Kaposi's Sarcoma-Associated Herpesvirus-Encoded Latency-Associated Nuclear Antigen Modulates K1 Expression through Its cis-Acting Elements within the Terminal Repeats

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K1 is the first open reading frame encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) and lies positionally to the immediate right of the terminal repeats. K1 is a transmembrane glycoprotein having a functional immunoreceptor tyrosine-based activation motif (ITAM) capable of activating B-cell receptor signaling. K1 is expressed mostly during the lytic cycle of the virus and its promoter lies within the terminal repeat which contains the binding sites for latency-associated nuclear antigen (LANA). The K1 promoter (K1p) having LANA binding sites assayed by reporter assay demonstrated that LANA is capable of down-regulating K1 promoter transcriptional activity. However, the KSHV replication transcription activator RTA up-regulates K1p transcriptional activity. The promoter deleted of LANA binding sites showed loss in LANA-mediated down-regulation but was unaffected for RTA-mediated up-regulation. Increasing amounts of RTA rescued LANA-mediated repression of K1p transcriptional activity in cotransfection experiments. Reporter assay data suggest that LANA binding to its cognate sequence is critical for LANA-mediated repression of K1p as a LANA construct lacking the DNA binding domain was unable to repress K1p transcription. Additionally, KSHV primary infection experiments suggest that K1 is expressed during early infection but is repressed on the establishment of latency and so follows an expression profile similar to that of RTA during infection. Analysis of the promoter sequence revealed the presence of Oct-1 transcription factor binding sites within the −116 to +76 region. Mutational analysis of the Oct-1 sites abolished RTA-mediated transcriptional activation, suggesting that RTA up-regulates K1p transcription through binding to this transcription factor.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is the causative agent of Kaposi's sarcoma, a complex endothelial neoplasm predominantly seen in AIDS patients (6, 7, 18, 49). KSHV has also been shown to be present in primary effusion lymphoma and multicentric Castleman's disease, rare B-cell lymphoproliferative disorders (5, 13, 44, 50). KSHV was initially identified from a Kaposi's sarcoma biopsy specimen using representation difference analysis. Sequence comparison identified a new virus having strong similarity to herpesvirus saimiri, a gammaherpesvirus subfamily member (7, 8). KSHV, like other herpesviruses, establishes both lytic and latent infections but predominantly persists latently in Kaposi's sarcoma-associated tumors and primary effusion lymphoma with the expression of an array of limited genes (3, 14, 24, 25, 51, 60).

K1, which is predominantly expressed during lytic infection (26), is the positional equivalent to herpesvirus saimiri transformation protein, R1 of rhesus rhadinovirus, and Epstein-Barr virus latent membrane protein 1 (LMP1) (11, 15, 23). KSHV, besides having a strong resemblance to herpesvirus saimiri, did not show similarity in amino acid sequence or structural organization to herpesvirus saimiri transformation protein but could replace its functional properties in terms of immortalization of rodent fibroblasts (33, 34, 55). Additionally, K1 transgenic mice have been shown to develop plasmablastic lymphoma with the feature of spindle cells (42).

K1 is a 46-kDa transmembrane glycoprotein with an N-terminal signal sequence, an extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic tail containing an immunoreceptor tyrosine-based activation motif (ITAM) capable of activating signaling pathways (26, 33, 34, 57, 61). K1 protein is related to the immunoglobulin receptor family and has similarity to the B-cell receptor (33). K1 is constitutively active through oligomerization via conserved, extracellular cysteine residues leading to the phosphorylation of the ITAM and recruitment of the major B-cell kinase Syk for the phosphorylation of cellular proteins, mobilization of intracellular calcium, and activation of transcription factors such as NFAT and AP-1 (21, 27, 28, 30, 33, 38, 58). The amino-terminal domain of K1 interacts with the μ chain of the B-cell receptor complex leading to the inhibition of intracellular transport of B-cell receptor and resulting in retaining B-cell receptor complexes in the endoplasmic reticulum, preventing their intracellular transport to the cell surface which implies a role for K1 in the survival of KSHV-infected cells (26, 30, 32).

The expression profile of K1 during the KSHV life cycle in not restricted to only the lytic cycle. Previous studies performed by Lagunoff and Ganem demonstrate that a 1.3-kb transcript is expressed only when BCBL-1 cells were induced with tetradecanoyl phorbol acetate (26), whereas Samaniego et al. have shown the presence of a 1.3- and a 3-kb K1 transcript in uninduced BC-3 cells as well as their up-regulation in tetradecanoyl phorbol acetate-induced BC-3 cells (47). The K1
FIG. 1. LANA down-regulates K1 promoter transcriptional activity. A. Strategy for cloning K1p into pGL3B. The NotI-BamHI fragment of cosmid Z6 containing K1 and the terminal repeat was excised and cloned into BSpuro at the indicated sites, generating pBSpuroB. The coding sequence of K1 was deleted by digesting with BamHI and PstI followed by blunting both ends and religating it to make pBSpuroBB. K1p was...
transcript has also been detected in Kaposi’s sarcoma tumors as well as in multicentric Castleman’s disease tissues (39, 47).

