59 (polymerase processivity factor; PPF), ORF6 (single stranded DNA binding protein), ORF56 (primase), ORF40/41 (primase-associated factor), and ORF44 (helicase). Upon transfection of these six genes along with K8 into Vero cells, replication compartment (RC) –like structures were formed (198). RC structures are sub-nuclear domains which are filled with viral replication proteins and display active viral DNA synthesis (198). Furthermore, K-bZIP localized with PPF in the RC structures. These results suggest that K-bZIP may be involved in KSHV lytic DNA replication.

The EBV Rta protein is not only involved in transactivation of the EBV genome, it is also an origin binding protein which is required for lytic replication (183, 184). Lin *et al.* used a DNA binding site selection assay to identify KSHV genome sequences that bound K-bZIP either directly or indirectly (199). They identified an approximately 600 bp fragment that bound to K-bZIP from induced BCBL-1 cells as well as 293 transfected cells. This sequence was previously identified by others as a possible KSHV origin of lytic DNA replication (ori-lyt) (200) and had features of an origin of replication including several A-T rich palindromes (199). The presumed ori-lyt was cloned into a plasmid and used in a transient transfection replication assay in BCBL-1 cells. The two perfect A-T rich palindromes in ori-lyt, 16 and 18 bp long, were replicated in BCBL-1 cells transfected with an Rta expression vector used to induce lytic gene expression and replication. Deletion of the 18 bp palindrome inhibited DNA replication and reduced the ability of K-bZIP to bind (199). These results indicate that K-bZIP is a KSHV origin of replication binding protein and does mimic some of the functions of the EBV Zta protein.

4.4. Interaction with cellular proteins

Due to its similarity with the EBV Zta transactivator and the presence of a bZIP domain, K-bZIP was expected to be the second KSHV transactivator. However, K-bZIP only exhibits sequence-specific DNA binding activity to an origin of lytic replication and not to viral promoters and, therefore, may mediate transactivation by binding to cellular transactivators. K-bZIP has been shown to interact with several cellular proteins, including CBP; hSNF5, a component of the chromatin remodeling complex; and p53. Moreover, K-bZIP interacts with Rta and C/EBP-alpha to regulate viral gene expression as discussed below.

4.4.1. CBP

The EBV Zta protein interacts with CBP to enhance Zta-mediated transactivation and control cellular transcription (201, 202). Given that K-bZIP is presumed to be the evolutionary equivalent of Zta, Hwang *et al.* determined whether K-bZIP interacted with CBP and the functional consequence of this interaction (203). K-bZIP was found to interact with the C/H3 domain of CBP and co-localized with CBP in the nucleus of TPA-induced BCBL-1 cells. The C/H3 domain is a conserved region of CBP that has been shown to interact with the adenovirus E1A protein (204). CBP was demonstrated to bind to the basic region of K-bZIP but not to the leucine zipper (203). Although Zta interacts with CBP to mediate transcriptional activation and K-bZIP exhibits 37% sequence similarity with Zta in the bZIP region, K-bZIP has been shown to have properties that are distinct from Zta. Similar to E1A, which inhibits CBP-mediated activation of transcription (204), K-bZIP inhibited expression from the AP1 promoter and the HIV LTR in a CBP-dependent manner (203). CBP, similar to K-bZIP (198, 205), has been shown to bind to the promyelocytic leukemia bodies and be recruited to PML oncogenic bodies (206, 207). Furthermore, PML is a strong activator of nuclear hormone receptors (206). These results suggest that the interaction of K-bZIP with PML and CBP may be one mechanism by which K-bZIP may mediate transcriptional activities.

