Identification of the Determinants of Latency and Replication for Rhesus Rhadinovirus (RRV)

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Abstract

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Kaposi's sarcoma herpesvirus (KSHV), the causative agent of Kaposi's sarcoma (KS), establishes a characteristic latent infection both in tumor cells in vivo and in cultured cells in vitro. The switch from latency to replication is a critical event in the KSHV life cycle, and the viral replication and transactivator (Rta) protein is sufficient to initiate the replicative program of gene expression leading to virus production. In contrast, rhesus rhadinovirus (RRV), a related virus of macaques, establishes a characteristic replicative program of infection in tumor cells and cultured cells. To understand the basis for the different outcomes of KSHV and RRV infection we have characterized the promoter of the RRV Rta homolog and compared it to the KSHV Rta promoter in the context of viral latency and replication. Using cell lines permissive for RRV replication and novel systems of latency in human salivary gland (HSG) and adenocarcinoma gastric (AGS) epithelial cells, we found that the RRV Rta promoter was strongly active in permissive cells and minimally active in non-permissive cells. An Sp1 element located at nucleotide -113 was required for Rta promoter activity in permissive cells and butyrate responsiveness in non-permissive cells. Ectopic expression of the latency-associated nuclear antigen (LANA) inhibited Rta promoter activity and RRV replication in permissive cells,

suggesting that LANA inhibition of Rta expression could play a role in RRV latency. However, our results suggest that LANA inhibitory activity is restricted in RRV-infected cells. RRV lacks the Rta-inducible LANA promoter that is critical for the establishment of KSHV latency. We also identified an arginine-glycine rich motif that targets RRV LANA to the nucleolus, which may limit the inhibitory potential of LANA in infected cells. Based on these findings we propose a model in which host factors, such as Sp1, determine high-level RRV Rta promoter activity that correlates with cellular permissivity for RRV replication.

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CHAPTER I

Introduction

Four epidemiological forms of Kaposi's sarcoma

The Hungarian dermatologist Moritz Kaposi first described an "idiopathic multiple pigmented sarcoma of the skin" among elderly Mediterranean men in the mid-19th century (Kaposi 1872, Sternbach and Varon 1995). The disease was designated Kaposi's sarcoma (KS) in 1891 (Boshoff and Chang 2001). The form of KS described by Dr. Kaposi, now called classical KS, commonly presents as multifocal skin lesions on the extremities and is relatively benign. A highly aggressive form of KS emerged in the 1980s at the onset of the HIV/AIDS epidemic in association with viral immunosuppression. Unlike the classical manifestation of the disease, early cases of AIDS-associated KS frequently involved lymph nodes, viscera and mucosa in addition to skin and were frequently fatal. Though highly active anti-retroviral therapy (HAART) in HIV patients has decreased its incidence, KS remains one of the most common malignancies in AIDS patients (Gates and Kaplan 2002, Portsmouth, Stebbing et al. 2003). An endemic form of KS occurs in Sub-Saharan Africa, and widespread AIDS-associated immunosuppression has exacerbated the burden of disease in these areas. Among adults, endemic KS shows the same male predominance seen in the classical form of the disease. However, KS is quite common in children in endemic areas, and rates among male and female children are approximately equal (Ziegler and Katongole-Mbidde 1996, Boshoff and Weiss 2002). KS also occurs in immunosuppressed transplant patients (Lebbe, Legendre et al. 2008). This form of the disease, known as iatrogenic or transplant-associated KS, typically coincides with the onset of immunosuppressive therapy and subsides with immune reconstitution (O'Brien,

Kedes et al. 1999). Classical, epidemic (AIDS-associated), endemic, and iatrogenic KS all present with similar histological findings in skin and other frequent sites of involvement such as the gut and lymph nodes. The hallmark of KS lesions is the spindle-shaped endothelial tumor cell, and lesions may display patch, plaque or nodule (raised) morphology. Early patch lesions are typically confined to the upper dermis but can contain irregular vascular architecture and a marked inflammatory infiltrate. Older, plaque lesions are dominated by spindle cells and are characterized by significant angiogenesis. Leakage of red blood cells from the slits in the vasculature gives the lesions their characteristic red-purple color. Nodular lesions are commonly raised and typically develop late in the progression of KS disease (Safai, Johnson et al. 1985, Ganem 2010).

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of KS and two lymphoproliferative disorders

The strong association with host immunosuppression and the multifocal nature of the lesions suggested that KS had an infectious etiology. Using a subtractive hybridization technique called representational difference analysis, herpesvirus-like sequences were first identified in KS lesions from an HIV-positive patient in 1994 (Chang, Cesarman et al. 1994). These sequences were distinct from all known herpesvirus sequences, although they were related to two tumorigenic gammaherpesviruses: Epstein-Barr virus (EBV), the human lymphocryptovirus, and herpesvirus saimiri (HVS), the prototypical rhadinovirus of New World primates (Russo, Bohenzky et al. 1996). The novel virus was designated Kaposi's sarcoma-associated herpesvirus (KSHV). Based on sequence analysis, KSHV is classified as a gamma-2

herpesvirus or a rhadinovirus. A phylogenetic tree showing the relatedness of KSHV to alpha, beta, and gamma herpesviruses is shown in Figure 1-1.

The KSHV genome contains a 140kb coding region flanked by GC-rich terminal repeats (TRs) and has considerable genomic colinearity with EBV and HVS (Renne, Lagunoff et al. 1996, Russo, Bohenzky et al. 1996). KSHV encodes homologs of many cellular genes, such as viral interferon regulatory factors (vIRFs), viral G-protein coupled receptor (vGPCR), viral cyclin (vCyc), viral interleukin-6 (vIL-6), and viral FLICE-inhibitory protein (vFLIP). These genes dysregulate a number of signaling pathways in host cells, including cell cycle control, apoptosis, and anti-viral responses, and contribute to tumorigenesis (Choi, Means et al. 2001).

The availability of the KSHV genomic sequence facilitated the development of PCR-based and serological assays to study the natural history of KSHV infection in individuals and populations. Epidemiological studies have now confirmed that KSHV is the causative agent of all forms of KS. KSHV infection precedes development of KS in all cases, and KSHV DNA shows a strong association with KS tumors (Verma and Robertson 2003, Ganem 2006). Furthermore, population-wide studies have shown that KSHV seroprevelance matches with the geographical distribution of KS disease. Unlike other herpesviruses infections that are ubiquitous worldwide, KSHV has a low seroprevalence (between two and five percent) in most of the world. However, rates of KS are unusually high in the Meditterranean, where the seroprevalence of KSHV is approximately 20%. In some regions of Africa, where KS is endemic, over 60% of the population have antibodies against KSHV (Boshoff and Weiss 2001, Parkin 2006). In endemic areas, KSHV is commonly transmitted through saliva, and most primary infections occur in childhood prior to the onset of puberty (Mayama, Cuevas et al. 1998, Olsen, Chang et al. 1998). A strong family association also exists for KSHV in endemic areas

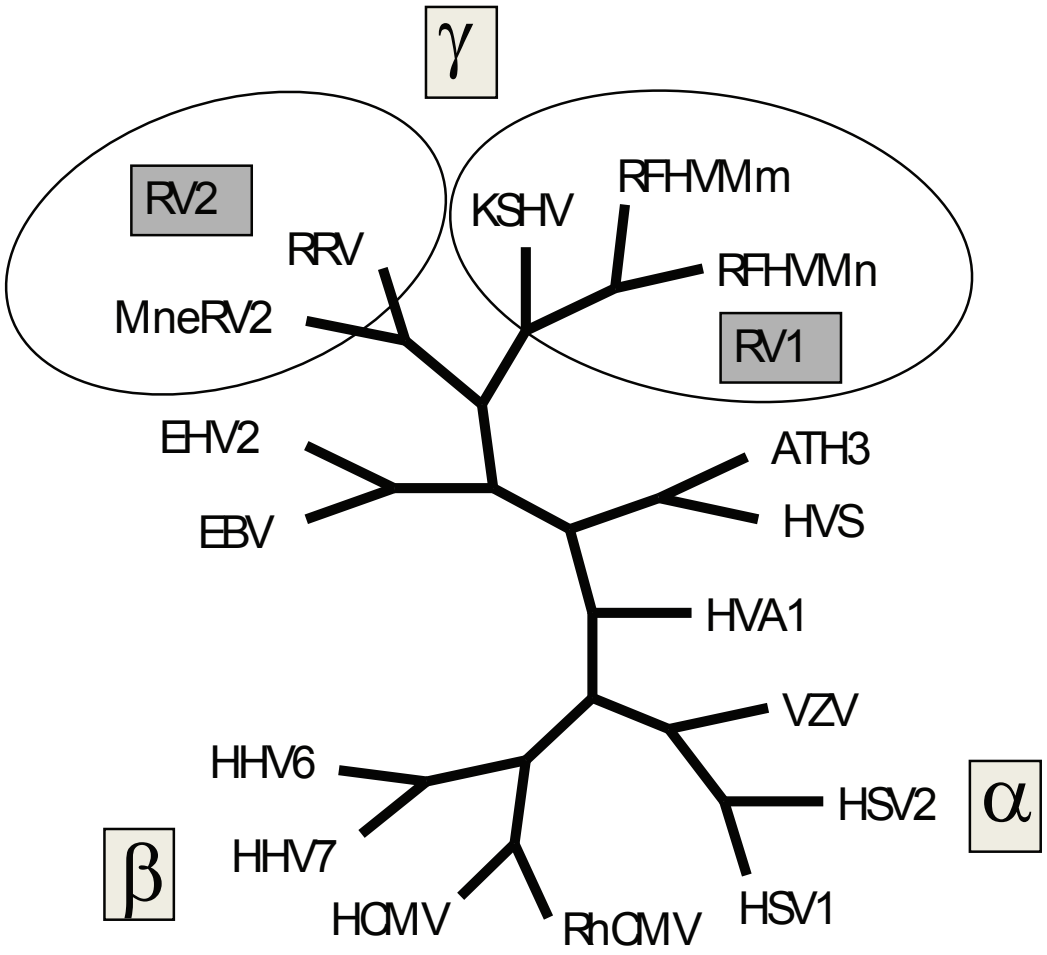

Figure 1-1: Phylogenetic tree of alpha, beta and gamma herpesviruses based on amino acid sequence of the DNA polymerase gene. Adapted from Schultz, Rankin et al., 2000.

(Plancoulaine, Abel et al. 2000). This closely resembles what has been reported for other herpesviruses that are transmitted through saliva. In the HIV-positive population, epidemiological studies showed that men who have sex with men (MSM) had the highest seroprevalence of KSHV and were at highest risk for developing KS. Sexual transmission is thought to be a major route of transmission for KSHV in the MSM population.

KSHV has also been found to be the causative agent of two AIDS-associated lymphoproliferative disorders: multicentric Castleman's disease and primary effusion lymphoma. Multicentric Castleman's disease (MCD) involves a polyclonal expansion of B cells and high level expression of interleukin 6 (IL-6). Patients with MCD exhibit high KSHV viremia and elevated serum levels of IL-6 and IL-10 (Polizzotto, Uldrick et al. 2012). Primary effusion lymphoma (PEL) is an aggressive B cell lymphoma that commonly presents in the peritoneal, pleural and pericardial cavities of late-stage AIDS patients. The prognosis for patients with PEL is poor, with a median survival of 6 months (Chen, Rahemtullah et al. 2007). All PEL cells are infected with KSHV and most are also EBV-positive, and the programs of infection of these viruses are thought to play an important role in PEL tumorigenesis. PELs contain immunoglobulin gene rearrangements and express marker of activated B cells like CD30 and CD38 (Matolcsy, Nador et al. 1998). However, PEL cells lack surface expression of the common B cell markers CD19 and CD20 (Nador, Cesarman et al. 1996). Unlike KS tumor cells that lose the viral episome after passage in culture, PEL cells maintain the KSHV genome through serial passage in vitro. For this reason, PEL cells are an important resource for in vitro studies of KSHV biology (Cesarman, Chang et al. 1995).

KSHV entry

Viral entry is a complex process involving multiple virus-host interactions that trigger virion uptake by endocytosis or membrane fusion (Burckhardt and Greber 2009). Many viruses utilize surface heparan sulfate (HS) glycosaminoglycans for initial attachment to target cells (Liu and Thorp 2002, Oh, Akhtar et al. 2010). Specific HS binding domains have been identified in the surface glycoproteins of several herpesviruses, including HSV-1, CMV, and VZV (Laquerre, Anderson et al. 1998, Mardberg, Trybala et al. 2001, Shukla and Spear 2001). Following the initial binding step, interactions with an additional cellular receptor are often required for viral entry. Multiple cell surface proteins have been proposed to play a role in KSHV entry, including HS, CD98/xCT, DC SIGN, integrins α3ß1 and αVß3, and ephrinA2 (Akula, Pramod et al. 2002, Kaleeba and Berger 2006, Rappocciolo, Jenkins et al. 2006, Garrigues, Rubinchikova et al. 2008, Veettil, Sadagopan et al. 2008, Chakraborty, Veettil et al. 2012). The role of integrin signaling has been an area of particular interest for KSHV entry studies (Sharma-Walia, Naranatt et al. 2004). Integrins are a family of transmembrane glycoprotein receptors that mediate interactions with the extracellular matrix (ECM). Each integrin heterodimer has distinct ligand specificity that is determined by the pairing of one alpha and one beta subunit. Eighteen alpha chains and 8 beta chains have been identified that combine to form 24 integrin molecules (Hynes 2002). Many integrins are utilized by viruses for entry (Stewart and Nemerow 2007, Grove and Marsh 2011). Previous studies in our lab have shown that the glycoprotein B (gB) of KSHV contains an N-terminal arg-gly-asp (RGD) motif that specifically binds αVß3 integrin. We demonstrated that specific RGD antagonists inhibit KSHV infection, suggesting that the binding of viral gB to αVß3 is required for viral entry (Garrigues, Rubinchikova et al. 2008). However, since target

cells often express several of the putative KSHV receptors, it has been difficult to identify the role of each surface receptor in KSHV entry.

KSHV latency

The life cycle of herpesviruses involves a latent and a replicative program of infection, and the switch from latency to replication is a critical event in the viral life cycle. In KS tumors and KSHV infected cells in vitro the vast majority of cells are latently infected, with only a small percentage of cells undergoing active replication. The viral genome persists in the nuclei of latently-infected cells as a circular episome and is copied by host replication machinery during mitosis. Only a limited set of genes are expressed during KSHV latency, including the latency-associated nuclear antigen (LANA/ORF73), viral FLICE-inhibitory protein (vFLIP/ORF71), viral Cyclin D (vCycD/ORF72), Kaposins A-C and 12 microRNAs. LANA, vFLIP and vCycD are encoded by co-terminal monocistronic and polycistronic transcripts expressed under the control of the major latency promoter (Staskus, Zhong et al. 1997, Dittmer, Lagunoff et al. 1998, Glenn, Rainbow et al. 1999, Sarid, Wiezorek et al. 1999, Talbot, Weiss et al. 1999, Pearce, Matsumura et al. 2005). A different promoter controls expression of the Kaposin genes and miRNAs (Li, Komatsu et al. 2002, Pearce, Matsumura et al. 2005, Cai and Cullen 2006).

The major latency antigen LANA is a large nuclear protein that contains three distinct domains: a serine-proline rich N-terminal region with a functional nuclear localization signal (NLS), a central repeat region of variable size, and a C-terminal domain that mediates dimerization and transcriptional regulation (Schwam, Luciano et al. 2000, Piolot, Tramier et al. 2001, Cherezova, Burnside et al. 2011). The size of LANA isolates ranges from 1003 to 1162 amino acids depending on the length of the central repeat region (Gao, Zhang et al. 1999).

LANA regulates a number of viral and host genes, including several involved in cell survival and proliferation (Choi, Means et al. 2001). LANA modulates the apoptosis pathway through post-translational modification of the p53 and retinoblastoma (pRB) tumor suppressors (Friborg, Kong et al. 1999, Radkov, Kellam et al. 2000, Si and Robertson 2006). Beta-catenin, a cellular protein that activates cellular proliferation by promoting entry into S phase, is also regulated by LANA (Fujimuro, Wu et al. 2003). In infected cells LANA plays a critical role in the establishment and maintenance of KSHV latency. LANA binds the terminal repeat (TR) region of the viral genome and tethers the viral episome to host cell chromatin through interactions with histones 2A and 2B (Ballestas, Chatis et al. 1999, Barbera, Ballestas et al. 2004). This tethering ensures the faithful separation of the viral episome during cell division and thus allows latent virus to persist in host cells. When bound to chromatin and TR regions, LANA exhibits a characteristic punctuate morphology (Schwam, Luciano et al. 2000, Ballestas and Kaye 2001). This punctuate pattern of expression can be seen in tumor cells in vivo and in latently infected tissue culture cells (Rainbow, Platt et al. 1997).

KSHV replication and transcriptional activator (Rta): the master regulator of reactivation

The replication program of herpesviruses proceeds in distinct waves of viral gene expression. The first genes expressed are the immediate-early genes, which do not require de novo expression of viral or cellular proteins but instead are regulated by the pre-existing balance of host factors present upon infection. The expression of immediate-early genes is therefore resistant to cycloheximide, an inhibitor of protein synthesis. Generally, immediate-early genes encode transcriptional regulators that activate downstream viral gene expression of early and late lytic genes. (Sun, Lin et al. 1999).

While latency is considered the default program of infection for KSHV, it is not believed to be sufficient for viral persistence in vivo. Clinical studies have found that treatment with specific inhibitors of KSHV replication, such as ganciclovir, reduced the incidence of KS lesions (Glesby, Hoover et al. 1996, Mocroft, Youle et al. 1996, Martin, Kuppermann et al. 1999). These findings indicated that low levels of ongoing viral replication in vivo play a critical role in the maintenance of KS tumors, presumably by supplying angiogenic factors and replenishing the latent reservoir (Grundhoff and Ganem 2004). Dissecting how replication is regulated in rhadinoviruses, therefore, is essential to understanding the biology of these infections and developing new antiviral therapies. However, our understanding of KSHV replication is somewhat limited due to the lack of a natural system of replicative infection. In vitro, high levels of KSHV replication can be induced by treating latently infected cells with histone deacetylase (HDAC) inhibitors such as sodium butyrate or phorbol esters like TPA. BC-1 and BCBL-1 PEL cells are latently infected with KSHV and contain high copy numbers of the viral genome. Treatment of these cells with sodium butyrate or TPA results in viral reactivation and production of infectious virions. KSHV replication can also be induced by overexpressing the viral replication and transcriptional activator (Rta) encoded by ORF50. Rta was initially identified as a positional homolog of the EBV transactivator BRLF1, and it has been shown that Rta is the only gene that is necessary and sufficient for reactivation of latent KSHV (Lukac, Renne et al. 1998, Sun, Lin et al. 1998, Lukac, Kirshner et al. 1999). Rta activates expression of viral genes both by direct binding of viral promoters and through interactions with cellular transcription factors. Studies of KSHV gene expression revealed that Rta transcription is resistant to cycloheximide, and therefore displays immediate-early kinetics. Rta has been identified as a component of the KSHV virion, and it is thought that virion-associated Rta may

activate its own promoter early in infection (Bechtel, Winant et al. 2005, Harrison and Whitehouse 2008).

Because of its central role in reactivation, Rta expression is considered the critical step in initiating the KSHV replication cascade. A number of cis-acting regulatory elements have been identified in the Rta promoter including binding sites for the cellular transcription factors Oct1, C/EBP, Sp1, Ap1, XBP-1, and YY1 (Sakakibara, Ueda et al. 2001, Lu, Zhou et al. 2003, Wang, Wu et al. 2003, Wang, Wu et al. 2004, Ye, Shedd et al. 2005, Wilson, Tsao et al. 2007, Yu, Feng et al. 2007, Chang, Chen et al. 2011). Additionally, viral factors, such as LANA and miRNAs have been shown to negatively regulate Rta expression during latency (Lan, Kuppers et al. 2004, Orzechowska, Powers et al. 2008).

