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The Rta/Orf50 Transactivator Proteins of the Gamma-Herpesviridae

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Abstract The replication and transcription activator protein, Rta, is encoded by *Orf50* in Kaposi sarcoma-associated herpesvirus (KSHV) and other known gammaherpesviruses including Epstein-Barr virus (EBV), rhesus rhadinovirus (RRV), herpesvirus saimiri (HVS), and murine herpesvirus 68 (MHV-68). Each Rta/Orf50 homologue of each gammaherpesvirus plays a pivotal role in the initiation of viral lytic gene expression and lytic reactivation from latency. Here we discuss the Rta/Orf50 of KSHV in comparison to the Rta/Orf50s of other gammaherpesviruses in an effort to identify structural motifs, mechanisms of action, and modulating host factors.

1 Introduction

As all members of the *Herpesviridae*, the gammaherpesviruses can establish either a latent or lytic life cycle within host cells. During latency, only a few viral genes are transcribed and the virus exists as a nonintegrated circular episome within the nucleus of the infected cell (Fakhari and Dittmer 2002; Jenner et al. 2001; Paulose-Murphy et al. 2001; Sarid et al. 1998; Zhong et al. 1996). B cell latency can be disrupted by host cell signaling, such as B cell receptor cross-linking, which leads to the sequential expression of several subsets of lytic genes: immediate-early genes (IE) that encode viral transcriptional regulators; delayed-early genes (DE) that encode proteins involved in viral DNA replication, and late genes (L) that encode viral structural proteins. Herpesvirus lytic gene expression follows this temporal and sequential cascade, ultimately resulting in the production of progeny virions and destruction of and egress from the infected host cell. The Rta/Orf50 switch protein is essential to initiate lytic reactivation of all gammaherpesviruses: Epstein-Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV), rhesus rhadinovirus (RRV), herpesvirus saimiri (HVS), and murid herpesvirus 68 (MHV-68).

2 Immediate-Early Genes

Immediate-early genes define mRNAs that are transcribed in the presence of protein synthesis inhibitors, such as cycloheximide. This applies to herpesvirus genes after de novo infection of permissive cells (Roizman 1996) but also to cellular genes after serum stimulation. Lau and Nathans identified cellular immediate-early genes (*jun* / *fos*) because they constituted the first wave of mRNAs after serum stimulation of mouse fibroblasts (Lau and Nathans 1985, 1987). *jun/fos* mRNA levels were induced within 10 min after addition of serum

and declined shortly thereafter. By comparison, the induction of c-myc, a cellular early mRNA, was delayed. c-Myc mRNA peaked at 20–45 min, after the wave of immediate-early mRNAs subsided, and stayed induced for longer periods of time. The definition of early genes for herpesviruses is more strict: Early gene transcription is dependent on immediate-early transactivators independent of the time frame. Herpesvirus immediate-early transactivators are necessary and sufficient to initiate viral replication (McKnight et al. 1987; Triezenberg et al. 1988).

Rta/Orf50 is an immediate-early protein of rhadinoviruses. It is necessary and sufficient to drive lytic replication for KSHV, HVS, RRV, and MHV-68. Ectopic expression of Rta/Orf50 will reactivate virus from latency (sufficient); deletion of Rta/Orf50 or inhibition by a dominant-negative mutant will prevent lytic reactivation and replication (necessary). Although other rhadinovirus mRNAs are transcribed in the presence of cycloheximide (CHX) (Orf57/Mta, K8/Zta, Orf45) and are therefore considered immediate-early genes, their gene products are not sufficient to reactivate virus from latency. Whether any of these are necessary for lytic replication remains to be established.

Figure 1 shows an array analysis of RRV transcription at 6 h after de novo infection in the presence or absence of permissive fibroblasts (from Dittmer et al. 2005). Here, the levels for each viral mRNA were measured by quantitative real-time RT-PCR, and for each viral mRNA the number of cycles that were required to obtain a fixed amount of product was plotted. Rta/Orf50 is the most abundant mRNA at early times after infection and is unaffected by CHX. In contrast, the majority of RRV transcripts are not transcribed that early in the infection process (requiring more than 40 cycles of PCR to detect a signal under either condition) or are significantly inhibited by CHX. The latter includes mRNAs driven by Rta/Orf50-responsive promoters. But there are also a significant number of mRNAs that were transcribed in the presence of the protein synthesis inhibitor. By definition these are immediate-early genes, and their transcription is dependent only on preformed (i.e., immediate early) cellular regulators or RRV virion transactivators. Yet at the same time many of these genes are also Rta/Orf50-responsive. Rta/Orf50's own promoter falls into this class. In the case of the Rta/Orf50 promoter, transactivation by Rta/Orf50 protein establishes a direct positive feedback loop that locks the lytic transcription cascade into place. On the basis of extensive transcriptional profiling, we would speculate that the gamma herpesviridae evolved a more plastic, less ridged transcriptional control program than the alpha and beta herpesviridae to cope with the various signaling events, cytokine exposures, and growth stimuli in the life of a latently infected lymphocytes.

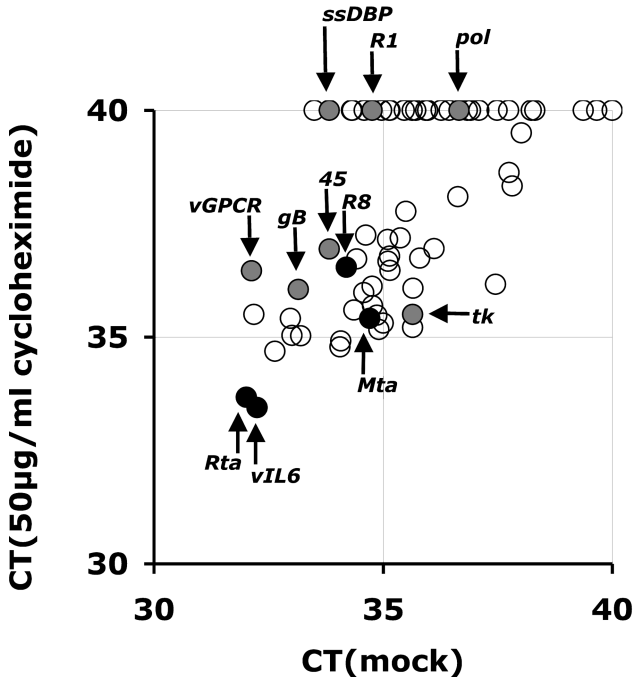

Fig. 1 Array analysis of RRV transcription at 6 h after de novo infection in the presence (*vertical*) or absence (*horizontal*) of cycloheximide in permissive fibroblasts (from Dittmer et al. 2005). Shown are relative mRNA levels on a log2 scale (*CT*). Known KSHV immediate-early genes are in *black*, known Rta targets in KSHV in *gray*, and all others in *open circles*