4.4.2. hSNF5

During a yeast two-hybrid screen of proteins that may interact with K-bZIP, K-bZIP was shown to have weak transactivating functions (208). Amino acids 1-115 of K-bZIP were identified as having the strongest transactivating activity. To facilitate the identification of proteins that bound to K-bZIP, a construct containing only amino acids 1-75 was used and the hSNF5 protein was identified (208). hSNF5 is a component of the SWI/SNF complex (209), an ATP-dependent chromatin remodeler which alters chromatin structure (40-42). The interaction of K-bZIP with hSNF5 was confirmed in 293T cells and TPA-treated BCBL-1 cells. In yeast, the interaction of K-bZIP with hSNF5 mediated transactivation (208); although, this has not been confirmed in mammalian cells and the functional consequence of K-bZIP binding to SWI/SNF was not determined. However, due to similarity with the EBV transactivator, Zta, and the fact that SWI/SNF is an important mediator of chromatin remodeling (40-42), K-bZIP may facilitate viral gene expression by targeting SWI/SNF to viral promoters and mediating the repositioning of nucleosomes.

4.4.3. p53

Similar to Zta (210), K-bZIP has been shown to bind to p53 (205, 211) and inhibit p53 transactivation (211). Mutational analysis of K-bZIP and p53 demonstrated that the interaction was localized to the bZIP region of K-bZIP and the C-terminal domain of p53. Furthermore, expression of only the bZIP domain of K-bZIP inhibited p53 transactivation (211). The functional significance of the ability of K-bZIP to bind to and inhibit p53 is not known. However, activation of p53 is known to induce growth arrest and apoptosis (212). K-bZIP inhibition of p53 to mediate growth arrest appears to be counter-productive to the ability of K-bZIP to induce growth arrest by p21/cip-

1 and C/EBP-alpha. However, the ability of K-bZIP to suppress the pro-apoptotic properties of p53 would be more critical to viral replication. Induction of apoptosis by p53 occurs via sequence-dependent transactivation as well as independent pathways (212), suggesting that K-bZIP inhibition of p53 transactivation may suppress apoptosis in infected cells allowing for increased production of viral progeny prior to cell death.

5. RTA AND K-bZIP INTERACT TO REGULATE VIRAL GENE EXPRESSION

Although the exact function of K-bZIP in KSHV infection is still not known, its positional and structural similarities with EBV Zta and its ability to interact with cellular proteins involved in transcription suggests that K-bZIP would likewise modulate transcription. K-bZIP has been shown to bind to Rta (155, 213, 214) and co-localize with Rta in the nucleus (213, 214). Numerous viral promoters contain Rta response elements (RRE), including K8, PAN, and ORF57 (109, 117, 119, 124, 127). Some RREs bind to Rta directly (119), while others may interact with Rta indirectly (122). K-bZIP can suppress Rta transactivation of the K8 (213) and ORF57 promoters (214) but not the PAN promoter (214). These results indicate that the suppression of the ORF57 and K8 promoters is mediated either by interaction of K-bZIP with a cellular protein or by interfering with the ability of Rta to interact with a cellular protein. Furthermore, stable expression of K-bZIP in the KSHV infected cell line, BCBL-1, repressed TPA-induced viral gene expression as well as viral DNA replication (214). Interestingly, Izumiya *et al.* determined that the basic region (aa 122-189) of K-bZIP bound to Rta (214), while Liao *et al.* identified the ZIP domain (aa 190-237) as the region that interacted with Rta (213). Each region, respectively, was shown to be involved in the suppression of Rta transactivation (213, 214). However, additional studies have demonstrated that K-bZIP could mediate transactivation of its own promoter and ORF50 with C/EBP-alpha alone or in combination with Rta (155, 156). Additionally, Rta, K-bZIP, and C/EBP-alpha bound to the ORF50 promoter *in vivo* as demonstrated by ChIP assays (156). Binding of K-bZIP and Rta to the ORF50 promoter required C/EBP-alpha as pre-clearing extracts prior to ChIP prevented precipitation of ORF50 DNA with either K-bZIP or Rta antibodies (156). Previous studies have demonstrated that K-bZIP and Rta each interact with C/EBP-alpha (154, 156). These results indicate that binding of Rta and K-bZIP requires C/EBP-alpha binding to its cognate sites in the ORF50 promoter. Moreover, modulation of Rta activity by K-bZIP may be required to ensure proper timing of viral DNA replication.