Rta expression is silenced during latency, and it is thought that epigenetic regulation is involved in the transcriptional repression of Rta. In latently infected PEL cells, the region surrounding the Rta promoter has been shown to be associated with repressive chromatin patterns, such as the trimethylation of histone 3 lysine 27 (H3K27-me3) (Lu, Zhou et al. 2003). H3K27-me3 is a marker of heterochromatin and transcriptional repression. A recent study of the Rta promoter indicated that several repressive histone modifications accumulate in this region following de novo KSHV infection, although these epigenetic patterns may not be established for several days. Interestingly, it was noted that the Rta promoter region contains bivalent chromatin structure that is characterized by both activating and repressive histone markings (Gunther and Grundhoff 2010). Similar chromatin markings have been shown to keep promoters “poised” for activation during key stages embryonic development (Bernstein, Mikkelsen et al. 2006, Mikkelsen, Ku et al. 2007). It is believed that the bivalent chromatin structure of the Rta promoter, therefore, reflect a readiness for viral reactivation. DNA methylation may also play a

role in Rta repression during latent infection, though reports have been mixed on the requirement of these modifications for latency. Rta promoter DNA has been shown to be highly methylated in some latently infected cells in vitro (Sun, Lin et al. 1998, Chen, Ueda et al. 2001). However, Gunther et al. have recently reported that this region is unmethylated in PEL cell lines, suggesting that methylation of Rta promoter DNA is not essential for latency but may instead stabilize latency in some instances (Gunther and Grundhoff 2010).

Gene expression kinetics in early KSHV infection

Two studies have reported that Rta is expressed immediately following de novo KSHV infection (Krishnan, Naranatt et al. 2004, Lan, Kuppers et al. 2004). However, instead of inducing active viral replication, Rta levels decline rapidly shortly after infection. It was also reported that LANA levels steadily increase over this time period, peaking at 24 hours post-infection. At this time point, LANA protein has readily accumulated in the nuclei of infected cells and viral latency is established. Because LANA functions as an inhibitor of Rta expression, the steep decline in Rta levels is thought to result from LANA-mediated repression of the Rta promoter (Lan, Kuppers et al. 2004, Jin, He et al. 2012). However, the spike in Rta levels during early KSHV infection prompted investigation into the role of Rta in latency. A series of recent reports have demonstrated that Rta induces LANA expression early in infection through an Rta-inducible promoter (LANApi) and that Rta-mediated activation of LANApi is critical for the establishment of latency (Matsumura, Fujita et al. 2005, Lu, Verma et al. 2011). LANApi is distinct from the constitutive promoter (LANApc) that regulates LANA expression in latently infected cells and is located in the intron region that is spliced out during maturation of the latent transcript. Rta activates LANApi through an interaction with the recombinant signal binding

protein for immunoglobulin kappa J region (RBP-Jk) transcription factor, a downstream effector of the Notch signaling pathway (Lan, Kuppers et al. 2005). Rta and RBP-Jk form a complex at LANApi, and DNA binding is mediated by an Rta-responsive element (RRE) and two head-to-head RBP-Jk binding sites in the LANA promoter region (Hilton and Dittmer 2012). Deletion of either RBP-Jk binding site abolished LANApi activity and resulted in a diminished ability to establish latency (Lu, Verma et al. 2011, Hilton and Dittmer 2012). Therefore, LANA and Rta together comprise a regulatory loop early in KSHV infection that leads to the establishment of viral latency. As such, early expression of Rta activates the LANApi promoter and induces high-level expression of LANA. LANA shuts off Rta expression through transcriptional repression, ultimately resulting in the establishment of latency in the infected cell. Interestingly, Hilton et al. found that expression of the Notch activator ICN alone did not induce the LANApi promoter. LANApi activation required Rta, suggesting that the promoter is Rta-specific and does not respond to broad Notch signaling (Hilton and Dittmer 2012). It was suggested that the close proximity of the RRE and head-to-head RBP-Jk sites found in LANApi may account for its unique requirement for Rta and the insensitivity of the promoter to Notch activation.

Macaques and other Old World primates are host to two distinct lineages of KSHV-like rhadinoviruses

Early attempts to identify an animal model for KS revealed that KSHV infection of non-human primates elicited a host immune response but failed to produce evidence of persistent infection or disease (Dittmer, Stoddart et al. 1999, Renne, Dittmer et al. 2004). Because of the lack of a tractable animal model for KSHV infection and disease, studies of KSHV biology have relied on in vitro infection systems and comparative analyses of closely related viruses. Two

distinct lineages of KSHV-like rhadinoviruses have been identified in Old World primate species, as shown in Figure 1-1 (Searles, Bergquam et al. 1999, Greensill, Sheldon et al. 2000, Schultz, Rankin et al. 2000, Strand, Harper et al. 2000, Lacoste, Mauclere et al. 2001). The RV1 rhadinovirus lineage consists of KSHV and its close evolutionary homologs from non-human primate species. Retroperitoneal fibromatosis herpesvirus (RFHV), the macaque homolog of KSHV, was initially detected in KS-like lesions in different species of immunosuppressed macaques (Rose, Strand et al. 1997). The isolates were designated RFHVMm, from rhesus macaques (Macaca mulatta), and RFHVMn, from pig-tailed macaques (Macaca nemestrina). These viruses cluster with KSHV in the RV1 lineage of Old World rhadinoviruses. The complete sequences of the ORF8 DNA polymerase and ORF9 glycoprotein B genes of RFHV, as well as the four adjacent genes of divergent locus B (DL-B), were obtained using consensus degenerate hybrid oligonucleotide primers (CODEHOPs) targeting regions highly conserved across members of the herpesvirus family (Rose, Schultz et al. 1998, Rose, Ryan et al. 2003). Additionally, our laboratory cloned and sequenced the LANA/ORF73 homolog of RFHVMn. Sequence analysis revealed that RFHV LANA contained a serine-proline rich N-terminal domain of ~300 amino acids, a central acidic repeat region, and a C-terminal domain of ~225 amino acids (Burnside, Ryan et al. 2006). This structure was highly conserved with KSHV LANA. Like KSHV LANA, RFHV LANA localizes to the nucleus in transfected cells (Burnside, Ryan et al. 2006). RFHV LANA was also detected in the nuclei of retroperitoneal fibromatosis (RF) tumor cells, indicating that RFHV, like KSHV, establishes latent infection in tumor cells in vivo (Bruce, Bakke et al. 2006). These studies have provided important insights into the structural evolution of RV1 rhadinoviruses, but the lack of an in vitro culture system for RFHV has limited our understanding of its biology.

The RV2 rhadinovirus lineage is evolutionarily distinct from KSHV and the RV1 homologs. Members of the RV2 lineage have been detected in the same non-human primate species that are host to the RV1 rhadinoviruses, and individual animals can be co-infected with both RV1 and RV2 viruses (Greensill, Sheldon et al. 2000, Strand, Harper et al. 2000). Though RV2 lineage viruses have been identified in several species of macaque, African green monkeys, mandrills, gorillas and chimpanzees, an RV2 lineage rhadinovirus has not yet been detected in humans. Rhesus rhadinovirus (RRV), the prototypical RV2 lineage virus, was identified by two groups in immunocompromised (strain 17577) and immunocompetent (strain 26-95) rhesus macaques (Desrosiers, Sasseville et al. 1997, Searles, Bergquam et al. 1999). Full genome sequencing of RRV isolates 17577 and 26-95 identified few strain differences and showed that the genome of RRV was highly homologous and colinear with KSHV and RFHV, although several differences were noted. One major difference was the lack of the K3 and K5 immune evasion genes that are believed to be important for maintaining latency in KSHV and the expansion of the viral interferon regulatory factors (vIRFs) that control the host immune response to RRV infection (Alexander, Denekamp et al. 2000, Robinson, Estep et al. 2012). Unlike KSHV, most tissue culture cells are permissive for RRV replication and produce high titers of infectious virus (Desrosiers, Sasseville et al. 1997, DeWire, Money et al. 2003). Studies of captive macaques have shown that the seroprevalence of RRV in these populations is high and increases with age (White, Todd et al. 2009, Ryan and Rose in press). Furthermore, we have recently demonstrated that RRV is associated with a distinct group of macaque T cell and B cell lymphomas that express markers of viral replication (Bruce, Bielefeldt-Ohmann et al. 2012). Because of its replicative phenotype in vitro and in vivo, RRV represents a useful system to study the determinants of rhadinovirus replication.

An Rta homolog was identified in RRV that is positionally conserved with KSHV Rta and displays high homology across the DNA binding and activation domains (Lin, Robinson et al. 2002). Studies have confirmed that RRV Rta is expressed early in infection and functions as a potent activator of viral promoters, including R8p, ORF57p, and bZIPp (Damania, Jeong et al. 2004, Dittmer, Gonzalez et al. 2005). RRV also encodes a LANA/ORF73 homolog that localizes to the nuclei of transfected cells (Burnside, Ryan et al. 2006). However, a system of natural RRV latency has not been identified, and thus the role of RRV LANA in latent infection is still unknown. Functional studies have shown that ectopic expression of RRV LANA inhibits replication in rhesus fibroblast cells, and it was suggested that the inhibition occurred through repression of Rta-mediated transcriptional activity (DeWire and Damania 2005). Sequence analysis indicated that the C-terminal domain of RRV LANA is highly conserved with the RV1 LANAs, but RRV LANA lacks the central acidic repeat region conserved in KSHV and RFHV LANA (Burnside, Ryan et al. 2006).

Though RV2-specific reagents are not commercially available, our laboratory has developed several reagents and assays to follow RV2 infections in vitro and in vivo. We have previously characterized replicative RRV infection using a rabbit polyclonal antibody against the RV2 ORF59 DNA polymerase processivity factor, a commonly used marker of herpesvirus replication (Bruce, Bakke et al. 2009). Using this antibody, we showed that rhesus primary fetal fibroblast (RPFF) and African green monkey kidney epithelial (Vero) cells are permissive for RRV replication. The infected cultures contained rapidly expanding foci of cells expressing ORF59 and produced high titers of infectious RRV. Gene expression studies showed the accumulation of transcripts of replication-associated genes, including early expression of Rta (ORF50), followed by ORF59, DNA polymerase (ORF9), and glycoprotein B (ORF8)

expression. These results suggest a central role for Rta in the replicative program of infection of RRV.

Hypothesis

Latency is considered the default program of infection for KSHV but can be disrupted by over-expression of the Rta gene or treatment with biochemical activators. Recent studies have demonstrated that interplay between Rta and LANA during early KSHV infection is critical for the establishment of latency in infected cells. Whereas KSHV infection is predominantly latent, RRV replicates efficiently in cultured cells in vitro and in vivo. KSHV and RRV, therefore, exhibit a differential ability to establish latency and replication in infected cells. Based on these phenotypes, I hypothesize that the Rta transactivator is differentially regulated in KSHV and RRV. In order to understand how Rta expression is regulated during latency and replication of RRV, I will compare the activity of the RRV Rta promoter in non-permissive and permissive cells and identify cis-acting regulatory elements in the Rta promoter region. I will also examine whether the viral factor LANA regulates the Rta promoter in RRV. These studies will inform our understanding of how KSHV latency and RRV replication are determined in infected cells.

CHAPTER II

A critical Sp1 element in the RRV Rta promoter confers high-level activity that correlates with cellular permissivity for viral replication

BACKGROUND

Sequence analysis has revealed that RV1 and RV2 lineage rhadinoviruses are closely related and share similar genomic architecture. However, KSHV and RRV, the prototypical RV1 and RV2 rhadinoviruses, respectively, display a differential ability to establish latency and replication in infected cells. Both in vitro and in vivo, KSHV establishes latent infection, with only a small percentage of cells undergoing active replication. In contrast, RRV infection of cultured cells is permissive for replication leading to a cascade of lytic gene expression resulting in the production of infectious virus. We have also reported the expression of lytic genes in RRV-associated lymphomas, suggesting that the replicative phenotype observed in cultured cells is a hallmark of RRV infection and not an artifact of in vitro culture (Bruce, Bielefeldt-Ohmann et al. 2012).

Because of its role as the master regulator of viral replication, the KSHV Rta promoter has been extensively studied. A transcriptional start site (TSS) was identified 36 nucleotides upstream of the translational start site (+1), and a TATA-like element (TTAAAA) located at nucleotide -67 has been shown to be critical for basal activity (Lukac, Kirshner et al. 1999, Ye, Shedd et al. 2005). A number of binding sites for cellular transcription factors have also been identified in the KSHV Rta promoter, including binding sites for Ap1, C/EBP, Oct1, Sp1, and XBP-1 (Sakakibara, Ueda et al. 2001, Lu, Zhou et al. 2003, Wang, Wu et al. 2003, Wang, Wu et al. 2004, Wilson, Tsao et al. 2007, Yu, Feng et al. 2007, Orzechowska, Powers et al. 2008).

However, since a natural system of KSHV replication has not been identified, it has only been possible to study KSHV Rta promoter activation in non-permissive cell lines following treatment with butyrate or TPA. Using these systems, Wang et al. reported that TPA reactivation of KSHV involves the Ap1 signaling pathway, and the Ap1-binding site in Rta promoter is required for TPA-mediated activation (Wang, Wu et al. 2004). Similarly, the Sp1 site located at -112 was shown to be critical for butyrate-responsiveness of the Rta promoter (Lu, Zhou et al. 2003, Ye, Shedd et al. 2005). Auto-activation of the Rta promoter requires the Oct1-binding site at nucleotide -257 (Harrison and Whitehouse 2008). Whether these specific host factors are involved in reactivation of latent KSHV in the host is unknown.

An Rta homolog has been identified in RRV that is highly conserved with KSHV Rta across the DNA-binding and activation domains. We and others have reported that RRV Rta, like KSHV Rta, is expressed early following de novo infection, and can activate the promoters of several viral genes including its own (DeWire and Damania 2005, Dittmer, Gonzalez et al. 2005, Bruce, Bakke et al. 2009). These studies indicate that RRV Rta is a positional and functional homolog of KSHV Rta and plays a central role in viral replication. However, whereas KSHV Rta expression is quickly shut off following infection, RRV Rta continues to be expressed, driving expression of replication-associated genes and the production of infectious virus (Krishnan, Naranatt et al. 2004, Lan, Kuppers et al. 2005, Bruce, Bakke et al. 2009). In this chapter, we have identified a novel system of latency in epithelial cells and characterized the RRV Rta promoter in cells that are permissive and non-permissive for viral replication. We demonstrate that the RRV Rta promoter is highly active in permissive cells but only minimally active in non-permissive cells. A critical Sp1 element that is conserved in KSHV was found to be required for high-level RRV Rta promoter activity in permissive cells and butyrate-

responsiveness in non-permissive cells. We also present evidence of an in vivo correlation between Sp1 and RRV replication in salivary gland tissue from an infected macaque. These findings suggest that host factors, like Sp1, regulate the RRV Rta promoter and that high-level Rta promoter activity strongly correlates with cellular permissivity for replication.

MATERIALS AND METHODS

Plasmids. The pGL2-R-Rta plasmid containing 502 nucleotides of the RRV Rta promoter sequence upstream of the firefly luciferase reporter gene was a kind gift from Dr. B. Damania. Truncated fragments of the Rta promoter were prepared from pGL2-R-Rta and inserted upstream of the firefly luciferase gene in pGL2 basic (Promega) using KpnI and SacI restriction sites and the following forward primers: 487 (5' ATAAGGTACCTCGCGTGATCTTTT 3'), 443 (5' ATATGGTACCTAACAAACCTC ACTCCCTGTAA 3'), 414 (5' ATATGGTACCATAAGGTCCGTTCTTTCTATC 3'), 385 (5' ATATGGTACCTGAATCTATAGTTACATCTTTAAG 3'), 321 (5' ATAAGGT ACCTTAAAAAATCGCAAAAGCGAC 3'), 299 (5' ATAAGGTACCGATGGCTCTA TCCGCGTT 3'), 263 (5' ATAAGGTACCTAGTCACGATGGATCTCCAGT 3'), 216 (5' AATAGGTACCTTAACTGGAATGGAAACAGC 3'), 187 (5' ATATGGTACCGTGA ACTTCCTGATGTCTCCTA 3'),153 (5' ATAAGGTACCAAACAGAGCTAAATACC AATGAC 3'), 136 (5'ATAAGGTACCAATGACTGTCACCCCTACCC 3'), 102 (5' ATAAGGTACCGTACTATTAGACCAGGGGTGAG 3'), 73 (5' ACTTGGTACCCTA TCCTTTAAAAACCCATACG 3'). The same reverse primer was used for all RRV Rta promoter clones: (5' ACTTGAGCTCTTTATGACAGGCGTC 3'). Mutagenesis of the Sp1 site at nucleotide -113 in pGL2-R-Rta was done by site-directed mutagenesis (Mutagenex). pGL2-

K-Rta, containing the homologous region of the KSHV Rta promoter upstream of the luciferase reporter gene, was constructed by amplifying promoter sequence from BCBL-1 DNA using the following primers: forward (5' ATTAG AGCTCGCTGTTGCCTGGCATTTTGC 3'), reverse (5' ATTAACGCGTTTTTGTGGC TGCCTGGACAG 3'). KSHV Rta promoter sequence was inserted into pGL2 using the SacI and MluI sites. All constructs were verified by sequencing. The pRL-CMV plasmid, containing the CMV IE promoter driving a Renilla reporter gene was obtained from Promega.

Cells and virus stocks. Human salivary gland (HSG) epithelial cells were a kind gift of Dr. K. Izutsu (Shirasuna, Sato et al. 1981). Owl monkey kidney (OMK) epithelial cells were provided by Dr. S. Barcy. Gastric adenocarcinoma (AGS) epithelial cells were provided by Dr. N. Salama (Pinto-Santini and Salama 2009). Rhesus primary fetal fibroblast (RPFF) cells and RRV strain 17577 were kindly provided by Drs. M. Axthelm and S. Wong. 293 human kidney epithelial cells and African green monkey kidney epithelial (Vero) cells were obtained from the ATCC. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% cosmic calf serum, 1% penicillin-streptomycin and 1% HEPES. RRV virions were harvested by ultracentrifugation of supernatants from infected RPFF cell cultures as previously described (Bruce, Bakke et al. 2009).

Antibodies. ORF59 expression was detected using rabbit polyclonal antiserum with specific reactivity against RV2 ORF59. Briefly, 89 amino acids from the C-termini of RRV and MneRV2 were cloned into the 6xHis expression vector pQE30. This region of ORF59 showed high conservation among RV2 viruses but little homology with RV1 sequences. Recombinant

His-tagged proteins were expressed in E. coli, purified, and used to immunize rabbits. Serum with specific reactivity to RV2 but not RV1 ORF59 protein was selected and used in IFA assays (Bruce, Bakke et al. 2009). Antibody binding was visualized with Alexa-488 conjugated secondary goat anti-rabbit antibody. The Sp1 antibody (clone 1C6) was obtained from Santa Cruz and used according to the manufacturer's recommendations for immunohistochemistry.

