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Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression

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Abstract

A hallmark of all herpesvirus is the ability to exist in either a latent, or lytic, state of replication, enabling the lifelong infection of its host. Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) can efficiently establish a latent infection in a variety of cell types in vitro, making it a valuable model for the study of latency and reactivation. To facilitate the identification of KSHV lytic replication, and allow subsequent experiments with live cells, a recombinant virus, rKSHV.219, was constructed using JSC-1 cells that expresses the red fluorescent protein (RFP) from the KSHV lytic PAN promoter, the green fluorescent protein (GFP) from the EF-1α promoter, and with the gene for puromycin resistance as a selectable marker. rKSHV.219 from JSC-1 cells was used to infect Vero cells for purification of the recombinant virus. Vero cells were also used for the production of rKSHV.219 at levels of 10^5–10^6 infectious units (IU) of virus per milliliter using a combination of KSHV/RTA expressed from a baculovirus vector, BacK50, and butyrate. Virus produced from Vero cells was used to infect human fibroblasts (HF), 293, DU145, T24, HaCaT, and HEp-2 cells, and in all cells except 293 cells, only a latent infection was established with GFP expression, but no RFP expression. In 293 cells, 10–15% of cells showed lytic gene expression. Both primary and immortalized microvascular endothelial cells (MVEC) were also infected with rKSHV.219, and reduced spontaneous lytic replication was found in immortalized cells. In all cells used in this study, rKSHV.219 efficiently established latent infections from which the virus could be reactivated to productive lytic replication. This work also demonstrated strong synergy between KSHV/RTA and butyrate for the activation of KSHV lytic replication and the production of infectious virus.

Keywords: Herpesvirus; Red fluorescent protein; Vero cells

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is the eighth human herpesvirus (HHV-8) to be identified, and as with all herpesviruses, KSHV can exist in both latent and lytic replicative states (Jenner et al., 2002; Moore and Chang, 2001). In the latent state, herpesviruses have limited gene expression and reside quiescently within the cell without causing cytopathic effect. With the appropriate stimulation, the cascade of herpesvirus lytic cycle genes, consisting of immediate early (IE), early (E), and late (L) kinetic classes, are expressed resulting in the production of mature infectious virions. In vivo, KSHV apparently exists predominately in a latent state, with periodic reactivations necessary for such aspects of viral biology as transmission, and dissemination within the host. Lytic replication has also been associated with the pathogenesis of KSHV. Increased virus was detected in PBMC preceding KS (Whitby et al., 1995), and treatment with gancyclovir, which inhibits lytic replication, was reported to reduce the incidence of KS (Martin et al., 1999). In addition, KSHV encodes many lytic genes that may impact pathogenesis, such as v-IL6, viral chemokines, and v-GPCR; and the low percentage of cells with lytic gene expression in KS lesions may play a role in mediating features such as angiogenesis, immune infiltration, and the provision of paracrine functions that are involved with KS (Dourmishev et al., 2003; Jenner et al., 2002, #1459).

In vivo, many cell types have been identified with KSHV latent or lytic replication including monocytes, B cells, endothelial cells, and epithelial cells (Blasig et al., 1997; Boshoff et al., 1995; Diamond et al., 1998; Dupin et al.,

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1999; Huang et al., 1996; Sturzl et al., 1997). KSHV lytic and latent replication has also been identified in the three KSHV-related malignancies, Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and AIDS-related multicentric Castleman's disease (MCD). In KS and PEL, essentially all cells are infected, and greater than 95% of cells contain latent virus, while in MCD not all cells are KSHV-positive in the proliferative mass, and there is a higher percentage of cells with lytic virus than in KS or PEL (Boshoff et al., 1995; Dupin et al., 1999; Sturzl et al., 1997, 1999).

In vitro, KSHV has been reported to infect more than 20 cell types, including B cells, human fibroblasts (HF), microvascular endothelial cells (MVEC), 293 cells, human umbilical vein endothelial cells (HUVEC), DU145, rat endothelial cells, mouse fibroblasts, and Loukes (Bechtel et al., 2003; Blackbourn et al., 2000; Friborg et al., 1998; Mesri et al., 1996; Panyutich et al., 1998; Renne et al., 1998; Vieira et al., 2001). Of the many cell types infected by KSHV in vitro, MVEC, which are considered one of the precursors of KS tumor cells, have proven a valuable experimental system in that KSHV infection results in most infected cells harboring latent virus, with a few percentage of cells undergoing lytic replication with the production of infectious virus, a situation thought to mimic in vivo replication (Ciufo et al., 2001; Flore et al., 1998; Lagunoff et al., 2002; Moses et al., 1999; Panyutich et al., 1998; Poole et al., 2002). While KSHV can infect many cell types, it predominately goes latent, and long-term latently infected cultures have been established (Vieira et al., 2001). The reasons why KSHV primarily establishes a latent infection in cell culture are not as yet understood, but this has limited the generation of significant amounts of virus for experimental studies from any but PEL-derived lines, and has hampered the production purified recombinant virus.

The activation of lytic replication is a vital aspect of herpesvirus biology, and while the cellular stimuli that cause the switch from latent to lytic replication are not well understood, it is well documented that lytic gene expression of herpesviruses is initiated by expression of the IE class of viral proteins that transactivate the expression of the full complement of viral genes necessary for viral replication and virion production. KSHV contains a homologue of the EBV transactivator BRLF1 gene, RTA (replication and transcription activator), encoded by ORF 50, except for the first six amino acids which are encoded by an exon 5′ of ORF 50 (Sun et al., 1998). Ectopic expression of the KSHV/RTA gene can activate lytic gene expression (Lukac et al., 1998; Sun et al., 1998), and KSHV/RTA can activate the promoters of KSHV early genes (Chang et al., 2002; Zhang et al., 1998). While this viral gene can activate lytic replication, what cellular and viral factors control the expression and function of KSHV/RTA are only beginning to be understood. It has been reported that cellular stress, such as hypoxia, can induce the RTA promoter (Davis et al., 2001). NF-kB was indicated as inhibiting lytic replication, and thereby potentially functioning to maintain latency (Brown et al., 2003). A cellular factor, downstream of the activation of RTA expression, required for lytic replication has also been identified (Liang and Ganem, 2003). Additionally, clues to cellular factors that activate KSHV may be suggested by the fact that KSHV lytic replication can be induced with chemical agents, such as phorbol esters and butyrate (Miller et al., 1996; Renne et al., 1996). Phorbol esters are activators of protein kinase C (PKC), and butyrate can inhibit histone deacetylase, suggesting that pathways initiated by PKC, or chromatin state of the viral genome, may play a role in the natural cellular processes leading to the promotion of lytic replication.

The alternation between latent and lytic replication is a vital feature of herpesvirus biology, allowing the permanent infection of the host. Because KSHV can efficiently establish a latent infection in many cell types in vitro, it provides a valuable system for the study of latent replication, as well as the factors effecting the switch from latency to lytic replication. To facilitate the study of the switch from latent to lytic replication, we have developed a recombinant KSHV that expresses the Ds Red fluorescent protein (RFP) as a lytic gene, and have used this virus to establish long-term infected cultures for the examination of latent and lytic replication in many cell types. The activation of KSHV lytic replication, and the production of infectious virus by both KSHV/RTA and chemical reagents have been examined, and significant synergy in activation by KSHV/RTA and a histone deacetylase inhibitor was found. In addition, methods for the purification and production of recombinant KSHV are presented.

Results

Generation of rKSHV.219

To facilitate the identification of KSHV lytic replication, a recombinant KSHV, rKSHV.219 (Fig. 1A), was constructed containing the RFP under the control of the lytic PAN RNA promoter, along with the GFP gene expressed by the human elongation factor 1-α promoter, and the *pac* gene for resistance to puromycin expressed by the RSV promoter. This recombinant virus was created by transfecting JSC-1 cells with pQ219, a plasmid which contains a 4.8-kb segment of KSHV DNA with the RFP/GFP/PURO cassette inserted between ORF K9 and ORF 57 at a site that sequence data indicates does not contain a gene. Once cultures were established under puromycin selection, they were examined for GFP and RFP expression with, and without, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, and a line showing good induction of RFP with TPA is shown in Fig. 1B, although it should be noted that there was wide variation in the expression of RFP following TPA induction of the different JSC lines generated from the transfections. The JSC cultures that had grown under puromycin selection were induced with TPA to produce