Northern hybridization and microarray data analyzing KSHV’s transcriptional program during lytic reactivation in primary effusion lymphomas and real-time PCR analysis indicate that K1 expression was up-regulated during the lytic cycle (16, 22, 25, 40, 48). However, analysis of gene clusters based on transcriptional program groups K1 with the latency-associated nuclear antigen (LANA) gene cluster of Orf73, Orf72 (cyclin), and Orf71 (vFLIP) (16). Based on the previous data it is believed that K1 is clearly up-regulated during the lytic cycle but there is a possibility of a low level of K1 expression during latent infection. A previous published report shows that ITAM-dependent signaling by K1 can boost lytic reactivation in KSHV-infected primary effusion lymphoma cells, determined by introducing K1 mutants into BCBL-1 cells, which diminished lytic cycle gene expression (27). Therefore it is believed that K1 might have multiple roles in cellular signal transduction and viral lytic reactivation (27).

Since the expression of K1 was mostly up-regulated during the lytic cycle Bowser et al. evaluated the role of RTA on a K1 promoter cloned by PCR amplification of K1p spanning the −325 to +76 region including part of the terminal repeat sequences (4). In this report we have evaluated the effect of LANA on K1p activity by cloning the promoter region including the LANA binding sequence. We demonstrate that the K1 promoter element was down-regulated in the presence of LANA, which explains the reduced or minimal levels of K1 during latent infection. We have further demonstrated that RTA can rescue LANA-mediated down-regulation of K1p in a dose-responsive manner and thus may contribute to control the lytic reactivation of KSHV.

**MATERIALS AND METHODS**

**Promoter cloning and plasmids.** We digested previously described cosmid Z6 with BamHI and NotI and precisely excised a 2,315-bp band which includes the region of the first terminal repeat and the entire K1 open reading frame (ORF) (9, 45). The fragment was cloned into pBSpurO, resulting in pBSpurOB, which was further digested to remove the K1 ORF region with PseI (enzyme site immediately after translation start site) and BamHI. The plasmid was end blunted and ligated to generate pBSpurOB containing the K1 promoter region. The K1 promoter region (−650 to +76), including the untranslated region, was excised by NotI, followed by end-filling and HindIII digestion. This fragment was cloned into pGL3Basic vector that had been digested with BglII, end filled to blunt the site using the Klenow fragment, and then digested with HindIII, generating pGL3B K1p (−650 to +76) (Fig. 1A).

The promoter element deleted of the LANA binding sequence was generated by deleting the SmaI fragment from the above promoter and designated pGL3B K1p (−350 to +76). pGL3B K1p (−116 to +76) deleted of potential RTA binding sequence (interleukin-6 promoter region) was generated by digesting the above −350 to +76 construct with Ascl, followed by blunting this site and digesting the vector backbone again with Smal to remove the region of the promoter from −350 to −115. Self-ligation of the remainder of the vector yielded pGL3B K1p (−116 to +76). The promoter region from −116 to +76, including the untranslated region, was analyzed for transcription factor binding motifs using an online motif search tool (http://motif.genome.jp). Oct-1 transcription factor binding sites were mutated by site-directed mutagenesis using PCR primers containing the desired mutations. All the clones were sequenced and confirmed for the presence of indicated mutations.

pA3M LANA and RTA Myc were used as expression vectors for LANA and RTA and were described previously (29, 37, 56). LANA mutants LANA N (amino acids 1 to 340), LANA C (amino acids 762 to 1162), and LANA ΔIR (amino acids 327 to 929 deleted) used in reporter assay were described previously (54). pBSpurO was made by cloning the puro cassette at the SalI and Chrl sites of pBS (Stratagene). The expression vector containing K1p under control of its endogenous promoter, pBSpurOΔLBS, was made as described above. Plasmid pBSpurOΔLBS, with K1 gene expression under the control of endogenous K1p deleted of LANA binding sites (LBS), was generated by removing the NotI and Smal fragment of pBSpurO. pCDA3.1 K1 was generated by PCR amplification using pBSpurO as template DNA.

**Cell lines.** BCBL-1 is a KSHV-positive primary effusion lymphoma cell line. BJAB, KSHV-negative cells were cultured in RPMI supplemented with 7% fetal bovine serum, 2 mM l-glutamine and penicillin-streptomycin (5 U/ml and 5 μg/ml, respectively). Human embryonic kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and penicillin-streptomycin (5 U/ml and 5 μg/ml, respectively).

**Antibodies.** Myc-tagged proteins were detected using mouse hybridoma 9E10 described previously (56). Beta-actin was detected using rabbit anti-β-actin antibody (Cell Signaling, Beverly, MA). Lyn and phospho-Lyn (SS07) were detected using specific antibodies (Cell Signaling, Beverly, MA).

**Reporter assay.** Reporter assays were performed in 293 and BJAB B cells; 10 million 293 and BJAB cells were transfected using a Bio-Rad electroporator (Bio-Rad Laboratories); 5.0 μg of the indicated reporter plasmids was transfected along with indicated amounts of LANA and RTA expression vectors. pA3M empty vector was used as filler DNA. Transfection efficiencies were normalized using the green fluorescent protein-containing vector pEGFP1 (Clontech Inc., Palo Alto, CA). At 24 h posttransfection cells were harvested, washed in phosphate-buffered saline and lysed in cell lysis buffer (Biovision); 50 μl of cell lysate was used for the reporter assay using a Lumimterometer LMX384 (Molecular Devices, CA).