Immunofluorescence and confocal microscopy. Cells were plated onto 17mm spots as previously described (Garrigues, Rubinchikova et al. 2008). Following overnight culture cells were incubated with RRV for 3 hours at 37°C. To determine if the RRV infections were permissive, a low MOI (~0.005) was used and the cultures were evaluated at 3 days post infection for the presence of spreading ORF59-positive foci. To determine if RRV infections were latent, infected cultures were treated 3 hours post-infection with 4mM sodium butyrate (Millipore) and the cultures were evaluated 3 days later for the presence of ORF59-positive foci. To detect ORF59 expression, cells were fixed in 4% paraformaldehyde and incubated with a polyclonal rabbit 425 anti-RV2 ORF59 antiserum as previously described (Bruce, Bakke et al. 2009). For comparison purposes, BCBL-1 cells latently-infected with KSHV were incubated with a monoclonal antibody reactive with KSHV ORF59 (Advance Biotechnologies) before and after treatment with 4 mM sodium butyrate for 24 hours to identify sites of replication. Cell nuclei were stained with TO-PRO (Molecular Probes). Images were acquired with a Zeiss LSM 5 Pascal confocal microscope.

Reporter assays. Cells were cultured on 6-well plates and transfected with 3 ug of the firefly reporter gene construct (pGL2-R-Rta or pGL2-K-Rta) and 10 ng of Renilla luciferase pRL-CMV

construct as an internal control for transfection efficiency using LT1 reagent (Mirus). Lysates were harvested 24 hours post-transfection using the Dual Luciferase kit (Promega). Background activity of empty pGL2 vector was subtracted and the mean luciferase activity from triplicate wells was determined. Relative luciferase activity is expressed as the ratio of firefly luciferase activity to Renilla activity.

Immunohistochemistry. Parotid salivary gland tissue from M. nemestrina M04203 and K93344 were provided by S.L. Hu, Washington National Primate Research Center (WaNPRC). Tissues were collected at necropsy and fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemistry was performed using the Envision system (Dako). Briefly, sections were cut, deparaffinized and rehydrated in a graded series of alcohol solutions. Antigen retrieval was performed in Tris-EDTA buffer at 95°C for 25 minutes, and samples were subsequently allowed to cool to room temperature. Tissue was blocked with 0.15 M glycine (pH 7.4) for 15 minutes and 2% normal goat serum/TBS for 30 minutes and incubated with primary antibody overnight at 4°C. Bound antibody was visualized with peroxidase labeled polymer and AEC chromagen substrate according to the manufacturer's instructions, followed by counterstaining with hemotoxylin.

RESULTS

Comparison of the KSHV and RRV Rta promoters. In order to identify regulatory elements conserved between KSHV and RRV Rta promoter sequences, the regions directly upstream of the translational start sites were aligned. These regions correspond to nucleotides 71170-71697 of the KSHV genome (NC_009333.1) and nucleotides 66858-67364 of the RRV strain 17577

genome (NC_003401.1) (Searles, Bergquam et al. 1999, Alexander, Denekamp et al. 2000). Although the two promoter regions did not show a high level of sequence homology, an optimal alignment was obtained that showed a close relationship between the Rta translational and transcriptional start sites of KSHV and RRV, with scattered regions of conserved sequences extending to the upstream ORF48 translational start site and coding sequence. A number of binding sites for cellular transcription factors identified in the KSHV Rta promoter were mapped to the sequence (Figure 2-1). A GC box containing nine consecutive guanines and cytosines located between -112 and -103 of the KSHV Rta promoter has been shown to be a binding site for the Sp1 transcription factor (Lu, Zhou et al. 2003, Ye, Shedd et al. 2005). Similar proximal Sp1-binding sites have been identified in a wide variety of eukaryotic promoters (Lania, Majello et al. 1997). The transcription factor binding prediction program PROMO (Messeguer, Escudero et al. 2002) identified a positionally conserved Sp1 site in the RRV Rta promoter, from nucleotides -113 to -103. Eight of nine nucleotides in the GC box were identical between the KSHV and RRV Rta promoter sequences. The other transcription factor binding sites identified in the KSHV Rta promoter, including those for Oct1, Ap1, XBP-1 and C/EBP, were not well conserved in the RRV Rta promoter.

Vero and 293 kidney epithelial cells are permissive for RRV replication. RRV has been widely reported to replicate in rhesus fibroblast cells (Alexander, Denekamp et al. 2000, DeWire, Money et al. 2003). We have also shown that RRV replicates efficiently in Vero cells, a kidney epithelial cell line of African green monkeys. The RRV infected Vero cultures contained rapidly expanding foci of cells expressing ORF59, the DNA polymerase processivity factor replication marker, and produced high titers of infectious virus (Bruce, Bakke et al. 2009). We examined

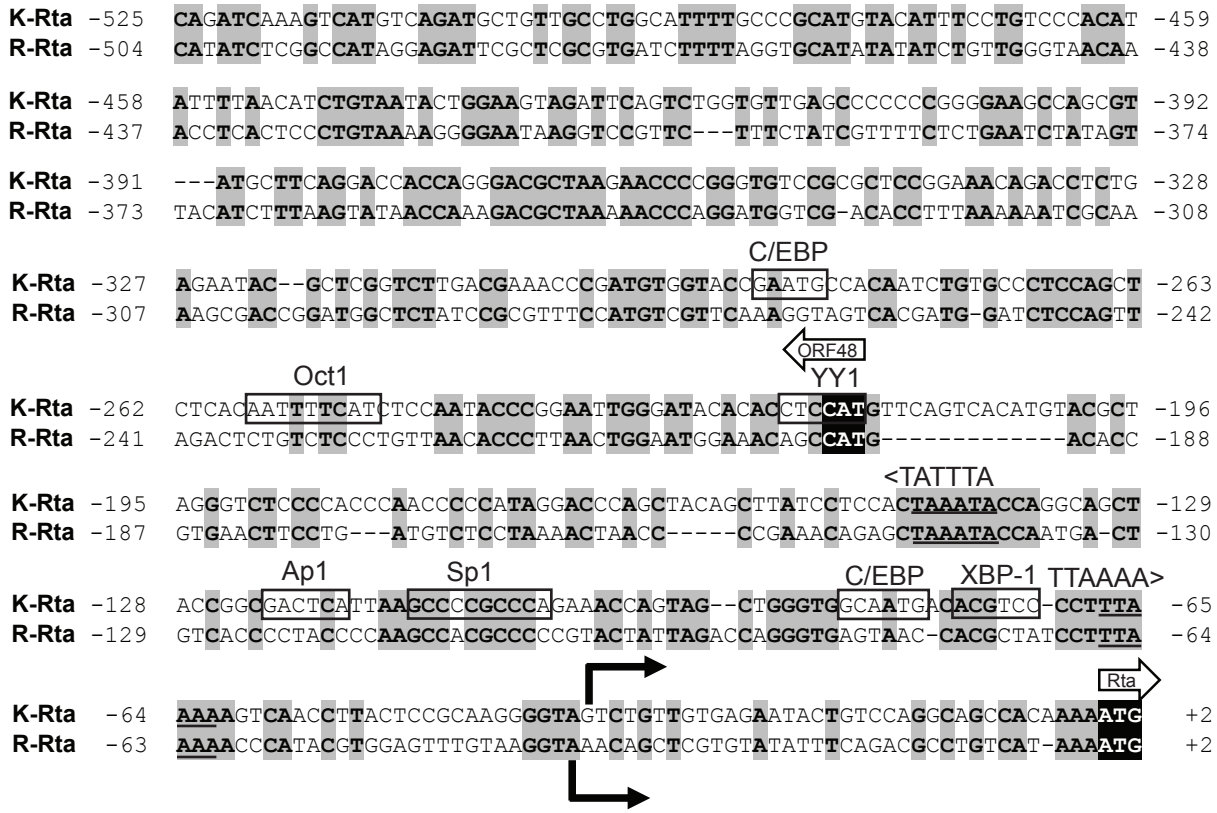

Figure 2-1: Comparison of the promoter regions of K-Rta and R-Rta, the replication and transactivator genes for KSHV and RRV, respectively. Alignment of nucleotides 71170 to 71697 of KSHV (NC_009333.1) and 66858 to 67364 of RRV (NC_003401.1) genomic sequence. Nucleotide numbering indicates the position relative to the translational start site. Identical nucleotides are highlighted in gray. Known transcription factor binding sites for C/EBP, Oct1, YY1, Ap1, Sp1 and XBP-1 in the KSHV Rta promoter are indicated. Transcriptional start sites for KSHV Rta and RRV Rta are identified by solid arrows. Translational start sites for Rta and ORF48 are indicated. TATA-like elements upstream of ORF48 and Rta are shown.

the permissivity of other epithelial cell lines using expression of ORF59 as an indicator of replication. We first evaluated the ability of RRV (strain 17577) to infect 293 cells, a human kidney epithelial cell line. A previous study using RRV strain 26-95 suggested that 293 cells were not permissive for RRV replication (DeWire and Damania 2005). We incubated 293 cultures with purified RRV virions and evaluated the cells for replication. Three days post-infection, ORF59 expression was detected using the rabbit polyclonal antibody we have developed against the ORF59 homologs of macaque RV2 rhadinoviruses. Permissive RPFF and Vero cell cultures were included as positive controls for infection. As expected, infected RPFF and Vero cultures contained expanding foci of ORF59-positive cells (Figure 2-2A,B), as reported previously (Bruce, Bakke et al. 2009). Similar ORF59-positive foci were also detected in the RRV infected 293 cultures (Figure 2-2C), indicating that 293 cells were permissive for RRV (strain 17577) replication and that the infection had spread from an initial site of infection to surrounding cells in the course of three days. To test for the presence of newly replicated infectious RRV virions, supernatant from 293 cultures was removed and incubated with uninfected RPFF and Vero cell cultures. Analysis of these cultures two days post-infection revealed new ORF59-positive foci demonstrating the presence of infectious virions in the supernatant of RRV infected 293 cells (data not shown). Thus, like primary fibroblasts, kidney epithelial cells from both human and non-human Old World primate species are permissive for RRV infection leading to replication and the production of infectious virions.

Human salivary gland (HSG) and adenocarcinoma gastric (AGS) epithelial cells are not permissive for RRV replication. The human salivary gland (HSG) epithelial cell line was tested for permissivity for RRV replication. HSG cells were originally isolated from an

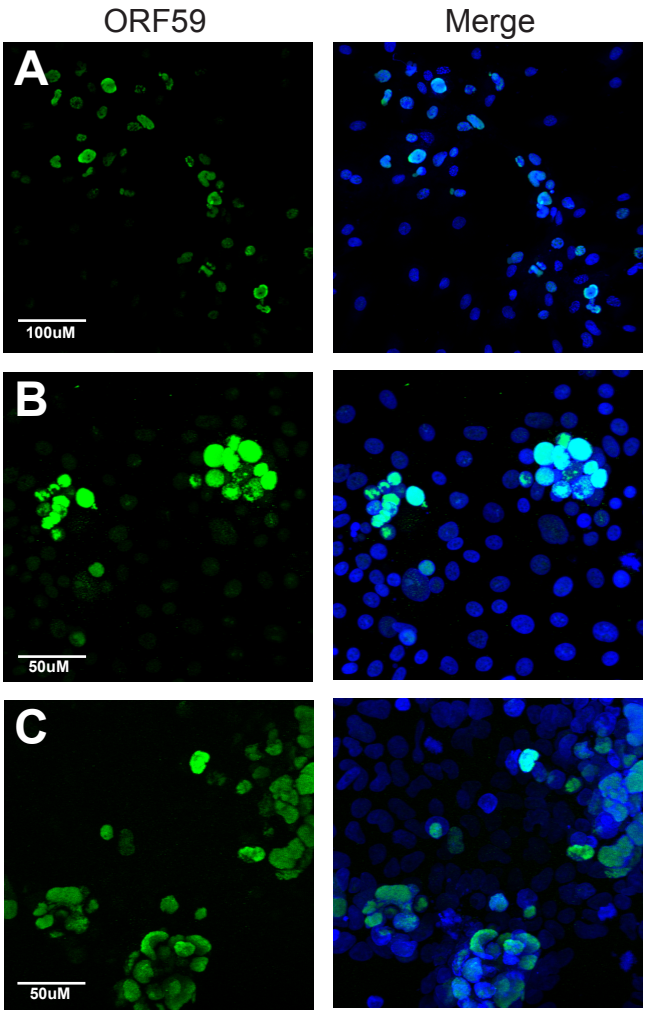

Figure 2-2: RRV replicates efficiently in a variety of cell lines from human and non-human Old World primate species. (A) Rhesus primary fetal fibroblast (RPFF), (B) African green monkey kidney epithelial (Vero), and (C) human embryonic kidney epithelial (293) cells were infected with RRV and evaluated three days post-infection for expression of ORF59, the DNA polymerase processivity factor, using rabbit anti-RV2 ORF59 antiserum. Cell nuclei were visualized with TO-PRO stain and are shown in blue (merge). Images were acquired by confocal microscopy and are shown as projections of the z-stack.

irradiated submandibular gland and grow as an undifferentiated monolayer with cuboidal morphology under normal culture conditions (Shirasuna, Sato et al. 1981). HSG cell cultures were incubated with RRV as described for the kidney epithelial cell lines. Three days post-infection the cell cultures were examined for ORF59 expression using the anti-RV2 ORF59 antibody to identify sites of replicative infection. No ORF59 expression was detected in these cultures (Figure 2-3A), indicating that HSG cells were not permissive for RRV replication. To determine if RRV had established a latent infection in these cells, we tested whether sodium butyrate could induce active RRV replication in the infected HSG cells. ORF59-negative, RRV infected HSG cells were treated with sodium butyrate immediately following infection and assayed for ORF59 expression at three days post-infection. Foci of ORF59 positive nuclei were detected in the butyrate-treated HSG cell cultures (Figure 2-3B), indicating that the initial RRV infection of HSG cells was latent and could be reactivated by butyrate treatment. Further testing revealed that RRV latently infected other cell lines, including human gastric adenocarcinoma (AGS) and owl monkey kidney (OMK) epithelial cells, and could be reactivated with sodium butyrate (data not shown). These systems recapitulate the butyrate-mediated reactivation of latent KSHV in BCBL-1 cells as shown in panels C and D of Figure 2-3. As others have shown, all BCBL-1 cells in the culture are infected with KSHV, but this infection is predominantly latent with only a small percentage of cells undergoing replication. Only minimal numbers of ORF59-positive cells were detected in uninduced BCBL-1 cultures (Figure 2-3C). However, butyrate treatment induced widespread reactivation of the latent KSHV infections leading to large numbers of ORF59-positive cells that produce infectious virions (Figure 2-3D).

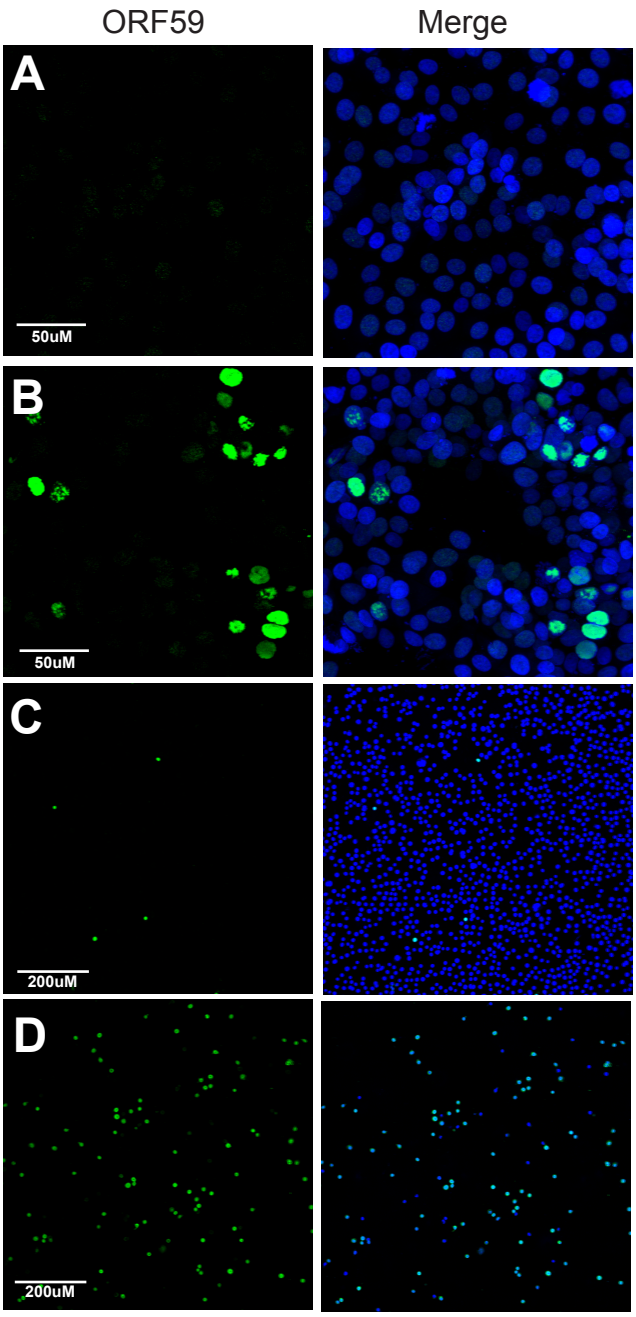

Figure 2-3: RRV latently infects HSG cells and is reactivated by sodium butyrate. (A) HSG cells were incubated with RRV and assayed for ORF59 expression three days post-infection using the anti-RV2 ORF59 antiserum. (B) Infected HSG cultures were treated with sodium butyrate and evaluated for ORF59 expression three days post-infection. (C) BCBL-1 cells containing latent KSHV and (D) butyrate-treated BCBL-1 cells containing replicating KSHV were analyzed for ORF59 expression using an anti-KSHV ORF59 antibody.

The RRV Rta promoter is highly active in permissive epithelial cells. The identification of permissive and non-permissive cell lines allowed us to examine RRV Rta promoter function in natural lytic and latent systems. To evaluate RRV Rta promoter activity in permissive cell lines, a pGL2-R-Rta construct containing 502 nucleotides of RRV Rta promoter sequence upstream of the firefly luciferase gene was obtained from Dr. B. Damania and transfected into 293 and Vero cells. Cells were co-transfected with pRL-CMV, a construct expressing Renilla luciferase under the control of the CMV IE promoter, to normalize for transfection efficiency. We found that the pGL2-R-Rta construct showed an 18- and 60-fold increase in luciferase activity over empty pGL2 in 293 and Vero cells, respectively, indicating that this promoter region contains positive regulatory elements (Figure 2-4).

Sodium butyrate induces high-level RRV Rta promoter activity in HSG cells. We evaluated the activity of the RRV Rta promoter in non-permissive HSG cells. Because sodium butyrate induced RRV replication in HSG cells (Figure 2-3), we analyzed Rta promoter activity in HSG cells in the presence and absence of sodium butyrate. 293 and HSG cells were transfected with pGL2-R-Rta with or without butyrate treatment and luciferase activity was evaluated at 24 hours. While the RRV Rta promoter was highly active in permissive 293 cells, pGL2-R-Rta had minimal activity in non-permissive HSG cells (Figure 2-5). We found that the activity of the RRV Rta promoter in butyrate-treated HSG cells was comparable to that seen in 293 cells. These results indicate that Rta promoter activity is low in HSG cells but that high-level activity can be induced by sodium butyrate in this cellular background. These data also suggest a strong correlation between RRV Rta promoter activity and cellular permissivity for RRV replication.

