CTMI (2006) 312:157–183

Regulation of KSHV Lytic Gene Expression

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Abstract The life cycle of KSHV, latency versus lytic replication, is mainly determined at the transcriptional regulation level. A viral immediate-early gene product, replication and transcription activator (RTA), has been identified as the molecular switch for initiation of the lytic gene expression program from latency. Here we review progress

on two key questions: how RTA gene expression is controlled by viral proteins and cellular signals and how RTA regulates the expression of downstream viral genes. We summarize the interactions of RTA with cellular and other viral proteins. We also discuss critical issues that must be addressed in the near future.

1 Introduction

Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), was discovered by its association with Kaposi sarcoma (KS), the most common form of cancer in human immunodeficiency virus type 1 (HIV-1)-infected patients (Chang et al. 1994). Subsequently, KSHV was found to be associated with two lymphoproliferative diseases related to HIV-1 infection: primary effusion lymphoma (PEL, a non-Hodgkin lymphoma) and multicentric Castleman disease (MCD) (Cesarman et al. 1995a; Soulier et al. 1995; Knowles and Cesarman 1997). The genomic DNA of KSHV (Russo et al. 1996) consists of a 140.5-kb-long unique coding region flanked by multiple 801-bp GC-rich terminal repeat sequences. The long unique coding region encodes over 80 open reading frames (ORFs), including many cellular gene homologues implicated in viral pathogenesis (Russo et al. 1996). Based on its genomic organization and other properties, KSHV has been classified as a member of the gamma 2-herpesvirus subfamily (Neipel and Fleckenstein 1999; Schulz and Moore 1999).

Like other herpesviruses, KSHV has two distinct phases in its life cycle, latency and lytic replication. Latency is characterized by persistence of the viral genome with expression of a limited set of viral genes. Once the virus is reactivated from latency and enters the lytic cycle, most viral genes are expressed in an orderly fashion (immediate-early, early, and late), leading to the production of infectious virions. KSHV gene expression patterns in latency and lytic phase have been studied with biopsies from KS/MCD/PEL, cell lines derived from PEL, and de novo infection of cultured cells in vitro. In situ hybridization studies of KS biopsies showed that the majority of tumor cells in the lesions contain KSHV genomic DNA and express viral latent transcripts, but in a subpopulation of tumor cells (1%–3%), viruses spontaneously enter the lytic cycle as evidenced by the expression of lytic transcripts (Staskus et al. 1997; Sun et al. 1999). Notably, these transcripts include those encoding for viral macrophage inflammatory protein-I (vMIP-I), viral interleukin-6 (vIL-6), viral Bcl-2 homologue, as well as an unusual polyadenylated nuclear RNA (PAN). Colocalization experiments have also shown that it is the same subpopulation of cells in KS lesions that spontaneously express both the

early (e.g., PAN RNA and vMIP-I) and late [e.g., major capsid protein (MCP) and small viral capsid protein (sVCA)] viral transcripts. These observations suggest a novel paradigm for pathogenesis and tumorigenesis by an oncogenic herpesvirus. Whereas transforming function of certain viral latent gene products expressed in a majority of the tumor cells may play direct roles in tumorigenesis, production of viral and cellular cytokines by adjacent infected cells in which KSHV undergoes lytic replication may create a favorable microenvironment to enhance the growth of latently infected cells and hence also contribute significantly to tumor development.

A number of human B cell lines derived from PEL have greatly facilitated the study of KSHV gene expression, gene function, and viral pathogenesis. Similar to KS biopsies, these PEL cell lines predominantly carry the virus in a latent state, with a small subset of cells in which the virus undergoes spontaneous reactivation (Cesarman et al. 1995b; Arvanitakis et al. 1996; Renne et al. 1996; Said et al. 1996; Staskus et al. 1997; Boshoff et al. 1998; Sun et al. 1999). Viral lytic replication in the whole cell population can be further activated by treating cells with inducing chemical agents such as phorbol esters or sodium butyrate (NaB) (Renne et al. 1996; Miller et al. 1997; Nicholas et al. 1997a; Sarid et al. 1998). With these cell lines (e.g., BC-1, BCBL-1, and BC-3), KSHV gene expression patterns have been more extensively examined through Northern blot analysis (Zhong et al. 1996; Sarid et al. 1998; Sun et al. 1999). These studies showed that the kinetics of KSHV gene expression is similar to those of other herpesviruses, that is, viral gene expression is restricted in latency, and lytic genes are expressed in a cascade fashion (immediate-early, early, and late), leading to the production of infectious virions (Renne et al. 1996; Vieira et al. 1997; Gradoville et al. 2000).

More recently, the temporal pattern of KSHV gene expression has been examined on a genomic scale with custom-built DNA microarrays (Jenner et al. 2001; Paulose-Murphy et al. 2001). These studies confirmed previous results on the kinetics of viral gene expression and identified a correlation between stages of gene expression and the function of the gene in viral replication. For instance, early genes such as those encoding proteins involved in viral DNA replication are expressed at relatively early time points, whereas those involved in virion assembly are expressed at later time points. Gene expression regulation of Epstein-Barr virus (EBV) has been extensively studied, providing information that is instructive for KSHV studies. In this review, we mainly discuss some of the regulatory steps in KSHV lytic gene expression and their implications in viral replication and pathogenesis.