A portion of the cell lysate was used for Western blotting. Transferred proteins were detected by Odyssey infrared scanning technology (LiCor, Lincoln, NE.) using Alexa Fluor 680 and Alexa Fluor 800 (Molecular Probes, Carlsbad, CA, and Rockland, Gilbertsville, PA, respectively). Empty vector pGL3B showed some up-regulation with RTA expression and therefore the fold change in reporter plasmid with RTA is normalized with the empty vector. All the transfections were done in duplicate and the results shown represent the mean of the data from three independent experiments.

**Reportet assay.** Reporter assays were also performed by transfecting the above plasmids into 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA); 300 ng of indicated reporter plasmids was transfected with 500 ng of LANA and RTA expression vectors using 1.0 μl of Lipofectamine as per the manufacturer’s instructions. At 24 h posttransfection cells were harvested and subjected to the reporter assay and Western blot analysis.

**Quantitation of K1 transcript.** pBSpurO, pBSpurOB (−650 to +76 K1p and ORF) and pBSpurOΔLBS (−350 to +76) was cotransfected into 10 million HEK293 or BJAB cells with LANA, RTA or both by electroporation; 36 h posttransfection cells were harvested and divided into two sets. The first set was
for the isolation of total RNA and the second set was used for Western detection of Lyn, phospho-Lyn, and β-actin using specific antibodies. Lyn and phospho-Lyn were subsequently detected with Alexa Fluor 680 and Alexa Fluor 800 secondary antibodies using Odyssey Infra-Red scanning technology (LiCor, Lincoln, NE). Band intensities were quantified using Odyssey software v1.2 (LiCor, Lincoln, NE) and relative intensities were calculated considering lane 1 of each set as the reference.

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was synthesized using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) as per the manufacturer’s recommendations. K1 gene-specific primers Forward (5'-CCCAACGGACGAAATGTGAAAC-3') and Reverse (5'-ACGTGTGGTTGCATGGATTAA-3') were used for amplification and quantitation of K1 transcripts in synthesized cDNA. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with specific primers (Forward, 5'-TGCACCAACACCTGCTTAG-3', and Reverse, 5'-GATGCAGGATGATGTTC-3') was used for the normalization of Ct values in different samples.

Amplification was performed using SYBR Green real-time master mix (MJ Research Inc., Waltham, MA). 1 mM each primer, and 1 μl of the cDNA product in a total volume of 20 μl. Thirty cycles of 1 min at 94°C, 1 min at 56°C, and 30 s at 72°C, followed by 5 min at 72°C, were performed in an MJ Research Opticon II thermal cycler (MJ Research Inc., Waltham, MA). Each cycle was followed by two plate reads, the first at 72°C and the second at 82°C. A melting curve analysis was performed to verify the specificity of the amplified product. K1 transcripts were calculated using pCDNA3.1 K1 purified plasmid as a standard. The experiment was done in triplicate and the data presented here are the means of two independent experiments.

Primary infection of HEK293 cells with KSHV in vitro. KSHV was purified from BCBL-1 as described previously (29). Approximately 50 million cells were induced with 20 ng of triacetadecanol phorbol acetate/ml and 1.5 mM sodium butyrate (Sigma, St. Louis, MO) for 5 days. Medium containing virion particles was centrifuged to remove cell debris, followed by filtering through 0.45-μm syringe filters. Virions were pelleted at 20,000 rpm for 2 h and resuspended in 1× phosphate-buffered saline; 10 million 293 cells were infected at the 25% confluency stage and cells were harvested at the indicated time points. Total RNA was extracted and used for cDNA synthesis using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Gene-specific primers were used for the amplification of K1 used in the previous experiment. The LANA- and RTA-specific primers for real-time quantitation used were described previously (29). The values for the relative copies were calculated by the ΔΔCt method using the β-actin gene as the control. Each sample was analyzed in triplicate.

Electrophoretic mobility shift assay. Oligonucleotides containing the Oct-1 site or mutated Oct-1 site were synthesized and annealed to the respective antisein strand by gradient cooling. The double-stranded DNA probe was filled in with a 32P-labeled dCTP, dATP, dGTP, and dTTP using the Klenow fragment (New England Biolabs, Beverly, MA). The labeled probes were purified on a Nuc Trap probe purification column (Stratagene Inc., La Jolla, CA) according to the manufacturer’s instructions. The total incorporation of [γ-32P]dCTP was measured, and approximately 75,000 cpm of probe was used per reaction.

Nuclear extracts prepared from BCBL-1 cells were used as a source of Oct-1 transcription factor. In vitro-translated RTA prepared by the coupled in vitro transcription/translation system (TNT) of Promega Inc. (Madison, WI) according to the manufacturer’s instructions was used in the indicated lanes. Anti-Oct-1 antibody (Santa Cruz Biotechnology) was used for supershift analysis.

Nuclear extract (5.0 μg of total protein) was used for binding to the indicated probe in electrophoretic mobility shift assay reactions. Unlabeled competitor (100-fold) was added 5 min prior to the addition of radiolabeled probes; 1.0 μg of rabbit anti-Oct-1 was used to supershift the complex. The protein-DNA binding reaction mixture was incubated at 25°C for 15 minutes. The bound complex was resolved onto a 6.0% polyacrylamide gel containing 0.5× M Tris-borate, pH 8.2, 1 mM EDTA. The gel was resolved in 0.5× TBE for 4 h at 150 V, dried, and autoradiographed using a Phosphorimager plate (Molecular Dynamics, Inc.).