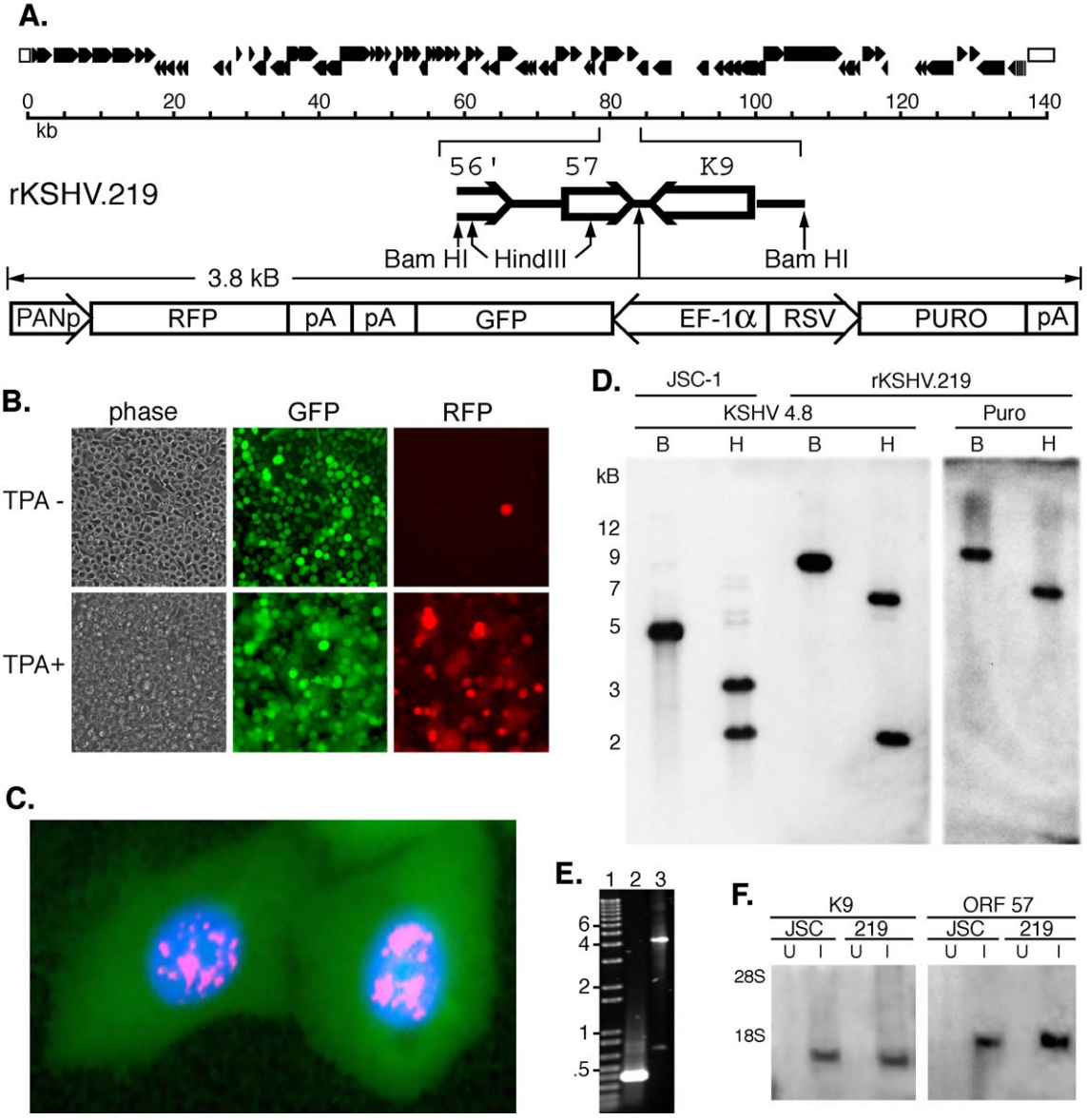


Fig. 1. (A) Schematic diagram of the KSHV genome showing an expansion of the 4.8-kb *Bam*HI fragment used to make the recombinant virus, and the structure and insertion site of the RFP/GFP/PURO construct. The *Bam*HI and *Hin*dIII sites of the 4.8-kb fragment are indicated. (B) Photomicrograph of JSC-1 cells transfected with pQ219 and selected with puromycin. Top row: no treatment; bottom row: treated with TPA. Left column: phase; middle column: fluorescence for GFP; right column: fluorescence for RFP. (C) Photomicrograph of Vero cells infected with rKSHV.219 with the computer-combined images of fluorescence for GFP (green), DAPI nuclear staining (blue), and Alexa 594-labeled antibody detection of LANA (red). (D) Autoradiograph of the hybridization analysis of DNA isolated from JSC-1 cells (JSC-1), and from Vero cells infected with rKSHV.219 (KSHV.219). The probes were the 4.8-kb *Bam*HI fragment (KSHV 4.8) of KSHV used to make the recombinant, or the puromycin resistance gene (Puro). The DNAs were digested with *Bam*HI (B) or *Hin*dIII (H) before electrophoresis. The position of markers in kb is indicated. (E) Ethidium bromide-stained agarose gel following electrophoresis of the PCR products from amplification of viral DNA from JSC-1 cells, or rKSHV.219, using primers 5′ TTGCCAAACCCCATGGCAGAGT and 5′ TTGCGGCGAGGTGCAG-TAATTTC, which flank the insertion site, and amplify a 0.4-kb fragment from JSC-1 DNA, and a 4.2-kb fragment from rKSHV.219. The size in kb of markers is shown on the left. (F) Northern analysis of total RNA isolated from JSC-1 cells (JSC), uninduced (U) or induced (I) with 15 ng/ml TPA, and Vero cells containing rKSHV.219 (219) uninduced (U) or induced (I) with RTA and sodium butyrate as described below. The probes were specific for the K9 transcript (K9) or the ORF 57 transcript (ORF 57).

virus, the virus was passaged to Vero cells using a low multiplicity of infection, and the infected Vero cells were grown with puromycin selection. Vero cells were chosen because in previous work with rKSHV.152 (Vieira et al., 2001), it was found that KSHV efficiently establishes a latent infection in Vero cells with no production of infectious virus (J. Vieira, unpublished observations). Puromycin-resistant Vero cells resulting from the infection with rKSHV.219 exhibited the coincident expression of GFP and the latent nuclear antigen (LANA), which was detected by immunofluorescence and showed the typical punctate nuclear staining pattern of latently infected cells, but RFP

expression was not observed (Fig. 1C). Individual GFP-positive Vero colonies resulting from the puromycin selection were isolated from the cultures and expanded for further analysis of recombinant virus. Next, the structure of the recombinant virus was examined, and Fig. 1D shows the hybridization analysis of total cellular DNA from Vero cells containing rKSHV.219 and JSC-1 cells with the KSHV 4.8-kb *Bam*HI fragment that was used in constructing the recombinant virus, and the hybridization analysis of rKSHV.219 with the puromycin resistance gene. In the hybridization analysis using the KSHV probe it can be seen that in the digests of rKSHV.219 there is no wild-type 4.8-kb *Bam*HI fragment, or 3-kb *Hin*dIII fragment detectable, but fragments unique to the recombinant virus of the size expected for the addition of the 3.8-kb insert are present in both the *Bam*HI and *Hin*dIII digests of rKSHV.219. The analysis with the puromycin resistance gene probe identified the same size fragments as the unique fragments of the recombinant virus detected by the KSHV probe. This demonstrated that the recombinant virus, rKSHV.219, is purified free of wild-type virus, and that the insertion had recombined at the expected location in the viral genome. As an additional test that wild-type virus was not present, PCR reactions were performed with primers flanking the insertion site, and no 0.4-kb band corresponding to wild-type virus was amplified from rKSHV.219, but a 4.2-kb band was amplified (Fig. 1E). To confirm that the region of the insert did not contain a transcribed gene as indicated by sequence data, and flanking transcripts were not disrupted, Northern analysis of RNA from JSC-1, and Vero cells containing rKSHV.219, was carried out, and similar patterns of transcription with probes for ORFs K9 and 57 were seen (Fig. 1F).

rKSHV.219 contains the GFP expressed from the elongation factor 1-α (EF 1-α) promoter and there are two points about the promoter and GFP that are important to the work to be described below. Firstly, the EF 1-α promoter was chosen for making recombinant viruses because it is a strong cellular promoter without an identified strong enhancer, which if present could impact the expression of nearby viral genes. However, needs to be noted that while the EF 1-α promoter expressed GFP in 15 different cell types that have been infected by rKSHV.219, there can be variation in the level and percentage of infected cells that express GFP. In some cell types, essentially all infected cells express GFP, as illustrated by 293 cells in Fig. 2A, where 2 days postinfection the expression of GFP and the detection of LANA by immunofluorescence are coincident. In other cell types, not all infected cells express detectable levels of GFP, as demonstrated by GFP and LANA detection in infected MVEC (Fig. 2B), where LANA-positive nuclei (red) are present that are not associated with GFP-positive cells. The silencing of the EF 1-α promoter has also been observed in some cells, such as HF or MVEC, where in cultures with mostly GFP-positive cells that had been passaged in the presence of puromycin, there were are also cells that did not express detectable levels of GFP unless they were treated with inducers of gene expression such as sodium butyrate, or activators of lytic gene replication such as KSHV/RTA, or infection with HCMV (J. Vieira, unpublished observations). Secondly, in many experiments described below, the expression of GFP by rKSHV.219 infection of 293 cells was used as an assay for the presence of infectious virus. 293 cells were chosen for this assay because as demonstrated in Fig. 2A, 293 cells exhibit easily detectable levels of GFP expression in all infected cells 2 days postinfection. In addition to the fact that the coincident expression of GFP and LANA demonstrate that the presence of GFP is due to viral infection, two other controls were carried out that demonstrate the GFP detected is due to gene expression resulting from viral infection. Firstly, that GFP expression requires gene expression is indicated by the fact that in 293 cultures infected with rKSHV.219 there are no GFP-positive cells 4 h postinfection, but GFP-positive cells are evident 1 day postinfection. Secondly, in the experiments described below using infectious rKSHV.219, virus was harvested from cell-free media, and the cells were not sonicated, so there was very little GFP or RFP present in virus stocks. However, we have examined the incubation of 293 cells with an inoculum containing a much greater amount of GFP than would be present in rKSHV.219 preparations, but that contained no infectious virus. This inoculum was prepared by sonicating Vero cells containing latent rKSHV.219 (which express GFP but do not produce infectious virus), and after removing the cellular debris by centrifugation, the supernatant was used to inoculate 293 cells. At 1 and 2 days post inoculation with this material, no GFP-positive cells were observed (data not shown).