2
Transcriptional Regulation of Immediate-Early Genes

2.1
Overview

Immediate-early genes are the first group of genes expressed during herpesvirus lytic replication (de novo infection or reactivation) and are usually defined by their transcription without requiring de novo protein synthesis. They generally encode regulatory proteins that either activate the downstream viral lytic gene expression cascade and/or modulate host cellular environment to facilitate viral replication. In early studies, because of a lack of an efficient permissive cell system for de novo infection, KSHV lytic gene expression was generally studied by treating latently infected PEL cell lines with chemical inducers, such as TPA or NaB, to activate the viral lytic cycle. Recently, studies on viral transcription in infected TIME, HFF cells, and 293 cells have been initiated, which provide additional information (Moses et al. 1999; Krishnan et al. 2004; Sharma-Walia et al. 2005). With chemical inducers and the protein synthesis inhibitor cycloheximide, several immediate-early genes of KSHV have been identified (Sarid et al. 1998; Sun et al. 1998; Lukac et al. 1999; Zhu et al. 1999; Gradoville et al. 2000; Haque et al. 2000; Saveliev et al. 2002). These include ORF50, ORF45, ORF K4.2, and a 4.5-kb mRNA species that corresponds to the viral genomic region between nt. 49419 and 54688. ORF K5 has also been reported as an immediate-early gene, even though its transcription is only resistant to cycloheximide at a low concentration (10 μg/ml) and its promoter is moderately activated (~5-fold) by another immediate-early protein, RTA (Haque et al. 2000).

The best-characterized immediate-early gene is RTA (replication and transcription activator, a homologue of EBV RTA, also called regulator of transcription activation, ART, Lyta, or ORF50). The major transcript is a 3.6-kb multiply spliced bicistronic message containing ORF50 and K8 (Sun et al. 1998; Zhu et al. 1999). The RTA protein is mainly encoded within ORF50. A splicing event upstream of ORF50 introduces a new methionine initiation codon and adds a coding region for an additional 60 amino acids (aa) to the N terminus of ORF50. The resulting RTA protein of 691 aa has a N-terminal DNA binding and dimerization domain, a C-terminal activation domain, and two nuclear localization signals (Lukac et al. 1998; Sun et al. 1998). The splicing event as well as the domain organization are highly conserved among the RTA homologues of gamma-herpesviruses including EBV and herpesvirus saimiri (HVS). The strongest homologous sequence with other RTA is found in between residues 103 and 202 within the DNA binding and dimerization domain (Sun et al. 1998). The activation domain also shows limited sequence

conservation with other RTA homologues as well as several cellular and viral transcription factors such as the herpes simplex virus-1 VP16 (Lukac et al. 1998). RTA protein has a predicted molecular mass of 73.7 kDa; however, when it is expressed in mammalian cells its migration pattern on Western blot reveals an apparent molecular mass of approximately 110 kDa, suggesting posttranslational modifications (Lukac et al. 1999; Song et al. 2002). Indeed, the RTA protein sequence contains numerous potential sites for phosphorylation. However, whether and how these sites are utilized and what other modification mechanisms are responsible for the observed increase of RTA molecular mass remain to be investigated.

Several lines of evidences have demonstrated that RTA serves as the "molecular switch" for KSHV life cycle. Expression of RTA alone in latently infected PEL cells disrupted latency and activated the expression of viral lytic genes such as vIL-6, PAN RNA, and ORF59; induction of viral late protein synthesis, ORF65 and K8.1, by RTA indicated that RTA drives the lytic cycle to completion (Lukac et al. 1998; Sun et al. 1998). Moreover, ectopic expression of RTA increased the production of DNase-resistant encapsidated viral DNA, providing the ultimate proof that RTA is capable of initiating and driving the complete viral lytic cycle, leading to the release of newly produced viral particles (Gradoville et al. 2000). On the other hand, introduction of dominant-negative mutant RTA proteins that lacked the C-terminal activation domain into latently infected cells reduced spontaneous viral reactivation, suggesting that RTA function is necessary for lytic reactivation (Lukac et al. 1999). A similar conclusion was reached by using the ribozyme approach to inhibit RTA expression (Zhu et al. 2004). Finally, deletion of RTA generated by the KSHV BAC system further confirmed the essential roles of RTA in viral lytic reactivation (Xu et al. 2005). Taken together, these data have demonstrated that RTA is both necessary and sufficient to mediate the switch from latency to lytic replication of KSHV in vitro. Because of the lack of an animal model for KSHV infection, several groups utilized a murine gammaherpesvirus-68 (MHV-68) to study the functions of RTA in vivo. The results suggested that RTA of MHV-68 also plays a central role in initiating the lytic replication cycle during infection of mice (Wu et al. 2001; Pavlova et al. 2003; Boname et al. 2004; Rickabaugh et al. 2004).

Because RTA serves as the molecular switch for viral life cycle, activation of the RTA promoter becomes the key to understanding the mechanisms controlling KSHV latency and reactivation. The activity of the RTA promoter on the viral genome should be determined by a number of factors: activators and repressors (both viral and cellular) as well as chromatin structures of the viral genome. The remainder of this section will be devoted to discussing the recent progress in understanding the transcriptional regulation of this key molecule.