3 Lytic Reactivation

All gammaherpesviruses encode an Rta/Orf50 homologue, and each has been shown to play a pivotal role in the initiation of viral lytic gene expression and lytic reactivation from latency. Although the gene product of *Orf50*, named Rta (replication and transcription activator), is the only essential latent/lytic switch protein for the gamma-2-herpesviruses (rhadinoviruses), two proteins, Zta and Rta, independently can reactivate EBV, a gamma-1 or lymphocryptovirus, from latency. This difference in viral lytic switch proteins between the lymphocryptoviruses and rhadinoviruses indicates a marked difference in the precise molecular mechanisms of virus-mediated lytic reactivation of the two related subgroups of gammaherpesviruses.

3.1 Lymphocryptovirus—EBV

Many excellent reviews exist that describe EBV lytic reactivation in detail. Hence, we will only recount the basic tenets here to compare them vis-à-vis rhadinovirus lytic reactivation. The major viral lytic switch protein of EBV is considered to be Zta (also known as Zebra, *BZLF-1*, or Z protein) (Chen et al. 1999; Cox et al. 1990; Quinlivan et al. 1993; Ragoczy et al. 1998; Zalani et al. 1996). Zta is a bZIP-type transcriptional transactivator. The EBV Orf50 homologue, the *BRLF1* gene product Rta, is also a sequence-specific DNA-binding protein known to function as a transcriptional activator (Quinlivan et al. 1993; Ragoczy et al. 1998; Ragoczy and Miller 2001; Russo et al. 1996). Independently, both Zta and Rta initiate the expression of lytic genes and, in a somewhat cell type-specific manner, Rta can lead to the activation of DE promoters. It is believed that Zta and Rta proteins of EBV act synergistically (although Zta can suppress the transactivation function of Rta) to induce viral reactivation in latently infected B cells. However, EBV Rta alone can activate a subset of lytic promoters that do not require Zta, and EBV Zta alone can activate a subset of lytic promoters that do not require Rta (Ragoczy and Miller 1999). A deletion mutant of either Zta or Rta is defective for viral reactivation (Feederle et al. 2000). The KSHV Rta/Orf50 is the sequence homologue of the EBV Rta/BRLF1, whereas the KSHV KbZIP/K8 protein is the sequence homologue of EBV Zta/BZLF1. In contrast to EBV, the KSHV Rta/Orf50 is considered to be the only essential lytic switch protein to date. A KSHV Rta/Orf50 deletion mutant is incapable of reactivation from latency (Xu et al. 2005), although a deletion mutant of KSHV KbZIP remains to be evaluated. In this regard the two subclasses of gammaherpesviruses (lymphocryptovirus and rhadinovirus) differ in their molecular mechanisms of reactivation in that to date only one viral protein, Rta/Orf50, has been shown to mediate viral reactivation and induce lytic gene expression in the rhadinoviruses (KSHV, HVS, RRV, and MHV-68). In fact, expression of KSHV Rta/Orf50 precedes expression of K8 (K-bZip) and transactivates the K8 promoter (Lukac et al. 1998; Sun et al. 1998). Therefore, in an effort to minimize complexity and derive general principles for homologous molecular mechanisms, we will only discuss the Rta/Orf50 proteins of the rhadinoviruses.

4
Rhadinoviruses—KSHV, HVS, RRV, and MHV-68

4.1
Experimental Considerations

Before we delve into a detailed molecular description of the rhadinovirus Rta/Orf50 transactivator, it seems prudent to highlight some experimental constraints that affect the general conclusions we can draw from the many studies on Rta/Orf50 and rhadinovirus reactivation. KSHV, RRV, and MHV68 establish latency in B lymphocytes and are associated with B-cell hyperplasia, but unlike EBV and primate lymphocryptoviruses, KSHV, RRV, and MHV68 do not immortalize primary B cells with any efficiency in culture. Rta/Orf50 is sufficient to reactivate latent virus in each case, but the exact B-cell compartment and lineage-specific transcription factor makeup may be different for each virus. Only RRV and MHV68 establish a robust de novo infection in primary fibroblasts, which amplifies input virus. Hence, Rta/Orf50's role in primary infection under low MOI conditions can only be investigated in these lytic model systems. KSHV infects primary and immortalized endothelial cells, but to date only a single plaque has been published (Boshoff et al. 1995; Ciufo et al. 2001). Although KSHV can be serially propagated, input virus is not amplified and high MOI infection in the presence of polybrene is required to initiate the culture (Foreman et al. 1997; Lagunoff et al. 2002; Renne et al. 1998). Reactivation from latency in response to biological signals or chemical inducers such as phorbol ester is a low-frequency event for KSHV (Chang et al. 2000; Renne et al. 1996) and sets up an interesting paradox. In a latent culture every single cell is infected with KSHV, carries 10–50 copies of the viral episome, and expresses the latency-associated nuclear antigen (LANA/Orf73). LANA is necessary and sufficient for latent viral replication, which is analogous to Rta/Orf50's requirement for lytic viral replication (Ballestas et al. 1999; Godfrey et al. 2005). If a latent cell culture, for instance, BCBL-1 cells, is treated with phorbol ester, 100% of the cells receive the drug and are subject to drug action; however, not all cells will express Rta/Orf50, and of the cells that express Rta/Orf50 not all cells express delayed-early genes. Furthermore, within the subset of cells that express delayed-early genes even fewer yet express true late viral mRNAs, such as Orf29, a capsid component (Zoeteweij et al. 1999). Therefore, we speculate that additional constraints exist that regulate viral replication at each junction (IE, DE, E, L) in the regulatory cascade.