Activation of KSHV lytic replication in Vero cells

It had previously been found in work with rKSHV.152 (Vieira et al., 2001) that ectopic expression of KSHV/RTA from transfected plasmid, or expression by infection with a recombinant Baculovirus (BacK50) expressing KSHV/RTA from the HCMV major immediate promoter, could activate latent KSHV in Vero cells to a productive lytic replication, and that sodium butyrate could activate lytic KSHV gene expression alone, and also significantly augmented virus production by KSHV/RTA (J. Vieira, unpublished observations). In Fig. 3, the activation of lytic gene expression in Vero cells containing rKSHV.219 by the treatment with sodium butyrate, the expression of RTA by infection with BacK50, or the combination of RTA and sodium butyrate, is demonstrated by RFP expression, and the antibody detection of the early lytic ORF 59 protein with MAb 11D1 (Chan et al., 1998), or the antibody detection of the late lytic K8.1 protein with MAb A4 (Zhu et al., 1999). This experiment demonstrated that the lytic antigens were only found in cells that are RFP-positive and not in cells only expressing GFP. In addition, both ORF 59 and K8.1 antigens were present in cells activated by all three conditions,

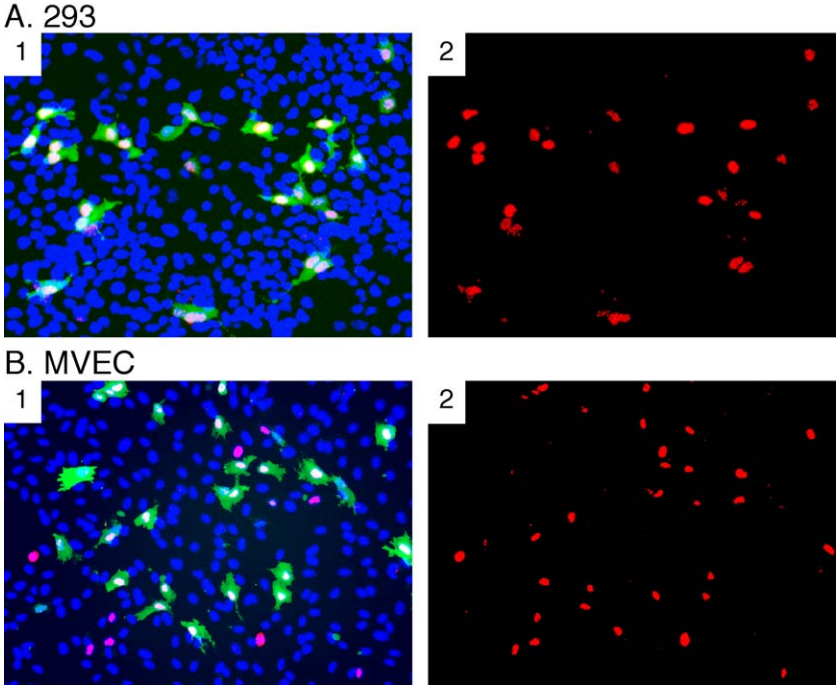


Fig. 2. Detection of GFP and LANA in cells infected by rKSHV.219. (A1) 293 cells, 2 days postinfection with rKSHV.219, showing GFP expression (green), LANA detection by immunofluorescence with Alexa 633 (red), and DAPI staining of nuclei (blue). (A2) Image of the immunofluorescence detection of LANA in 293 cells, alone. (B1) MVEC, 5 days postinfection with rKSHV.219 showing GFP expression (green), LANA detection by immunofluorescence with Alexa 594 (red), and DAPI staining of nuclei (blue). (B2) Image of the immunofluorescence detection of LANA in MVEC alone. The green, red, and blue channels were imaged separately and combined.

with the least amount of lytic antigen-positive cells present with butyrate alone, and the greatest amount of cells expressing lytic antigens present in cultures activated by both sodium butyrate and KSHV/RTA. There was no induction of RFP expression by infection with a Baculovirus vector not carrying the KSHV/RTA gene (data not shown).

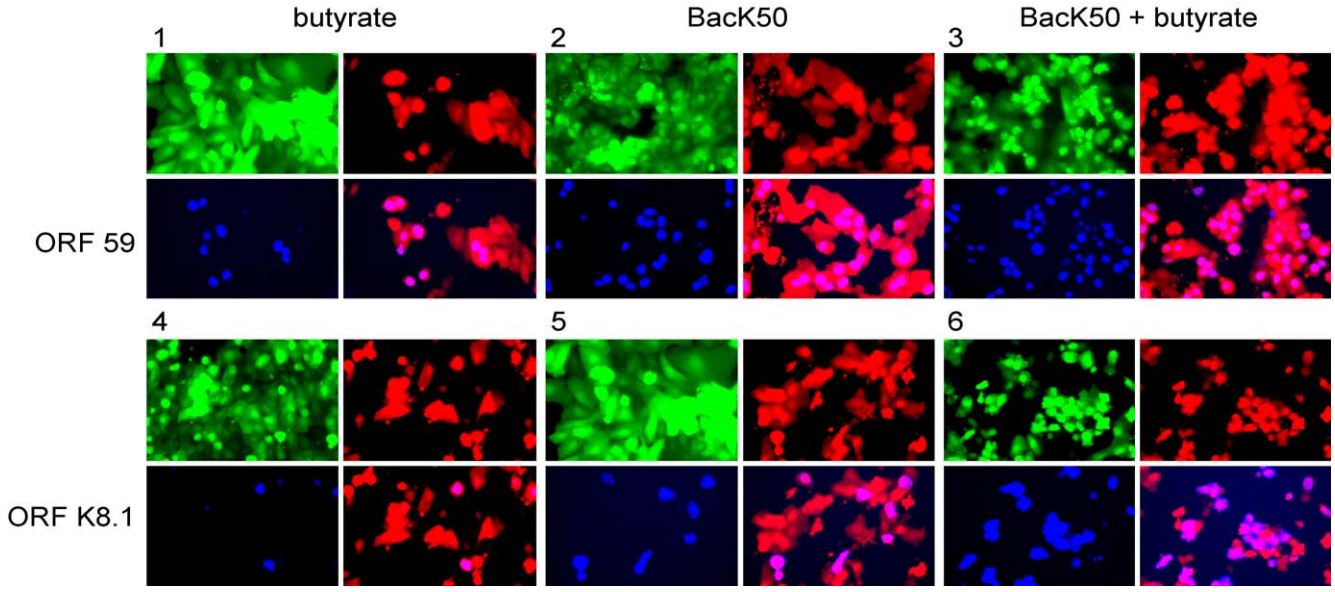


Fig. 3. The six panels are photomicrographs of Vero cells containing rKSHV.219 showing the detection of GFP, RFP, and ORF 59 protein or ORF 8.1 protein 2 days post induction. The induction conditions for the different panels were the following: 1 and 4, sodium butyrate; 2 and 5, BacK50; 3 and 6, BacK50 and sodium butyrate. KSHV proteins were detected using immunofluorescence with Alexa 388 (blue). The quadrants of each panel are: upper left, GFP; upper right, RFP; lower left, immunofluorescence for KSHV protein; lower right, overlay of RFP and immunofluorescence for KSHV protein. The detection of ORF 59 is in panels 1–3, and the detection of OFK K8.1 is in panels 4–6.

Because of the differences seen in lytic gene expression with the different induction conditions it was of interest to determine if this was predictive of differences in the amount of infectious virus being produced. To investigate this, the GFP gene carried by rKSHV.219 was utilized for the detection of infectious virus by infecting 293 cells with cell-free culture supernatants from cultures with rKSHV.219, and counting GFP-positive cells 2 days postinfection of the 293 cells. The number of GFP-positive cells produced per milliliter of media was used as the number of infectious units (IU) of KSHV per milliliter. The amount of infectious virus produced from Vero cells containing rKSHV.219 uninduced, and 3 days post induction with sodium butyrate, RTA expressed by BacK50, or BacK50 in combination with sodium butyrate, was determined by titering cell-free media from the cultures on 293 cells and counting GFP-positive cells. Fig. 4A shows the IU produced by the different activation processes and demonstrates that while no virus is produced by uninduced cultures, all three activation conditions resulted in the production of infectious rKSHV.219. Moreover, in agreement with the number of cells detected expressing lytic proteins, sodium butyrate alone produced the least amount of infectious virus, with the combination of RTA and butyrate resulting in the highest level of virus production. To assess that the action of sodium butyrate in augmenting KSHV production was not specific to baculovirus expression of KSHV/RTA, Vero cells with rKSHV.219 were transfected with a plasmid expressing KSHV/RTA from the HCMV IE promoter, and then cultured minus or plus sodium butyrate, and as with BacK50, sodium butyrate increased virus production (Fig. 4A). While RFP expression was visible 1 day post induction, it did not indicate when infectious virus is produced. To determine the timing of the production of infectious KSHV from Vero cells, a time course of rKSHV.219 production from Vero cells activated with RTA and sodium butyrate was carried out, and it was found that infectious virus was produced as early as 36 h post induction (Fig. 4B).