2.2 Regulation of RTA Gene Transcription by KSHV Proteins

As a powerful transcriptional activator, RTA activates the expression of many viral genes, including itself (Seaman et al. 1999; Deng et al. 2000; Gradoville et al. 2000). Autoactivation of the immediate-early RTA gene represents an important strategy for KSHV to effectively respond to environmental stimuli and maximally activate the virus lytic cycle. A 0.5-kb RTA promoter sequence was activated 2.7-fold by RTA expression (Seaman et al. 1999). With a construct with a much longer upstream sequence, it was shown that a 3-kb RTA promoter is highly responsive to TPA and NaB. In addition, RTA autoactivated the 3-kb promoter reporter construct up to 144-fold, independent of other viral factors or B cell-specific factors. Furthermore, ectopic expression of RTA in latently infected cells activated the expression of the RTA gene from endogenous viral genomes as demonstrated by either ribonuclease protection assay of the 5′ untranslated region (Deng et al. 2000) or Northern blot analysis of the RTA bicistronic transcripts using a probe derived from the ORF K8 region (Gradoville et al. 2000).

Several studies were conducted to map the RTA response element (RRE) in RTA promoter. A luciferase reporter with the 950-bp fragment upstream of the RTA coding region was weakly activated four- to eightfold by RTA, and the response element was mapped to an octamer-binding site (Sakakibara et al. 2001). Electrophoretic mobility shift assay (EMSA) showed that cellular Oct1 protein binds to an octamer-binding site; however, an RTA-Oct-1 complex was not observed, suggesting that the RTA protein may not associate with the octamer-binding site strongly and therefore an indirect mechanism may be involved. Nevertheless, this 950-bp promoter supports only mild activation by RTA (up to 8-fold) in comparison to a 3-kb promoter (Deng et al. 2000) that can be highly activated (144-fold), indicating that other unidentified RREs may exist further upstream. Sequence analysis of this region reveals multiple binding sites for the cellular transcription factor RBPJ. Using a luciferase construct with 3-kb promoter sequences, Liang et al. revealed that autoactivation of this RTA promoter is significantly lowered in RBPJ-null cells (10-fold) but much stronger in wild-type cells (up to 70-fold) (Liang and Ganem 2003). These observations suggested that RBPJ, a known cellular partner of RTA (Liang et al. 2002) (see more details below), is involved in the autoactivation of RTA promoter. Indeed, Chang et al. have shown that a DNA-binding-defective RTA mutant is still competent to induce the ORF50 transcription in vivo and that the RTA promoter with multiple RBPJ-binding sites was autoactivated at a higher level than that lacking RBPJ-binding sites (Chang et al. 2005c). These lines of evidence suggested that autostimulation

of RTA promoter is mediated mainly through indirect non-RTA-binding mechanisms and involves at least two cellular proteins, RBPJ and Oct-1.

Several viral proteins have been reported to inhibit the expression of RTA (Cannon et al. 2006). Interestingly, RBPJ-binding sites are also involved in the repression of RTA expression by latency-associated nuclear antigen (LANA). LANA is a multifunctional protein that is essential for latency establishment and maintenance. LANA mediates viral episomal DNA persistence during latency by tethering the viral episomes to cellular mitotic chromosomes (Ballestas et al. 1999; Hu et al. 2002; Fejer et al. 2003; Grundhoff and Ganem 2003). It also promotes cell survival and regulates cell cycle progression by modulating various cellular targets, including p53, Rb, and Wnt pathways (Friborg et al. 1999; Fujimuro and Hayward 2003; An et al. 2005). More recently, it has been shown that LANA suppresses lytic reactivation by repressing not only the basal level expression of the RTA promoter but also RTA-mediated autoactivation (Lan et al. 2004). Intriguingly, LANA-mediated repression of RTA promoter depends on the RBPJ-binding sites (Lan et al. 2005) that are also involved in RTA autoactivation (see above). By directly interacting with RBPJ protein, LANA may not only recruit additional corepressors to suppress the transcription of the RTA gene but also repress RTA autoactivation activity by competing with RTA in RBPJ-binding. The fact that both positive and negative regulators of RTA gene expression use the same RBPJ-dependent mechanism suggests that the switch between latency and lytic reactivation is finely controlled by the levels of LANA and RTA proteins in virus-infected cells. Furthermore, RTA also induces LANA expression (Lan et al. 2005; Matsumura et al. 2005), providing a negative feedback in keeping viral lytic reactivation under check.

Although RTA expression is strictly controlled in latency, RTA and a subset of lytic genes are transiently expressed very early after viral entry and quickly replaced by latent gene expression (Krishnan et al. 2004). The mechanisms and physiological significance for such transient expression and repression are still unknown. One possibility is that some viral factors are directly brought into the infected cells in the form of virion proteins to effectively modulate the intracellular environment to facilitate viral replication, as suggested by data from different perspectives (Lu et al. 2005). An advantage of bringing viral regulatory factors into infected cells as virion-associated proteins is that these factors can directly interact with the host cellular environment as soon as they are delivered into the cells by virus entry, independent of transcription and translation. Alphaherpesviruses and betaherpesviruses are known to incorporate a number of regulatory gene products as tegument proteins. For example, herpes simplex virus-1 virion carries transcription factor VP-16 and virion host shut-off (VHS) proteins. However, little is known about the physiological

functions of any gammaherpesvirus virion proteins immediately after infection of the cell. RTA turns out to be one of the KSHV virion-associated proteins (Bechtel et al. 2005; Lan et al. 2005). It may be involved in triggering transient expression of some lytic genes that modulate the host cell for the benefit of viral infection and/or induce the expression of latency proteins (Lan et al. 2005; Matsumura et al. 2005) that eventually shut down the lytic gene expression and establish latency in some types of cells. Further studies are needed to address the roles of KSHV virion proteins in establishing viral infection.