6. K9/v-IRF-1

The viral homolog of interferon regulatory factor (K9/vIRF) was first characterized when genomic sequencing was performed on a KSHV clone isolated from BC-1 cells (15). K9 is one of four IRF homologs (vIRF-1 to 4) that are tandemly arranged between ORFs 57 and 58 within the KSHV genome (215). Most vIRFs (1, 2, and 4) were found to be inducible upon NaB or TPA treatment. However, K9 has been the most intensely studied of the four homologues.

Early studies found vIRF-1 to be only 13% homologous to its cellular IRF counterparts but contained a conserved domain (aa 88-121) derived from the IRF family (216). vIRF-1, when compared to IFN-alpha-stimulated gene factor 3-gamma (ISGF3-gamma) and IFN consensus sequence binding protein (ICSBP), was found to contain a tryptophan-rich DNA binding domain within its N-terminus. However, this domain contains only two of the five conserved tryptophans whose positions are conserved in cellular IRFs. Three of these five tryptophan repeats have been shown to be critical for DNA recognition and contact (217). Therefore, this viral IRF homologue is unable to bind to the IFN-stimulated response elements (ISREs) located within the promoters of IFN-dependent genes (218). Within the C-terminus of vIRF-1 is an IRF transactivator/repressor region that is important for a number of functional interactions.

vIRF-1 has been shown to inhibit interferon type I gene promoters in infected cells by interfering with the transactivating activity of cellular IRFs. Thus, vIRF-1 may have more functional similarity to IRF-2, the antagonistic repressor of IRF-1, a transcriptional activator. As shown in a study by Li *et al.* (219), vIRF-1, when stably expressed, was able to inhibit IFN-mediated transcription similarly to IRF-2. Furthermore, vIRF-1 was able to transform NIH 3T3 cells, which displayed features of malignant fibrosarcomas in nude mice (218). GRIM19, a novel cell death-associated gene induced by IFN/retinoic acid (RA) treatment, was targeted for inhibition by vIRF-1, conferring a growth advantage to IFN/RA-treated cells (220). Lastly, vIRF-1 has been shown to confer resistance to tumor necrosis factor alpha (TNF-alpha)-induced apoptosis (221). However, a caveat to this potential oncogenic role is that loss of K9 in BCBL-1 cells does not alter the growth rate (219). This may be due to the presence of other viral oncogenes, such as v-Cyclin and v-Bcl2, or additional vIRF transcripts that have both distinct and overlapping functions.

Given the ability of vIRF-1 to repress transcription of IRF-1 and IRF-3-dependent promoters, vIRF-1 is thought to play a significant role in molecular mimicry. The mechanism of inhibition of transcription appears to occur through the sequestration of transcriptional co-activators, such CBP and p300. While vIRF-1 has been shown to be within the same complex as IRF-1, this interaction does not appear to be direct (222). vIRF-1 was demonstrated to interact with the C-terminus (aa 1623-2414) of p300 (221, 223) and compete out the binding of the p300/CBP-associated factor (P/CAF) (224). The consequence of this association was decreased histone acetyltransferase (HAT) activity and reduced expression of CBP/p300-dependent genes. In a related report, Lin *et al.* (223) revealed that vIRF-1 could block the formation of an IRF-3-DNA-CBP, but not IRF-3-DNA complex. This may be due, in part, to the interaction of vIRF-1 with the C-terminus of CBP. However, similar observations have demonstrated that increasing the amount of vIRF-1 reduces

the affinity of IRF-1 for DNA (221), suggesting that vIRF-1 may act as a dominant negative factor and alter the conformation of IRF targets.