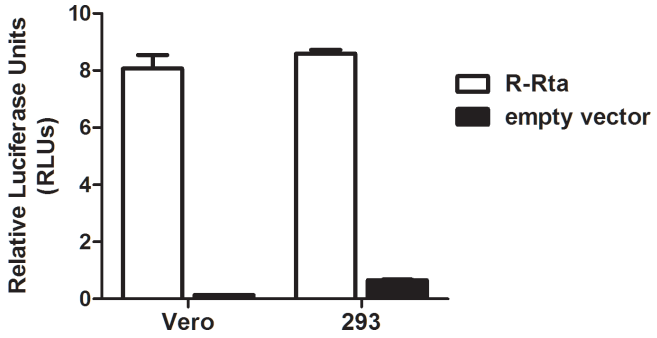

Figure 2-4: The RRV Rta promoter is highly active in epithelial cells permissive for viral replication. Vero and 293 cells were transfected with the either the pGL2-R-Rta promoter construct, containing 502 nucleotides of RRV Rta promoter sequence driving luciferase expression, or empty pGL2 vector, and assayed for luciferase activity 24 hours post-transfection. Luciferase activity was normalized to the activity of pRL-CMV, containing the CMV IE promoter driving Renilla expression, which was co-transfected to control for transfection efficiency.

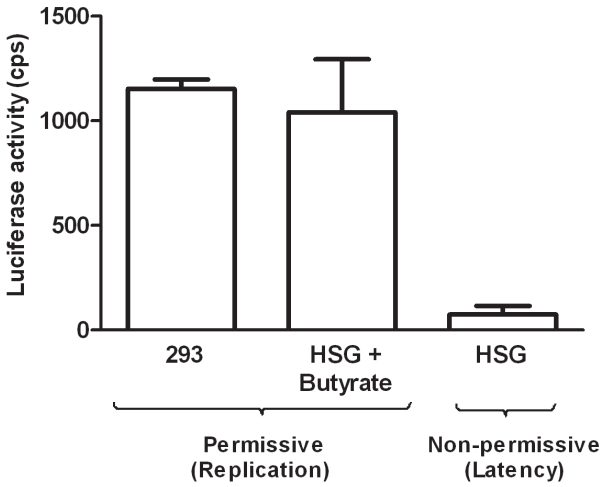

Figure 2-5: The RRV Rta promoter has high activity in permissive cells but minimal activity in non-permissive cells. 293 cells were transfected with pGL2-R-Rta and luciferase activity was evaluated at 24 hours. HSG cells were transfected with pGL2-R-Rta with or without butyrate treatment. Promoter activity is shown as counts per second (cps) luciferase activity above background. Error bars represent the standard deviation from triplicate wells.

An Sp1 site in the RRV Rta promoter confers high-level activity in permissive epithelial cell lines and butyrate-responsiveness in non-permissive cells. To identify critical regulatory regions within the RRV Rta promoter, we prepared 5' truncations of the Rta promoter region beginning 502 nucleotides upstream of the translational start site. Serial truncations were cloned into the pGL2 firefly luciferase backbone, and the activity of each truncation construct was evaluated in Vero cells, which are permissive for RRV replication. Deletion of nucleotides -502 to -136 did not significantly impact promoter activity (Figure 2-6). This region contained the upstream sequences encoding ORF48 and its putative TATA-like element (see Figure 2-1). However, further deletion of nucleotides from -136 to -102 resulted in a dramatic decrease in Rta promoter activity (Figure 2-6B). Similar results were obtained with the 293 human epithelial cells, which are also permissive for RRV infection (data not shown).

The truncation analysis suggested the presence of a critical regulatory element between nucleotides -136 and -102. Because this region contained the Sp1 element conserved between KSHV and RRV, we tested whether the Sp1 site was required for RRV Rta promoter activity. The Sp1 element was eliminated in pGL2-R-Rta by site-directed mutagenesis (Figure 2-7A), and the activities of the wild type and Sp1 mutant constructs were analyzed in Vero, 293 and HSG cells. HSG cells were treated with sodium butyrate to induce high-level Rta promoter activity. In 293 and Vero cells we found that the Sp1 mutation abolished RRV Rta promoter activity (Figure 2-7B). These results indicate that the Sp1 site at -113 is critical for high-level RRV Rta promoter activity in permissive cells. We also found that the Sp1 mutant was not induced by sodium butyrate in HSG cells, suggesting that the Sp1 element was required for butyrate-responsiveness of the promoter in non-permissive cells. This mirrors previous reports that

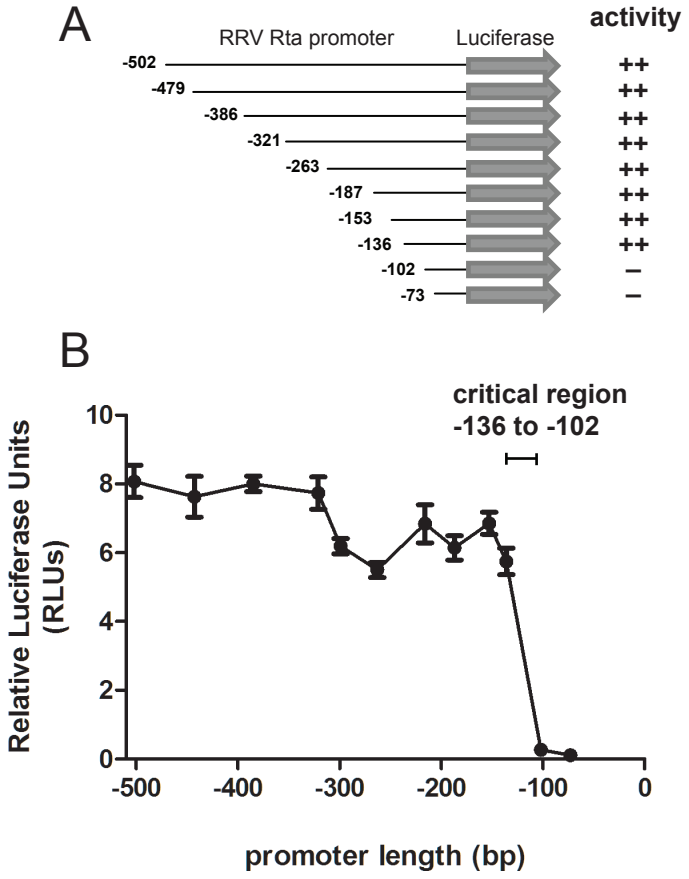

Figure 2-6: Deletion analysis of the RRV Rta promoter reveals a critical regulatory region. (A) 5' serial truncation mutants of the RRV Rta promoter region were constructed in the pGL2 firefly luciferase backbone. (B) Luciferase activity was measured in extracts from permissive Vero cells transfected with pGL2 R-Rta promoter deletion clones and normalized using pRL-CMV as described in the legend for Figure 2-4. Error bars represent the standard deviation of results from triplicate wells.

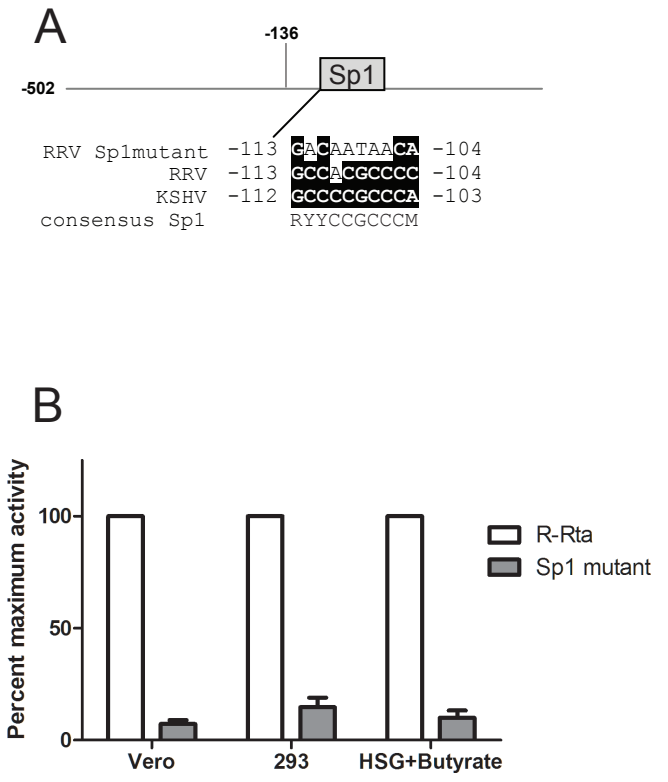

Figure 2-7: The conserved Sp1 element is critical for RRV Rta promoter activity. (A) The Sp1 site in the RRV Rta promoter was eliminated by site-directed mutagenesis in the pGL2-R-Rta construct. Nucleotides matching the Sp1 consensus sequence are highlighted. (B) Permissive Vero, 293, and butyrate-treated HSG cells were transfected with either wild type pGL2-R-Rta or the Sp1 mutant and evaluated for luciferase activity. Promoter activity is shown as percent of maximum Rta promoter activity (100%).

showed that the Sp1 site in the KSHV Rta promoter was critical for butyrate induction in non-permissive cells (Lu, Zhou et al. 2003, Ye, Shedd et al. 2005).

RV2 replication correlates with high-level Sp1 expression in ductal cells of the salivary gland in infected macaques. To determine the relevance of these findings in vivo, we investigated sites of viral replication in various tissues, including the salivary gland, of RV2-infected macaques. Epidemiological studies indicate that saliva is an important route of transmission for RV2-lineage rhadinoviruses in captive macaque colonies (White, Todd et al. 2009). K99344, an adult male pig-tailed macaque, was previously reported to be seroreactive against RV2 ORF59 antigen (Bruce, Bakke et al. 2009). This animal was consistently negative for RFHV by PCR in saliva and PBMCs, but shed high levels of RV2 virus in saliva at the time of necropsy. We evaluated viral replication in the salivary gland of K99344 using the polyclonal rabbit antibody against RV2 ORF59. Paraffin-embedded sections from the parotid gland tissue were prepared and analyzed for ORF59 expression as described in Materials and Methods. Rabbit serum collected prior to immunization with ORF59 peptide ("pre-immune") was used as a negative control. Cell nuclei were visualized with hematoxylin stain to assess cellular morphology. In these tissues we identified distinct duct structures with epithelial cells lining the lumen space. These ductal epithelial cells displayed strong nuclear ORF59 staining (Figure 2-8), indicating RRV replication in these cells.

We also evaluated Sp1 expression in macaque salivary gland tissue. Though Sp1 is constitutively expressed, it has been shown that Sp1 levels can vary up to tenfold across cell types and during differentiation (Saffer, Jackson et al. 1990, Saffer, Jackson et al. 1991). As described above, parotid tissue sections were prepared from M04203, an adult pig-tailed

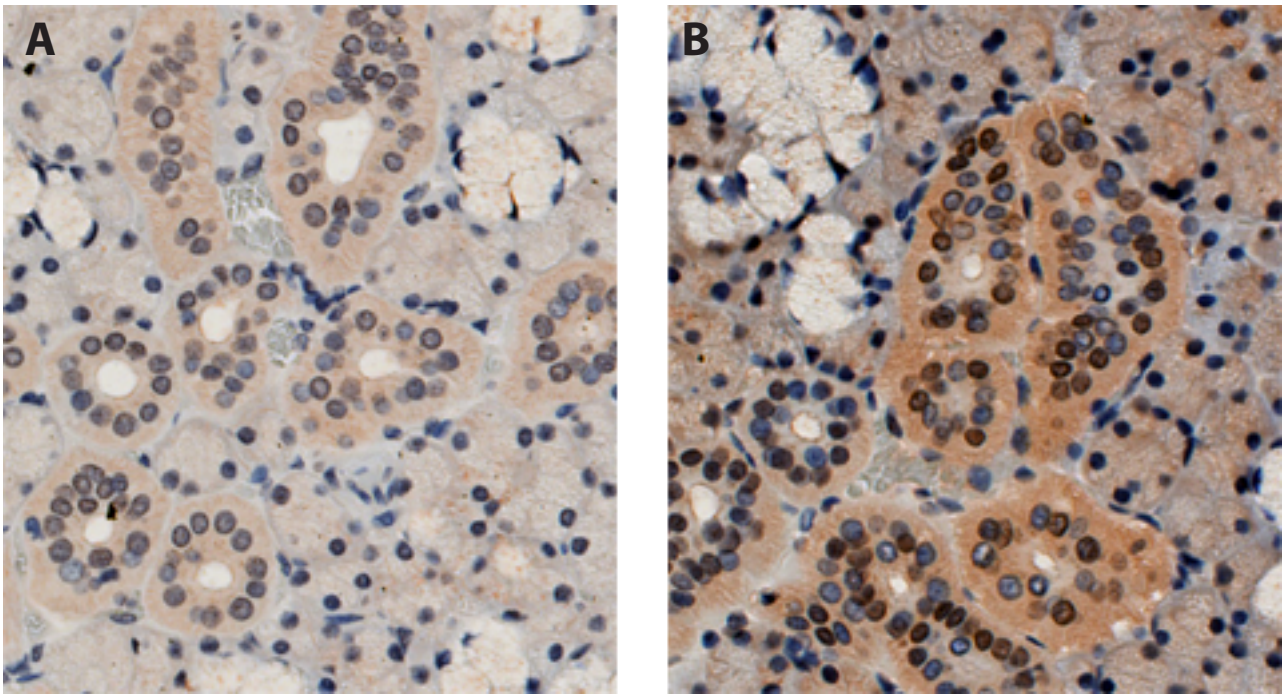

Figure 2-8: RRV replicates in the ductal salivary gland cells of an infected macaque. Paraffin-embedded sections from the parotid gland of an RV2-infected pig-tailed macaque were evaluated for viral replication using an antibody against ORF59. Tissue was incubated with (A) pre-immune rabbit serum as a negative control and (B) poly-clonal rabbit anti-RV2 ORF59 antiserum. Nuclei were visualized with hemotoxylin. Stained tissue sections were analzyed using NDP viewer.

macaque that was positive for both RV1 and RV2 viruses. Like K99344, M04203 also showed evidence of RV2 replication in salivary gland ductal cells and shed virus in saliva (data not shown). We evaluated Sp1 expression in the salivary gland of M04203 using a monoclonal antibody against Sp1. Distinct nuclear Sp1 staining was observed, and the strongest signal was detected in the cells associated with ductal structures throughout the tissue (Figure 2-9). Together, these data indicate that ductal salivary gland epithelial cells expressed higher levels of Sp1 than surrounding cells and were strongly associated with replication of RV2 virus.

Comparison of KSHV and RRV Rta promoter activity in AGS, HSG, 293 and Vero epithelial cells. While RRV replicates efficiently in 293 and Vero cells, KSHV only establishes a latent infection in these cell lines (Vieira, Huang et al. 1997, Zhou, Zhang et al. 2002, Vieira and O'Hearn 2004). To compare the activities of the KSHV and RRV Rta promoters in epithelial cell lines, a 503 bp fragment of the KSHV Rta promoter was cloned upstream of the firefly luciferase reporter gene in pGL2-K-Rta, as described in Materials and Methods. The pGL2-R-Rta and pGL2-K-Rta constructs were transfected into AGS, HSG, 293 and Vero cells and treated with sodium butyrate as indicated. As previously reported, we found that the RRV Rta promoter had minimal activity in non-permissive HSG cells and high-level activity in 293 and Vero cells, which are permissive for viral replication. In the gastric epithelial (AGS) cells, which were non-permissive for RRV replication, we found that the RRV Rta promoter had minimal activity and was induced by sodium butyrate (Figure 2-10). These data mirror what was reported for HSG cells in Figure 2-5. Furthermore, we found that the KSHV and RRV Rta promoters behaved similarly in all the epithelial cell lines tested. Like the RRV Rta promoter, the KSHV Rta promoter had minimal activity in AGS and HSG cells and high-level activity in 293 and Vero

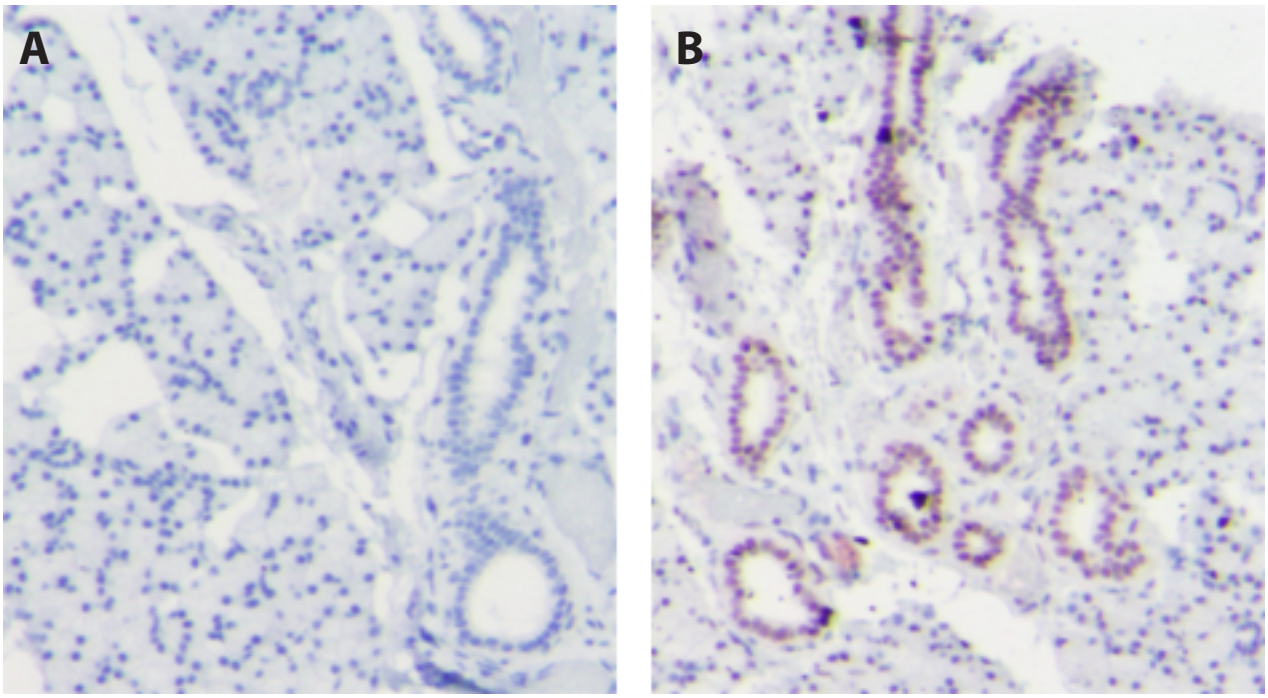

Figure 2-9: High-level Sp1 expression in cells lining salivary gland ducts of a pig-tailed macaque. Parotid gland sections from a pig-tailed macaque were analyzed with (A) negative control mouse IgG or a (B) monoclonal antibody against Sp1. Tissue sections were prepared and analyzed as described in Figure 2-8.