2.3 Cellular Signaling Pathways That Control RTA Expression

A critical question that must be addressed in KSHV biology is what host cellular signals control RTA expression and thus the balance between viral latency and lytic replication. Chemical agents such as phorbol esters, NaB, 5′-azacytidine, and glycyrrhizic acid can reactivate KSHV in latently infected cells in culture (Cesarman et al. 1995b; Miller et al. 1996; Renne et al. 1996; Chen et al. 2001; Curreli et al. 2005). These agents, although not necessarily natural physiological inducers, provide valuable tools for studying the mechanism of KSHV reactivation and may reveal potential cellular pathways that may be involved. Phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) can activate protein kinase C (PKC) (Castagna et al. 1982), suggesting that signaling pathways initiated by PKC may contribute to physiologically relevant KSHV reactivation. Experiments of overexpression and selective inhibition indicated an essential but not sufficient role for PKCδ isoform in KSHV reactivation (Deutsch et al. 2004). In addition, activation of Ap-1 pathway has also been suggested to be involved in TPA-induced KSHV reactivation (Wang et al. 2004). A different group of chemical inducers such as NaB and 5′-azacytidine implicates the roles of chromosomal modulation in KSHV reactivation, which is discussed in Sect. 2.4.

Another approach to define the mechanism controlling the reactivation is to explore suspected physiological stimuli, such as inflammatory cytokines (Chang et al. 2000; Mercader et al. 2000; Wang et al. 2005) and hypoxia (Haque et al. 2003). Hypoxia has been proposed to be at the location where KS occurs frequently. Several neuron transmitters associated with stress responses have also been found to reactivate KSHV. One example is that epinephrine and norepinephrine efficiently reactivated lytic replication of KSHV in latently infected PEL cells via β-adrenergic activation of the cellular cyclic AMP/protein kinase A (PKA) signaling pathway (Chang et al. 2005b).

Many cellular signaling pathways have been found to positively regulate herpesvirus reactivation, but negative regulators have been less defined. It

has been found that the cellular transcription factor NF-κB, which is highly active in lymphocytes, is required for maintaining viral latency of KSHV and EBV (Brown et al. 2003, 2005; Chang et al. 2005b). Downregulation of NF-kB resulted in viral reactivation in latently infected B cell lines. On the other hand, overexpression of NF-κB in epithelial or fibroblast cells inhibited MHV-68 lytic replication and allowed the virus to establish persistent infection. NF-κB also inhibited the activation of viral lytic genes by RTA in reporter assays. The inhibition was also reversible by I-κB and regulated by the relative amount of RTA versus NF-κB in the cell. These data suggest that high levels of NF-κB activity in lymphocytes play a direct role in the establishment and maintenance of viral latency in these cells. NF-κB can be upregulated by a viral latent protein, vFLIP (An et al. 2003; Guasparri et al. 2004). In addition, MTX was found to inhibit the lytic cycle of KSHV (Curreli et al. 2002). Interestingly, MTX, a well-known DHFR inhibitor, acts in a DHFR-independent fashion in this case. The underlying mechanism is not clear, but potentially bears novelty. The potential link between transcriptional regulation and signaling originating from cellular metabolism is a topic to be explored.

2.4 Effect of Chromatin Architecture on RTA Gene Expression

It is well known that both DNA methylation and histone acetylation play critical roles in gene regulation through chromatin remodeling (Wu and Grunstein 2000). Not surprisingly, expression of the RTA gene on the KSHV genome is also regulated at the chromatin level. Treatment of BCBL-1 cells with 5-azacytidine, a DNA methyltransferase inhibitor, induced KSHV reactivation. Bisulfite genomic sequencing analysis confirmed that the RTA promoter region is methylated and the promoter of a latent gene LANA lacks methylation in latently infected cells. These results suggested that methylation status is critical for controlling the RTA promoter activity, and hence viral reactivation (Chen et al. 2001).

In addition to DNA methylation, the chromatin structure of the RTA promoter is also regulated by histone acetylation. Positively charged histones bind tightly to the phosphate backbone of DNA, keeping chromatin in a transcriptionally silent state. Acetylation of histones by histone acetylase (HAT) neutralizes the positive charges on histones and therefore disrupts the higher-order chromatin structures for easy access by transcription factors and RNA polymerase complex to initiate transcription. Histone deacetylation mediated by histone deacetylases (HDACs) restores a positive charge on histones, leading to a tightly supercoiled chromatin structure that is associated with transcription repression. It has been known that inhibitors of HDACs such as

NaB and trichostatin A can induce lytic reactivation of the KSHV in latently infected PEL cell lines (Riggs et al. 1977; Cousens et al. 1979; Miller et al. 1996). The responsive element was mapped to a GC box that binds Sp1/Sp3, over which transcriptional initiation site a nucleosome is located (Lu et al. 2003). In latently infected cells, the RTA promoter is associated with multiple HDACs (including HDACs 1, 5, and 7), whereas NaB treatment resulted in the rapid binding of the SWI/SNF chromatin remodeling complex. Cyclic AMP-response element binding protein (CREB)-binding protein (CBP) HAT was also shown to stimulate RTA transcription from a plasmid (Lu et al. 2003). Taken together, these studies suggest that chromatin remodeling of the RTA promoter region, including histone acetylation and DNA demethylation, is a critical step in the switch from latency to lytic reactivation.