Another important cellular target of vIRF-1 is the tumor suppressor protein, p53. vIRF-1 was shown to interact with p53 through its putative DNA binding region and the central region (225). This interaction leads to suppressed phosphorylation and acetylation of p53, and functional inactivation of p53. The consequence of repression of IRF family members and p53 by vIRF-1 is the decreased transactivation of several factors important for an immune response, cell cycle regulation, and apoptosis, including RANTES, IP-10, IFN-beta, p21/cip1, Bax, and CD95 (APO-1/Fas) (223, 225, 226). Suppression of host defenses contributes to the close interplay of immune evasion and tumorigenesis. This effect may be compounded by the ability of vIRF-1 to increase its own transcription (227) and that of several other viral proteins (219, 228). An early report suggested that expression of vIRF-1 may positively regulate the expression of several KSHV proteins such as vIL-6 and the viral polypeptides, p40 and p60 (219). Recently, Wang and Gao (227) demonstrated that the addition of exogenous vIRF-1 could transactivate the vIRF-1 promoter in a dose-dependent manner via two cis elements, Vac1 and Vac2. Similarly, GAL-vIRF (vIRF-1 tethered to the GAL4 DNA-binding domain) was able to transactivate the UAS_{GAL}-CAT reporter in a manner comparable to the GAL-VP16 construct (228). However, further studies are needed to establish whether vIRF-1 directly affects these promoters or if this increase is a downstream result of IRF and p53 inactivation.

7. K3 AND K5

Based on their sequence similarity with the major immediate early (IE1) gene of bovine herpesvirus 4, K3 and K5 were initially classified as immediate early gene IE1-B and IE1-A, respectively (229). Nevertheless, their classification as immediate early or early genes was not clear. Indeed, depending on the method of analysis, the PEL cell line (BCBL-1 or BC3 versus BC1) studied, as well as the chemical inducer used (NaB versus TPA) to induce viral reactivation, different groups classified these genes as immediate early (79, 80, 230, 231) or early (76, 77, 80). Extended analyses focusing on the transcription pattern of these two genes clearly showed their immediate-early expression profile (230, 231). Several features of the RNA molecules encoding the K3 protein, comprising multiple transcription start sites, multiple splice donor sites and potential alternative ATG usage, may explain the difficulty in classifying this gene (231). From these results, Rimessi *et al.* concluded that K3 is expressed as both immediate early and early transcripts (231). Discrepancies between studies on K5 gene expression have been suggested to be due to EBV co-infection of certain PEL cell lines such as BC-1, which could interfere with the expression of some KSHV transcripts following virus reactivation and may explain their delayed detection (230, 231). Finally, classification of the K5 gene as an immediate early transcript was confirmed by Okuno *et al.* who demonstrated that while K5 expression was not detected earlier than Rta by western blotting, the number of K5 positive cells was higher than that of Rta positive cells at all times after TPA treatment (232).

The K3 and K5 proteins share 40% amino acid identity with each other and belong to a recently recognized family of viral proteins found in several gamma-2 herpesviruses, as well as poxviruses (233). This family of proteins has an amino terminal zinc finger domain, which is homologous to the plant homeodomain (PHD), followed by two transmembrane domains and a cytoplasmic carboxy-terminal tail varying in size. The PHD domains, also known as leukemia-associated protein (LAP) zinc fingers, are characterized by seven cysteines and one histidine in the order C_4HC_3, which have been implicated in protein-protein interactions (234, 235). These domains are predicted to be structurally similar to RING domains found in the E3 ubiquitin ligases, which have been shown to participate in the transfer of ubiquitin to other proteins and to themselves (236). K3 and K5 are predominantly localized in the endoplasmic reticulum and their C-terminal tails possess a highly conserved short sequence termed the conserved region (CR) (230, 237).