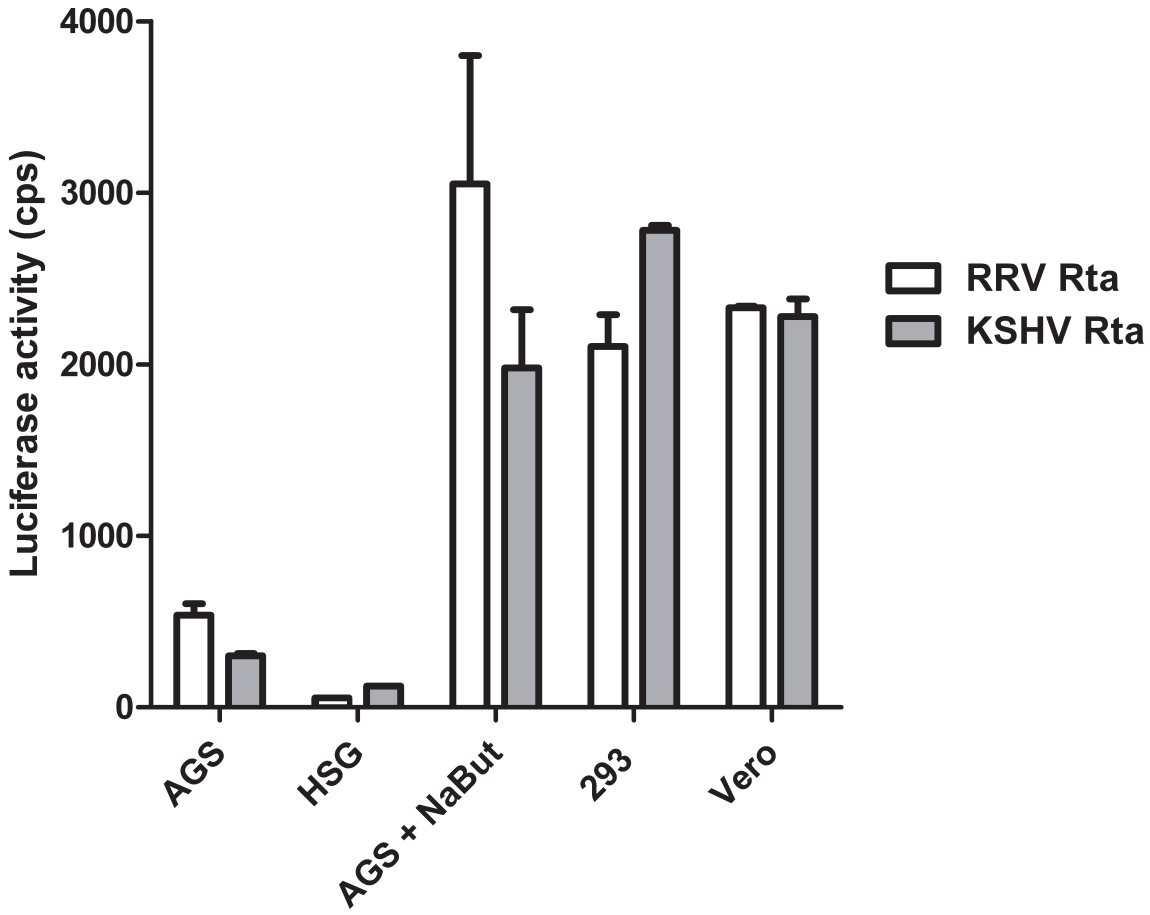

Figure 2-10. Summary of KSHV and RRV Rta promoter activity in AGS, HSG, 293 and Vero epithelial cell lines. EIther pGL2-R-Rta or pGL2-K-Rta was transfected into AGS, HSG, 293 and Vero cells and luciferase activity was measured at 24 hours. Cells were treated with sodium butyrate as indicated. Error bars indicate results from triplicate wells.

cells. Butyrate induced high-level activity of the KSHV Rta promoter in AGS cells, similar to what was seen with the RRV Rta promoter. These results suggest that the KSHV and RRV Rta promoters are similarly regulated by host factors in these epithelial cell backgrounds. Interestingly, we found that the KSHV and RRV Rta promoters both had high activity in 293 and Vero cells (Figure 2-10), even though these cells are non-permissive for KSHV and permissive for RRV replication. Therefore, the different programs of infection exhibited by KSHV and RRV in these cells is not due to variable Rta regulation by host factors, but rather suggests that Rta is differentially regulated by viral-associated factors such as LANA.

DISCUSSION

In this chapter, we investigated the differential ability of KSHV and RRV to establish latent and replicative infection in cells by comparing the regulation of the promoter of the viral transcriptional activator Rta. Our study was designed to identify cis-acting regulatory elements that determine Rta promoter activity and to analyze promoter activity in the context of latency and replication. Here, we demonstrated that the RRV Rta promoter is highly active in permissive cells, in which RRV replicates, and minimally active in non-permissive cells, in which RRV infection is latent. In non-permissive cells, sodium butyrate induced high-level RRV Rta promoter activity and viral replication. We found that high-level RRV Rta promoter activity in permissive cells is dependent on an Sp1 element located 113 nucleotides upstream of the translational start site. This Sp1 element is required for the butyrate induction of the RRV Rta promoter in non-permissive AGS and HSG cells and is highly conserved with an Sp1 site in KSHV that is critical for butyrate induction of the KSHV Rta promoter in non-permissive cells.

Our results suggest that host factors, such as Sp1, regulate RRV Rta promoter activity and that high-level Rta promoter activity correlates with viral replication.

Our study adds to a growing number of reports linking butyrate treatment and Sp1 activity (Yang, Kawai et al. 2001, Han, Zhong et al. 2008, Zhang, Wang et al. 2009). How sodium butyrate alters Sp1 binding and activity is not completely understood. Sp1 is widely expressed, and its activity is known to be highly regulated by several mechanisms including transcriptional control, post-translational modification, and degradation (Merchant, Du et al. 1999, Fojas de Borja, Collins et al. 2001, Yang, Su et al. 2001, Majumdar, Harmon et al. 2003). A previous report by Ye et al. showed that butyrate augments Sp1 binding to the KSHV Rta promoter, consistent with the model that butyrate enhances the functionality of Sp1 or Sp1-containing complexes (Ye, Shedd et al. 2005). It has also been suggested that the Sp1 site plays an important role in epigenetic silencing of KSHV Rta in latently infected cells. Studies in PEL cells have detected repressive histone markings surrounding the KSHV Rta promoter, suggesting that condensed chromatin structure stabilizes viral latency in these cells (Gunther and Grundhoff 2010). The Sp1 site in the KSHV Rta promoter was found to be critical for nucleosome positioning over the Rta transcriptional start site (Lu, Zhou et al. 2003). Our study suggests that Sp1 plays an important role in initiating RRV replication, but the role of epigenetic regulation in RRV latency and reactivation is unknown.

We have previously developed a polyclonal antibody reactive with the RRV ORF59 DNA polymerase processivity factor and have shown that ORF59 is a marker of virus replication. In the current study, we have used this antibody to show that butyrate treatment of RRV-infected HSG and AGS cells recapitulates the butyrate-induced reactivation of latent KSHV in non-permissive cells. The HSG and AGS systems, therefore, represent a novel system for

understanding RRV latency. It was previously reported that 293 cells were non-permissive for RRV replication, and that RRV can persist in 293 cells treated with ganciclovir, an inhibitor of viral replication that specifically targets the viral DNA polymerase (DeWire and Damania 2005). Our results indicate that 293 kidney epithelial cells are permissive for RRV replication and show strong expression of the ORF59 replication marker in expanding foci of infected cells with concomitant production of infectious virus. At present, the reason for these different findings is unknown. Differences in the 293 cell line or passages used in the two studies could contribute to the differences in infection outcome. Additionally, our study used the 17577 RRV strain, while the 26-95 RRV strain was used in the DeWire study.

Salivary transmission is an important route of transmission for herpesviruses like CMV, EBV and KSHV. Here, we report expression of ORF59 in the ductal cells of salivary gland tissue from an RV2-infected macaque. Our results suggest that the salivary gland is a site of active replication for RV2 viruses. Salivary duct cells also expressed high levels of Sp1, suggesting an association between Sp1 and RV2 replication in vivo. Whereas the beta-herpesvirus CMV has been widely reported to replicate in the salivary gland, studies on KSHV replication in the salivary gland have had mixed results. Two reports failed to detect KSHV DNA in the salivary gland of KS patients by PCR, though KSHV was detected in lymph nodes and the prostate gland (Corbellino, Poirel et al. 1996, Corey, Brodie et al. 2002). However, a study by Klussman et al. reported the expression of lytic cycle genes in the ductal cells of the salivary gland of a KSHV positive patient using an antibody against the ORF26 capsid protein (Klussmann, Muller et al. 2000). Notably, these were the same cells in which we observed RV2 replication. Additional longitudinal studies are needed to characterize KSHV shedding and better understand the source of infectious virus in saliva.

We found that the KSHV and RRV Rta promoters had minimal activity in cultured HSG cells. Because both these promoters contain Sp1 elements, this suggests that the HSG cells have low Sp1 activity. The low Sp1 activity in cultured HSG cells, therefore, contrasts with the elevated Sp1 expression observed in vivo. This may be because HSG cells and tissue duct cells represent different stages of differentiation of salivary gland epithelial cells. The HSG cell line grows as an undifferentiated monolayer under normal culture conditions, but can be induced to undergo differentiation under specific conditions to form ductal structures (Jung, Hecht et al. 2000, Lam, Zhang et al. 2005). Furthermore, the differentiation of salivary gland cells in vitro has been shown to involve Sp1-responsive genes (Omotehara, Kawamata et al. 2002). Based on the morphology of the HSG cells and their low Rta promoter activity, the HSG cells may be similar to the Sp1-low epithelial cells surrounding the ductal structures in the salivary gland. Further studies are needed to determine the impact of ductal differentiation on RRV infection in HSG cells. It may be that the process of cellular differentiation in salivary gland epithelial cells, which has been shown to involve Sp1, is linked to RRV replication, similar to what has been reported for KSHV in keratinocytes (Seifi, Weaver et al. 2011).

Interestingly, we found that the KSHV and RRV Rta promoters had similar activity in AGS, HSG, 293 and Vero cells (Figure 2-10). Both promoters were minimally active in AGS and HSG cells and were induced by sodium butyrate. The finding that the KSHV Rta promoter was highly active in 293 and Vero cells was unexpected since these cells are non-permissive for KSHV replication. KSHV establishes latency in 293 and Vero cells despite high-level Rta promoter activation by host factors. These results suggest that the Rta promoter is strongly down-regulated by viral factors following KSHV infection of 293 and Vero cells. Others have shown that this inhibition is largely determined by the activity of KSHV LANA. In the

following chapter we examine the impact of the viral factor LANA on Rta promoter activity and RRV replication.

CHAPTER III

Analysis of the inhibitory potential of RRV LANA in infected cells

BACKGROUND

Two studies of early gene expression kinetics during KSHV infection have shed light on the events that lead to the establishment of latency. Both studies reported a discrete but transient increase in Rta expression early after infection (Krishnan, Naranatt et al. 2004, Lan, Kuppers et al. 2005). This finding was notable given the propensity of KSHV for latent infection and suggested a role for Rta in the establishment of latency. Recent reports have shown that Rta induces LANA expression through activation of the LANApi promoter (Matsumura, Fujita et al. 2005). The LANApi promoter is located in the intronic region that is excised during maturation of the latent transcript and is distinct from the constitutive promoter (LANApc) that regulates LANA expression in latently infected cells. Rta activates LANApi through interaction with the cellular transcription factor RBP-Jkappa (RBP-Jk), a downstream effector of the Notch signaling pathway (Lan, Kuppers et al. 2005). Rta and RBP-Jk form a complex at LANApi, and DNA binding is mediated by an Rta-responsive element (RRE) and two RBP-Jk binding sites in the LANA promoter region. LANA then inhibits the Rta promoter, effectively shutting off Rta expression in the infected cell (Lan, Kuppers et al. 2004). This Rta-mediated induction of LANA has been reported to be critical for the establishment of latency, as deletion of the RBP-Jk binding site abolishes LANApi promoter activity and results in a more highly lytic virus (Lu, Verma et al. 2011, Hilton and Dittmer 2012).

In the previous chapter, we characterized host-mediated activity of the Rta promoter in cells permissive and non-permissive for RRV replication. We found that RRV Rta promoter

activity was high in permissive cells but only minimally active in non-permissive cells. We also found that both the KSHV and RRV Rta promoters are highly active in Vero and 293 cells. This result was unexpected, given that these cells are permissive for RRV but are non-permissive for KSHV replication. Whereas RRV Rta is able to drive the full cascade of replication-associated gene expression in Vero and 293 cells, KSHV establishes a latent infection in these cellular backgrounds. This is consistent with the current understanding of KSHV latency, in which LANA-mediated shutoff of Rta expression determines latency in an infected cell. In order to understand the role of LANA in RRV latency we have characterized RRV LANA activity using infection systems and reporter assays. In this chapter we show that ectopic expression of RRV LANA inhibits Rta promoter activity and viral replication, demonstrating that these functions are conserved between KSHV and RRV. Given that most cell types are permissive for RRV replication, we hypothesize that the inhibitory activity of RRV LANA is restricted in infected cells, by transcriptional regulation and/or nucleolar targeting.

MATERIALS AND METHODS

Plasmids. pcDNA N-Flag RRV LANA, containing an N-terminal flag tag and the ORF73 coding sequence from RRV strain 17577, was created by inserting the RRV LANA sequence amplified from pRRV73GFP into pcDNA3 using HindIII and XbaI sites and the following primers: forward (5’ TAGCAAGCTTGCCACCATGGATTACAAGGATGACGACGATAAGTGGGGCAGCCGGC AA 3’, Flag coding sequence underlined) and reverse (5’ GCTATCTAGATTAGT GCTGAATTGGTAGTCCTCTG 3’). The pcDNA N-Flag KSHV LANA expression plasmid was constructed by ligating an insert with a Flag tag and the fourteen N-terminal amino acids of

KSHV LANA (up to the AscI site in the LANA coding sequence) and an insert containing the remaining 989 C-terminal amino acids (downstream of the AscI site) into pcDNA3 vector. Briefly, overlapping oligos containing an initiating methionine, Flag sequence and the first fourteen amino acids of KSHV LANA were denatured at 95°C for 4 minutes in 100 mM potassium acetate, 2 mM magnesium acetate, and 20 mM HEPES and then annealed at 70° C for 10 minutes. The oligos were designed such that the annealed insert contained KpnI and AscI overhangs. KSHV LANA coding sequence (minus the fourteen N terminal amino acids) was excised from pcDNA3.1 V5/HISA orf73 (Renne, Barry et al. 2001) with AscI and XhoI. Both inserts were subcloned into pUC19 using KpnI and XhoI sites. The resulting pUC19/NFlag KSHV LANA plasmid was confirmed by diagnostic digest. The entire N-Flag KSHV LANA sequence was then excised and cloned into pcDNA3 using KpnI and XhoI sites (Burnside, unpublished results). LANA expression constructs were verified by sequencing.

pDsRed-B23 was a kind gift of Dr. Denis Archambault (Gomez Corredor and Archambault 2009).

Immunofluorescence and confocal microscopy. In LANA inhibition studies cells were transfected with 200 ng pcDNA3-N-Flag RRV LANA and infected with RRV eight hours post-transfection. Cells were fixed 2 days post-infection and assayed for ORF59 expression as described in Chapter II using anti-RV2 ORF59 antiserum. Flag-tagged RRV LANA was visualized using the M2 anti-Flag antibody (Sigma) and Alexa594 conjugated goat anti-mouse secondary antibody (Molecular Probes).

For localization studies, LANA constructs were transfected with 250 ng of pcDNA N-Flag RRV LANA, pEGFP RRV $LANA_{1-45}$, or pEGFP RRV $LANA_{34-45}$ (Burnside, unpublished

results). 250 ng of pDsRed-B23 was co-transfected as a nucleolar marker. Cells were fixed in 4% paraformaldehyde 24 hours post-transfection. Flag-tagged RRV LANA expression was analyzed as described above. Nuclei were visualized with TO-PRO stain. Images were acquired with a Zeiss LSM 5 Pascal confocal microscope and are shown as projections of the z-stack.

Reporter assays. In the LANA inhibition studies, cells were co-transfected with reporter plasmids and LANA expression vectors using LT1 reagent (Mirus). The total volume of DNA was held constant using empty pcDNA3 vector. Luciferase activity was measured at 24 hours using Promega passive lysis buffer, as previously described in Chapter II.

RESULTS

RRV LANA inhibits viral replication in permissive epithelial cells. Previous studies have shown that KSHV LANA plays a major role in preventing the Rta-induced cascade of lytic gene expression and virus replication (Lan, Kuppers et al. 2004). To determine whether ectopic expression of RRV LANA inhibited viral replication, we transfected permissive Vero cells with pcDNA3 N-Flag RRV LANA, a construct that expresses Flag-tagged RRV LANA from the highly active CMV IE promoter. Transfection of empty pcDNA3 vector was included as a control. The transfected cells were subsequently infected with RRV and evaluated for replication two days post-infection using the anti-ORF59 antibody. Expression of LANA was detected using an anti-Flag antibody. Nuclei were visualized with TO-PRO (Figure 3-1A,B). No LANA staining was detected in the RRV-infected cells transfected with the empty pcDNA3 construct (Figure 3-1C). These cultures showed high levels of ORF59 expression, indicating widespread viral replication (Figure 3-1E). Cells transfected with pcDNA3 N-Flag RRV LANA

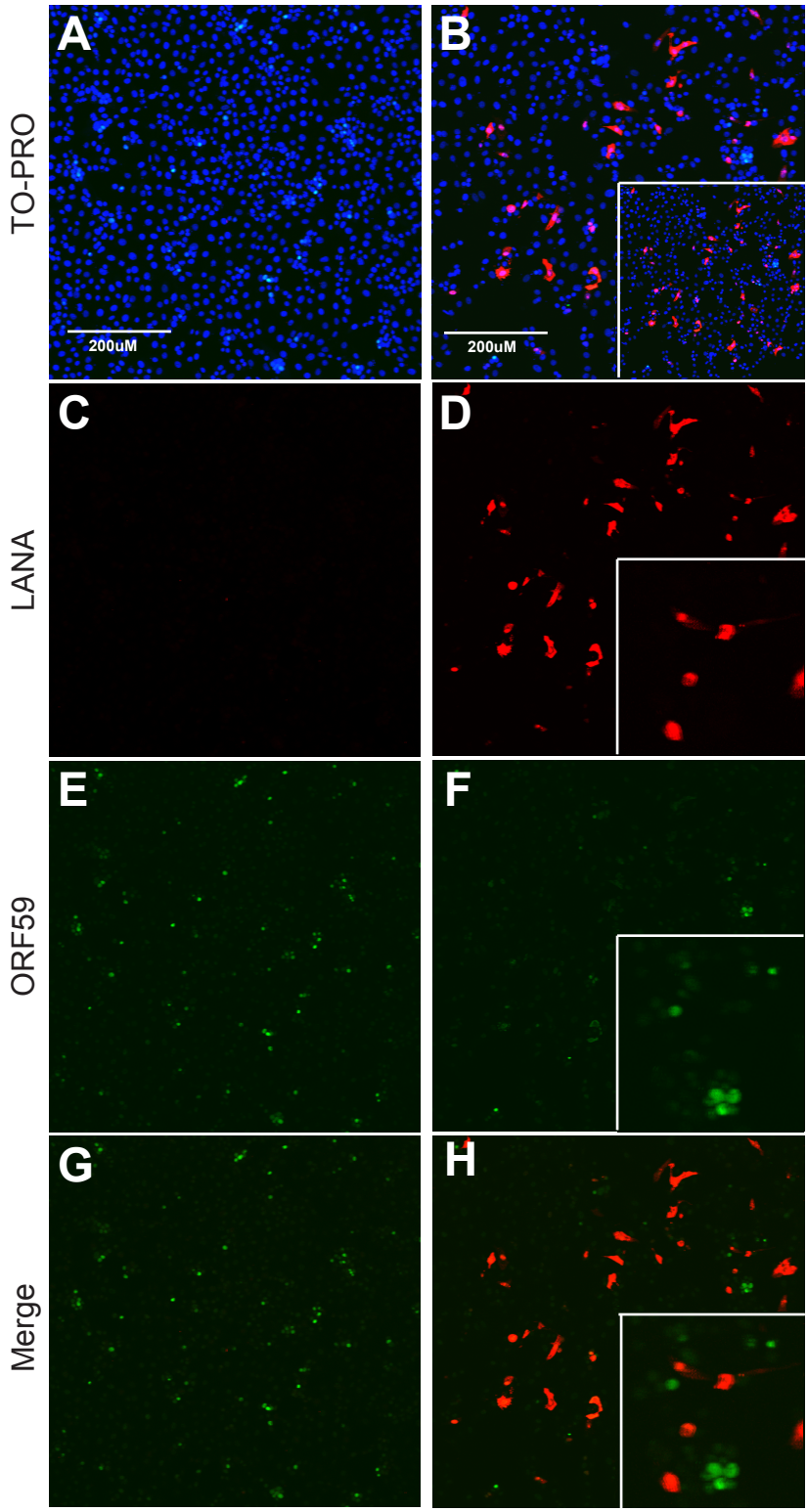

Figure 3-1. RRV LANA inhibits viral replication in Vero cells. Cells were transfected with either empty pcDNA3 or pcDNA3-N-Flag RRV LANA, infected with RRV, and evaluated 2 days post-infection for evidence of viral replication. (A-B) TO-PRO-stained cells. (C-D) RRV LANA expression was evaluated with an anti-Flag antibody. (E-F) ORF59 expression was evaluated with an anti-RV2 ORF59 antibody. (G) Merge of panels C and E. (H) Merge of panels D and F.

showed abundant expression of RRV LANA (Figure 3-1D). No cells expressing LANA showed evidence of ORF59 expression and virus replication (Figure 3-1H). Only a few ORF59-positive nuclei were detected in the LANA-transfected cultures, but none of these cells expressed LANA indicating that LANA expression inhibited RRV replication in Vero cells. Subsequent butyrate treatment of the RRV infected, LANA-transfected cultures revealed a strong activation of ORF59 expression in LANA-positive cells (data not shown). This indicates that cells expressing LANA were susceptible to RRV infection and that sodium butyrate could overcome LANA-mediated inhibition, leading to the viral replication cascade.