Because the blocking of histone deacetylases (HDACs) is considered the major mechanism by which sodium butyrate activates gene expression, another inhibitor of HDACs, trichostatin A (TsA), was also tested for its ability to activate KSHV. Treatment with TsA led to RFP expression and the production of infectious KSHV (Fig. 5). It was noted that TsA was more toxic to Vero cells than sodium butyrate, which may have contributed to the narrow range for TsA induction, below 0.05 μm gave very little induction, and above 0.1 μm had significant toxicity. While the level of infectious virus was lower than that produced by sodium butyrate induction, the TsA data supported the role of histone state in the maintenance of latency.

The experiments with butyrate and TsA indicated an important role of histone acetylation, which can be closely linked with DNA methylation (Bestor, 1998), in the activation of KSHV. In addition, previously published work indicated that DNA methylation plays a role in controlling expression from the KSHV/RTA promoter, and that the KSHV/RTA promoter is hypomethylated following TPA induction of KSHV in a PEL line (Chen et al., 2001). For these reasons, we next examined an inhibitor of DNA methylation, 5-azacytidine, for the activation of rKSHV.219. Vero cells containing latent KSHV were treated with 5-azacytidine and assessed for RFP expression and virus production. 5-Azacytidine treatment induced RFP expression, although there was little CPE, and infectious virus was not detected (Fig. 5). While compounds effecting HDACs and DNA methylation could activate KSHV in Vero cells, TPA did not (data not shown).

The previous experiments showed that KSHV/RTA could activate productive KSHV lytic replication in Vero cells, with sodium butyrate considerably increasing virus production; therefore, Vero cells were used as a convenient

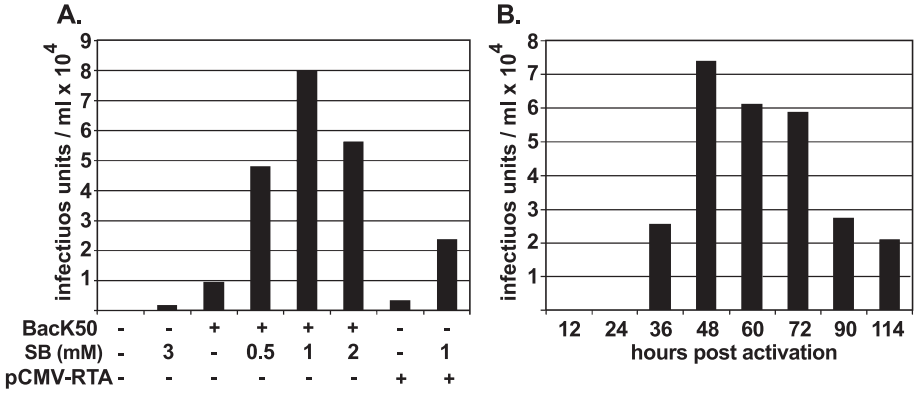


Fig. 4. (A) The production of infectious rKSHV.219 from latently infected Vero cells, with no activation, or 3 days post activation of lytic gene expression. The induction conditions included sodium butyrate (SB), infection with BacK50 (BacK50), or a plasmid construct (pCMV-RTA) expressing KSHV/RTA from the HCMV IE promoter as indicated beneath the graph. The graph represents the infectious units of rKSHV.219 as were determined by titering cell-free supernatants from the cultures on 293 cells and counting GFP-positive cells. (B) The timing of production of infectious rKSHV.219 from latently infected Vero cells following induction with BacK50 and sodium butyrate (1 mM) at the time points indicated beneath the graph.

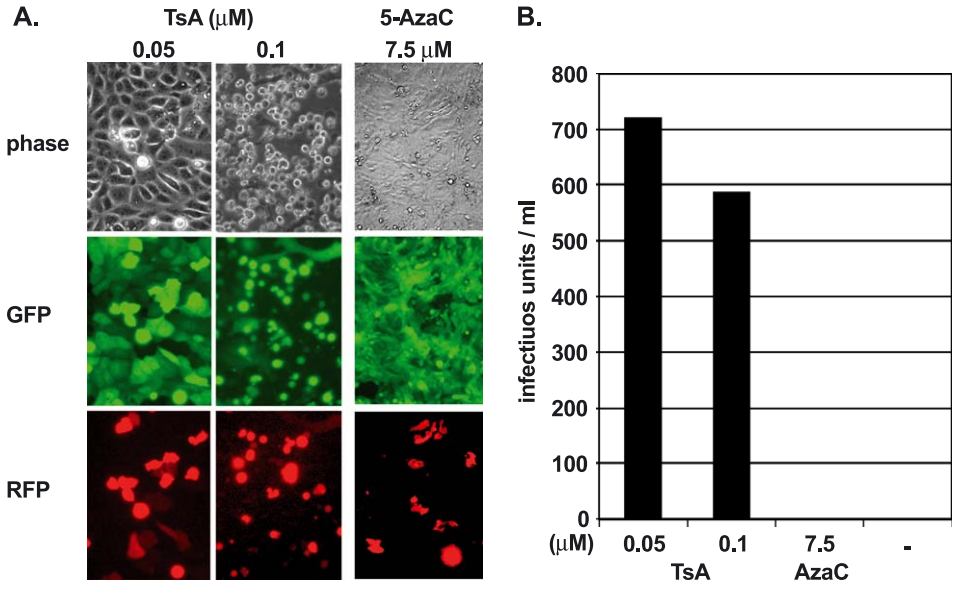


Fig. 5. (A) Photomicrograph of Vero cells containing rKSHV.219 induced with TsA or 5-AzaC at the indicated concentrations showing images of phase, GFP fluorescence, and RFP fluorescence 2 days post induction. (B) Graph of the detection of infectious units of KSHV from cultures induced by TsA or 5-AzaC 3 days post activation by titering cell-free culture supernatants on 293 cells.

cell line for the production of purified recombinant virus. Vero cells with rKSHV.219 in 150 cm^2 flasks were used for the production of viral stocks, and photomicrographs of cells, pre-activation and 2 days post-activation, are shown in Fig. 6A. While the uninduced cultures exhibited normal morphology, and no RFP expression, the induced culture had RFP expression plus significant CPE. IU of rKSHV.219 were determined by the titering of media from induced cultures on 293 cells and counting GFP-positive cells 2 days postinfection, and the activation of rKSHV.219 containing Vero cells with BacK50 and sodium butyrate in 150 cm^2 flask with 25 ml of media resulted in the production of 10^5–10^6 infectious units per milliliter of rKSHV.219. An example of a titering procedure is shown in Fig. 6B with a photomicrograph of 293 cells plated in a 3.83 cm^2 well inoculated with 0.025 ml of cell free media from the induced Vero cells containing rKSHV.219 with a titer of approximately 7×10^5 IU of virus.

rKSHV.219 produced from Vero cells was used to infect 293 cells, human fibroblasts, microvascular endothelial

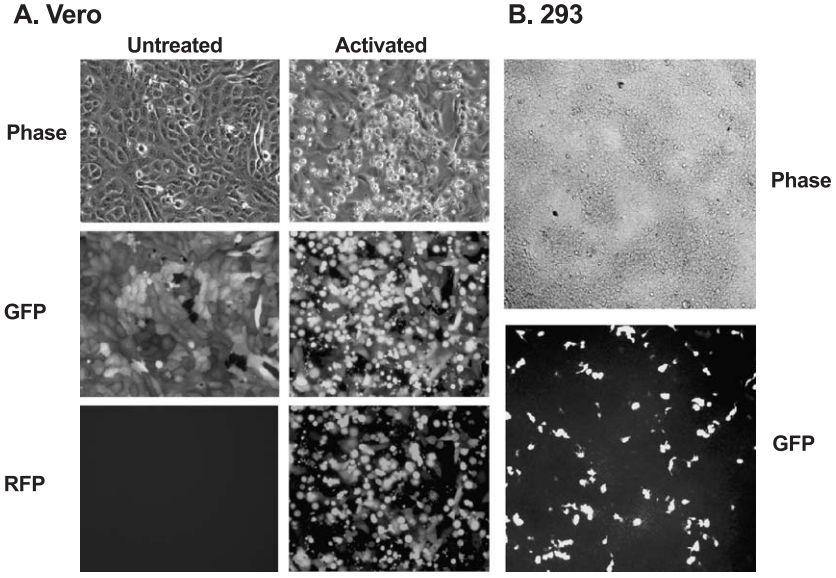


Fig. 6. (A) Photomicrograph of Vero cells infected with rKSHV.219 pre-activation (Untreated), and 2 days post-activation (Activated) with BacK50 and sodium butyrate showing images of phase, GFP fluorescence, and RFP fluorescence, as indicated. (B) Photomicrographs of the phase image and GFP fluorescence of an example of the titering of rKSHV.219 on 293 cells showing GFP expression 2 days postinfection with 0.025 ml of cell free media harvested from Vero cells containing rKSHV.219 activated with BacK50 and butyrate. The titer of the rKSHV.219 was approximately 7×10^5 IU/ml.

cells, HaCaT cells, DU145, HEp-2, and T24. All cell types could be infected as indicated by GFP expression, and these cultures were then evaluated for production of infectious KSHV, without or with activation as presented below.