K3 and K5 have been shown to function in immune evasion through down-regulation of major histocompatibility complex class I (MHC-I) but not MHC-II molecules (237-239). While K3 down-regulates all four allotypes, K5 only down-regulates histocompatibility leukocyte antigen A (HLA-A) and HLA-B2 allotypes (238). Furthermore, K5 also down-regulates the co-stimulatory molecules, ICAM-1 and B7.2, ligands for natural killer (NK) cell-mediated cytotoxicity receptors (240, 241). B7.2 is a cell surface protein involved in helper T cell activation, while ICAM-1 (intracellular adhesion protein-1) is an adhesion protein important for formation of the immunological synapse (242-245). Both K3 and K5 reduce the cell surface expression of these immune molecules by enhancing their endocytosis and not by interfering with their expression or their intracellular transport (237, 238, 246). During down-regulation of HLA molecules, phosphorylation of the highly conserved serine residues of the carboxy-terminal cytoplasmic tail of MHC-I, which has been implicated in intracellular trafficking, is inhibited (247). For these reasons, K3 and K5 have been termed Modulators of Immune Response 1 and 2 (MIR-1 and MIR-2), respectively (248). Mutational analysis of the zinc finger region of MIR-2, as well as deletion of the C-terminal cytoplasmic tail, have been shown to abrogate its activity (241). Sanchez *et al.* have also shown, by mutational analysis, that the C-terminal conserved region within MIR-2 is necessary but not sufficient for the down-regulation of MHC-I proteins. These results suggest that the PHD and the CR domains are involved in the possible recruitment of cellular machinery. On the other hand, generation of chimeras between MIR-1 and MIR-2 revealed that their transmembrane domains are responsible for the target specificity of MIR activity (248).

MIR-1 and MIR-2 have been shown to function, via their PHD domain, as membrane-bound E3 ubiquitin ligases. These ligases ubiquitinate the cytosolic

tail of their substrates by recruiting E2 ubiquitin-conjugated enzymes, and target substrates for endolysosomal degradation (249, 250). Coscoy and Ganem further demonstrated that the cytoplasmic tails of the substrate proteins require the presence of lysine residues, which are targeted for the addition of ubiquitin (249). To decipher the mechanisms leading to endocytosis and subsequent lysosomal degradation of the MIR-1 targeted molecules, Means *et al.* showed that these activities were functionally and genetically distinct (251). Indeed, they observed that the N-terminal zinc finger motif, the YAAV and NRTV sequences in the central region, as well as two di-acidic clusters at the C-terminal region of MIR1, were required for efficient down-regulation of MHC-I molecules. They then further showed that, while the N-terminal and central motifs of MIR-1 are involved in triggering internalization of MHC-I molecules and in redirecting them to the trans-Golgi network, the di-acidic cluster motifs in the C-terminal region of MIR-1 targeted these molecules to the lysosomal compartment. Finally, endosomal degradation of the ubiquitinated MHC-I molecules targeted by MIR-1 depended on the tumor susceptibility gene 101 (TSG101) pathway (252). TSG101 is an ubiquitin enzyme 2 variant protein involved in late endosomal sorting (253). Depletion of this gene by small interfering RNA in MIR1 expressing cells prevented MHC-I degradation and preserved the cell surface expression of MHC-I molecules, further demonstrating that MIR-1 induced ubiquitination of MHC-I molecules provides a signal for internalization and degradation (252). The ability of MIR-1 and MIR-2 to impair the host antiviral immune responses of virus-infected cells is believed to be an important factor in the *in vivo* persistence of KSHV-infected cells (254).

8. ADDITIONAL KSHV IMMEDIATE EARLY GENES

A number of IE transcripts identified are still uncharacterized or just beginning to be examined. These IE transcripts include K4.2, ORF45, ORF29b, and ORF57. While sequence analysis with GCG Blast could not identify homologues of K4.2, a Kyte-Doolittle hydrophobicity analysis suggests this IE protein may be a membrane protein as indicated by hydrophobic regions at the N- and C-termini (77).