RRV LANA is a potent inhibitor of Rta promoter activity. A previous study showed that RRV LANA inhibits the Rta-induced transactivation of viral promoters (DeWire and Damania 2005). To determine the impact of RRV LANA on the RRV Rta promoter, we performed luciferase reporter assays in permissive Vero cells expressing RRV LANA. The RRV Rta promoter construct pGL2-R-Rta was transfected with increasing amounts of pcDNA3 N-Flag RRV LANA and luciferase activity was measured at 24 hours. To compare with KSHV, we also tested the impact of KSHV LANA expression on KSHV Rta promoter activity by transfecting Vero cells with pGL2-K-Rta and pcDNA3 N-Flag KSHV LANA, as described in Materials and Methods. We observed a dose-dependent inhibition of KSHV Rta promoter activity by KSHV LANA, with a maximal inhibition of 50% at the highest plasmid concentration tested (Figure 3-2). This level of inhibition is similar to that reported previously (Lan, Kuppers et al. 2004). Interestingly, RRV LANA strongly inhibited the RRV Rta promoter with greater than 60% inhibition at the lowest plasmid concentration tested. A dose-dependent response was detected with increasing inhibition to a maximal level of approximately 80% (Figure 3-2). These data

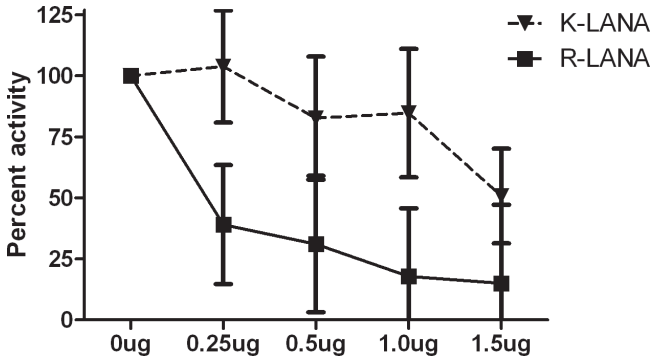

Figure 3-2: RRV LANA, like KSHV LANA, is a potent inhibitor of Rta promoter activity. Vero cells were transfected with either the pGL2-K-Rta promoter construct and increasing amounts of pCDNA3-NFlag KSHV LANA or pGL2-R-Rta and increasing amounts of pCDNA3-NFlag RRV LANA. Luciferase values were measured 24 hours post-transfection and are shown as percent of maximal activity. The amount of total DNA in each experiment was held constant with empty pcDNA3 vector.

indicate that RRV LANA, like KSHV LANA, is a potent inhibitor of Rta promoter activity. Furthermore, our results suggest that any LANA protein present in an RRV infected cell could mediate shutoff of Rta.

RRV LANA lacks the Rta-inducible LANApi promoter, which is conserved in the RV1 rhadinoviruses. In KSHV, the constitutive LANApc promoter regulates expression of a spliced LANA transcript that originates from the adjacent K14/R14 coding region (Figure 3-3A). However, in the presence of Rta, the inducible LANApi promoter drives expression of an unspliced LANA transcript. A spike in Rta expression following de novo KSHV infection induces LANA expression from the LANApi promoter. The newly-synthesized LANA shuts off the Rta promoter and induces additional LANA expression from the spliced latency associated mRNA transcript derived from the constitutive LANApc promoter (Jeong, Orvis et al. 2004). High-level expression of LANA coupled with the shutoff of Rta expression leads to the establishment of KSHV latency. The Rta-inducible LANApi promoter is located within the intronic region of the latent LANA transcript and contains two head-to-head RBP-Jk binding sites and an Rta-response element (RRE) adjacent to a TATA box that are critical for promoter activity (Hilton and Dittmer 2012). We found that the basic structure of this Rta-inducible lytic LANA promoter is conserved in RFHV, the macaque RV1 homolog of KSHV, with a conserved RBP-Jk binding site immediately adjacent to a putative TATA box (Figure 3-3B). In both the KSHV and RFHV sequences, the conserved LANApi promoter is located within the intron region spliced from the latent LANA transcript.

Though the RRV LANA promoter region has not been fully characterized, a study in permissive rhesus fibroblast cells reported a lytic transcriptional start site (TSS) located within

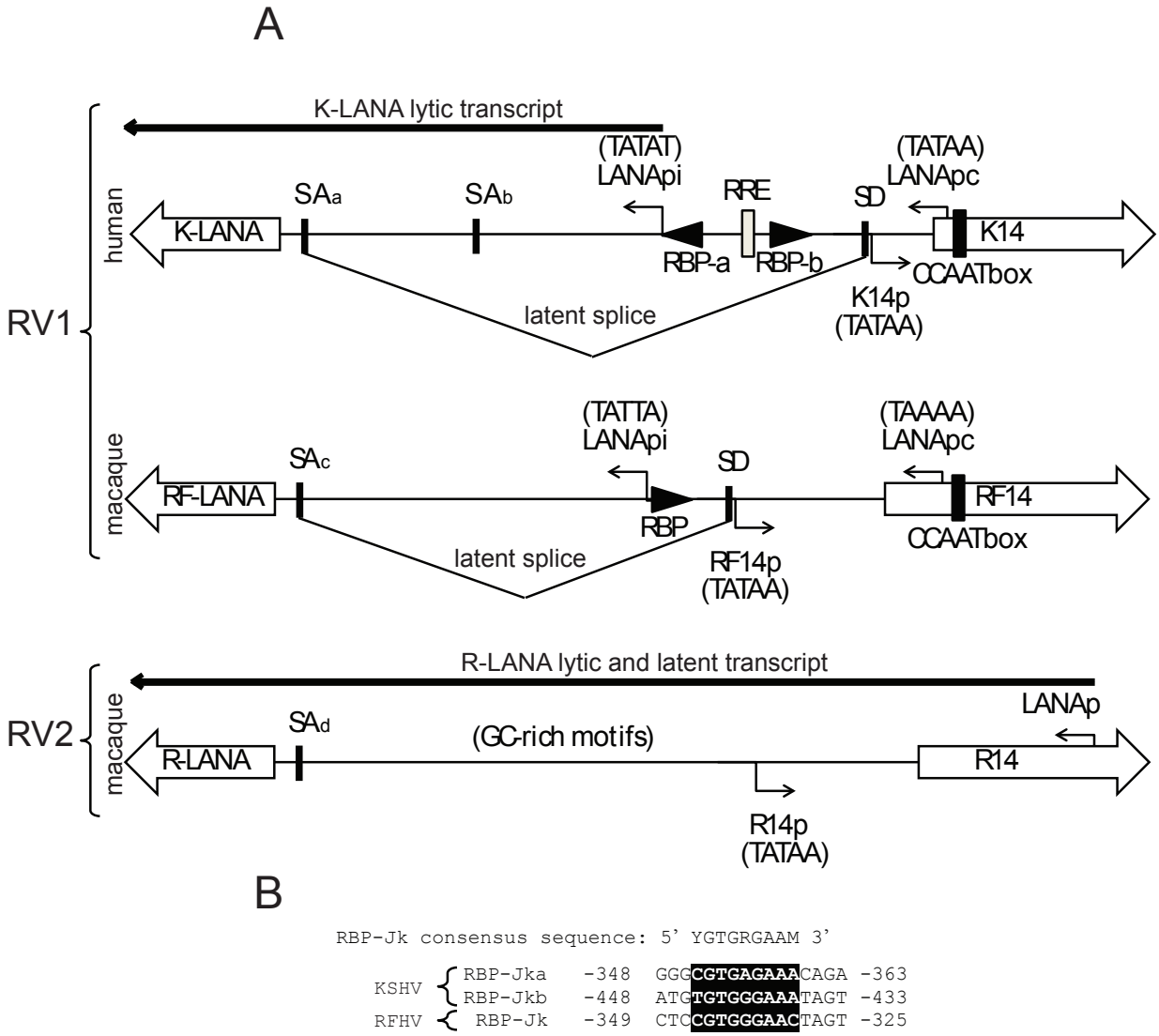

Figure 3-3. Comparison of the promoter regions of the LANA homologs of the RV1 and RV2 rhadinoviruses. (A) Schematic of the bi-directional promoter region for the LANA (leftward) and K14 (rightward) homologs of the human (KSHV) and macaque (RFHVMn) RV1 rhadinoviruses and the macaque RV2 rhadinovirus (RRV). The coding regions for the ORF73 homologs (K-LANA; RF-LANA; R-LANA) and ORF74 homologs (K14; RF14; R14) are indicated as large open arrows. The homologous ORF74 "TATAA" promoter elements (K14p; RF14p; R14p) are shown. The latency-associated constituitive LANA promoter (LANApc) and Rta-inducible LANA promoter (LANApi) are shown for KSHV. Splice acceptor (SAa , SAb) and splice donor (SD) sites utilized to produce the latent spliced LANA transcript are indicated (Dittmer, Lagunoff et al. 1998, Sarid, Flore et al. 1998, Talbot, Weiss et al. 1999). RBP-Jkappa and RRE binding sites in LANApi are shown. Arrowheads indicate directionality of RBP-Jk binding sites. The putative homologous latent promoter, splice sites (SD, SAc) and inducible lytic promoter with adjacent RBP-Jkappa binding site are shown for the closely related LANA of RFHV. (B) The consensus binding site for RBP-Jkappa (Nellesen, Lai et al. 1999) is compared to the RBP-Jkappa binding sites upstream of the LANApi promoter for both KSHV and RFHV LANA.

the upstream R14 coding region (DeWire and Damania 2005). Notably, this TSS is located near the latency-associated LANApc of KSHV (Figure 3-3A). This suggests that RRV LANA transcripts originate from a distal promoter in the presence of Rta and not a LANApi-like promoter as has been described for KSHV LANA. Furthermore, we did not find evidence of a RBP-Jk-sensitive, Rta-inducible promoter for RRV LANA. The region of RRV that corresponds to the conserved Rta-inducible promoters in KSHV and RFHV is GC-rich and contains no obvious TATA-like sequences. Furthermore, analysis of the entire RRV LANA promoter region revealed no RBP-Jk binding sites, which are required for Rta-mediated induction of LANA expression in KSHV. Whereas the LANApi structure in KSHV is conserved in RFHV, our analysis suggests that RRV lacks an inducible LANApi.

RRV LANA lacks the classical nuclear localization signal conserved in KSHV and RFHV LANA homologs. The ability of LANA to traffic to the nuclei of infected cells is critical for its function as a transcriptional repressor. Previous work in our lab mapped a N-terminal classical bipartite nuclear localization signal (NLS) that is conserved in KSHV and RFHV LANA (Cherezova, Burnside et al. 2011). The RV1 LANA proteins contain the "K(K/R)X(K/R)" consensus motif required for binding to importin α, the intermediate binding partner utilized in the classical nuclear import pathway (Lange, Mills et al. 2007). RRV LANA lacks the K(K/R)X(K/R) motif that is critical for the nuclear localization of KSHV and RFHV LANA. We have identified a non-classical NLS within the N-terminal region of RRV and MneRV2 LANA. Our studies show that the N-terminal 44 amino acids of RRV LANA target the heterologous EGFP protein to the nucleus in transfected cells, suggesting the presence of a functional NLS in this region (L. Cherezova, unpublished results). This region contains an

arginine/glycine-rich motif (Figure 3-4), which is typical of proteins that bind importin ß and utilize the non-classical pathway for nuclear import (Lee and Aitchison 1999, Kumari, Singhal et al. 2007). Our data indicate that RRV $LANA_{1-44}$ mediates direct binding with members of the importin ß family, without an importin α intermediate, and that this N-terminal region is sufficient for nuclear localization of RRV LANA (L. Cherezova, unpublished results).

The Arg/Gly-rich N-terminal region targets RRV LANA to the nucleolus. A previous study reported that RRV LANA localized to nucleoli in transfected rhesus fibroblast cells (DeWire and Damania 2005). This nucleolar targeting is unique among rhadinoviruses, as KSHV and RFHV LANA are both excluded from the nucleolus (Burnside, Ryan et al. 2006). To evaluate the localization of RRV LANA in permissive Vero cells, the pcDNA N-Flag RRV LANA construct was transfected and LANA expression was evaluated using an anti-Flag antibody. pDsRed-B23, a construct expressing RFP-tagged B23 protein, was co-transfected as a nucleolar marker (Gomez Corredor and Archambault 2009). Cell nuclei were stained with TO-PRO, and LANA and B23 expression were evaluated by confocal microscopy. Consistent with previous findings, we found that RRV LANA strongly localized to discrete regions of the nucleus (Figure 3-5A). LANA signal co-localized with B23, confirming that these regions are nucleoli.

RG-rich regions like those identified in RRV and MneRV2 LANA are common in nucleolar localization signals (NoLS) (Creancier, Prats et al. 1993). To identify specific domains within the N terminus of RRV LANA that mediate nucleolar targeting, we developed EGFP fusion constructs containing the first 45 amino acids (R-$LANA_{1-45}$) and amino acids 34-45 (R-$LANA_{34-45}$) of RRV LANA (Burnside and Cherezova, unpublished results). The R-$LANA_{1-45}$ construct contained the entire RG-rich region of the NLS, while the R-$LANA_{34-45}$ construct

Figure 3-4. N-terminal regions of RV2 LANA proteins are arginine-glycine rich. Alignment of the N-terminal 45 amino acids of the LANA homologs in RRV isolate 17577, RRV isolate 26-95, and MneRV2. Arginine (R) and glycine (G) residues are highlighted. Putative RNA-binding "RGG" motifs are indicated.

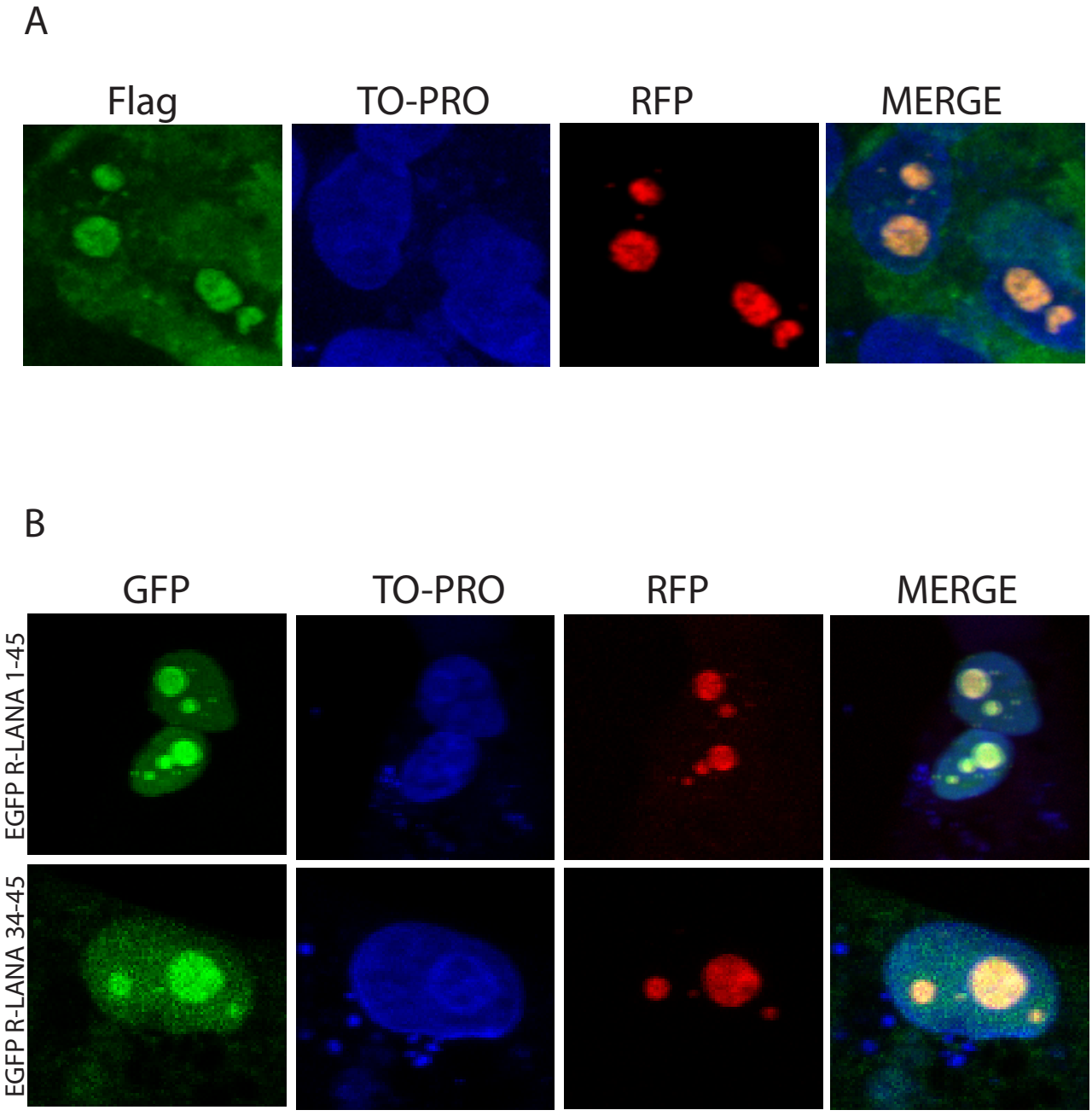

Figure 3-5. RRV LANA localizes to the nucleolus. (A) Vero cells were transfected with pcDNA3-NFlag-RRV LANA and pDsRedB23, which expresses RFP-tagged B23, a nucleolar marker. LANA expression was detected using a Flag antibody as previously described in Figure 3-1. (B) The pEGFP-RLANA1-45 and pEGFP-RLANA34-45 constructs were co-transfected with pDsRedB23.

contained a stretch of eight consecutive arginine and glycine residues (See Figure 3-4). The pDsRed-B23 was co-transfected as a nucleolar marker. We found strong nucleolar localization of both R-LANA$_{1-45}$ and R-LANA$_{34-45}$ (Figure 3-5B). These results indicate that amino acids 34-45 of RRV LANA are sufficient to target the nucleolus, suggesting that these residues function as a NoLS.