Human fibroblasts

It was previously demonstrated that KSHV establishes a latent infection in HF as demonstrated by viral protein expression and gardella gel analysis (Vieira et al., 2001). The infection of HF with rKSHV.219 resulted in GFP expression, but not RFP expression, although it should be noted that an occasional RFP-positive cell (<100000) was seen in HF cultures. HF carrying latent rKSHV.219 were tested for virus activation by KSHV/RTA without, or with, sodium butyrate. While RFP was not expressed in HF, the infection of HF containing rKSHV.219 by BacK50 induced significant RFP expression (Fig. 7A) and the production of infectious virus (Fig 7B). The combination of BacK50 and sodium butyrate led to RFP expression, more CPE than BacK50 alone (Fig. 7A), and resulted in a significant increase in the level of virus produced as compared to BacK50 alone (Fig. 7B). As with Vero cells, TPA did not activate virus (data not shown). These results demonstrated that fibroblasts with an established latent infection are fully permissive for the complete lytic replication cycle of KSHV following activation by KSHV/RTA in agreement with previous work (Bechtel et al., 2003; Vieira et al., 2001), and again showed the impact of butyrate on increasing the level of infectious KSHV.

293 cells

In 293 cells infected with rKSHV.219, while no GFP was detected on the day of infection, GFP expression was evident as early as 1 day postinfection, and high-level GFP expression was evident 2 days postinfection (Fig. 8A), but RFP expression was not (although not present in Fig. 8A, it should be noted that occasional RFP-positive cells could be present at 2 days postinfection). In long-term rKSHV.219-infected cultures, which were established with puromycin selection, a relatively stable mixture of cells expressing only GFP, and cells expressing both GFP and RFP, were present 1 month postinfection (Fig. 8B), indicating some level of lytic gene expression. Although the cells shown in Fig. 8B were grown with puromycin, the induction of RFP expression occurred even without antibiotic selection (data not shown). To confirm that the RFP expression indeed signified lytic gene expression, 293 cells infected with rKSHV.219 were examined for ORF 59 expression using monoclonal antibody 11D1 (Chan et al., 1998), and ORF 59 was present in cells expressing RFP (Fig. 8C). Long-term rKSHV.219-infected 293 cells were examined for the production of infectious KSHV without any treatment, or treated with BacK50 or sodium butyrate by titering culture supernatants on 293 cells and counting GFP-positive cells (Fig. 8C). Even with the high level of spontaneous lytic activation in KSHV-infected 293 cultures, there were only

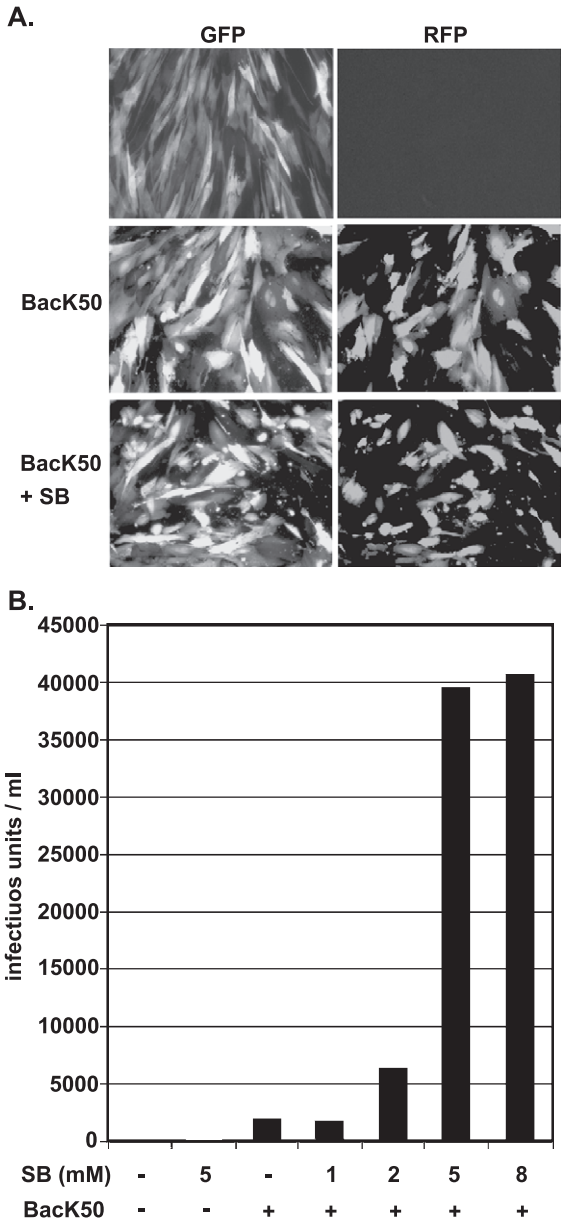


Fig. 7. (A) HF infected with rKSHV.219. Photomicrographs of GFP and RFP fluorescence in untreated cells (top row), and cells 2 days post activation with BacK50 (middle row), or 2 days post activation with BacK50 plus sodium butyrate (bottom row). (B) Infectious units of rKSHV.219 produced by latently infected HF without and treatment, or treated with sodium butyrate (SB) or infected by BacK50 (BacK50), as indicated below the graph determined 3 days post activation.

low levels of virus produced. Increased virus production was seen with activation by sodium butyrate or KSHV/RTA, with the highest levels of infectious virus produced with both, but the levels were still lower than Vero cells or fibroblasts. Treatment of rKSHV.219-infected 293 cells with phorbol esters, or 5-azacytidine increased the level of lytic gene expression as indicated by RFP expression, and there was approximately a 3-fold increase in virus production with TPA, but not a detectable increase in virus yield with 5-azacytidine (data not shown).

Microvascular endothelial cells

Microvascular endothelial cells have proven a valuable cell system for the study of KSHV, although because of the limited life span of primary MVEC, immortalized MVEC have been developed and used for KSHV experimentation (Lagunoff et al., 2002; Moses et al., 1999). While immortalized cells are of significant convenience, the comparison of primary cells with immortalized cells derived from them, for the growth of KSHV, has not been examined. As a preliminary examination of this question, pooled MVEC were immortalized with hTert, or with hTert combined with papilloma virus E7 (Halbert et al., 1991; Kiyono et al., 1998). Both the hTert and the hTert/E7 cell lines continued to proliferate beyond the life span of the primary cells. During the passage of these lines, the hTert line suffered a minor crisis of slowed growth, while the hTert/E7 line did not, similar to that reported for keratinocytes (Dickson et al., 2000). The primary MVEC, and the immortalized lines were infected with rKSHV.219, and all three lines were efficiently infected with the majority of cells becoming GFP-positive. The examination of the three cultures for RFP expression revealed that in the primary cells there were frequent RFP-positive cells, including groups of RFP-positive cells; while in the immortalized lines, there were far fewer RFP-positive cells, which were most often single cells. Examples of GFP and RFP expression in primary, and hTert/E7 immortalized cells, are shown in Fig. 9A. The primary and immortalized MVEC were then tested for the production of infectious KSHV without activation. Because with the same cells, noticeable well to well variation in RFP expression was observed, virus titers were determined from five wells of culture initiated at the same time and split 1:2 once (Fig. 9B). As predicted by the level of RFP expression, primary cells produced infectious virus at higher levels than the immortalized cell lines. To test if the reduced virus production by immortalized cell lines was simply due to a lack of spontaneous activation and not an inability to produce virus, primary and immortalized KSHV-infected cultures were treated with HCMV, TPA, or BacK50 and butyrate to activate KSHV lytic replication. The activation of KSHV lytic gene expression in primary and hTert/E7 cells by BacK50 and butyrate, as indicated by RFP expression, is shown in Fig. 9C, demonstrating a high percentage of activation. The level of infectious virus produced by unactivated and activated cultures was determined 3 days post activation (Fig. 9D). In these experiments, it was found that KSHV was activated from primary or immortalized cells by HCMV, or BacK50/butyrate, to similar levels of infectious virus. While treatment with TPA increased virus yield by more then 10-fold in immortalized cells, the yield of virus was below the amount of virus produced by TPA-induced primary cells.