RESULTS

LANA down-regulates the K1 promoter in the reporter assay. KSHV-encoded K1 is the first open reading frame shown to be expressed predominantly during the lytic cycle of the virus with the exception of low levels during latency (16, 26, 37). The role of K1 in tumorigenesis is clearly established by various laboratories but the regulation of K1 expression during latent and latent/lytic switch is not well understood (34, 42). One study done so far has demonstrated that the −125 to +76 region was sufficient and responsive to RTA expression (4), but did not explore the regulation of K1 expression during latent infection.

Sequence analysis of the K1 promoter region cloned from the Z6 cosmid revealed the presence of LANA binding sequence upstream of bp −325 previously described as a promoter (4). Since LANA is expressed in almost all KSHV-positive cells and important for tethering of the viral genome by binding to the
LANA binding sequence (2, 10, 19, 20, 26), we hypothesized that LANA may have a role in regulation of K1 expression. We digested cosmid Z6 of KSHV with BamHI and NotI and cloned K1p as described in the schematic (Fig. 1A). In order to determine the effect of LANA on K1p, we cotransfected pGL3B K1p(-650 to +76) with the LANA expression vector in HEK293 as well as in BJAB cells and assayed promoter activity as relative luciferase units. Reporter assays performed using pGL3B K1p(-650 to +76) with LANA showed a decrease in promoter activity which was further decreased in a dose-dependent manner with increasing LANA expression (Fig. 1C). LANA-dependent down-regulation of K1p was similarly observed in BJAB cells with increasing amounts of LANA (Fig. 1E). However, RTA, which was previously shown to up-regulate K1p (4), showed dose-dependent up-regulation of the full-length K1 promoter activity in both the 293 and BJAB cell lines (Fig. 1C and E). RTA expression was detected using the anti-Myc antibody, as these expression plasmids were carboxyl-terminally fused with the Myc epitope.

LANA binding sequence in K1p is important for LANA-mediated down-regulation. It has clearly been demonstrated that LANA binds to its cognate sequence within the terminal repeat with high affinity to at least one binding site, LBS1 (19). Therefore, we were interested in determining the effect of LANA on K1p deleted for the LANA binding sequence, pGL3B K1p ΔLBS (-350 to +76) (Fig. 1B). Cotransfection of reporter plasmid with the LANA expression vector did not show any down-regulation of the promoter activity in either of the cell lines tested, 293 or BJAB (Fig. 1D and F). Increasing amounts of LANA expression with the pGL3B K1p ΔLBS (-350 to +76) reporter plasmid revealed a small but noticeable up-regulation in promoter activity with increasing LANA expression (Fig. 1D and E). However, transcriptional activity of LBS-deleted K1p (pGL3B K1p ΔLBS) in the presence of RTA was similar to that on full-length K1p(-650 to +76) (Fig. 1D and E). LANA and RTA expression was detected using the anti-Myc antibody, as these expression plasmids were carboxyl-terminally fused with the Myc epitope.

RTA prevents LANA-mediated down-regulation of K1p transcriptional activity. Our reporter assay data suggested that LANA can down-regulate K1 expression and it has been clearly shown that LANA is expressed in almost all infected cells during latent infection and tethers the viral genome to the host chromosomes (1, 9). Therefore, it is most likely that binding of LANA to its cognate sequence can repress K1 expression. In order to determine whether RTA is able to up-regulate K1 expression when LANA is bound to its cognate sequence we cotransfected pGL3B K1p(-650 to +76) along with constant amounts of LANA and RTA with LBS-deleted K1p (-350 to +76) showed enhanced up-regulation in K1p, suggesting a cooperative effect of LANA and RTA.
FIG. 4. Coexpression of LANA and K1 driven by its native promoter showed down-regulation in K1 transcript level and Lyn phosphorylation. A, 293 and BJAB B cells, pBSpuroB containing the K1 ORF under the control of K1p (−650 to +76) was transfected alone (lane 1) and also cotransfected with either LANA (lane 2) or RTA (lane 3) or both LANA and RTA (lane 4). Total RNA extracted 36 h posttransfection and analyzed for K1 transcripts showed its reduction in LANA-expressing cells (lane 2) but elevation in RTA-expressing 293 as well as BJAB cells (lanes 3). LANA- and RTA-coexpressing cells also showed slight increases in K1 transcripts (lane 4). The level of phospho-Lyn was down-regulated in LANA-expressing cells (lane 2, relative density was 1.13 versus 0.87) relative to that in cells without LANA (lane 1, relative density was 1). In contrast, RTA showed slight up-regulation in phospho-Lyn levels (lane 3, relative density 1.25 versus 1.16), which was also slightly up-regulated in LANA- and RTA-expressing cells compared to the LANA only-expressing cells (lane 4). The level of phospho-Lyn was also not modulated in LANA-expressing cells (compare lanes 1 and 2, phospho-Lyn and Lyn panels). RTA, which showed increased K1 transcripts, showed slightly increased phospho-Lyn levels (lane 3, Lyn and phospho-Lyn panels). Lane 4 also showed modulations in phospho-Lyn levels. The pBSpuro empty vector showed very little or no change in Lyn and phospho-Lyn levels in cells transfected with LANA, RTA, or both, indicating moderate effects of these proteins on Lyn phosphorylation. LANA and RTA expression was detected using anti-Myc antibody. BJAB cells cotransfected with
on the truncated promoter (Fig. 2B). LANA and RTA in all these reporter assays were detected by Western blot using the anti-Myc antibody.