DISCUSSION

Previous studies have shown that KSHV LANA can repress Rta promoter activity and block downstream Rta-mediated activation of the viral replication cascade. We confirmed the LANA-mediated block of the KSHV Rta promoter and showed that RRV LANA can repress the transcriptional activity of the RRV Rta promoter as well (Figure 3-2). Furthermore, we showed that ectopic expression of RRV LANA inhibited viral replication in permissive Vero cells. These findings indicate that RRV LANA, like KSHV LANA, is a functional inhibitor of lytic cycle gene expression. However, RRV exhibits a strong propensity for replication, suggesting that LANA activity is highly restricted in infected cells, either at the level of transcription or by an alternative mechanism.

Little is known about the regulation of LANA expression during RRV infection. A study in permissive fibroblast cells detected RRV LANA transcripts that originated from a distal promoter located within the R14 coding region (DeWire and Damania 2005). This suggests that RRV LANA is transcribed from an upstream promoter during replication and not from a proximal promoter like LANApi as has been described for KSHV. Our analysis indicated that RRV lacks an Rta-inducible LANApi promoter, which KSHV utilizes to increase LANA expression during early infection. Given the absence of a LANApi promoter in RRV, the LANA

levels in RRV-infected cells may not be sufficient to overcome the activity of Rta, thus tipping the balance towards the replicative program of infection.

In this chapter, we provide evidence of another potential mechanism that may restrict LANA activity in RRV-infected cells. We have identified a strong NoLS that targets RRV LANA to the nucleolus of transfected cells. We found that RRV $LANA_{34-45}$, which contains a stretch of glycine and arginine residues, is sufficient to translocate EGFP to the nucleolus. This may be an important mechanism of regulation in infected cells, as LANA protein that is sequestered in the nucleolus would be unable to inhibit Rta promoter activity. Whether RRV LANA localizes to the nucleolus in infected cells is unknown. We are currently developing antibodies specific to RRV and MneRV2 LANA to answer this question. RV2 LANA proteins may also have additional, distinct functions in the nucleolus. Both RRV and MneRV2 LANA contain RGG motifs (Figure 3-4), which are common in RNA-binding proteins. The role of RRV LANA in the nucleolus may be important in the viral life cycle or in tumorigenesis and, therefore, merits further investigation.

CHAPTER IV

Characterization of KSHV infection in cells with low Rta promoter activity: requirement of integrin αVβ3 and implications for latency

BACKGROUND

In Chapter II we demonstrated that 293 and Vero cells are permissive for RRV replication, leading to expression of the full replication cascade and production of infectious virus. We also showed that the RRV Rta promoter was highly active in these cell lines. Even though KSHV Rta promoter activity is high in 293 and Veros, KSHV establishes latency in these cellular backgrounds. As a result of LANApi activation, high levels of LANA in infected cells repress the KSHV Rta promoter and shut off the Rta-mediated replication cascade. It has been suggested that the induction of LANApi by Rta is a critical step in the establishment of KSHV latency. In this chapter, we examine the ability of KSHV to infect HSG and AGS cells, two cell lines with low Rta promoter activity. Such cells would have diminished Rta expression early in infection, and would therefore have limited or no induction of the LANApi pathway.

In this chapter, we report that HSG cells are refractory to KSHV infection due to the absence of the critical cellular receptor αVβ3 integrin. Ectopic expression of αVβ3 rendered HSG cells susceptible to latent KSHV infection, similar to the latent phenotype we reported for RRV in HSG cells in Chapter II. We also tested the susceptibility of AGS cells to KSHV infection. We found that AGS cells expressed high levels of surface αVβ3 and that KSHV was able to establish latent infection in AGS cells. Our results indicate that integrin αVβ3 is required for KSHV entry and raise important questions about the establishment of latency in rhadinoviruses.

MATERIALS AND METHODS

Detection of KSHV antigens in infected HSG and AGS cells using tyramide signal amplification (TSA). KSHV virions were prepared from the supernatant of tetradecanoyl phorbol acetate (TPA)-treated BCBL-1 cells. Supernatant was ultracentrifuged onto a 50% Optiprep cushion as previously described (Garrigues Rubinchikova 2008). HSG, Vero and AGS cells were plated to 60 mm dishes and infected with KSHV for 3 hours at 37°C. At 24 hours post-infection, cells were fixed in 4% paraformaldehyde and permeabilized with TNEN/Tween. Endogenous peroxidase was quenched with 1% H_20_2 for 30 minutes at room temperature. Expression of latent and lytic viral antigens was evaluated using specific monoclonal antibodies against KSHV LANA and ORF59 (Advanced Biotechnologies). To visualize antibody binding, cells were incubated with either HRP-conjugated donkey anti-rat or HRP-conjugated goat anti-mouse (Jackson) for 1.5 hours at room temperature and labeled with Alexa-488 tyramide (Invitrogen) according to the manufacturer's instructions. Nuclei were stained with TO-PRO. Images shown are projections of the z-stack. Fluorescence in LANA-positive cells was analyzed using the frequency histogram function of LSM software. Individual nuclei were selected as a region of interest (ROI) and LANA expression is reported as the number of pixels in each ROI above a threshold intensity of 150.

Detection of surface receptors in live cells. Live HSG cells were incubated with monoclonal antibodies against CD98 (Serotec, clone 4F2), integrin α3 (Chemicon, clone P1B5), αVβ3 (Chemicon, clone LM609), ephrinA2 (Millipore), αVβ5 (Chemicon, clone P1F6) and heparan

sulfate (Northstar Bioproducts) for 1 hour at 4°C. Following fixation, antibody binding was detected using an HRP-conjugated donkey anti-mouse (Jackson) and TSA488 substrate.

Detection of B3 expression and KSHV infection in transfected HSG cells. The pcDNA3-β3 construct was kindly provided by Dr. M.H. Ginsberg. HSG cells were plated to 60 mm dishes and transfected with 1ug pcDNA3-β3. Following overnight culture, transfected cells were incubated with purified KSHV virions for 3 hours at 37°C. 24 hours post-infection, live cells were incubated with mouse anti-β3 antibody (Chemicon) for 1 hour on ice. Cells were then washed with DMEM and fixed in 4% paraformaldehyde/sucrose. Endogenous peroxidase activity was quenched with 1% H_20_2. Bound β3 antibody was detected with HRP-conjugated donkey anti-mouse (Jackson) and Alexa-488 tyramide. Cells were then permeabilized and evaluated for LANA expression with TSA594.

RESULTS

HSG cells are highly resistant to KSHV infection. To determine their susceptibility to KSHV infection, HSG cells were incubated with purified KSHV virions and evaluated for latent and lytic infection using a tyramide signal amplification (TSA)-based assay for LANA and ORF59, respectively. TSA improves the sensitivity of traditional immunofluorescence assays, allowing us to detect even low-level infection. Vero cells were included as a control for KSHV infection. At 24 hours post-infection punctuate LANA expression was detected in the nuclei of Vero cells, indicating that these cells were readily infected by KSHV and that infection of these cells was latent, as previously reported. However, we found that HSG cells had an unusually low susceptibility to KSHV infection (Figure 4-1A). No ORF59-positive cells were detected in the

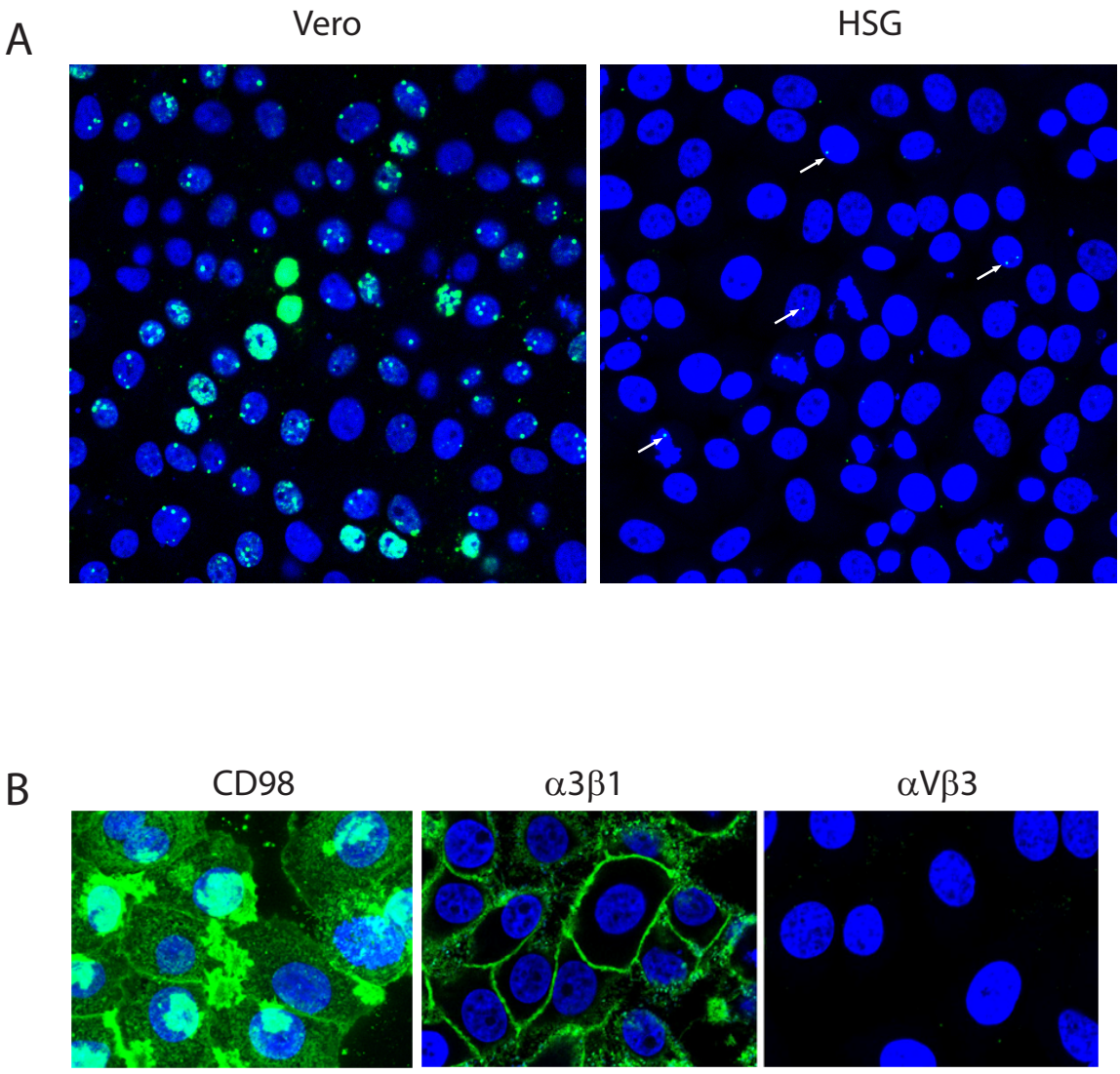

Figure 4-1. HSG cells are not susceptible to KSHV infection and lack surface aVb3 integrin. Vero and HSG cells were incubated with KSHV and evaluated for LANA expression 24 hours post-infection. TSA-488 was used to visualize LANA in fixed cells. Nuclei were visualized with TO-PRO stain. Arrows indicate low-level LANA expression in HSG cells. (B) Live cell receptor staining of HSG cells. HSG cells were incubated at 4 degrees with antibodies against CD98, integrin α3β1and αVβ3. Antibody binding was detected with TSA-488 assay.

cultures (data not shown), indicating that HSG cells were not permissive for KSHV replication. Suprisingly, only approximately 2 percent of HSG cells expressed LANA. In the representative images shown, 81 of 94 Vero cells expressed nuclear LANA, while only 4 of 89 HSG cells infected in parallel were positive for LANA (Figure 4-1A). These results show that HSG cells are highly refractory to infection with cell-free KSHV. Additionally, LANA expression was quantified in HSG and Vero cells using Zeiss LSM software. It has been shown previously that the number and size of LANA dots is an indicator of how many viral genomes are in an infected cell (Adang, Parsons et al. 2006). We found that the few infected HSG cells expressed very little LANA protein. The level of LANA expression in HSG cells averaged 9 pixels per nucleus compared to an average 292 pixels per nucleus in Vero cells. These results suggest that HSG cells are highly refractory to KSHV infection and that the few infected cells support only a low level of infection.

Limited susceptibility of HSG cells to KSHV infection correlates with levels of αVβ3 and not α3β1, xCT, CD98, HS, αVβ5, or ephrin A2. Because they were uniquely resistant to KSHV infection, we characterized the surface expression of putative KSHV entry receptors on HSG cells. To determine the receptor profile of HSG cells we incubated live cells with specific antibodies against the putative KSHV receptors CD98, xCT, α3β1, αVβ3, αVβ5, EphA2, and heparan sulfate. Antibody binding was visualized with TSA488 as described in Materials and Methods. We found that HSG cells expressed high levels of CD98 and α3β1 integrin (Figure 4-1B). We also detected surface expression of heparan sulfate, xCT, αVβ5 and EphA2 (data not shown). However, αVβ3 expression was undetectable in HSG cells (Figure 4-1B). The limited susceptibility of HSG cells to KSHV infection, therefore, strongly correlates with levels of αVβ3,

but not the other putative KSHV entry receptors, on these cells. Furthermore, our results indicate that CD98/xCT, α3β1, αVβ5, and EphA2 expression are not sufficient for KSHV entry in HSG cells.

HSG cells expressing αVβ3 support latent KSHV infection. To further evaluate the requirement of αVβ3 for KSHV entry, HSG cells were transiently transfected with a β3 expression construct and infected with purified KSHV as described in Materials and Methods. αVβ3 expression was evaluated on live cells at 24 hours post-infection using an antibody against β3. Because β3 pairs exclusively with the αV subunit in cells other than platelets, β3 serves as a marker of surface αVβ3 heterodimer. Following live cell staining with the β3 antibody, HSG cells were fixed and evaluated for LANA expression using TSA594. We found that many cells in the culture expressed αVβ3 at the cell surface and that αVβ3 localized to fibrillar projections, lamellipodia and cell-cell junctions (Figure 4-2A). LANA was detected in cells that expressed αVβ3 integrin, indicating that transfection of β3 rendered HSG cells susceptible to latent KSHV infection. In contrast, essentially none of the αVβ3-negative HSG cells expressed LANA, consistent with the refractory phenotype described for the parental HSG cells. LANA expression and TO-PRO staining are shown for several individual nuclei to illustrate the difference in the levels of LANA expression between αVβ3-positive cells (cells I and II) and αVβ3-negative cells (cells III and IV). We also quantified LANA expression in the HSG cultures. The number of LANA pixels in αVβ3-positive cells ranged from 134 to 1640 pixels/nucleus, with an average of 607 pixels/nucleus (Figure 4-2B). The majority (6 of 10) of the αVβ3-negative cells in the representative field were negative for LANA pixels, and the remaining cells contained an average of 4 pixels/nucleus. Whereas parental HSG cells are refractory to KSHV infection, our

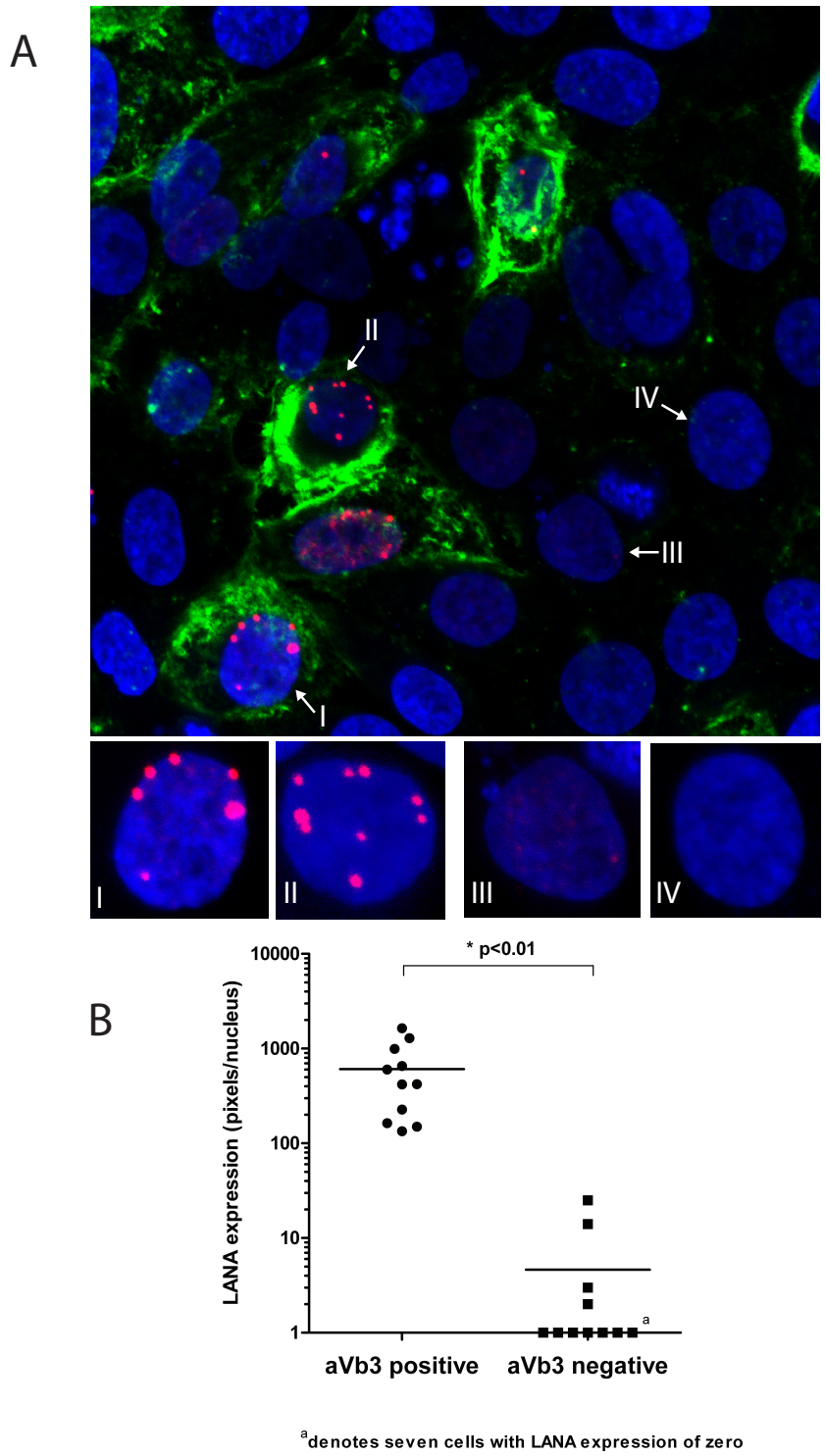

Figure 4-2. Transfection of b3 integrin renders HSG cells susceptible to latent KSHV infection. HSG cells were transiently transfected with pcDNA3-β3 and subsequently infected with KSHV. β3 surface expression was evaluated by live cell assay using TSA-488. LANA expression was evaluated after fixation with a TSA-594 assay. Enlarged images of LANA expression in individual nuclei of cells positive (I, II) and negative (III, IV) for αVβ3 expression are shown. (B) Quantitative analysis of LANA in cells positive and negative for integrin αVβ3. Bar indicates mean LANA expression. Statistcal significance was determined using student's t-test.

results indicate that HSG cells expressing αVβ3 are susceptible to KSHV infection and support latency, providing further evidence that αVβ3 is required for KSHV entry.