McAllister et al. showed that the cell cycle state influences how competent each host cell is to support viral replication (McAllister et al. 2005). At this point it is unresolved whether the host cell cycle state simply reflects responsiveness of the PKC downstream targets that activate the Rta/Orf50

promoter, the effectiveness of Rta/Orf50 to function as a transactivator, or steps dependent on viral DE genes. Alternatively, cells in S phase may remain viable for a longer time and can, therefore, accumulate more viral mRNA and proteins before dying because of the effects of viral capsid maturation and egress. Many cell lines do not tolerate stable Rta/Orf50 expression, but recently exciting new tools have become available to investigate Rta/Orf50 function and add to the existing tools of cDNA expression (Sun et al. 1998; Lukac et al. 1998) and Rta/Orf50 dominant-negative plasmids (Lukac et al. 1998): an *Orf50* k/o virus for MHV68 and KSHV, a KSHV-inducible system in BCBL-1 and 293 cells (Nakamura et al. 2003; Xu et al. 2005), a KSHV Rta/Orf50 recombinant adenovirus (Liang et al. 2002), and novel DNA-binding mutants of KSHV Rta/Orf50 (Chang et al. 2005). These tools will yield exciting new insights into the molecular function of Rta/Orf50 and the biology of the rhadinoviruses, although they may also lead to discrepancies with existing studies due to differences in the experimental approach.

4.2 Chemically Induced Viral Reactivation of Gammaherpesviruses

Reactivation of rhadinoviruses in latently infected cells can be achieved by treatment with chemicals that mimic BCR signaling such as *n* -butyrate, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or calcium ionophores. These types of treatment lead to expression of viral lytic genes, foremost among them *Orf50*. Although the entire cellular signaling cascades that lead to viral reactivation in response to these chemical treatments are unknown, we do know that induction of the KSHV *Orf50* promoter by TPA involves the cellular AP1 pathway and induction by sodium butyrate (NaB) involves cellular Sp1 (Wang et al. 2004a, 2003a, 2003b). Cannon et al. have recently shown that overexpression of the KSHV lytic protein vGPCR/~~orf~~74 indirectly resulted in a decreased efficiency of chemical induction of the ORF50 transcript (Cannon et al. 2006). Because the KSHV vGPCR/~~orf~~74 is known to modulate cellular signaling pathways, which, in extreme instances, can lead to transformation (Bais et al. 1998; Polson et al. 2002) this adds credence to a model of multilayered cross talk between KSHV and the host. In RRV, the histone deacetylase inhibitor *trichostatin A* (TSA) is also capable of reactivating RRV from latently infected cells, presumably by de-repressing the *Orf50* promoter (DeWire et al. 2002). Like TSA and butyrate, valproic acid (2-propylpentanoic acid) also has potent histone deacetylase activity. At the same time it is FDA approved as antiseizure medication. Treatment of KSHV-infected primary effusion lymphoma (PEL) cells with valproate induced lytic reactivation in culture (Shaw 2000; Klass et al. 2005) and is currently in clinical trials for the treatment of Kaposi sarcoma.

4.3
Viral Induction of Lytic Reactivation of Rhadinoviruses

Although many viral proteins were assayed for their ability to reactivate KSHV in PEL cells, only ectopic expression of Rta/Orf50 was sufficient to disrupt latency and activate lytic replication, resulting in a complete productive viral life cycle (Gradoville et al. 2000; Lukac et al. 1998; Sun et al. 1998). The KSHV Zta and Mta homologues by themselves were not able to induce lytic reactivation, although, as in EBV, they may transactivate some DE promoters independently of Rta/Orf50. Expression of HVS Orf50a protein and MHV-68 Rta/Orf50 also induces lytic reactivation and production of infectious viral progeny in HVS and MHV-68 models of latency, respectively (Goodwin et al. 2001; Wu et al. 2000). The RRV Rta/Orf50 and MHV-68 Rta/Orf50 can reactivate KSHV from latency (Damania et al. 2004), and the KSHV Rta/Orf50 protein can reactivate the murine viral homologue, MHV-68, from latency (Rickabaugh et al. 2005). These studies demonstrate a strong conservation of function across evolution of the rhadinovirus Rta proteins. Although RRV Orf50 has been shown to be a potent transactivator of RRV DE promoters, it has not yet been demonstrated to reactivate RRV from latency (DeWire et al. 2002), because lytic replication rather than latency is the default pathway following primary RRV infection of cells in culture. Recently, a latently infected RRV system has been established, and we can expect formal demonstration of this in the near future (DeWire and Damania 2005).

5
Rta/Orf50 Transcription

The Orf50 transcripts from all gamma-2-herpesviruses share a similar architecture that is essentially comprised of two exons separated by one intron. A major IE transcript is observed after reactivation of KSHV that is a 3.6-kb tricistronic mRNA encoding the Orf50, K8, and K8.1 reading frames (Zhu et al. 1999). ~~The Orf50 transcript architecture of two exons separated by one intron is a characteristic not only of KSHV, but also the other rhadinovirus.~~ Splicing results in the major Orf50 transcript; however, other alternatively spliced mono-, bi-, and polycistronic Orf50-containing transcripts have been found (Saveliev et al. 2002; Tang and Zheng 2002; Wang et al. 2004). Presently, the significance of these isoforms is unclear. Splicing of the Orf50 transcript is a characteristic of gamma-2-herpesviruses and is in contrast to the Orf50 cDNA of the gamma-1 herpesvirus EBV, which is identical to its genomic open reading frame structure (Manet 1989). In the rhadinoviruses, Orf45 is located within the Rta/Orf50 intron in opposite orientation and the rhadinovirus

Orf45 promoter is presumably located within the Rta/Orf50 open reading frame. Similarly in EBV, Orf45 (BRRF1/Na) is located 5′ of the Rta/Orf50 open reading frame, also in the opposite orientation and also within the first intron (the first exon of EBV Rta is noncoding) (Hong et al. 2004).