Carboxyl terminus of LANA is sufficient for down-regulation of K1p (−650 to +76). Previous studies have demonstrated that the C terminus of LANA contains the DNA binding domain; therefore we wanted to determine whether the binding domain by itself is sufficient for K1p down-regulation. Thus, we cotransfected different truncated mutants of LANA for the reporter assay along with full-length K1p (−650 to +76) as well as LBS-deleted K1p (−350 to +76). Our reporter assay data showed that the C terminus of LANA (amino acids 762 to 1162) had down-regulatory effects similar to those of full-length LANA, suggesting that the carboxyl terminus is sufficient to down-modulate K1p by binding to its cognate sequence of the promoter (Fig. 3A).

The N terminus of LANA did not have any noticeable effect on transcriptional modulation, whereas the LANA truncation mutant deleted of the middle glycine/glutamic acid region (ΔIR; amino acids 327 to 929 deleted but containing both the amino- and carboxyl-terminal domains) up-regulated K1p (Fig. 3A). K1p deleted of the LBS (−350 to +76) upon transfection with LANA showed similar up-regulation in promoter activity; whereas neither the amino nor the carboxyl terminus of LANA was able to modulate its activity to any noticeable level above that of the control (Fig. 3B). The LANA truncation mutant (ΔIR) up-regulated the promoter like full-length LANA, suggesting that both the amino and carboxyl termini of LANA are important for up-regulation but binding of the LANA carboxyl terminus to the K1p region is important for down-regulation.

LANA and RTA modulate K1 transcription level from the native endogenous viral promoter element. We used pBSpuro B containing the entire K1p (−650 to +76) and its coding sequence for cotransfection with expression vectors of LANA, RTA, or both together into 293 and BJAB cells. Cells were harvested 36 h posttransfection for the isolation of total RNA followed by cDNA synthesis. Transcripts detected using real-time quantitative PCR showed almost 1.5-fold-reduced numbers of K1 transcripts in LANA- and pBSpuro B-cotransfected 293 cells (Fig. 4A, lanes 1 and 2). BJAB cells cotransfected with LANA and the above promoter (pBSpuro B) showed an almost twofold reduction in K1 transcripts at 36 h posttransfection (Fig. 4B, lanes 1 and 2). This corroborated our reporter assay data for both the 293 and BJAB cell lines. RTA, which was shown previously to up-regulate the K1 promoter as well as in our reporter assay, showed 1.5- to 2.5-fold increases in K1 transcript levels in 293 and BJAB cells, respectively. Cotransfection of the LANA and RTA expression vector together with pBSpuro B showed a moderate reduction in K1 transcripts which may be due to the antagonistic role of LANA on RTA-mediated K1p transcriptional up-regulation.

We also used LANA binding site-deleted K1p as the native viral promoter upstream of the K1 ORF and cotransfected it with LANA and RTA for detection of K1 transcripts. LANA-transfected 293 and BJAB cells showed moderate increases in K1 transcripts, whereas RTA transfection showed a significant (twofold) increase in the levels in both cell lines (Fig. 4A and B). Cotransfection of LANA and RTA together with LBS-deleted K1p showed significant increases in levels of K1 transcripts in 293 as well as BJAB cells, corroborating the reporter assay data. cDNA made from vector (pBSpuro)-transfected 293 and BJAB cells did not show any amplification using gene-specific primers for K1 (data not shown).

Further, we wanted to determine the phosphorylation state of Lyn in cells expressing K1 from its endogenous promoter and the effect of LANA and RTA on K1 expression and Lyn phosphorylation. K1 was previously shown to be increased in K1 transgenic mice (42). Protein extracted from 293 and BJAB cells cotransfected with pBSpuro B and LANA or RTA showed various levels of Lyn phosphorylation (relative band intensities are shown at the bottom of each band). Detection of phospho-Lyn in pBSpuro B- and LANA-transfected 293 as well as BJAB cells showed reduced levels of Lyn phosphorylation (Fig. 4A and B, relative change in band intensity of lanes 1 and 2 in the Lyn and phospho-Lyn panels). 293 and BJAB cells cotransfected with RTA and pBSpuro B showed a slight increase in the phosphorylation of Lyn which may be due to the increased K1 expression, thus corroborating the above data (Fig. 4A and B, relative change in band intensity of lanes 1 and 3 in the Lyn and phospho-Lyn panels). Expression of LANA and RTA along with pBSpuro B did not show a significant difference in phosphorylation of Lyn (Fig. 4A and B, lane 4 in the Lyn and phospho-Lyn panels).

The K1 gene under control of the deleted LBS (−350 to +76) promoter showed slight increases in the phosphorylation of Lyn upon cotransfection with either LANA or RTA compared to no LANA or RTA, suggesting that increased K1 might have enhanced the phosphorylation of Lyn (Fig. 4A and B, −350 to +76 panels, compare lane 1 with lanes 2 and 3). Expression of LANA and RTA together with the above promoter showed enhanced Lyn phosphorylation, corroborating the above data (Fig. 4A and B, lane 4). Transfection of LANA and RTA with the pBSpuro vector control in 293 and BJAB showed little or no change in relative levels of Lyn and phospho-Lyn in cells transfected with LANA, RTA, or both. This suggests that modulations in the phosphorylation of Lyn were due to the various levels of K1 expression. Expression of LANA and RTA was detected by Western blot analysis using anti-Myc antibodies. Blots were stripped and probed for β-actin as a control for protein loading.