3
Transcriptional Regulation of Early Genes

3.1
Overview

KSHV early genes generally encode proteins that are involved in nucleic acid metabolism and modulation of cellular functions. They are usually under the control of the immediate-early proteins. A number of KSHV early lytic genes have been shown to be activated by RTA through direct or indirect mechanisms. They include PAN RNA (also called nut-1 or T1.1), Kaposin (Kpsn; also called K12), ORF57 (a posttranscriptional activator), K-bZIP (the KSHV homologue of ZEBRA encoded by EBV; also called K8), vIL-6, K5, K9 [viral interferon (IFN) regulatory factor or vIRF], K14 (viral OX-2), K15, ORF6 (single-stranded DNA binding protein), ORF59 (DNA polymerase-associated processivity factor), ORF21 (viral thymidine kinase or vTK), ORF74 (viral G protein -coupled receptor) (Zhang et al. 1998; Chang et al. 2000; Chen et al. 2000; Haque et al. 2000; Duan et al. 2001; Jeong et al. 2001; Lukac et al. 2001; Song et al. 2001; Deng et al. 2002b; Wong and Damania 2006). Interactions of RTA with some of these viral promoters have been characterized in detail. In this section, we will use examples to illustrate how RTA differentially activates the downstream gene expression through various mechanisms.

3.2
RTA Activation of PAN and Kaposin Genes

PAN RNA is a novel 1.1- to 1.2-kb noncoding polyadenylated transcript, forming a speckled pattern in the nucleus and colocalizing with cellular Sm pro-

tein. Therefore, PAN RNA possesses features of both U snRNA and mRNA (Sun et al. 1996; Zhong and Ganem 1997). It is the most abundant transcript expressed during KSHV lytic phase, comprising approximately 80% of the total polyadenylated RNA in infected cells. Although its function in KSHV replication and pathogenesis has remained a mystery, PAN RNA serves as a good model to define the mechanism of RTA activation. With a transient transfection reporter assay, it was demonstrated that RTA activates the PAN promoter up to 7,000-fold and that this activation is independent of other KSHV or B cell-specific factors (Song et al. 2001). Deletion analysis further mapped the RTA-responsive element (RRE) to a 31-bp region of the PAN promoter. EMSAs showed that RTA directly binds to the PAN RRE and forms a highly stable complex (Song et al. 2001). Independently, Chang et al. also mapped PAN RRE to a 25-bp region (Chang et al. 2002), which is contained within the 31-bp RRE mapped by Song et al. previously (Song et al. 2001). A detailed analysis of specific interactions between RTA and the PAN promoter showed that RTA has a strong affinity for the RRE in the PAN promoter, which is reflected in the K_d at the nanomolar range. In addition, the minimal length for RTA binding was mapped to a 30-bp region spanning from −74 to −45 of the PAN promoter (with transcription initiation site of PAN RNA at nt 28667 on KSHV genome set as +1). Results from methylation interference assay, deletion analysis, and extensive mutagenesis study using both reporter assays and EMSAs correlated with one another and revealed base pairs critical for both RTA binding in vitro and RTA transactivation in vivo (Song et al. 2002). These studies were performed with the N-terminal half of RTA protein purified from *Escherichia coli* because the full-length protein was less efficient in binding, a problem that hindered many investigators. Only recently has the Miller lab overcome the hurdle by discovering that the C-terminal end of RTA inhibits the DNA binding activity (Chang and Miller 2004).

A sequence analysis of the KSHV genome revealed that a region in Kaposin promoter bears remarkable homology to the PAN RRE sequence. The Kaposin gene is abundantly expressed during latency and strongly induced during the viral lytic cycle. Kaposin A (K12) was reported to possess cellular transformation ability (Muralidhar et al. 1998, 2000; Kliche et al. 2001). Kaposin B increases the production of cytokines via activating the p38/MK2 pathway that is known to stabilize the AU-rich element-containing transcripts as seen for most cytokines (Sadler et al. 1999; McCormick and Ganem 2005). This 25-bp homologous region in the Kaposin promoter contains a consecutive stretch of 16-bp matches and additional 5-bp matches downstream. When tested in EMSA, this region conferred binding to RTA, although at a lower affinity. Mutation of two nucleotides (CC→TG) so that the mutated Kaposin promoter sequence (Kpsn/TG) has 19 consecutive matches plus additional

4-bp matches showed that its binding affinity for RTA is higher than that of the Kaposin promoter but still lower than that of the PAN promoter (Song et al. 2003). This result indicated that the sequences downstream of the 16-bp homology are also important for binding by RTA, consistent with the previous analysis of the PAN promoter (Song et al. 2003). Deletion analyses of the Kaposin promoter sequences have further confirmed that the 16-bp homologous region is essential for RTA binding and *trans* -activation and that the 5′ and 3′ flanking sequences also play important roles (Chang et al. 2002).

3.3 RTA Activation of ORF57 and vMIP1 Genes

ORF57 encodes a posttranscriptional regulator that is conserved in herpesviruses. Its expression is upregulated by RTA expression (Kirshner et al. 1999, 2000; Duan et al. 2001). Using luciferase reporter assays, Lukac et al. identified a core 25-bp region in the ORF57 promoter that is responsive for RTA activation (Lukac et al. 2001). When Liang et al. first discovered the protein-protein interactions between RTA and a cellular DNA-binding factor RBPJ, they noticed an authentic RBPJ-binding site GTGGGAA within this 25-bp ORF57 RRE and showed that mutation of this site almost completely abolished RTA activation (Liang et al. 2002). Furthermore, RTA fails to activate ORF57 promoter in RBPJ-null cells while such activation can be restored by cotransfection of an RBPJ-encoding vector (Liang et al. 2002). These observations clearly demonstrated that RTA activation of ORF57 gene mainly depends on the cellular protein RBPJ bound to the RBPJ-binding site within the identified 25-bp RRE. This conclusion was further supported by a recent study from the Miller lab (Chang et al. 2005c), which showed that RBPJ rather than RTA binds ORF57 promoter in EMSA and that DNA-binding-deficient mutants of RTA can still activate ORF57 promoter but not promoters of PAN and Kaposin. Moreover, an RRE with similarity to that of ORF57 was identified in the promoter of vMIP-I (a virus-encoded chemokine homologue), and studies using EMSA and mutational analysis demonstrated that it too contains a real RBPJ-binding site and that RTA activates vMIP-I through protein-protein interaction with the bound RBPJ protein (Chang et al. 2005c).