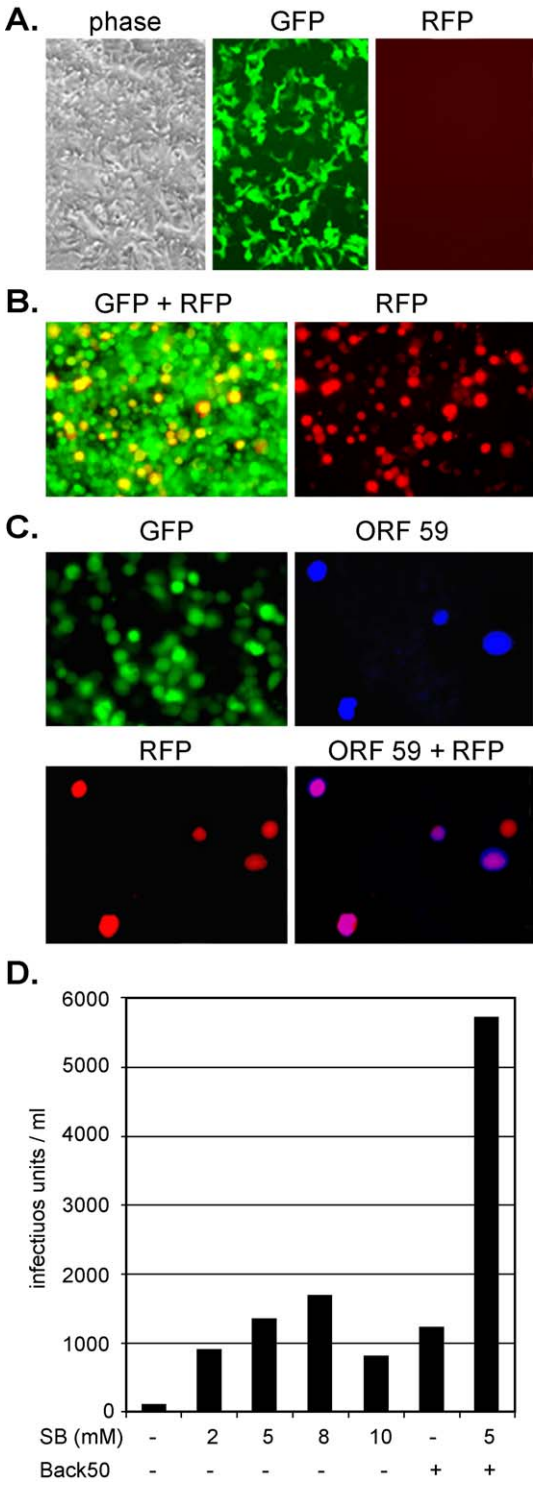
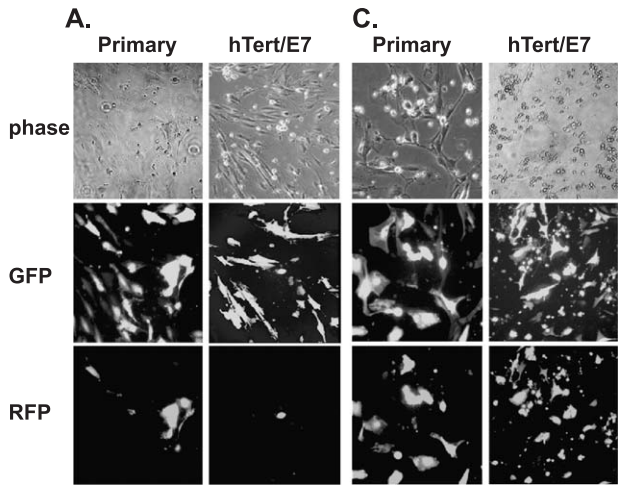


Fig. 8. (A) Photomicrographs of 293 cells infected with rKSHV.219 2 days postinfections showing phase, GFP fluorescence, and RFP fluorescence. (B) Long-term rKSHV.219-infected culture of 293 cells showing the overlay of GFP and RFP fluorescence (left panel), and RFP fluorescence (right panel). (C) GFP, RFP and ORF 59 protein expression in 293 cells, as indicated above each image. The ORF 59 protein was detected with monoclonal antibody 11D1 by immunofluorescence with Alexa 388 (blue) as the fluorescent marker. The lower right panel is the computer-generated overlay of RFP and the immunofluorescence for the ORF 59 protein. (D) Graph of the production of infectious rKSHV.219 from long-term infected 293 cells, without activation, or 3 days post activation by sodium butyrate (SB) or BacK50 (BacK50), as indicated beneath the graph.



B.

	mean IU/ml	(range, n=5)
Primary	418	(157 - 876)
hTert	*	(0 - 6)
hTert/E7	*	(0 - 4)

* because most wells were negative, an average was not determined

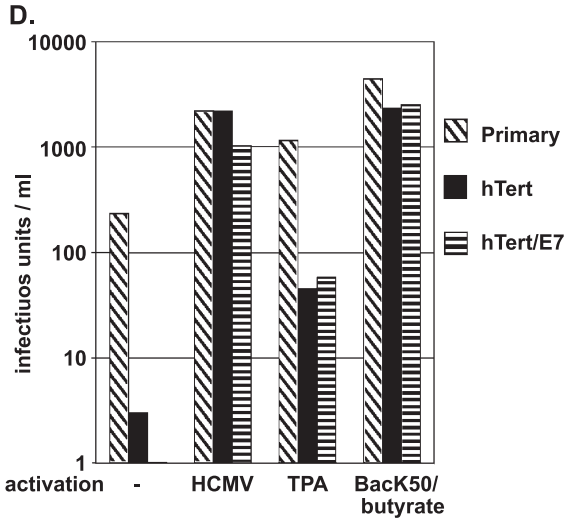


Fig. 9. (A) Photomicrographs of primary and hTert/E7 immortalized MVEC infected with rKSHV.219 showing phase, and GFP and RFP expression. (B) Production of infectious units of rKSHV.219 by primary and immortalized MVEC cultures with no activation. The *n* refers to five separate 3.83 cm^2 wells, and the range is the range of titers of IU from the separate wells. (C) Photomicrographs of primary and hTert/E7 immortalized cells infected with rKSHV.219 and activated with BacK50 and butyrate (3 mM) showing phase, GFP fluorescence, and RFP fluorescence. (D) Graph of infectious units of rKSHV.219 produced by primary, hTert immortalized, and hTert/E7 immortalized MVEC without activation (−), or activated by HCMV infection (HCMV), treatment with 15 ng/ml TPA (TPA), or infection with BacK50 in combination with 3 mM butyrate (BacK50/butyrate).

T24, DU145, HEp-2, and HaCaT

To determine if other cell lines supporting only latent replication of KSHV were proficient for the production of infectious virus following activation, four additional cells were tested. Latent infection of the bladder carcinoma cell line T24, and the prostate carcinoma line DU145 by KSHV was previously documented by gardella gel analysis (Vieira et al., 2001) In this work, rKSHV.219 was used to infect T24, DU145, HaCaT, a spontaneously immortalized epithelial cell line, and HEp-2 cells, derived from a larynx carcinoma. rKSHV.219 efficiently infected all four cell types and latently infected lines were established. No virus production was detected from any of the four, and all maintained normal cell morphology, although occasional RFP-positive cells were observed in T24 and Hep2 cells. To determine if these cells were permissive for virus production, they were treated with KSHV/RTA, in combination with sodium butyrate, to activate lytic replication. In Fig. 10A, photomicrographs of the four cell types 2 days post induction are shown, and all cell types expressed RFP, indicative of lytic gene expression. To determine if infectious virus was produced, virus in cell-free media was titered on 293 cells and the level of infectious units was determined (Fig. 10B). While the yield of virus was lower

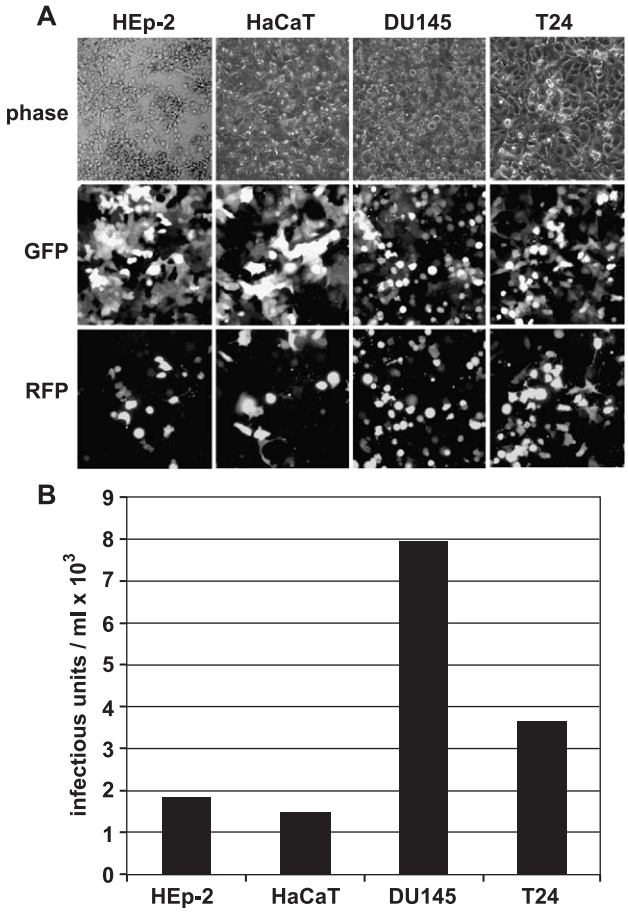


Fig. 10. (A) Photomicrographs of long-term rKSHV.219-infected cultures of HEp-2, HaCaT, DU145, and T24 cells 2 days posttreatment with BacK50 and sodium butyrate (3 mM) showing: top row, phase image; middle row, GFP fluorescence; and bottom row, RFP fluorescence. (B) Graph of infectious units of rKSHV.219 produced by the indicated cell types 3 days post treatment with BacK50 and butyrate.

than HF or Vero cells, all cells were capable of producing virus.