The major 3.6-kb Orf50 transcript is induced within 4 h of *n*-butyrate treatment of latently infected PEL cells and is resistant to treatment with the protein synthesis inhibitor, cycloheximide (CHX), thus displaying IE kinetics (Sun et al. 1999; Zhu et al. 1999). Lukac at al. also observed expression of Orf50 mRNA within 1 h of inducing viral reactivation in latently infected BCBL-1 cells by treatment with the phorbol ester TPA (Lukac et al. 1998). MHV-68 *Orf50* is also an immediate-early gene, as is the RRV *Orf50*, further demonstrating conservation of *Orf50* transcription kinetics among the rhadinoviruses (Rochford et al. 2001; DeWire et al. 2002; Dittmer et al. 2005). Of the rhadinoviruses, lytic reactivation of HVS results in transcription of two distinct Orf50 mRNA species, called *Orf50a* and *Orf50b* (Whitehouse et al. 1997). The Orf50a transcript is identical to that described for the other rhadinoviruses and is detected at early times during viral replication. The HVS Orf50b transcript is expressed at later time points during replication and is produced from a promoter within the second exon. Its function remains to be elucidated. Because abolishing Rta/Orf50 function inhibits lytic replication at IE times, it has not been possible to determine experimentally whether Rta/Orf50 has additional functions at DE or late times in any of the rhadinoviridae.

5.1
Regulation of the KSHV Rta/Orf50 Promoter

Because Rta/Orf50 protein is the key regulator of KSHV lytic reactivation, much attention has focused on the promoter that regulates Rta/Orf50 expression. The KSHV Rta/Orf50 protein autoregulates its own promoter via an indirect mechanism, because no obvious Rta/Orf50-responsive element (RRE) or RBP-J-κ consensus binding sites are present within the *Orf50* promoter (these mechanisms are described in detail in later sections of this review) (Chang and Miller 2004; Deng et al. 2000; Gradoville et al. 2000). By comparison to other Rta/Orf50-responsive promoters, the *Orf50* promoter is only marginally activated by Rta/Orf50 protein expression. Within the *Orf50* promoter, a binding sequence for the cellular transcription factor octamer-binding protein (Oct-1) was shown to mediate autoregulation by Rta/Orf50 protein. Oct-1 bound to a specific region of DNA within the *Orf50* promoter, as demonstrated by electrophoretic mobility shift assay (EMSA) (Sakakibara et al. 2001). In addition, both Sp1 and Sp3 cellular transcription factors appear

to be involved in Rta/Orf50 autoactivation (Chen et al. 2000; Zhang et al. 1998). This is similar to the EBV Rta protein, which also mediates autoregulation of its own promoter (Rp) via a non-DNA-binding mechanism involving cellular Sp1 and Sp3 proteins (Ragoczy and Miller 2001). KSHV Rta/Orf50 also interacts with the CCAAT/enhancer binding protein α(C/EBP-α) to upregulate Rta/Orf50 expression (Wang et al. 2003b).

The Orf50 promoter is heavily methylated in latently infected PEL cells, and treatment with TPA leads to demethylation of the promoter (Chen et al. 2001). In vivo, several biopsies from KSHV-related diseases, including Kaposi sarcoma, multicentric Castleman disease (MCD), and PEL, showed decreased methylation of the *Orf50* promoter, although this promoter was still heavily methylated in samples obtained from a latently infected KSHV carrier. This evidences an additional layer of regulation of Rta/Orf50 expression and, as such, KSHV lytic reactivation. Progressive methylation of viral lytic promoters could skew KSHV infection of host cells toward latency. This is consistent with the decoration of the viral episome and Rta/Orf50 promoter with inhibitory histone complexes (Lu et al. 2003). Because much of the investigation of Rta/Orf50's function is based on transient transfection assays of unmethylated, histone-free promoter-reporter plasmids, we do not know how Rta/Orf50's *general* transactivation function (via TAFs and regulation of HDACs) may work together with Rta/Orf50's *specific* transactivation functions (via direct DNA binding or RBP-J-κ binding sites).

The propensity of KSHV to establish latency on primary infection (Ciufo et al. 2001; Grundhoff and Ganem 2004; Krishnan et al. 2004; Moses et al. 1999) is in contrast to the propensity of RRV and MHV-68 to establish lytic infection. The bias of KSHV toward the establishment of latency after primary infection may be due to Rta/Orf50's interaction with species-specific chromatin modules, because Rta/Orf50 proteins of any rhadinovirus are able to transactivate unmethylated viral promoters in transient transfection assays. However, it is interesting to note that both MHV-68 and RRV are impaired in their ability to reactivate KSHV in the context of viral gene expression (Damania and Jung 2001).

Micrococcal nucleosome mapping techniques by Chen et al. reported a nucleosome positioned on the Orf50 promoter that overlapped the transcription start site and a GC-rich region bound by both Sp1 and Sp3 (Lu et al. 2003). The Sp1/Sp3 region of the Orf50 promoter was also mapped as highly responsive to two chemical compounds known to inhibit histone deacetylases, NaB and TSA (Lu 2003). In addition, NaB treatment led to the rapid recruitment of Ini1/Snf5, a component of the Swi/Snf family of chromatin remodeling proteins. These data describe complex, multitiered levels of transcriptional regulation of the Orf50 promoter within latently infected host cells.

6
Rta/Orf50 Protein

6.1
KSHV Rta/Orf50

The KSHV *Orf50* gene encodes a 691-amino acid protein that is highly phosphorylated and localizes to the nucleus of mammalian cells (Lukac et al. 2001, 1999; Seaman and Quinlivan 2003). All Rta homologues share a conserved C-terminal activation domain. Deletion of 160 amino acids in the C-terminal activation domain of the KSHV Rta/Orf50 results in production of a truncated, but stable, Rta/Orf50 protein that forms multimers with wild-type Rta/Orf50 in PEL cells and functions as a dominant-negative inhibitor of Rta/Orf50 transactivation (Lukac et al. 1998). Expression of this truncated Rta/Orf50 protein leads to suppression of both spontaneous and chemically induced viral reactivation. Expression of KSHV Rta/Orf50 and subsequent viral reactivation can also be efficiently knocked down by expression of human RNase P (Zhu et al. 2004). These data suggest that Rta/Orf50 is, indeed, the lytic switch protein of KSHV. Recently, Pari and colleagues reported genetic evidence for the Rta-lytic switch hypothesis by utilizing the KSHV genome cloned into a bacterial artificial chromosome (BAC36) (Gao et al. 2003; Zhou et al. 2002) and generating a deletion within the Orf50 open reading frame (Xu et al. 2005). After transfection of the Orf50-deficient BAC into HEK 293 cells, latent genes were expressed at wild-type levels; however, the virus was unable to reactivate on chemical treatment, unequivocally demonstrating that Rta/Orf50 is required for successful viral reactivation.