3.4 RTA Activation of IL-6 Genes

KSHV encodes homologues of several cellular cytokines and chemokines, one of which is viral IL-6 (vIL-6). vIL-6 is encoded by ORF K2 and shares 25% amino acid identity with human IL-6. Similar to its human counterpart, vIL-6

promotes growth and proliferation of IL-6-dependent human and murine hybridoma B cell lines (Moore et al. 1996; Nicholas et al. 1997b). vIL-6 also activates multiple signal transduction pathways, including JAK/STAT and Ras-MAP kinase pathways (Molden et al. 1997; Osborne et al. 1999). The vIL-6 transcript is one of the most abundant viral mRNAs expressed during lytic phase in PEL-derived cells (Sun et al. 1999), and its expression is under the control of RTA as well (Deng et al. 2002b). Through reporter assays and EMSAs, the vIL-6 RRE has been mapped to a 26-bp region, which, however, bears no similarities to either the PAN RRE or the ORF57 RRE.

Defining the vIL-6 transcription unit has also revealed the presence of two transcriptional initiation sites, and hence two promoters (Deng et al. 2002b). The existence of two promoters is intriguing, especially in light of the variable vIL-6 levels observed in KS, PEL, and MCD samples. In situ hybridization and immunohistochemistry studies demonstrated that vIL-6 is expressed at higher levels in PEL and MCD than in KS, and the average levels of vIL-6 expressed in individual infected PEL and MCD cells are greater by at least an order of magnitude than those in KS cells (Staskus et al. 1999). The presence of two promoters allows for differential regulation of vIL-6 gene expression in cell- and tissue-specific environments, which may lead to different manifestations in distinct malignancies associated with KSHV. In this aspect, it is interesting to note that the human cellular IL-6 (hIL-6) gene is also strongly upregulated by RTA (Deng et al. 2002a). It is remarkable that the virus takes multiple approaches to upregulate and maintain IL-6 levels during latency and lytic replication, which involve the roles from v-FLIP, RTA, and Kaposin, as well as encoding a viral homologue of IL-6. This strongly implicates the essential roles of IL-6-related functions in KSHV life cycle.

3.5
RTA Activation of Other KSHV Early Lytic Genes

A virally encoded G protein-coupled receptor (vGPCR) plays important roles in KSHV-induced angiogenesis. The most abundant vGPCR-containing transcripts are bicistronic RNAs with K14 (Nador et al. 2001) at the 5′ end and vGPCR at the 3′ end. This K14/vGPCR transcript is strongly induced during lytic reactivation (Kirshner et al. 1999, 2000; Nador et al. 2001; Chiou et al. 2002). The promoter governing this transcript is highly responsive to RTA activation, in which three putative RREs (sites A, B, and C) were identified through deletion and mutation mapping (Liang and Ganem 2004). None of these sites binds directly to RTA; rather, both sites A and C bind to RBP-J, whereas site B binds to yet-unknown cellular factors. The importance of RBP-J in the transcription of K14/vGPCR mRNAs is demonstrated by the observa-

tion that RTA activation of K14/vGPCR promoter is significantly inhibited in cells lacking RBP-J (Liang and Ganem 2004). Interestingly, a recent study (Matsumura et al. 2005) has shown that RTA uses these same RREs to activate the latency transcript (LT) locus, encoding LANA (ORF73), v-cyclin (ORF72), and v-FLIP (ORF71), which are transcribed in the opposite direction of K14/vGPCR. Another study (Zhang et al. 2005), however, has shown that RTA can directly bind to and activate through IFN-stimulated response element (ISRE) and that one such ISRE is present within the K14/vGPCR promoter, partially overlapping site A (Liang and Ganem 2004). RTA was further shown to selectively induce some cellular IFN-responsive genes (IRGs) including ISG-54, MxA, and STAF50 (Zhang et al. 2005), whose in vivo functions in viral replication and pathogenesis wait further studies.

The KSHV K-bZIP (KSHV basic leucine zipper) or K8 protein is a homologue of the EBV ZEBRA (Zta), one of the two immediate-early proteins that control the life cycle of EBV. However, the KSHV K-bZIP is an early gene, whose expression is activated by RTA (Sun et al. 1998; Lin et al. 1999) and has been shown to play an essential role in viral DNA replication (Lin et al. 2003) and cell cycle regulation. Wang et al. reported that K8 gene is differentially transcribed during immediate-early (IE) and delayed-early stages with different promoters (Wang et al. 2004). K8 IE promoter can be activated by butyrate, possibly through Sp1-binding sites. K8 delayed-early promoter is activated by RTA, the mechanism of which has been characterized in detail by several groups (Lukac et al. 2001; Seaman and Quinlivan 2003; Wang et al. 2003, 2004). A total of three RREs were identified. RRE-I, via cooperative binding of RTA, RAP (K-bZIP), and cellular factor C/EBPα, has a minor effect in B cells but more in 293 cells. RRE-II plays a major role via a non-DNA-binding mechanism in both 293 and B cells, the details of which are yet to be characterized. RRE-III contains a standard RBPJ-binding site, mutation of which causes a 2.5-fold transcriptional reduction in both cell types.

