Induction of Kaposi’s Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen by the Lytic Transactivator RTA: a Novel Mechanism for Establishment of Latency

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Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent contributing to development of Kaposi’s sarcoma, primary effusion lymphoma, and multicentric Castleman disease. Following primary infection, latency is typically established. However, the mechanism by which KSHV establishes latency is not understood. We have reported that the latency-associated nuclear antigen (LANA) can repress RTA (for replication and transcription activator) expression by down-regulating its promoter. In this study, we show that RTA is associated with the virion particle. We also show that RTA can activate the LANA promoter and induce LANA expression in transient reporter assays. Additionally, the transcription of RTA correlates with LANA expression in the early stages of de novo infection of KSHV, and induction of LANA transcription is responsive to induction of RTA with an inducible system. This induction in LANA transcription was dependent on recombination signal sequence binding protein Jc (RBP-Jc), as a RBP-Jc-deficient cell line was significantly delayed and inefficient in LANA transcription with expression of RTA. These studies suggest that RTA contributes to establishment of KSHV latency by activating LANA expression in the early stages of infection by utilizing the major effector of the Notch signaling pathway RBP-Jc. This describes a feedback mechanism by which LANA and RTA can regulate each other and is likely to be a key event in the establishment of KSHV latency.

Kaposi’s sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with a number of human malignancies, which include Kaposi’s sarcoma, primary effusion lymphoma, and multicentric Castleman disease (5, 8, 9, 54, 56, 57). Similar to other herpesviruses, KSHV is a large double-stranded DNA virus, which displays two alternate genetic life cycle programs upon infection of host cells (46). In latent infection, gene expression is limited to a small subset of viral latent genes and includes the latency-associated nuclear antigen (LANA) encoded by open reading frame 73 (ORF73), viral cyclin (v-cyclin) encoded by ORF71, viral interferon regulatory factors encoded by ORF72, viral Fas-associated death domain interleukin 1L-converting enzyme inhibitory protein encoded by ORF71, viral interferon regulatory factors encoded by K10, and kaposin encoded by K12 (16, 54, 60). During latency, the viral episome is maintained through successive generations, but no viral progeny are produced. In contrast, lytic replication leads to extensive viral gene expression, virion production, and death of the infected cell (60). Latently infected cells can be induced to enter the lytic cycle under specific physiological conditions (14, 25). Thus, the pool of latently infected cells represents a reservoir of viral persistence from which infectious virus can be later reactivated with production of viral progeny, which can spread to new target cells.

Generally, KSHV establishes latency within 48 h postinfection. To date, it is widely accepted that latent infection by the virus plays a central role in viral pathogenesis with the expression of select genes responsible for targeting and controlling selective cellular pathways (17, 32). Occasionally, lytic reactivation of the virus may be critical, as expression of viral cytokine homologues during this phase may function as paracrine factors in stimulating cell growth and proliferation (1, 2, 10, 58). The reduced gene expression pattern of latency minimizes the number of viral epitopes that are presented by infected cells to cytotoxic T lymphocytes and so contributes to the ability of the virus to escape immune surveillance and establishment of persistent infection (6, 7). In addition, a number of studies have suggested that the genes expressed during latency may be important contributors to the tumorigenic process in KSHV-associated cancers (17, 19, 22, 28, 51). Thus, establishment of latency is critical to the role of KSHV in human cancers. Nevertheless, the mechanism by which KSHV establishes latency postinfection is still poorly understood; in the past decade, numerous studies were focused on maintenance of latency or the mechanism by which KSHV switches to lytic reactivation from latency.

Two virus-encoded molecules LANA and RTA (for replication and transcription activator) are thought to play a central role in the switch between latency and lytic replication. Among the limited number of latent genes, the ORF73 gene, which encodes LANA, is critical for the establishment of latent KSHV infection through maintenance of the viral episome (3, 4, 47, 49). Recently, studies in our laboratory showed that
LANA is capable of suppressing lytic reactivation and maintaining viral latency through repression of the transcriptional activity of the RTA promoter (39). RTA, encoded by ORF50 of KSHV, is an immediate early protein and serves as the key master switch for viral lytic replication. It has been shown that exogenous expression of RTA from a heterologous promoter or induction of endogenous RTA expression can initiate lytic reactivation and drive the latent virus to undergo the full lytic replication cycle (44, 45). Most recently, it has been shown that a small number of lytic genes including RTA are transiently expressed very early after de novo infection of KSHV; however, this abortive lytic gene expression is terminated with the supervention of latency (36). These observations when placed in the context of our own studies led us to hypothesize that the switch between lytic and latent replication and establishment of latent infection may be regulated by a feedback mechanism. The expression of the lytic RTA gene may induce the expression of latent genes including LANA, and the accumulation of these latent gene products can contribute to quick establishment of latent KSHV infection.