A provocative recent report showed that Rta/Orf50 was present in KSHV virions (Bechtel et al. 2005b), which would make it a virion transactivator much like the herpes simplex virus VP16 and thus ensure lytic replication on primary infection. However, herpesvirus virions are notorious for capturing a variety of proteins, and even mRNAs (Bechtel et al. 2005a), simply as scaffolds during assembly or because of sloppy egress. The composition of nonstructural proteins in the rhadinovirus virions is highly variable (Zhu and Yuan 2003; Zhu et al. 2005; O'Connor and Kedes 2006; Trus et al. 2001) and may not necessarily have a function in the next infection cycle.

The different functions of KSHV Rta/Orf50 are subject to posttranslational modifications. KSHV Rta/Orf50 is highly phosphorylated, a modification that is mediated at least in part by the ability of Rta/Orf50 to bind to and be phosphorylated by the cellular Ste20-like kinase hKFC (Gwack et al. 2003b; Lukac et al. 2001,, 1999). The phosphorylation of Rta/Orf50 by hKFC as well as Rta's poly(ADP)-ribosylation by cellular PARP-1 protein both result in decreased ability of KSHV Rta/Orf50 to transactivate viral promoters (Gwack

et al. 2003b). Recently, KSHV Rta/Orf50 was shown to have E3 ubiquitin ligase activity and could direct polyubiquitination of cellular interferon regulatory factor 7 as well as polyubiquitination of itself (Yu et al. 2005). Point mutations in the Cys + His-rich N-terminal domain of Rta/Orf50 abolished the E3 ligase activity, which should be independent of Rta's transcriptional activity. Much more still needs to be learned, but the many posttranslational modifications of KSHV Rta/Orf50 most likely act to regulate the function, and possibly the stability, of this key viral transactivator protein.

KSHV Rta/Orf50 has been shown to modulate transcription of host genes as well. It has been reported to transactivate cellular interleukin 6 (IL-6) transcription (Deng et al. 2002a). KSHV Rta/Orf50 can also modulate the ability of cellular STAT3 to function as a transactivator, indirectly leading to modulation of host gene expression (Gwack et al. 2002). Furthermore, Rta/Orf50 interacts with RBP-J-κ (Chang et al. 2005) and may thereby regulate the transcription of RBP-J-κ-dependent host mRNAs.

6.2 MHV-68 Rta/Orf50

The Rta protein encoded by *Orf50* of MHV-68 is sufficient to induce lytic reactivation in latently infected cells (Wu et al. 2000). Investigations of the disruption of the MHV-68 Rta/Orf50 open reading frame demonstrated that Rta/Orf50 is also necessary for viral reactivation (Liu et al. 2000; Pavlova et al. 2005, 2003). The requirement for MHV-68 Rta/Orf50 in lytic reactivation was also demonstrated by other means, whereby a loss in viral reactivation was observed after efficient knockdown of MHV-68 Rta/Orf50 expression by RNAi (Jia et al. 2004). A mutant MHV-68 virus, called M50, was generated to constitutively express MHV-68 Rta/Orf50 by insertion of a new promoter element into the 5′-untranslated region (UTR) of the Orf50 promoter (May et al. 2004). Constitutive expression of Rta/Orf50 by the M50 mutant MHV-68 virus resulted in defective establishment of latency. The physiological relevance of this mutant phenotype was demonstrated by studies in which immunization of mice with the mutant M50 virus resulted in partial protection against challenge with wild-type virus, demonstrating the importance of proper transcriptional regulation of *Orf50* in the both the pathogenesis and establishment of latency by MHV-68 (Boname et al. 2004; May et al. 2004). Importantly, a Rta/ORF50-null mutant of MHV-68 established long-term latency in the lungs of infected mice but failed to vaccinate against a wild-type virus challenge, therefore implicating the necessity of lytic replication for generation of a protective immune response (Moser et al. 2006). In addition, gene array studies of a recombinant virus that overexpressed MHV-68 Orf50

found that nearly every MHV-68 gene assayed was upregulated by Rta/Orf50 overexpression. This phenotype is consistent with Rta/Orf50 being the first gene in the MHV-68 lytic transcriptional cascade and highlights the potent transactivating ability of the protein (Martinez-Guzman et al. 2003).

6.3 HVS Rta/Orf50

Much less is known about HVS Rta/Orf50, but what has been reported suggests that the properties of HVS Rta/Orf50 resemble those of the other rhadinoviruses. HVS Rta/Orf50 induces viral reactivation of latently infected cells (Goodwin et al. 2001), a function conserved throughout all rhadinoviruses. HVS Rta/Orf50 contains an AT-hook DNA binding domain that is required for transactivation of at least two delayed early viral promoters, Orf6 and Orf57 (Walters et al. 2004). Although this report demonstrates that HVS Rta/Orf50 can transactivate certain viral promoters via direct DNA binding, this viral transactivator most likely also works through indirect mechanisms as do the lytic switch proteins of the other gammaherpesviruses. HVS Rta was shown to bind TATA-binding protein (TBP) in vitro, which provides an alternative means to influence viral and cellular transcription (Hall et al. 1999). Of note, the amino acid sequence of both Rta/Orf50 isoforms, Orf50a and Orf50b of HVS A11 and HVS C488 (a low-passage transforming isolate), are among the most divergent open reading frames in these viral isolates (Ensser et al. 2003), with only ~70% amino acid identity compared to >90% amino acid identity for the other 60 of 75 (80%) open reading frames in the HVS genome. Whereas the Orf50b of A11 showed decreased transactivation capability compared to Orf50a of A11, the Orf50b of C488 demonstrated full transactivation capability. In contrast, both the A11 and C488 Orf50a proteins were capable of reactivating lytic replication in persistently infected cells, indicating a conservation of function for one Rta/Orf50 isoform and not the other. The same study reported that Rta/Orf50 allelic variation cosegregated with Stp and Tip in their ability to transform human T cells in culture. By inference based on this genetic data set alone, Rta/Orf50 could be considered an oncogene. On the other hand, there is no independent evidence that Rta/Orf50 can transform cells in culture or can cooperate with other oncogenes to do so. Rather, long-term stable expression of Rta/Orf50 seems incompatible with continued cell growth. No molecular mechanism has been described to support such classification of HVS Rta/Orf50 as an oncogene, but one can imagine that a promiscuous transactivator like Rta/Orf50 could reset the cellular transcription profile via interactions with RPB-J-κ or other via other mechanisms. This was recently explored by Nakamura et al. using a tetracycline-inducible