Levels of K1 transcript increase during early infection, followed by rapid reduction towards the establishment of latency. We used purified BCBL-1 virions to infect 293 cells and fol-

pBSpuro B and LANA showed reduced numbers of K1 transcripts as well as reduced levels of phospho-Lyn (lanes 1 and 2). RTA showed increased numbers of K1 transcripts as well as phospho-Lyn levels (lane 3). Cells transfected with both LANA and RTA showed similar slight modulations in Lyn phosphorylation in BJAB cells (lane 4). pBSpuro B ΔLBS showed enhanced Lyn phosphorylation in cells transfected with both LANA and RTA. The pBSpuro vector alone did not show much change in phospho-Lyn levels with LANA or RTA coexpression.
followed the pattern of K1 transcripts levels during early infection up until 72 h. Total RNA was extracted from these infected cells at the indicated time points and detection of LANA, RTA and K1 transcripts levels is shown in Fig. 5. The expression levels for K1 peaked at 24 h, followed by a steady decline in levels up to 72 h. The LANA levels were increased by 12 h and remained high after 24 h postinfection.

FIG. 5. KSHV-infected 293 cells show high K1 transcript levels during early infection and reduced levels towards the progression of latency. 293 cells infected with KSHV wild-type BCBL-1 virus showed similar relative copy numbers of K1 and RTA. The expression levels for K1 peaked at 24 h, followed by a steady decline in levels up to 72 h. The LANA levels were increased by 12 h and remained high after 24 h postinfection.

observed until 72 h postinfection. The LANA levels were increased by 12 h and remained at a high level after 24 h of infection. K1 and RTA mRNA levels were maintained at the same low level, likely due to the small subset of KSHV-infected cells which spontaneously undergo lytic replication. The rapid decrease in the levels of K1 and RTA transcripts is likely due to LANA activating its own expression and repression transcriptional activity of the lytic genes.

**LBS-deleted K1p(−350 to +76) contains an interleukin-6 promoter-like RRE element.** Our reporter assay data indicated that LANA-mediated repression of K1p is due to the presence of its cognate sequence and most probably LANA binding to this region. We further looked to map the RTA-mediated responsive region on K1p. We aligned the −350 to +76 sequence of K1p with the sequence of known binding sites of RTA (12). In our sequence alignment analysis we found a region on K1p having significant similarity to the RTA response elements (RRE) present in the interleukin-6 promoter (Fig. 6).

In order to evaluate the effect of RTA, we made a mutant promoter by deleting the potential RRE element [K1p(−116 to +76)]. Reporter analysis with RTA revealed that the −116 to +76 region of the promoter was sufficient for RTA-mediated up-regulation, suggesting some other mechanism of RTA-mediated K1p activation (Fig. 6). Coexpression of LANA with the −116 to +76 promoter region showed a slight increase in promoter activity (Fig. 6). Full-length K1p(−650 to +76) and
LBS-deleted K1p(-350 to +76) were used parallel to the -116 to +76 region in this experiment (Fig. 6). Expression of LANA and RTA was detected by Western blot analysis using anti-Myc antibody.

Sequence analysis of the -116 to +76 region of K1p revealed Oct-1 transcription factor binding sites. In order to determine the specific transcription factor binding sites located within the -116 to +76 elements we scanned the indicated region which also includes the first start codon using a web-based motif search tool. The result revealed the presence of multiple Oct-1 transcription factor binding sites shown in Fig. 7A (Oct-1 binding sites shown are shown in expanded boxes).

We mutated these potential Oct-1 transcription factor binding sites individually as well as all three together by site-directed mutagenesis (Fig. 7B, C, D, and E).Reporter analysis performed in 293 cells demonstrated that loss of the first Oct-1 site reduced RTA-mediated responsiveness to 50%, suggesting that this site contributes a significant amount of activity to the promoter (Fig. 7B, I Oct-1 mut). Mutation of the second Oct-1 site reduced RTA responsiveness to 30%, whereas mutation of the third Oct-1 site was unaffected by RTA-mediated up-regulation (Fig. 7C and D). Mutation of all three Oct-1 sites reduced the RTA-mediated responsiveness to 25%, suggesting that Oct-1 transcription factor binding sites contribute signifi-

FIG. 7. Oct-1 sites are important for RTA-mediated up-regulation of K1p. A, Schematic showing the presence of Oct-1 transcription factor binding sites in the -116 to +76 region of K1p. B, C, and D, Oct-1 transcription factor binding sequences I, II, and III. The top strand is the sequence in K1p and the bottom strand is the mutated sequence incorporated by site-directed mutagenesis. E, K1p -116 to +76 with all Oct-1 sites mutated. Reporter assays performed in 293 cells with pGL3B K1p -116 to +76 (pGL3B-A), Oct-1 site I mutated (pGL3B-B), Oct-1 site II mutated (pGL3B-C), Oct-1 site III mutated (pGL3B-D), and all three Oct-1 sites mutated (pGL3B-E) with LANA and RTA. All Oct-1 site-mutated promoters had significantly reduced (80%) relative luciferase activity (E). The Oct-1-mutated promoters had different basic transcriptional activities which were normalized, and relative fold changes are plotted here. Representative Western blots show expression of LANA and RTA in the above transfections.
cantly to the RTA-mediated up-regulation of the K1 promoter (Fig. 7E). Representative Western blots for LANA and RTA expression are shown in Fig. 7F.