To delineate the role of ORF45 in viral infection, Zhu *et al.* (255) initially screened a human lymphocyte cDNA library using the yeast two-hybrid system to determine ORF45 binding partners. IRF-7, a mediator of IFN-alpha gene expression, was found to reproducibly interact with ORF45. The authors observed predominately cytoplasmic co-localization of both IRF-7 and ORF45 within co-transfected cells. Additional analysis revealed that ORF45 was able to suppress virus-induced phosphorylation of IRF-7, an important initial step for homodimerization and translocation of IRF-7 to the nucleus upon viral infection. Thus, IRF-7 was sequestered within the cytoplasm in ORF45-expressing cells, leading to a concomitant decrease in the expression of the IFN-alpha gene. The association of ORF45 with IRF-7 may help to potentiate the expression of other viral proteins, especially within co-infections (256). Conversely, another study revealed ORF45 to be a cytoplasmic phosphoprotein that is associated with the KSHV virion as a possible tegument protein (257). This may well prove to be essential for *de novo* infections in order to repress the IFN pathway upon entry. Therefore, ORF45, an IE transcript (77, 80), appears to have a regulatory role in promoting viral escape from immunosurveillance.

Saveliev *et al.* (81) further mapped IE regions initially identified by Zhu *et al.* (77) and demonstrated that a 2.6-kb mRNA initiated upstream within IE region 1 (KIE-1) and extended into the KIE-4 region. This transcript was comprised of two exons, ORF48 and ORF29b, separated by a 20-kb intron. The ORF29b exon is the second exon of the ORF29 gene. While the ORF29 transcript encodes for one protein, the IE 2.6-kb mRNA appears to be a bi-cistronic transcript encoding both ORF48 and the predicted ORF29b protein. However, the role of these proteins in the KSHV life cycle has yet to be elucidated.

ORF57 of KSHV was found to be a nuclear protein homologous to other well-known post-transcriptional regulators in other herpesviruses, including ICP27 of HSV and ICP27-homologues from EBV and HVS (258, 259). ICP27 acts to regulate mRNA processing by inhibiting splicing of host transcripts and promoting the export of intronless viral transcripts from the nucleus (260, 261). ORF57 was demonstrated to act synergistically with Rta to activate transcription of the PAN/T1.1 and Kaposin promoters in luciferase reporter assays (258). While not a broad range activator, ORF57 was able to increase (40- to 50-fold) gene expression over that generated by Rta alone on the PAN promoter suggesting that ORF57 plays a potent role in the lytic transcription program. Further studies are needed to determine the exact mechanism of transactivation and selectivity of ORF57.

9. SUMMARY AND PERSPECTIVES

KSHV is the causative agent of three human proliferative disorders: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Herpesvirus gene expression and viral replication is a complex, tightly regulated process involving latent, immediate early, early, and late viral gene transcription. Typically upon *de novo* infection of host cells, herpesvirus proteins that are found in the tegument of the virus activate expression of the immediate early genes. One of the more well known herpesvirus tegument transactivators is VP16 from herpes simplex virus (HSV) (262). VP16 forms a complex with the transcription factor Oct-1 and the cell-proliferation factor HCF-1 to activate transcription of HSV immediate early genes. The tegument proteins of KSHV that may initiate viral gene expression are not known. However, several KSHV genes have been suggested as possible tegument genes, including ORFs 19, 63, 64, 67, and 75, based on sequence similarity with other known herpesvirus tegument transactivators (16) but additional

work is needed to determine what role they may play in the life cycle of KSHV.

Due to the lack of a suitable cell culture system, instead of using *de novo* infection to analyze KSHV IE gene expression, latently infected PEL cell lines, which are treated with NaB or TPA, have been used. During latent infection, the ORF50 promoter is silenced due to DNA methylation and a positioned nucleosome. Following induction, histone acetylation induced either by NaB, TSA, or ectopic expression of CBP, leads to nucleosomal remodeling and transcriptional activation of the ORF50 promoter. Additionally, based on the analysis of a number of reports, several candidate IE genes have been identified. They include ORF50, K8, K9, K3, K5, ORF57, ORF29b, ORF45, and K4.2.