KSHV Rta/Orf50 cell line (Chang et al. 2005). Among the host genes that were induced by Rta/Orf50 via RPB-J-κ were CD21 and CD23a, which are involved in lymphocyte activation. KSHV Rta/Orf50 also inhibits the p53 tumor suppressor protein (Gwack et al. 2001b). This capability is conserved among the primate rhadinoviruses, though not in MHV-68 (Damania et al. 2004). Overall, Rta/Orf50 is a key regulator of both viral and cellular transcription. An in-depth analysis of the molecular mechanisms of transactivation by Rta/Orf50 is described below.

7
Rta/Orf50 Function

7.1
Viral Promoters Transactivated by KSHV Rta/Orf50

KSHV Rta/Orf50 is a strong transactivator of many viral promoters in transient transfection assays. A summary of these promoters is reported in Table 1.

7.2
Viral Promoters Transactivated by MHV-68 Rta/Orf50

Ectopic expression of MHV-68 Rta/Orf50 in latently infected cells leads to expression of lytic proteins (as determined by Western blots of cell extracts probed with immune mouse serum) and is sufficient to drive viral reactivation of latently infected cells (Wu et al. 2001, 2000). The MHV-68 Orf57 promoter is transactivated by Rta/Orf50. MHV-68 RREs are found within the Orf57 promoter region of the viral genome, as they are in the KSHV ~~orf~~57 promoter (Pavlova et al. 2005). In addition, the MK3 promoter was reported to contain an MHV-68 Rta-responsive element (Coleman et al. 2003). Potential MHV-68 RREs bear significant homology to published KSHV RREs, but to date fewer MHV-68 promoters than KSHV promoters have been investigated in detail.

7.3
Viral Promoters Transactivated by RRV Orf50

RRV Orf50 has also been shown to transactivate promoter-reporter constructs in transient transfection assays. The R8, Orf57, and gB promoters of RRV were highly activated and the vIRF promoter was only slightly activated by Rta/Orf50 (DeWire et al. 2002; Lin et al. 2002). In contrast to KSHV, RRV Rta/Orf50 did not autoactivate its own promoter.

Table 1 Viral promoters transactivated by KSHV Rta/Orf50

KSHV viral promoter	Mechanism	Reference
Nut-1/PAN	C/EBP-α, DNA	Song et al. 2001; Wang et al. 2003b
Kaposin (K12)	DNA	Lukac et al. 1998; Song et al. 2003
K-bZip (K8)	Ap-1, DNA	Lukac et al. 1999; Wang et al. 2003a, 2004
MTA (ORF57)	RBP-J-κ, C/EBP-α, DNA	Duan et al. 2001; Liang et al. 2002; Lukac et al. 1999; Wang et al. 2003b
K6 (vMIP-1)	RBP-J-κ	Chang et al. 2005
K5	Unknown	Haque et al. 2000
K1	Unknown	Bowser et al. 2002
vIRF (K9)	Sp1	Chen et al. 2000; Ueda et al. 2002
ssDNA-binding (ORF6)	RBP-J-κ	Liang et al. 2002
DNA pol.-pross (ORF59)	Unknown	Nishimura et al. 2001
Thymidine kinase (ORF21)	Sp1	Zhang et al. 1998
vOX-2 (K14)	RBP-J-κ	Jeong et al. 2001; Liang and Ganem 2004
vGPCR (ORF74)	RBP-J-κ	Jeong et al. 2001; Liang and Ganem 2004
vIL-6 (K2)	DNA	Deng et al. 2002
LANA LT_i	RBP-J-κ	Matsumura et al. 2005; Lan et al. 2005; Staudt and Dittmer, ~~submitted~~
Rta (ORF50)	Oct-1, Sp1, Sp3, C/EBP-α	Chen et al. 2000; Deng et al. 2000; Gradoville et al. 2000; Sakakibara et al. 2001; Wang et al. 2003a; Zhang et al. 1998

7.4 Viral Promoters Transactivated by HVS Orf50

The HVS Rta/Orf50a does transactivate its own promoter, as well as the DE Orf6, Orf57, and Orf9 viral promoters of HVS (Whitehouse et al. 1997; Thurau

et al. 2000; (Walters et al. 2005, 2004; Byun et al. 2002). The HVS Rta/Orf50a contains an AT-hook DNA binding domain that is required for transactivation of the Orf6 and Orf57 promoters, and cellular C/EPB-α synergizes with HVS Rta/Orf50a to transactivate the Orf9 DNA polymerase promoter (Walters et al. 2004). HVS Rta/Orf50a was found to autoregulate its own promoter by use of a 36-bp RRE that has no significant homology to previously reported RREs of any of the gammaherpesviruses. The HVS RRE DNA sequence conferred Rta/Orf50a responsiveness to an enhancer-less SV40 minimal promoter (Walters et al. 2005).