Sp1 transcription factor binding sites were not responsible for RTA-mediated up-regulation but important in LANA-mediated up-regulation. Sequence-scanning analysis also demonstrated the presence of multiple Sp1 transcription factor binding sites within −116 to +76, similar to a previous report (4). We wanted to further determine whether these sites may in fact play a role in RTA-mediated up-regulation of the promoter. A deletion excising all Sp1 transcription factor binding sites was generated (Fig. 8). Reporter analysis of this construct performed in the presence of the RTA expression vector revealed that there was a negligible change in RTA-mediated responsiveness, suggesting that Sp1 sites were not the critical sites for modulation (Fig. 8). However, the low level of activation by LANA was abolished in the Sp1-deleted promoter construct, suggesting that LANA may have this activity through binding to Sp1 transcription factors. Deletion of Sp1 sites in the third Oct-1 mutated reporter construct (retaining the second Oct-1 site) −116 to +76 promoter region clearly demonstrated that most of the RTA-mediated response is due to the second Oct-1 transcription factor binding site (Fig. 8C).

Oct-1 transcription factor binds to cis-acting elements within the K1p promoter region. We performed electrophoretic mobility shift assays to demonstrate the binding of cellular Oct-1 to nucleotide sequences within K1p. We used oligonucleotide probes comprising the second Oct-1 site as well as a mutated site as the probe. Labeled probes were incubated with BCBL-1 cell nuclear extract which showed a specific shift in the mobility of probe (Fig. 9, lane 2). The shift in mobility was abolished using 100-fold specific unlabeled competitor, suggesting the specificity of binding (Fig. 9, lane 3). Mutated nonspecific unlabeled competitor was unable to abolish the Oct-1-specific shift, suggesting that the mutation in the Oct-1 site abolished binding of the Oct-1 transcription factor.

Oct-1-specific binding was further confirmed by using anti-Oct-1 antibody to supershift the Oct-1-DNA complex (Fig. 9, lane 5). Nonspecific immunoglobulin G antibody did not have any effect on the mobility of the Oct-1-DNA complex and Oct-1-specific antibody by itself did not show any binding to DNA (Fig. 9, lanes 6 and 7, respectively). Addition of in vitro-translated RTA to the Oct-1-DNA complex did not show any change in the mobility of the complex, suggesting that RTA does not have a strong affinity for Oct-1 bound to DNA. Similar amounts of rabbit reticulocyte lysate were also unable to modulate the shift in mobility. In vitro-translated RTA by itself also did not bind to the probes (Fig. 9, lane 10). Thus, the activity of K1p mediated by RTA is most likely occurring through the specific Oct-1 sites by the interaction of Oct-1 and RTA, or by recruitment of additional activators and coactivators. Further studies to elucidate this possibility are ongoing.

**DISCUSSION**

Kaposi’s sarcoma-associated herpesvirus persists episomally in infected cells by tethering the viral genome to the host chromosome (1, 9). During latent infection only a small subset
of genes important for maintaining viral latency by modulating various cellular pathways are expressed (3, 14, 24, 25, 51, 60). Latency-associated nuclear antigen is one of the prominent proteins detected in all forms of latency and shown to be critical for maintenance of latency (1, 9, 17, 25, 43). K1, a transmembrane glycoprotein expressed mostly during lytic infection, contains an extracellular domain and a C-terminal transmembrane glycoprotein expressed mostly during lytic infection (38, 58). Recently, Lee et al. have shown that K1 protein efficiently interacts with a number of cellular Src homology 2-containing signaling proteins through its ITAM and up-regulates signal transduction and intracellular calcium mobilization and NFAT activation (31). The activation of signal transduction by K1 within the host cells favors efficient viral lytic replication (38).

Cloning and sequence analysis of the NotI-BamHI fragment of cosmids Z6 revealed the presence of LANA binding sequences in the K1p region which was not previously explored for K1 expression (4). However, terminal-repeat-mediated transcriptional activity has been shown to be repressed by LANA expression (35). Also, K1 mRNA has been detected at low levels in latently infected primary effusion lymphoma cell lines (16). Our reporter assay data for the full-length promoter (−650 to +76) compared to LBS-deleted K1p showed a small increase in K1p activity compared to the wild type with LANA, suggesting that binding of LANA to its cognate sequence contributes to the down-modulation of K1p transcription. This may partially explain why K1 mRNA is at very low levels during latent infection as LANA binds to its cognate sequence to tether the viral genome to the host chromosomes (55).

It would be interesting to see the levels of K1 mRNA in primary effusion lymphoma cell lines lacking LANA using small interfering RNA or binding of LANA to its cognate sequence with the use of the dominant negative LANA carboxy terminus. However, knocking down LANA may not be conclusive for K1 expression because LANA depletion leads to the expression of RTA and related genes and RTA by itself has been shown to up-regulate K1p (4, 29). Thus, the up-regulation of K1p transcriptional activity in LANA-depleted primary effusion lymphoma cell lines will be mainly because of RTA.