Discussion

In this report, we describe a recombinant virus rKSHV.219 that expresses the RFP as a lytic gene, enabling the identification of lytic gene expression without the destruction of the culture as is needed for the antibody detection of lytic proteins. This virus also carries the GFP that is expressed in the majority of latently infected cells, allowing the identification of infected cells whether lytic or latent, and the gene for puromycin resistance that enables the selection of infected cells. In rKSHV.219, the RFP protein is expressed from the strong lytic PAN promoter (Sun et al., 1996; Zhong et al., 1996) that can be directly activated by KSHV/RTA (Chang et al., 2002; Song et al., 2001). The expression of RFP from the PAN promoter was found to be a sensitive assay for lytic gene expression, in that RFP was not seen in latently infected cells, all cells that expressed lytic antigens also expressed RFP, and infectious rKSHV.219 was only produced in cultures exhibiting RFP expression. While only RFP- positive cells were positive for antibody detection of lytic proteins, there were RFP-positive cells that did not stain for the ORF 59 or ORF 8.1 proteins. This could be due to the proteins being present, but at levels below detection, or that there are different levels of lytic activation for KSHV as previously indicated by a greater number of cells expressing early than late genes (Moses et al., 1999). Because the PAN promoter can be directly activated by RTA, it may be present in all cells responsive to RTA activation, while some lytic genes may require other viral or cellular functions, such as DNA replication for the expression of ORF 8.1, that may vary from cell to cell and impact lytic gene expression.

A novel aspect of this work was the use of Vero cells for the purification and production of recombinant KSHV at titers of 10^5–10^6 infectious units per milliliter. The production of infectious rKSHV.219 was carried out by propagating latent virus in Vero cells, and then inducing lytic replication. Virus isolated from Vero cells efficiently infected more than a dozen cell types, and could grow lytically in MVEC, and produce infectious virus that could be passaged to other cell lines, as well as back to Vero cells. Vero cells provide a convenient cell type for the production of KSHV that has robust growth characteristics and can be efficiently transfected. Therefore, Vero cells containing rKSHV.219 provide a relatively straightforward system for the generation of recombinant virus by using rKSHV.219 as the parental virus and the use of a second selectable marker for the disruption of a viral gene, or the introduction of other genetic modifications (J. Vieira, unpublished data). This system should also prove useful for the production of recombinant KSHV made in Bacmid vectors (Delecluse et al., 2001; Zhou et al., 2002), where such constructs can be transfected into Vero cells, selected for as latently growing virus and then activated for the production of recombinant virus for further experiments.

Since the identification of the KSHV RTA gene as being capable of transactivating lytic gene transcription (Sun et al., 1998), numerous studies have confirmed its vital role in the activation of latent to lytic replication (Gradoville et al., 2000; Lukac et al., 1998; Lukac et al., 1999). Previously, ectopic expression of KSHV/RTA has been achieved using plasmid transfection (Gradoville et al., 2000; Lukac et al., 1998; Sun et al., 1998), Tet inducible systems (Nakamura et al., 2003), Adenovirus vectors (Bechtel et al., 2003), and in this study we used a Baculovirus, BacK50, expressing the KSHV/RTA from the HCMV IE promoter. BacK50 provides a convenient system for the production of a high titer viral vector expressing KSHV/RTA that can infect a very wide variety of mammalian cells, with very minimal impact (Kost and Condreay, 2002). In the present study, we found that ectopic expression of KSHV/RTA from BacK50 was able to activate lytic replication from established latently infected cultures and result in the production of infectious virus from the cells used in this study, in agreement with the many previous studies showing that KSHV/RTA is an effective activator of KSHV lytic replication. The demonstration that KSHV/RTA has the ability to activate virus from a variety of long-term latently infected cultures indicates that the control of RTA expression is a crucial aspect of the establishment and maintenance of latency.

In this study, we also found that sodium butyrate was able to activate the lytic gene expression of KSHV, as well as significantly augment the level of infectious virus produced by KSHV/RTA activation. The ability of butyrate to inhibit HDACs, and thereby increase the level of histone acetylation that is correlated with transcriptional activity, is considered the mechanism by which it can activate gene expression (Kruh, 1982); and butyrate has been shown to activate KSHV lytic gene expression in PEL lines (Miller et al., 1996; Yu et al., 1999). The fact that butyrate, alone, can result in the production of infectious virus indicates the important role of chromatin state on the maintenance of latency, and its role in controlling lytic transcriptional activity of herpesviruses. While the addition of butyrate increased the yield of KSHV produced with RTA activation, the impact of butyrate on virus activation in conjunction with the ectopic expression of KSHV/RTA is difficult to fully assess because butyrate can also increase the expression of genes introduced by baculovirus infection, or transfection. It is likely though, that in addition to increasing ectopic expression of KSHV/RTA, butyrate treatment induces modifications of the chromatin structure of the viral genome that results in enhanced activation by KSHV/RTA. In support of a role for butyrate in lytic activation, besides increasing ectopic RTA expression, was that a 20-fold increase in the amounts of BacK50 or transfected plasmid could not compensate for the absence of butyrate in viral yield (J. Vieira, unpublished data). The interaction of KSHV/RTA with both HDAC (a transcription

repressor) and CREB-binding protein (a transcription enhancer) was reported, and because these two cellular proteins have opposite effects on transcription, it was suggested that the balance between these two proteins may play a role in controlling latency (Gwack et al., 2001). The inhibition of HDACs by butyrate may therefore shift the balance toward the action of CREB-binding protein, and lead to a better activation of KSHV lytic genes in combination with KSHV/RTA. It must also be considered that butyrate may induce the expression of a cellular gene that increases virus production.

For the study of KSHV replication, MVEC have proven a valuable cell system that provides both latent replication, as well as a low level of lytic replication with the production of infectious virus, and have been important to a variety of KSHV studies (Ciufo et al., 2001; Flore et al., 1998; Lagunoff et al., 2002; Moses et al., 2002; Panyutich et al., 1998). The limited life span of primary MVEC have resulted in the use of immortalized MVEC for KSHV research (Lagunoff et al., 2002; Moses et al., 1999). Because MVEC, and immortalized lines, are an attractive culture system for the use of rKSHV.219 in the study of factors activating lytic replication, we wanted to compare the replication of KSHV in primary cells and immortalized cells from the same source, a comparison that has not been done. Our examination of primary MVEC, and MVEC immortalized with hTert, or hTert and papiloma virus E7 gene found that the immortalized cells had spontaneous lytic growth much below that of the primary cells. While it is not known what features of immortalized cells results in the tighter maintenance of latency, it was found that when activated by KSHV/RTA, HCMV, or TPA, the immortalized cells could produce levels of infectious virus about the same as similarly induced primary cells, indicating there are no blocks to lytic replication once it is initiated. While much work is needed on understanding the differences between primary and immortalized cells, these preliminary experiments do indicate there can be differences that should be considered in the use of immortalized cells. It is also very likely that there will be differences between immortalized lines, and some may retain a greater degree of primary phenotype than others.

Although the production of infectious virus from unactivated cultures was only detected from 293 cells and MVEC, all cells presented here could produce virus when activated, and levels of infectious virus over 10^6 infectious units per milliliter have been achieved from Vero cells. This level of virus still only represents a few infectious units per cell, and if this is because only a low percentage of cells produce a high amount of virus, or because each cell only produces a few infectious virions, is yet to be determined.

These studies have demonstrated that the RFP expressed from the PAN promoter is a sensitive assay for KSHV lytic gene expression in living cells, and the easy identification of latent and lytic replication in living cells afforded by rKSHV.219 will facilitate the investigation of the questions involved in the reactivation of herpesviruses. In agreement with many previous studies, we also found that the KSHV/RTA is capable of activating lytic gene expression (Bechtel et al., 2003; Gradoville et al., 2000; Lukac et al., 1998; Nakamura et al., 2003; Sun et al., 1998;). That KSHV/RTA could activate virus from a variety of established long-term latent cultures indicates that the control of the KSHV/RTA promoter plays a critical role in the propensity of KSHV to establish a latent infection in vitro, and most likely in vivo. The impact of sodium butyrate on the activation of KSHV indicates that chromatin state also plays an important role in maintaining latency, and its significant synergy with RTA indicates that both are important components in the production of infectious virus. A better understanding of the factors influencing both the expression of RTA, and the state of viral associated chromatin, will provide important insight into the switch from latent to lytic replication, and the combined use of KSHV/RTA and butyrate will aid in the production of recombinant virus for a variety of studies.

Material and methods

Cells

Fibroblasts were of human foreskin origin. T24, HaCaT, Vero, Hep2, and DU145 cells were obtained from the American Type Culture Collection. HF, T24, HaCaT, Vero, Hep2, and DU145 were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine in a humidified 5% CO_2 37 °C incubator. JSC-1 cells were obtained from J.S. Cannon and R.F Ambinder, Johns Hopkins University School of Medicine, Baltimore, MD, USA, and cultured in RPMI supplemented with 10% FBS 0.1 mg streptomycin, 100 U penicillin, and 2 mM L-glutamine. Microvascular endothelial cells were from Clonetics (San Diego, CA, USA) and cultured in EGM-2 media from Clonetics as recommended. MVEC were immortalized with retroviral vectors expressing hTert or Papiloma E7 obtained from J.K. MacDougall, FHCRC, Seattle, WA, as previously described (Halbert et al., 1991; Kiyono et al., 1998).