8
Mechanisms of Rta/Orf50 Transactivation

8.1
KSHV Orf50-Responsive Elements and Direct DNA Binding

There have been many reports showing data that KSHV Rta/Orf50 protein transactivates viral promoters by direct DNA binding to sequences found within these promoters [termed Rta response elements (RREs) or Orf50 response element in *n* promoter (50RE$_n$)]. KSHV Rta/Orf50 can transactivate two delayed-early (DE) promoters, Orf57 and K8 (K-bZip), by direct DNA binding (Lukac et al. 2001; Duan et al. 2001; Song et al. 2002). The N-terminal 272 amino acids of Rta protein are sufficient to bind a 12-bp DNA sequence, 5′-AACAATAATGTT-3′, found within both DE promoters and termed the 50RE$_{57}$. KSHV Rta/Orf50 also transactivates the PAN/nut-1 and K12 promoters (Chang et al. 2002) and was shown to directly bind DNA within these promoters. Intriguingly, the sequences of the 50RE found within the Pan/nut-1 and K12 promoters (5′-AAATGGGTGGCTAACCCCTACATAA-3′, PAN DNA sequence shown, K12 promoter sequence underlined) and the 50RE$_{57}$ (5′-AACAATAATGTT-3′) share no significant homology. Yet another Orf50-responsive element was discovered in the vIL-6 promoter that contains a 26-bp sequence, 5′-AAACCCCGCCCCCTGGTGCTCACTTT-3′ (Deng et al. 2002b). Direct comparison of RRE-containing viral promoters revealed that the transcription initiation rate of these promoters, as opposed to transcript stability, is the major determinant of expression of these viral proteins (Song et al. 2003). Liao et al. reported that KSHV Rta/Orf50 forms oligomers and makes multiple contacts with a tandem array of phased A/T triplets in the configuration of $(A/T)_3$ $(G/C)_7$ repeats (Liao et al. 2003a). An RRE and TATA box was also found within the KSHV OriLyt, and this DNA region functioned as an Rta/Orf50-responsive promoter when cloned into a reporter vector (Wang et al. 2004b). This promoter regulated a late 1.4-kb polyadenylated mRNA that

was sensitive to the viral DNA polymerase inhibitor *foscarnet* and has coding capacity for a 75 aa open reading frame whose gene product is of unknown function.

HMGB1 is a cellular protein belonging to the high-mobility group (HMG) box protein subfamily that affects transactivation function of both EBV Zta and EBV Rta (Ellwood et al. 2000; Mitsouras et al. 2002). HMG proteins are large chromosomal proteins thought to function to promote higher-order DNA-protein complexes by changing DNA conformation to be more easily accessible to transcriptional machinery. HMGB1 was recently shown to enhance direct DNA binding of KSHV Rta/Orf50 to RREs in vitro and to enhance transactivation functions of both KSHV and MHV-68 Rta/Orf50 proteins in transient transfection assays (Song et al. 2004).

KSHV Rta/Orf50 was reported to contain a protein domain that seemed to act in an autoregulatory fashion. This autoregulatory domain of KSHV Rta/Orf50 is contained within amino acids 521–534 and functions to control the direct DNA binding ability of the protein as well as protein stability (Chang and Miller 2004). Deletion of amino acids 521–534 or mutation of a basic motif (KKRK) at aa 527–530 dramatically enhanced DNA binding of Rta/Orf50. Although the DNA binding ability of the KKRK mutant was enhanced, its ability to transactivate the PAN promoter was impaired, suggesting that these two functions do not correlate synergistically on the PAN promoter. In addition, expression of autoregulation-domain mutants led to appearance of an alternative form of Rta, termed Orf50b, which showed decreased posttranslational modifications. At this time, investigations into the structure and posttranslational modifications of KSHV Rta/Orf50 are in the early stages, and we can expect more insights in the near future.

8.2 Interaction of Rta/Orf50 with RBP-J-κ

Although KSHV Rta/Orf50 binds to DNA containing the RREs described above, no obvious consensus sequence was found among other Rta/Orf50-responsive promoters: an observation that prompted many to look for an alternate mechanism of Rta transactivation. Ganem and colleagues used a yeast-two-hybrid approach to assay possible cellular binding proteins and found that the cellular protein RBP-J-κ (also called CSL or CBF-1) interacted with KSHV Rta/Orf50 (Liang et al. 2002). RBP-J-κ is a sequence-specific DNA binding protein and is the downstream effector of Notch signal transduction (Mumm and Kopan 2000). In uninfected cells RBP-J-κ functions as a transcriptional repressor until ligand-mediated Notch signaling occurs, which leads to the conversion of RBP-J-κ from a repressor to a transactivator of

downstream cellular gene targets (such as HES, hair/enh of split genes). KSHV can usurp the function of cellular RBP-J-κ without the requirement for Notch-ligand interaction, as the binding of KSHV Rta/Orf50 protein to RBP-J-κconverts RBP-J-κ from a transcriptional repressor to a transactivator. This mechanism has been demonstrated for the KSHV ~~MTA~~/Orf57, SSB/Orf6, PAN/nut-1, vGPCR/K14, vMIP-1/K6, and LT_i promoters (Liang et al. 2002; Liang and Ganem 2003, 2004; Lan et al. 2005; Matsumura et al. 2005; Staudt and Dittmer ~~unpublished~~). Amino acids 170–400 of Rta/Orf50 mediate binding to RBP-J-κ, and there are two contiguous but distinct regions of RBP-J-κ to which Rta/Orf50 binds: one is within the central repressor domain and one is within the N-terminal domain of RBP-J-κ. It is striking that the central repressor domain of RBP-J-κ to which KSHV Rta/Orf50 binds is the same region to which Notch, the physiological effector protein of RBP-J-κ, binds as well. This suggests that Rta/Orf50 replaces Notch during lytic reactivation in B cells; however, this has yet to be demonstrated. Liang and Ganem propose that KSHV may employ the repressive function of RBP-J-κ bound to lytic promoters as a means of maintaining latency in the absence of appropriate reactivation stimuli (Liang and Ganem 2003). The interaction between KSHV Rta/Orf50 and cellular RBP-J-κ demonstrates an elegant mechanism the virus has developed to hijack an essential cellular signal transduction pathway as a means to obtain ~~one level of~~ control over viral latency and lytic reactivation.

8.3 Interaction of Rta/Orf50 with Other Cellular Transcription Factors

The ubiquitously expressed cellular transcription factor Sp1 plays an important role in Rta/Orf50 transactivation of promoters, although to date there have been no reports of direct binding between KSHV Rta/Orf50 and cellular Sp1 protein. Sp1 binding sites within the *Orf50* promoter are essential for butyrate-induced Rta/Orf50 expression and lytic replication (Ye et al. 2005). Sp1 is also involved in Rta/Orf50 transactivation of other viral promoters, including vIRF/K9, thymidine kinase/Orf21, and Rta/Orf50 (Chen et al. 2000; Ye et al. 2005; Zhang et al. 1998).