ORF50, encoding the Rta protein, is absolutely required for initiating KSHV gene expression. Rta binds to specific sequences in several viral promoters and interacts with a wide variety of cellular proteins to mediate transactivation. Many of these cellular proteins are known transcription factors, including Oct-1, RBP-Jkappa, Stat3, C/EBP-alpha. A newly identified protein, K-RBP has been shown to bind to Rta to help mediate transactivation. However, although it appears that K-RBP may be a transcriptional repressor, its function in cellular transcription is not known. Portions of the KSHV genome in latently infected cells have been shown to be associated with histones and nucleosomes. Rta binds to CBP and SWI/SNF, both of which mediate chromatin remodeling, suggesting that the latent state of infected cells may be mediated by a repressive chromatin structure on the KSHV genome.

K-bZIP does not have any transactivating functions, although it does demonstrate some sequence identity with the EBV IE gene Zta, a co-linear genome organization, similar 3' splicing patterns, and the presence of a basic leucine zipper motif. However, K-bZIP does appear to be a lytic origin of replication binding protein and may be involved in lytic viral replication. K-bZIP also mediates cell cycle arrest by inducing and stabilizing p21/cip-1 and C/EBP-alpha, and inhibiting the kinase activity of Cdk2, which induces a G_1 arrest.

The other IE genes, K9/vIRF-1, K3, and K5 suppress various pathways of the immune response. Suppression of the immune response will allow virally infected cells to evade immune surveillance to allow continued replication and spread of the virus. But it may also facilitate the pathogenesis of Kaposi's sarcoma. The pathogenesis of KS is multi-factorial. KS is characterized by being highly vascular and contains a mixture of spindle cells, lymphocytes, erythrocytes, and atypical endothelial cells (263-265). Approximately 10% of spindle cells in KS lesions express KSHV lytic genes, including viral cytokines (vIL-6 and vMIP-1) and an anti-apoptotic protein (v-bcl2) (263, 265), suggesting that the lytic life cycle of KSHV may contribute directly to the pathogenesis of KS and that expression of viral proteins that would help infected cells evade the host immune response may also affect pathogenesis.

The function of several IE genes is not fully understood. K4.2 may be a membrane protein as indicated by hydrophobic domains at the N and C termini. ORF45 interacts with IRF-7, an interferon response gene, and sequesters it in the cytoplasm, possibly contributing to immune evasion by KSHV. ORF45 has also been found in the tegument of KSHV virions, which may allow KSHV to counteract an early antiviral response in infected cells. ORF29b is encoded from a bi-cistronic transcript but its function in the KSHV life cycle is not known. Lastly, ORF57 is a transcriptional activator, which acts synergistically with Rta to activate PAN/T1.1 and Kaposin gene expression.

Application of microarray technology for simultaneous expression measurements of all ORFs of virus species was recently shown to be possible (79, 80, 266). Furthermore, microarray has been paired with chromatin immunoprecipitation (ChIP-chip) to identify every binding site of 106 transcription factors in the yeast genome (267). As a means of studying multiple viruses using the microarray technology, we have recently generated a DNA microarray comprised of PCR products representative of almost all of the genes of eight different human RNA and DNA viruses, including KSHV (Ghedin *et al.*, submitted). Generating chromatin structural protein as well as transcription factor binding profiles for KSHV under different environmental conditions and at different time points will bring us closer to deciphering the complexities of gene regulation of this virus at a genome-wide scale *in vivo*.

A herpesvirus origin for KS was first suggested over 30 years ago (268). Nevertheless, it wasn't until 1994 that KSHV was identified (13) and 1996 when the viral genome was fully sequenced (16). In that time, much has been ascertained about individual genes and the roles that they may play in various stages of the virus life cycle as well as their roles in disease pathogenesis. Future studies, however, will require the development of an adequate *in vitro* system to study the viral life cycle, especially for those events that occur soon after *de novo* infection. Additionally, how the various IE genes interact to promote viral replication will provide greater insight for understanding the viral life cycle and potential therapies.

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Send correspondence to: Dr. Anne Pumfery, The George Washington University, Department of Biochemistry and Molecular Biology, 2300 I St. NW, Room 559, Washington, DC, 20037, USA, Tel: 202-994-4579, Fax: 202-994-1780, E-mail: bcmamp@gwumc.edu