KSHV vIRF (K9) has differential transcription patterns during latency and lytic phase (Chen et al. 2000). Its lytic promoter is highly responsive to RTA activation, and the RREs were finely mapped to two regions that have no sequence homology and cannot directly bind RTA in vitro (Ueda et al. 2002). Multiple cellular factors were found to bind to these elements, and their binding correlated with the RTA responsiveness, suggesting an indirect mechanism for RTA to act through cellular factor(s) (Ueda et al. 2002). The response of the viral *tk* promoter to RTA appears to require Sp1-binding sites (Zhang et al. 1998) and also the RBPJ protein (Liang unpublished data).

4
Viral and Cellular Factors That Interact with RTA Protein

4.1
Overview

RTA must function through interactions via other proteins. Multiple viral and cellular proteins have been identified to interact with RTA, which is consistent with the observation that RTA is a multifunctional protein. However, the challenge is to define the biological relevance of such interactions. Current progress is summarized here.

4.2
RBP-J as a Coactivator of RTA

One of the best-characterized RTA-interacting cellular proteins is the DNA-binding transcription factor RBP-J (Liang et al. 2002). RBP-J is a downstream target of the cellular Notch signal pathway. It recognizes the specific DNA sequence GTGGGAA and acts as transcriptional repressor by recruiting a corepressor complex. On activation, the Notch molecule spanning the cell membrane is cleaved to release the intracellular domain, which translocates to the nucleus, where it binds RBP-J, replaces the corepressor complex, and activates the target promoters using its intrinsic activation domain. Viral proteins such as EBV EBNA2 and KSHV RTA have been shown to pirate this cellular pathway in order to activate target promoters containing RBPJ-binding sites. Like Notch molecule and EBNA2, RTA also binds to the central repressor domain of RBP-J, and, by competing off the corepressor complex, activates target promoters using its strong activation domain. So far the mechanism of RBPJ-dependent RTA activation has been utilized by multiple RTA target promoters, such as ORF57, TK, K14/vGPCR, RTA, K3, K5, vIL-6, and vMIP-1 (Liang et al. 2002; Liang and Ganem 2003, 2004; Chang et al. 2005a, 2005c; Lan et al. 2005). The biological functions of RBP-J in the KSHV life cycle have been evaluated with a mouse RBPJ-knockout cell line (Liang and Ganem 2003), in which KSHV can still establish latency but RTA-induced lytic reactivation is completely abolished. This is likely caused by the defective expression of multiple lytic genes that depend on RBPJ for activation by RTA.

4.3
Other Cellular Proteins Interacting with RTA

Other RTA-interacting cellular transcription factors include CCAAT/enhancer-binding protein-α (C/EBPα) (Wang et al. 2003) and STAT3 (Gwack

et al. 2002). C/EBPα is a member of the leucine zipper family of transcription factors. Potential C/EBPα-binding sites are found in the promoters of K-bZIP, PAN, ORF57 (MTA), and RTA, whose activations can be cooperatively activated by RTA and C/EBPα (Wang et al. 2003). STATs are a family of proteins that is phosphorylated and activated by signaling pathways of various cytokines and growth factors. Activated STATs form homo- or heterodimers and translocate to the nucleus, where they bind to specific DNA response elements. Gwack et al. showed that interaction between RTA and STAT3 leads to STAT3 dimerization and nuclear translocation in the absence of phosphorylation and that RTA is able to induce STAT3-mediated transcription (Gwack et al. 2002). However, the biological roles of STAT3 in KSHV replication and pathogenesis have not yet been characterized.

Using GST pull-down combined with mass spectrometry, Gwack et al. identified multiple cellular transcription cofactors that can interact with RTA, including the CBP HAT, SWI/SNF complex, and TRAP/Mediator complex (Gwack et al. 2003). These cofactors interact with the RTA activation domain and presumably function to modulate chromatin structures of viral promoters and recruit general transcription factors to initiate viral gene expression. RTA also interacts with the cellular poly(ADP-ribose) polymerase I (PARP-1) and a human homologue of Ste20-related kinase from chicken (hKFC) (Gwack et al. 2003) through its serine/threonine-rich region. It was shown that PARP-1 can poly(ADP)-ribosylate RTA and hKFC can phosphorylate RTA (Gwack et al. 2003). Both PARP-1 and hKFC repress RTA transcriptional activity in vitro and suppress RTA-mediated lytic reactivation in vivo. The KSHV RTA-binding protein (K-RBP, or MGC2663) contains potential zinc finger domains and was shown to cooperate with RTA in activation of target genes such as ORF57, K8, and vMIP-1 (Wang et al. 2001). Using nuclear extracts for EMSA, Chang et al. showed that cellular proteins such as YY1 and Sp1 also bind to the sequences within the identified RREs in the promoters of PAN, Kpsn, ORF57, and vMIP-I genes (Chang et al. 2002, 2005c; Chang and Miller 2004). However, whether these cellular proteins contribute to RTA activation of the respective promoters and, if so, whether they act by protein-protein interactions with RTA remain to be studied.