Construction of recombinant KSHV

JSC-1 cells were used as the source for KSHV. For the construction of recombinant virus, a 4.8-kb *Bam*HI fragment (positions 81046–85820 of the published sequence; Russo et al., 1996) was isolated from the cosmid Z8 (Russo et al., 1996) and cloned into pUC21 to create pQ131. At the *Dra*III site (position 83710 of the KSHV sequence) present in the *Bam*HI fragment, after the ends were made blunt with T4 DNA polymerase and dNTPs, a blunt-ended DNA fragment containing the enhanced green fluorescent protein (GFP) (Clontech, Palo Alto, CA, USA) gene expressed by the elongation factor 1-α promoter, the puromycin *N*-acetyltransferase (*pac*) gene expressed by the RSV promoter,

and the Ds red fluorescent protein gene (RFP) (Clontech) expressed by the PAN RNA promoter (position 28520–28690) was inserted to create pQ219. pQ219 was used to electroporate JSC-1 cells as previously described (Vieira et al., 2001), and each of the 16 transfections was plated to three wells of a 12-well plate, resulting in 48 cultures. Three days post electroporation, the cells were grown with 0.5 μg/ml puromycin (Calbiochem, San Diego, CA, USA) to select for recombinants. The cells in 38 of the 48 wells grew under puromycin selection and were expanded to six-well plates. For the production of virus from the 38 cultures, cells were grown to a density of 5×10^5 cells/ml and induced with 15 ng/ml TPA and grown for 4 days. To harvest virus, cells were pelleted at 300 × *g* for 10 min, passed through a 0.8-μM filter, and the supernatant was used as virus inoculum to infect Vero cells. As judged by GFP expression, the infection of Vero cells was typically at a multiplicity of infection (moi) of less than 0.001. Two days post inoculation, the infected Vero cells were grown with 5 μg/ml puromycin. It should be noted that even at 5 μg/ml puromycin, spontaneous Vero mutants arose that were resistant to puromycin.

It should be noted that even at 5 μg/ml puromycin spontaneous Vero mutants could arise that were resistant to puromycin. This could cause the occurrence in cultures of Vero cells with rKSHV.219 of cells that had gained puromycin resistance and had lost rKSHV.219 by segregation (and were therefore not GFP-positive). The occurrence of such cells could be reduced, but not eliminated, by always growing cultures with drug selection, and to passage cultures before they were confluent.

Eleven of the thirty-eight cultures produced virus that infected Vero cells and resulted in the generation of puromycin-resistant colonies. Individual GFP-positive Vero colonies that had grown under puromycin selection were isolated using cloning rings, removed from the plate by trypsin, transferred to a fresh 6-well plate, and expanded with puromycin selection for further analysis. Vero cultures with recombinant virus were tested for the production of rKSHV.219 by induction with RTA expressed by BacK50 and sodium butyrate (as described below), and the amount of rKSHV.219 present in cell-free media 3 days post induction was determined by titering on 293 cells and counting GFP-positive 2 days later (as described below). The four Vero cultures that produced the highest level of virus were used for hybridization analysis of the viral DNA. Two of these that showed the correct pattern were subsequently used for further experiments, including those presented here, and no significant differences were seen between the two. The data from one of these isolates is presented here.

Baculovirus

A recombinant baculovirus expressing the KSHV RTA was constructed using the Bac-to-Bac Baculovirus (Invitrogen, Carlsbad, CA, USA) system. The KSHV/RTA construct consisted of the *Dra*I–*Sal*I fragment, nucleotides 71531–74815, with a deletion between the *Sac*II sites (nucleotides 71755–72316), placed under control of the HCMV IE promoter. Virus was grown in SF9 cells in Baculo Gold media, 10% FBS. Virus inoculum for mammalian cells was prepared from infected cultures by removing SF9 cells by centrifugation (400 × *g*, 15 min) and passed through a 0.8-μM filter, and stored at 4 °C. Infection of mammalian cells was carried out for 2 h. Both RT and 37 °C were compared for baculovirus infection, and no significant difference was seen.

It was found that both the concentration of BacK50 and the amount used was critical to achieve high titers of rKSHV.219 production, and the two-step procedure detailed below was used to optimize BacK50 infections. Firstly, the titer of baculovirus for the infection of mammalian cells was determined by titering BacK50 stocks on Vero cells containing rKSHV.219 and counting RFP-positive cells 2 days post BacK50 infection. This was carried out by plating Vero with rKSHV.219 in a 24-well plate (1.88 cm^2) with 0.5 ml of media. The cells were inoculated with 2-fold dilutions of BacK50, where the 2-fold dilutions were started with 5 μl and carried out for 12 dilutions. Titers from this procedure should be 5×10^7 or higher to achieve optimal levels of KSHV production. Lower titers of virus could not be completely compensated for by just increasing the amount baculovirus inoculum added. Secondly, the optimal amount of BacK50 to use for maximal KSHV production was determined by the infection of Vero cells (80–90% confluent) containing rKSHV.219 in a 12-well plate (3.83 cm^2) with BacK50 starting with 0.005 ml, and increasing by 0.01 ml increments to 0.2 ml per well. Following BacK50 infection, media with 1.25 mM sodium butyrate was added and 3 days later the amount of rKSHV.219 produced was determined on 293 cells as described below. Typically, a multiplicity of infection of approximately 20–40 was found appropriate, and the amount of BacK50 for any sized culture vessel was extrapolated from the 3.83 cm^2 well using the number of cells, or the area of the culture vessel.

Production of rKSHV.219 and determination of infectious units (IU)

For the generation of rKSHV.219 stocks, Vero cells containing latent rKSHV.219 (80–90% confluent) were infected with BacK50 for 2 h in a minimum volume, the inoculum was removed, the cells washed once with PBS, and fresh media with 1.25 mM sodium butyrate was added. Twenty to thirty hours later, the media with sodium butyrate was removed and fresh media, without puromycin, was added. Any cells in the original media were collected by centrifugation (300 × *g*, 5 min) and returned to the flask. At 50–70 h post baculovirus infection, the media was collected, cells were removed by centrifugation (300 × *g*, 10 min), the supernatant was passed through a 0.45- or 0.8-μM filter, and was then used as KSHV inoculum. Infectious units (IU) of rKSHV.219 were determined by titering virus present in cell-

free medium on 293 cells and counting GFP-positive 293 cells 2 days postinfection. Centrifugation enhancement was used for infection by centrifuging the culture plate at 450 × *g* for 20 min, with replacement of media 2 h after centrifugation. This increased the infection level approximately 2- to 3-fold over cultures infected without centrifugation.

Experiments examining virus production from the different cell types by the various activation processes were carried out in 12-well plates with 3.83 cm^2 wells. The process was the same as for the production of stock virus from Vero cultures described above, except the media with sodium butyrate was left on the cultures for the entire time, as it was found that this did not effect virus yield.

For all experiments, a representative result from one of at least three experiments was used as data for graphs.

Infection of cells with rKSHV.219 and puromycin selection

rKSHV.219 produced from Vero cells was used to infect human fibroblasts, 293 cells, MVEC, HaCaT, DU145, T24, and Hep-2 were infected at a moi of approximately 1–5 as determined by virus titer on 293 cells. Three days postinfection, cells were grown in media containing puromycin at 1 μg/ml for 293, DU145, Hep2, and T24 cells, 0.5 μg/ml for HF, and 0.25 μg/ml for MVEC.

Antibody detection of viral proteins

The IFA detection of KSHV ORF 59 and ORF 8.1 proteins was carried out with MAb 11D1 (specific for ORF 59 protein) (Chan et al., 1998), or MAb A4 (specific for ORF 8.1 protein) (Zhu et al., 1999) diluted 1:10 in PBS–0.3% BSA as previously described (Vieira et al., 2001), except Alexa 350 (Molecular Probes, Eugene, OR, USA) was used for fluorescent detection. For the IFA visualization of the latent nuclear antigen (LANA), cells were fixed and reacted with rabbit polyclonal antibody to LANA as previously described (Moses et al., 1999), except that the antibody was preabsorbed to fixed and permeabilized cells not infected with KSHV for 30 min. For Vero cells, the polyclonal antibody to LANA was followed with biotin-conjugated F(ab′)2 fragment goat anti-rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) diluted 1:200 for 30 min at 37 °C. The samples were washed twice with PBS and reacted with Alexa 594 streptavidin for 10 min at 37 °C, and then washed twice with PBS, and counterstained with 1 μg/ml DAPI for 1 min and washed in PBS. For 293 cells, the polyclonal LANA antibody was followed by Alexa Fluor 633 F(ab′)2 fragment of goat anti-rabbit IgG (Molecular Probes) diluted 1:200 for 30 min, washed twice with PBS, and counterstained with 1 μg/ml DAPI for 1 min and washed in PBS.

Hybridization analysis

DNA hybridization analysis was carried out on total cellular DNA as previously described (Vieira et al., 2001) using the 4.8-kb *Bam*HI fragment of KSHV DNA and the puromycin resistance gene as probes labeled with ^{32}P using the Ready-To-Go labeling beads (Amersham, Piscataway, NJ). Fifteen micrograms of total cellular DNA was used for rKSHV.219 and 7 μg of JSC-1 DNA was used for each digest. The different amounts of DNA were used to equalize the amounts of viral DNA.

RNA hybridization analysis was carried out as described (Goda and Minton, 1995) using 10 μg of total cellular RNA isolated with the RNA easy kit (Qiagen, Valencia, CA). The K9 probe consisted of a *Bst*XI fragment (83794–85745) and the ORF 57 probe consisted of a *Acc*65I–*Dra*III fragment (82342–83710), and were labeled with ^{32}P using the Ready-To-Go labeling beads (Amersham).

Microscopy

Nikon Eclipse TE300 inverted fluorescent microscope equipped with filter sets TE300 FITC, TE300 Texas Red HYQ, and TE300 633. Images were acquired with a Photometrics CoolSnap cf digital camera and Metavue imaging software.

Acknowledgments

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