Binding of LANA to the LBS is likely to be the primary reason for its repressive activity, as LANA lacking the DNA binding domain did not repress K1p. Additionally, the C terminus of LANA, which is the binding domain for DNA, repressed K1p as full-length LANA did, confirming our hypothesis. Surprisingly, LANA containing both the amino and carboxyl termini fused in frame (LANA AIR) was unable to repress K1p transcriptional activity in our reporter assays even though this contained the DNA binding domain. This may be due the fact that LANA lacking glutamine and glutamic acid residues has been demonstrated to have higher transcriptional activity than full-length LANA (54, 59). Thus, the repressive effect due to the binding of LANA-C may be overridden by increased transcription regulation by LANA AIR (Fig. 4).

The effect of LANA on the transcriptional activity of K1p was further confirmed by using the K1 ORF under the control of its own promoter and determining the copies of K1 mRNA. This corroborated the luciferase reporter assay data showing that LANA reduced the transcription of the K1 gene. Additionally, RTA-mediated up-regulation of K1p transcription was confirmed at the mRNA level and was unaffected by deletion of the LBS sequence from the promoter region, unlike LANA, which lost its repression ability in the LBS-deleted promoter region.

Increased transcriptional activity of K1p and thus enhanced K1 expression was further confirmed by looking at levels of cellular Src homology 2-containing signaling protein Lyn. Increased K1 expression has been shown to induce phosphorylation and activation of cellular Src homology 2-containing
signaling protein Lyn and other cellular signaling kinases (38, 41). Analysis of active phospho-Lyn levels in 293 and BJAB cells in the presence of LANA and RTA along with the K1 ORF under the control of its own promoter showed modulation in phospho-Lyn levels. The reduction in phospho-Lyn levels is most likely due to the reduced levels of K1 expression as there were no significant changes in phospho-Lyn levels in the cells expressing LANA only.

Reduced levels of phospho-Lyn may repress the downstream signaling activities, including phospholipase Cγ/Ca2+ pathway activation, thus establishing latency (38). However, previous studies have demonstrated ITAM tyrosine phosphorylation-independent Syc phosphorylation and NFAT reporter activation, suggesting that Syc is not the only effector of K1 signal transduction (28, 38). Additionally, K1-transgenic mice have been shown to have increased active Lyn kinase but not Syc kinase in splenic B lymphocytes, supportive for Syc-independent phosphorylation of Lyn (38, 42). In contrast to LANA, RTA showed increased levels of active phospho-Lyn and thus possible enhancement of the lytic cycle (38).

Detection of increased phospho-Lyn levels in LANA- and RTA-coexpressing cells with the homologous promoter-driven K1 gene suggests that RTA has strong up-regulatory effects, thus preventing LANA-mediated down-regulation, which corroborates the reporter assay data. Interestingly, we have observed reduced levels of LANA in RTA-expressing cells even though they were expressed from the same heterologous promoter. Therefore one might argue that RTA-mediated rescue may be due to degradation or reduced amounts of LANA in the cells. This needs to be further addressed by exploring the stability of LANA in the presence of RTA.

Our infection assay and time course detection of K1 mRNA demonstrated that K1 transcripts can be detected at about the same time as RTA and the level is suppressed with the persistence of infection, similar to previous observations (37, 40). This indicates that K1 might be playing an important role during initial lytic replication for the establishment of latency by mediating signaling of a productive infection (38). K1 might also be important for signaling to produce cytokines which mediate paracrine effects, thus influencing neighboring uninfected as well as infected cells and KSHV-associated pathogenesis (38). During the progression of latency, LANA mRNA increases, producing sufficient molecules for tethering the viral genome to the host chromosome and thereby influencing the activity at the K1 promoter.

This study as well as previous studies have shown that RTA can modulate K1p (4). However, we were interested in delineating the promoter region responsive to RTA. Our reporter deletion analysis showed that the −116 to +76 region of the promoter was sufficient for RTA-mediated up-regulation. Interestingly, a previous study has also demonstrated that the −125 to +76 region of the promoter was efficiently modulated by RTA in various cell lines (4). Sequence analysis of the −116 to +76 region, which includes untranslated sequence up to the translation start site, revealed the presence of Oct-1 transcription factor binding sites as well as multiple sites for Sp1 transcription factor binding (4).

Oct-1 belongs to the POU family and both Oct-1 and -2 can specifically interact with the octamer binding sequence ATG CAAAT (53). Since Oct-1 was shown previously to be the important transcription factor binding site in the RTA promoter region responsible for RTA-mediated autoregulation (46), we focused on addressing their role by mutating these binding sites. The promoter region (−116 to +76) mutated for Oct-1 sites showed a marked decrease in RTA responsiveness, suggesting that binding of Oct-1 is important for RTA-mediated up-regulation. This again did not clearly demonstrate whether up-regulation is due to direct tight binding of RTA to Oct-1 or a likely weaker binding shown for RTA (46).

The herpes simplex virus type 1 VP16 and varicella-zoster virus ORF10 proteins form a tight complex easily detected by electrophoretic mobility shift assay, with Oct-1 for activation of the target genes (36, 52). However, RTA has been shown to up-regulate genes even without forming a tight complex with Oct-1 (46). Sequence analysis showed that the Oct-1 sites identified have approximately 80% similarity to the Oct-1 consensus sequence and are bound by Oct-1 transcription factors. Therefore these sites may be critical to or contribute a major portion of K1 up-regulation.

In conclusion, the LANA binding sites of K1p are important for limited expression of K1 during latent infection as well as during the latent/lytic switch. RTA reverses the LANA-mediated repression of K1p and up-regulates K1 and its mediated...
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