4.4 Viral Proteins Interacting with RTA

In addition to cellular factors, RTA also interacts with many viral proteins that function to modulate its activity. We have described that viral latency protein LANA interacts with RTA and antagonizes its autoactivation activity and thus keeps lytic reactivation under check (Sect. 2.2). A viral early protein KbZIP was

shown to interact directly with RTA and to repress RTA activation in promoter-dependent manner, that is, it suppresses RTA activation of promoters of ORF57 and KbZIP but not of PAN. There is evidence suggesting (Izumiya et al. 2005) that sumoylation plays an important role in KbZIP's transcriptional repression activity. KbZIP represses RTA activation of a subset of promoters, which was suggested to contribute to the highly controlled expression of viral lytic genes. However, the significance of such control during viral infection is not yet understood. Another viral early protein, ORF57 (MTA), recently shown to interact with RTA in vitro and in vivo (Malik et al. 2004), cooperates with RTA to enhance the expression of target genes at a posttranscriptional level (Kirshner et al. 2000). The recent finding that RTA contains ubiquitin E3 ligase activity and targets interferon regulatory factor 7 (IRF-7) suggested a novel role of RTA in downregulating immune responses (Yu et al. 2005).

4.5 Other Regulators of RTA Activity

In addition to binding RTA directly, some regulators of RTA activity may indirectly modulate RTA transcriptional activity. Song et al. has shown that HMGB1 (also called HMG-1) protein, a highly conserved nonhistone chromatin protein, promotes RTA binding to different RTA target sites in vitro and stimulates RTA activation of target genes in vivo. The importance of HMGB1 in viral gene expression and replication of gammaherpesvirus was highlighted by a study using MHV-68, whose viral gene expression, as well as viral replication, was significantly reduced in HMGB1-deficient cells but can be partially restored by HMGB1 transfection (Song et al. 2004). A recent study by Wang et al showed that cellular IRF-7, an essential gene for IFN-α and -β response, competes with RTA for binding to the RTA response element and thus negatively regulates RTA activation of target genes such as ORF 57 (Wang et al. 2005). This implicates that host interferon responses, on KSHV infection, may play a role in suppressing lytic gene expression by attenuating RTA transcriptional activity by IRF-7. In addition, the stability and the DNA binding activity of the RTA protein are autoregulated through its distinct domains (Chang et al. 2005c).

5 Perspectives

After the virus enters the cell, viral gene expression control is the main determinant of viral replication, especially in the case of reactivation. Identification

of RTA as the key molecular switch for KSHV gene expression serves as the starting point for defining the viral gene expression cascade. Despite significant progress during the last decade, we are still faced with gaps in our knowledge that are critical in understanding the viral gene expression program and its underlying mechanisms. Among these issues, we would like to discuss the following five challenges.

First, we know very little about signaling upstream of RTA that controls the initiation of the lytic cycle, in comparison to the events immediately downstream of RTA. Cellular signals that positively or negatively regulate RTA expression or activity have not been systematically identified. With the available genomic approaches, this question can be addressed in the near future. It is expected that multiple cellular signaling pathways affect the balance between latency and lytic replication. The next challenge is how to define the combinatory effects of these multiple signals that simultaneously act on the infected cells. The current approaches in molecular or cellular biology usually examine one or two signaling pathways at a time. New methods must be introduced to define the biological effects of multiple inputs. This advance will help us to understand the mechanism coupling viral reactivation with cellular differentiation and associated pathogenesis (Young et al. 1991; Longworth and Laimins 2004; Johnson et al. 2005). On a related issue, we expect to see significant progress in defining the effects of virion proteins on RTA expression during de novo infection and the effects of chromatin structure on reactivation from latency.

Second, the activation of early genes by RTA was mostly studied with reporter assays. It requires direct evidence for the interactions between RTA or its partners with their DNA targets. The temporal relationship among the activation of the viral downstream genes has not been defined, although viral gene array data provide some hints at a low resolution. Chromosomal immunoprecipitation should facilitate the construction of gene expression cascade with a clearer molecular definition. A similar approach should be applied to cellular genes regulated by RTA.

Third, some lytic genes are differentially expressed among the KSHV-associated diseases (KS, PEL, and MCD). Such differential expression may contribute to the tissue tropism and the diverse disease outcomes of KSHV infection. We expect to see more studies addressing how different cellular and microenvironmental signals affect the expression of viral genes and how these varied expressions may alter disease pathogenesis.

Fourth, a gap in the current understanding of KSHV gene expression cascade is how late gene expression is controlled. Because some early genes and most late genes are not directly activated by RTA, the intermediator(s) functioning downstream of RTA must be identified. A current assumption

is that the late genes will be expressed once the viral DNA is replicated. How viral late gene expression is coupled to viral DNA replication is a long-standing question. Our recent study with MHV-68 suggested that additional viral factors are required in addition to the viral DNA replication in *cis*. The mechanism controlling late gene expression is a less well-defined area in the herpesvirus field.

Finally, the biological relevance of the majority of the studies reviewed here must be validated in the context of viral replication. The availability of a BAC system to manipulate the KSHV genome will greatly facilitate such work (Zhou et al. 2002; Gao et al. 2003). The next challenge is to apply the knowledge to clinical applications for both tumor therapy and prevention. Based on the concept that KSHV lytic replication plays a direct role in tumor development by providing a favorable microenvironment, inhibition of lytic gene expression may have therapeutic values. On the other hand, intentional induction of lytic gene expression in the presence of a gancyclovir-type drug may drive the destruction of tumor lesions because of the elevated immune responses and the bystander killing effect of gancyclovir.

Acknowledgements The authors appreciate the careful review and editing by Dr. Hinh Ly and Ms. Joyce X. Wu.

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