KSHV establishes latent infection in AGS cells. We also tested the susceptibility of AGS cells to KSHV infection. AGS cells were incubated with purified KSHV and evaluated for LANA and ORF59 expression 24 hours post-infection. Vero cells were infected in parallel as a positive control for infection. No ORF59 expression was seen in AGS cells, but these cells did contain punctuate LANA (Figure 4-3A) indicating that AGS cells support latent infection. The level of LANA expression was comparable between Vero and AGS cells. We also evaluated αVβ3 expression in AGS cells using the antibody against β3, as previously described. We found that AGS cells express high levels of αVβ3 at the cell surface (Figure 4-3B). Therefore, these data are consistent with the model that αVβ3 is essential for KSHV entry and indicate that KSHV establishes latency in infected AGS cells.

DISCUSSION

Viral entry into target cells is a complex, multistep process involving specific virus-host interactions. Many herpesviruses utilize surface heparan sulfate (HS) proteoglycans for initial attachment to the host cell. Following initial attachment, secondary interactions between virus and cell surface receptors trigger virion uptake by endocytosis or entry by membrane fusion, ultimately resulting in infection of the host cell. Multiple cell surface proteins have been proposed to play a role in KSHV binding and entry, including HS, integrins α3β1 and αVβ3, CD98/xCT, DC SIGN, and ephrinA2 (Akula, Wang et al. 2001, Akula, Pramod et al. 2002, Kaleeba and Berger 2006, Rappocciolo, Jenkins et al. 2006, Garrigues, Rubinchikova et al. 2008,

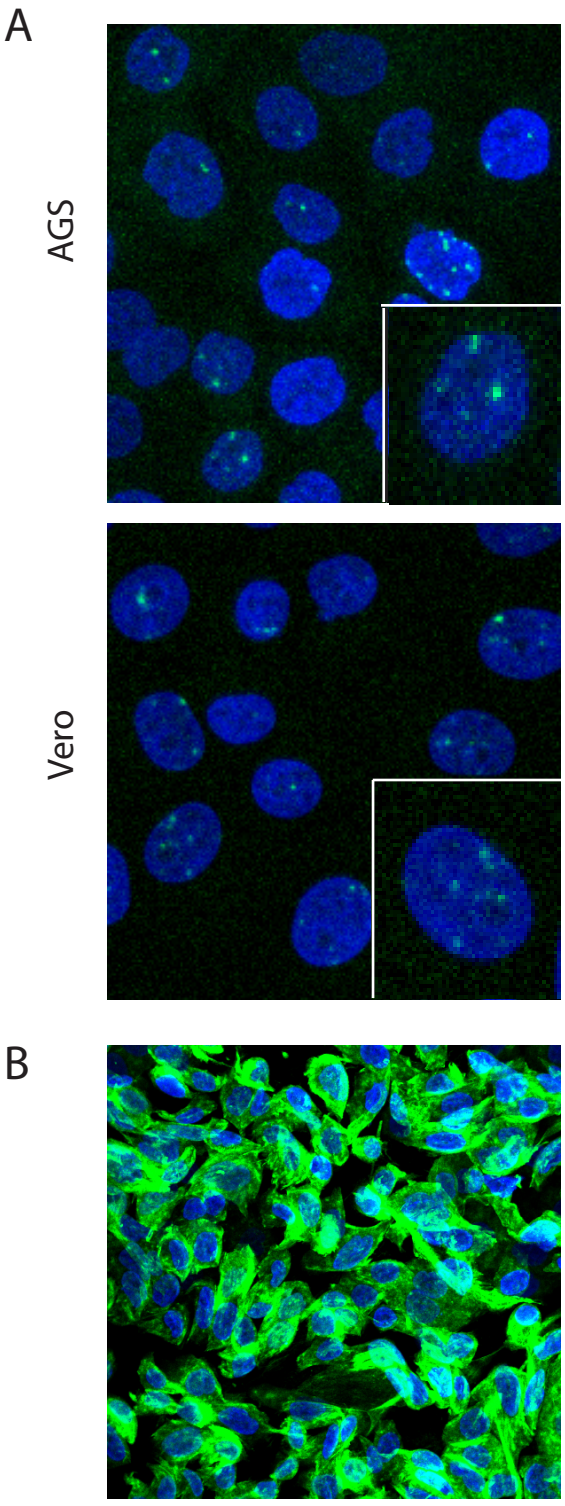

Figure 4-3. AGS cells are susceptible to KSHV infection and express surface αVβ3 integrin. (A) AGS cells were infected with KSHV. LANA expression was evaluated at 24 hours post-infection, as in Figure 4-1. Vero cells are shown as a control for infection. (B) Surface αVβ3 expression was analyzed on fixed AGS cells using an antibody against β3.

Veettil, Sadagopan et al. 2008, Chakraborty, Veettil et al. 2012). However, identifying the specific cellular machinery required for KSHV entry has proved to be a challenge because target cells often express several of the putative KSHV receptors. Furthermore, these receptors can co-localize to common microdomains of the plasma membrane, further complicating KSHV receptor studies (Prager, Feral et al. 2007, Kabir-Salmani, Fukuda et al. 2008). Previous studies in our lab showed that integrin αVβ3 binds an arg-gly-asp (RGD) motif in KSHV glycoprotein B (gB) (Garrigues, Rubinchikova et al. 2008). RGD motifs are common in a number of αVβ3-binding proteins including the ECM proteins fibrinogen and fibronectin (Plow, Haas et al. 2000). Additionally, we showed that RGD antagonists inhibited KSHV infection, indicating that the binding of gB to αVβ3 is a critical step in viral entry. Using highly sensitive confocal microscopy and 3-D surface rendering of cell surfaces, we have also found that KSHV binds to discrete regions of cell surface that are enriched for expression of several of the putative KSHV receptors including HS, αVβ3, and CD98/xCT (Garrigues et al., in press). Our previous studies suggest that αVβ3 functions as an RGD-dependent entry receptor for KSHV through binding to the viral glycoprotein B. Here, we report that αVβ3-deficient HSG cells were refractory to KSHV infection and became susceptible to latent infection following transfection of β3 integrin. Using this complementation approach we have demonstrated the requirement of αVβ3 for KSHV entry. HSG cells express high levels of the other putative KSHV receptors including α3β1 and CD98, suggesting that these molecules were not sufficient to mediate viral entry. The finding that infection requires αVβ3 provides further evidence that αVβ3 functions as an entry receptor for KSHV.

In Vero and 293 cells, in which KSHV Rta promoter activity is high, the establishment of latency has been reported to involve Rta-mediated induction of the LANApi promoter as

previously discussed. However, the LANApi pathway to latency would be limited or non-functional in cells with low Rta promoter activity, such as AGS and HSG cells. Here we have demonstrated that KSHV establishes latent infection in AGS and β3-expressing HSG cells. As such, the establishment of latency in AGS and HSG cells would occur in the absence of activation of the LANApi promoter. These findings raise the possibility of two distinct mechanisms for establishing KSHV latency, which will be discussed in the next chapter.

CHAPTER V

Conclusions and Future Directions

The induction of virus replication in permissive cells and the reactivation of latent infections in non-permissive cells are critical processes in the establishment, transmission, and pathology of herpesvirus infections. To more fully understand the regulation of these processes we have analyzed the promoters of the transactivator Rta in two related rhadinoviruses: KSHV, the prototype of the RV1 rhadinovirus lineage, and RRV, the prototype of the RV2 rhadinovirus lineage. These two viral lineages show differential ability to establish permissive and non-permissive infections in vitro and in vivo. Previous studies have identified and characterized in vitro culture systems to study latent KSHV infection and replicative RRV infection. Here, I have described additional cell lines that are non-permissive for RRV replication and recapitulate the latent infection systems and virus reactivation processes that have been characterized for KSHV. The latent and replicative RRV infection systems described in Chapter II availed us the unique opportunity to compare Rta promoter function in naturally permissive and non-permissive cell lines. Such a comparison has not been possible for KSHV due to the lack of a natural system permissive for replication. Using these systems, I have demonstrated that the RRV Rta promoter is highly active in permissive cells but only minimally active in non-permissive cells, in which RRV establishes latency.

A critical Sp1 element was identified in the RRV Rta promoter through truncational studies and mutational analysis. The Sp1 element was required for high-level promoter activity in permissive cells and butyrate-responsiveness in non-permissive cells. These results indicate that the Sp1 element is an important regulatory element in the Rta promoter and suggests that the

cellular transcription factor Sp1 plays an important role in the reactivation and replication of RRV. In vivo studies of macaque salivary gland tissue sections further suggested a correlation between Sp1 activity and RRV replication. We found that RRV replicated in salivary gland ductal cells of infected macaques and that these cells also expressed elevated levels of Sp1. These results provide in vivo evidence of the importance of Sp1 in replication and suggest the salivary gland as an important physiological site of replication for RRV, which, like KSHV, is thought to be transmitted through saliva.

Interestingly, several key regulatory elements in the Rta promoter, including the transcriptional start site, the TATA-box, and the critical Sp1 element, were highly conserved between KSHV and RRV. We also found that the KSHV and RRV Rta promoters behaved similarly in AGS, HSG, 293 and Vero cell backgrounds. Our results comparing KSHV and RRV Rta promoter activity in the context of latent and replicative infection of epithelial cells are summarized in Figure 5-1. Both promoters were minimally active in AGS and HSG cells, which are non-permissive for KSHV and RRV infection. At this low level of Rta promoter activation, neither virus is able to enter the replication cascade. We found that both promoters had high activity in 293 and Vero cells. Interestingly, this level of Rta promoter activity is sufficient to drive RRV replication, yet KSHV establishes latency in these same cellular backgrounds (Figure 5-1A). These data suggest that the ability of KSHV and RRV to establish latency and replication, respectively, in 293 and Vero cells is due to differential regulation of the KSHV and RRV Rta promoters by viral-associated rather than cell-associated factors.

In Chapter III I showed that several important inhibitory functions of KSHV LANA are conserved in RRV. Our results indicate that RRV LANA inhibits viral replication in permissive cells and that this inhibition is at least in part mediated by direct repression of the Rta promoter.

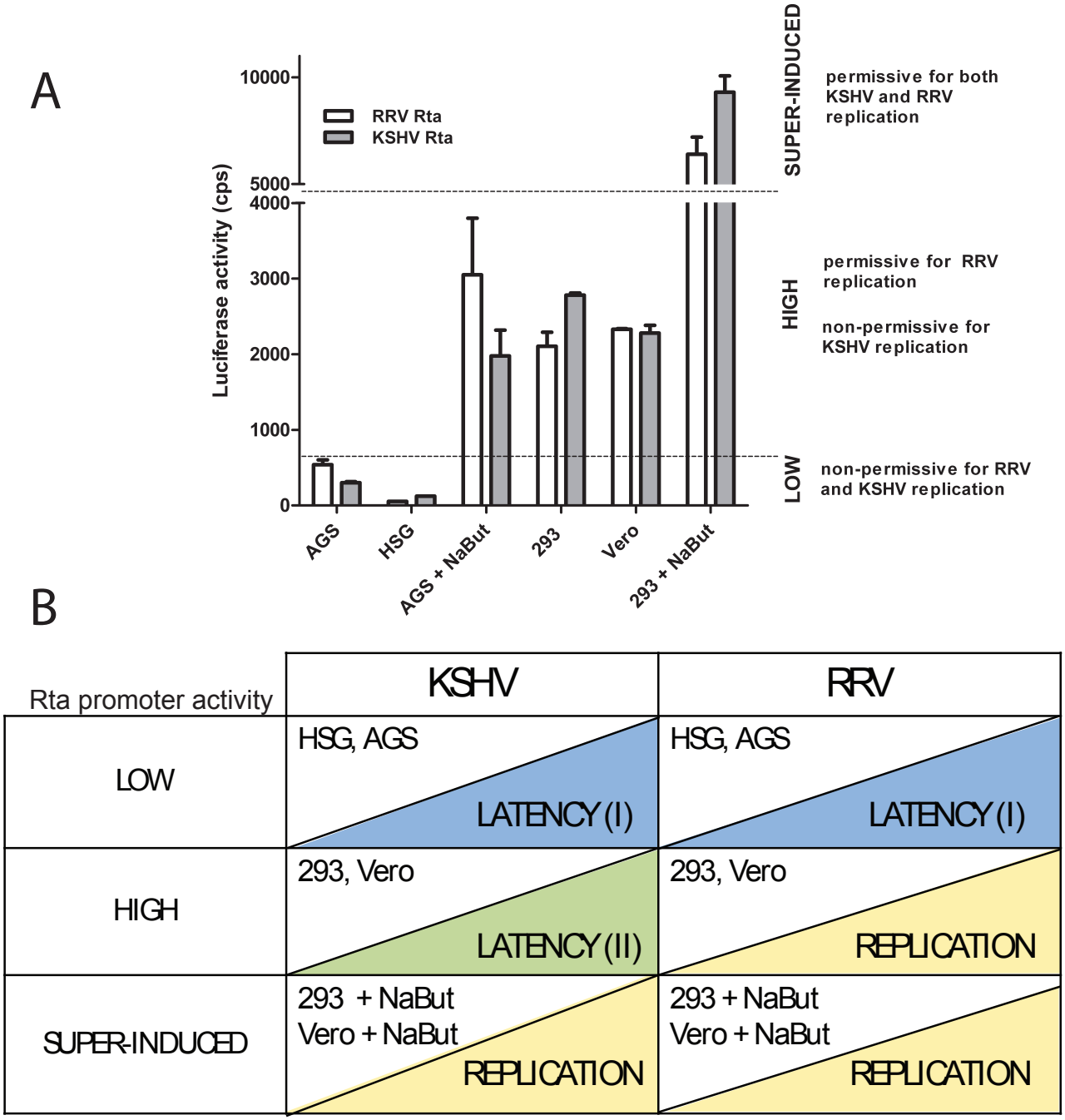

Figure 5-1. Summary of Rta promoter and cellular permissivity studies for KSHV and RRV. The activities of the KSHV and RRV Rta promoters in epithelial cell lines with or without butyrate treatment are shown as in Figure 2-10. Both viruses are non-permissive for replication in AGS and HSG cells which have LOW Rta promoter activity. Butyrate treatment of AGS cells, like HSG cells, induces HIGH Rta promoter activity and RRV replication. In cells with HIGH Rta promoter activity, such as 293 and Vero cells, RRV replicates efficiently while KSHV establishes latent infection. Because of the LANApi pathway, KSHV requires a higher threshold of Rta promoter activation to enter replication. When Rta promoter activity is SUPER-INDUCED, as in butyrate-treated 293 cells, KSHV is able to replicate.

We also showed that RRV LANA was as potent an inhibitor of Rta promoter activity as KSHV LANA. Therefore, the highly replicative phenotype of RRV is not due to a qualitative defect in LANA inhibitory activity. Instead, we propose that RRV LANA activity is highly restricted in infected cells either at the level of transcription or through a post-translational mechanism. Bioinformatic analysis suggested that RRV lacks the Rta-inducible LANApi promoter that has been described for KSHV. Without an inducible LANApi promoter, RRV LANA levels may not be sufficient to overcome Rta promoter activation in infected cells resulting in the full cascade of lytic gene expression. Another possible explanation for the lack of Rta shutoff in RRV infected cells involves the localization of RRV LANA. Our transfection experiments have identified an RG-rich N-terminal motif that targets RRV LANA to the nucleolus. The nucleolar localization of LANA appears to be RV2 specific, as RV1 LANAs are reportedly excluded from the nucleolus. RRV LANA that is sequestered in the nucleolus of infected cells would not be able to access viral DNA and would, therefore, be unable to repress the Rta promoter. Because antibodies to RRV LANA are not available at present, we have not yet been able to study the relevance of this finding in infected cells. Such a reagent would allow for a detailed investigation of RRV LANA localization during infection. Future studies may uncover novel functions for nucleolar LANA that inform our understanding RRV tumorigenesis.

A previous study reported that sodium butyrate increased KSHV Rta promoter activity in non-permissive cells 293 cells, resulting in the reactivation of latent virus (Lu, Zhou et al. 2003). It was assumed that host-mediated Rta promoter activity in 293 cells was too low to drive viral replication and that butyrate induced sufficient Rta promoter activity in these cells to drive replication. However, we have shown that the level of Rta promoter activity (Figure 5-1, “HIGH”) observed in 293 cells is sufficient to drive RRV replication in this cellular background.

This suggests that KSHV latency in 293 cells is determined by LANA-mediated inhibition of Rta promoter activity. The increased Rta promoter activity in 293s following butyrate treatment, therefore, represents a super-induction of the Rta promoter. Butyrate induces KSHV Rta promoter activity, thereby overcoming the repression of the Rta promoter by LANA and activating the lytic gene cascade.

Based on these data, we suggest a model in which LANA expression and/or the regulation of LANA activity is fundamentally different between KSHV and RRV. In this model the ability of LANA to inhibit Rta promoter ultimately determines the latent and replicative phenotypes of KSHV and RRV, respectively. Our studies, which are summarized in Figure 5-1, indicate that RRV establishes latency in cells with LOW Rta promoter activity (such as AGS and HSG cells) and replicates in cells with HIGH Rta promoter activity (such as 293 and Vero cells). In contrast, KSHV establishes latency both in cells with LOW Rta promoter activity and cells with HIGH Rta promoter activity. One interpretation of these results is that KSHV has evolved two pathways to latency, whereas RRV only has one. RRV only establishes latency in conditions where host-mediated Rta promoter activity is too low to drive viral replication. We have shown that KSHV is also able to establish latent infection in AGS and HSG cells, which have LOW Rta promoter activity. However, KSHV can also establish latency when Rta promoter activity is HIGH, as in 293 and Vero cells. KSHV has evolved a mechanism for Rta shutoff that involves an Rta-inducible LANApi promoter. This promoter demonstrates unusual specificity for Rta and is not activated by expression of intracellular Notch (ICN) (Hilton and Dittmer 2012).

Our studies raise interesting questions about the distinct advantages of latency and replication for rhadinoviruses. KSHV primarily establishes latent infection in the host, and

latency is considered the default program of infection for this virus. We have shown that KSHV can establish latency in both Rta LOW and Rta HIGH cells, suggesting that KSHV has multiple pathways to latency. It is also possible that KSHV can establish two distinct programs of latency, as has been described for EBV. If this is the case, each KSHV latency program may have unique advantages in the host. For the purposes of clarity, we have designated latency that occurs in cells with low Rta promoter activity "Latency (I)". The latency program in cells with high Rta promoter activity is designated "Latency (II)" (Figure 5-1B). Cells entering Latency (II) display a short burst of lytic gene activity prior to the establishment of latency. It is possible that transient expression of replication-associated genes increases the number of viral genomes contained in a latently infected cell. This would potentially lead to a more stable latency. Additionally, since many of the lytic cycle genes dysregulate host cell signaling, the short burst of lytic gene expression may contribute to KSHV tumorigenesis in critical ways. Latency (I), on the other hand, may yield fewer viral episomes per cell and be less stable, but could also provide a mechanism to evade the host immune system. A comparative analysis of gene expression following KSHV infection of Rta LOW and Rta HIGH cells is needed to determine if distinct latency programs exist and if so, to understand the consequences of each program.

In contrast, RRV has evolved a successful strategy for persistence in the host that involves a strong propensity towards replication. Because it lacks a LANApi pathway, the threshold Rta promoter activity required to drive replication is lower in RRV than in KSHV. As a result, RRV establishes latent infection in Rta LOW cells and replicates in Rta HIGH cells. The RRV latency program may, therefore, have important similarities to a KSHV Latency (I) program. Future studies will characterize the latent RRV systems described here and compare gene expression in these model systems to KSHV latency.

Our studies shed light on the differential ability of RRV and KSHV to establish latent and replicative infections in different cell types and how the interplay of Sp1, Rta, and LANA determine the outcome of infection for these viruses.

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