Using a proteomics approach, Wang et al. identified a novel cellular protein, MGC2663, that stably bound to Rta/Orf50 after tandem immunoaffinity chromatography (Wang et al. 2001). MGC2663 was found to bind KSHV Rta/Orf50 and specifically synergized with Rta/Orf50 to activate viral transcription. The MGC2663 protein was previously uncharacterized, but Wang et al. found that it was expressed in every primate cell line tested and that it enhanced transactivation by Rta/Orf50, hence assigning MGC2663 the name *K-RBP* for KHSV Rta binding protein.

KSHV Rta/Orf50 also binds CBP [cyclic AMP (cAMP)-responsive element binding protein (CREB)-binding protein], which is a transcriptional coactivator that contains intrinsic histone acetyltransferase (HAT) activity (Gwack et al., 2003a, 2001a). Acetylation of histones is associated with relaxing nucleosomal structures, thus rendering regions of tightly packed DNA open and accessible to the transcriptional machinery. Binding of CBP to KSHV Rta/Orf50 increased the ability of Rta/Orf50 to transactivate viral promoters. Binding between these two proteins is mediated by the N-terminal basic domain of Rta/Orf50, which contains a conserved LxxLL CBP-binding motif, and the C/H3 domain and C-terminal transactivation domain of CBP. In addition, other cellular CBP-binding proteins, including CBP-BP and c-Jun, enhanced the ability of Rta/Orf50 to transactivate viral promoters. The transactivation function of EBV Rta is also enhanced by binding cellular CBP (Swenson et al. 2001). KSHV Rta/Orf50 also binds HDAC1, a cellular histone deacetylase, and this binding decreases the ability of Rta/Orf50 to transactivate viral promoters (Gwack et al. 2001a). In addition, Sp1 binding sites are involved in Rta/Orf50 transactivation of many viral promoters (see Table 1 for references). Sp1 itself binds to the CBP/p300 coactivator complex, and the activity of Sp1 is repressed by HDAC1 in the absence of viral infection (Doetzlhofer et al. 1999), suggesting a complex interplay among these important modifiers of basal transcription.

The development and characterization of KSHV Rta/Orf50 DNA-binding mutants, which display either enhanced or abolished DNA binding to RREs, has enabled classifications of Rta/Orf50-responsive promoters into either of two subgroups: those where Rta/Orf50 directly binds promoter DNA and those where Rta/Orf50 does not directly bind promoter DNA but rather transactivates by protein-protein interactions with cellular transcription factors, including RBP-J-κ (Chang et al. 2005). These mutants will no doubt facilitate further clarification of Rta/Orf50-responsive promoters as to which mechanism Rta/Orf50 transactivates and which cellular pathways are involved.

9
Repression of Rta/Orf50 Transactivation

Rta/Orf50 interacts with the viral early protein K-bZip, encoded by the K8 open reading frame. This protein-protein interaction leads to repression of Rta's ability to transactivate in vitro (Liao et al. 2003a, 2003b). K-bZip is a homologue of EBV Zta, and accumulating evidence suggests a role for K-bZip in DNA replication and transactivation. K-bZip repressed Rta/Orf50 transactivation of the Orf57/~~MTA~~ and K8/Kb-Zip promoters but had no effect

on Rta/Orf50's transactivation of the PAN/nut-1 promoter, demonstrating promoter-specific repression by K-bZip. The leucine zipper domain (aa 190–237) of K-bZip seems to be required for Rta/Orf50 binding (Liao et al. 2003b).

In addition to viral proteins, cellular interferon response factor 7 (IRF7) was reported to decrease transactivation of the Mta/Orf57 promoter by competing with Rta/Orf50 for binding to the RRE. Interferon-α was also shown to decrease transactivation of the Mta/Orf57 promoter by Rta/Orf50, with this process still involving IRF7 (Wang et al. 2005). This is consistent with the observation that interferon-α inhibits KSHV reactivation in PEL (Chang et al. 2000; Zoeteweij et al. 1999; Pozharskaya et al. 2004). In contrast, Hayward and colleagues have reported an E3 ubiquitin ligase activity of KSHV Rta/Orf50 and that Rta directs polyubiquitination of cellular IRF7 leading to proteosomal degradation of IRF7 and blockage of IRF7-mediated expression of type I interferon transcripts (Yu et al. 2005). Because Rta/orf50 is the principal regulator of KSHV reactivation it seems logical that this protein serves as the nexus between virus and antivirus response.

The chemical compound methotrexate was shown to downregulate KSHV Rta/Orf50-mediated transactivation and inhibit lytic reactivation (Curreli et al. 2002). This is consistent with the previously reported antiviral activity of methotrexate for other herpesviruses (Lembo et al. 1999; Shanley and Debs 1989). Yet there are also reports that methotrexate induces EBV reactivation (Feng et al. 2004). Most likely the systemic antiviral activity of methotrexate is related to its action as an antimetabolite and dihydrofolate reductase inhibitor, while its effects on Rta/Orf50 transactivation may be a by-product of cellular stress signaling induced by imbalances in intracellular nucleotide pools.

Interestingly, the KSHV latency-associated nuclear antigen (LANA) protein is capable of repressing transcription of the Rta/Orf50 promoter (Lan et al. 2004). This repression is dependent on the presence of RBP-J-κ-responsive elements found within the Orf50 promoter (Lan et al. 2005). Lan et al. also showed that LANA bound Rta/Orf50 protein directly (Lan et al. 2005). RRV LANA (R-LANA), like KSHV LANA, represses the transactivation ability of RRV Rta/Orf50, and this repression is reversed by treatment with the histone deacetylase inhibitor TSA (DeWire and Damania 2005). The HVS C488 LANA also suppresses the HVS Rta/Orf50 (Schafer et al. 2003), which fits a model in which LANA, the latent transactivator, and Rta/Orf50, the lytic transactivator, counterbalance each other. This balance of power is evolutionarily conserved among the rhadinoviruses, although the governing molecular mechanisms may be different. The outcome of the Rta/Orf50–LANA/Orf73 power struggle eventually determines whether the virus persists latently or reactivates, which in turn results in a profound difference in overall viral persistence and pathogenesis within the infected host.

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