In this report, we demonstrated that RTA is detected in association with KSHV viral particles. RTA activates the LANA promoter and induces LANA expression in transient transfection experiments. Transcription of RTA correlates with that of LANA during the early stages of de novo infection of KSHV, and LANA transcription is responsive to induction of RTA expression. Additionally, we also found that the recombination signal sequence binding protein Jk (RBP-Jk) binding site within the LANA promoter is critical for regulation by RTA, known to be associated with a number of cellular regulatory proteins including RBP-Jk (Fig. 1A and B). In RBP-Jk knockout cells, KSHV was unable to establish latent infection when compared to wild-type cells. Our results therefore suggest that RTA can contribute to establishment of latency through activation of LANA expression in the early stages of infection. This represents a feedback loop whereby LANA and RTA regulate each other and is likely to be, at least in part, key events in establishment of KSHV latent infection.

**MATERIALS AND METHODS**

**Constructs, antibodies, and cell lines.** To construct the reporter plasmid of the LANA promoter, an 800-bp sequence upstream of the LANA translation initiation codon was amplified from total cellular DNA prepared from BC-1 cells, using primers KpnILANA (5’TCAAGGTACCCAGATGAACGCCACCCAAG 3’) and BglIIILANA (5’CGTAGATCTATCCTCGGGAAATCTGGTC 3’). The PCR fragment was cloned into the pGL2-basic vector (Promega), with KpnI and BglII as cloning sites, to derive pGLLANAp. The LANA promoter with mutated RBP-Jk binding site pGLLANAmp was obtained by PCR-based site mutagenesis. L54 phage DNA, which contains ORF73 encoding LANA and its regulatory sequences, was obtained from the National Institutes of Health Research and Reagent Program. The pCR3.1-RTA expression vector was described previously (39). All constructs and mutations were verified by DNA sequencing.

KSHV RTA rabbit polyclonal antibody was provided by Gary S. Hayward (Johns Hopkins University School of Medicine). KSHV RTA mouse monoclonal antibody was a kind gift from Koichi Yamanishi (Osaka University, Osaka, Japan). Human polyclonal serum, which recognizes LANA, was designated HS
and was provided by Gary Nabel (Vaccine Institute, National Institutes of Health, Bethesda, MD). KSHV ORF45 mouse monoclonal antibody was provided by Yan Yuan (University of Pennsylvania, Philadelphia, PA). KSHV gB rabbit polyclonal antiserum was a gift of Bala Chandran (The University of Kansas Medical Center, Kansas City, KS).

Human embryonic kidney fibroblast 293 and 293T cells, transformed with hTERT and T antigens, respectively, were obtained from Jon Aster (Brigham and Women’s Hospital, Boston, MA). BJAB is an Epstein-Barr virus (EBV)-negative B-cell line isolated from Burkitt’s lymphoma and was provided by Elliott Kieff (Harvard Medical School, Boston, MA). BCBL1 and BC3 are KSHV-positive body cavity-based lymphoma-derived cell lines obtained from Don Ganem and the American Type Culture Collection, respectively.

Human embryonic kidney fibroblast 293 and 293T cells were grown in high-glucose Dulbecco’s modified eagle medium (DMEM) supplemented with 5% bovine growth serum (BGS; HyClone, Inc., Logan, Utah), 2 mM l-glutamine, 25 μM penicillin, and 50 μg/ml streptomycin. BJAB, BCBL1, and BC3 were grown in RPMI 1640 medium supplemented with 10% BGS, 2 mM l-glutamine, 25 μM penicillin, and 25 μg/ml streptomycin. RTA-inducible BCBL1 and control cell lines were kindly provided by Jae Jung and grown in RPMI 1640 medium supplemented with 10% BGS, 2 mM l-glutamine, 25 μM penicillin, 25 μg/ml streptomycin, and 20 ng/ml hygromycin. Mouse RBP-Jκ (OT11) and wild-type (OT13) fibroblast cell lines were kindly provided by T. Honjo and were grown in high-glucose DMEM supplemented with 10% BGS and 100 U of mouse gamma interferon (PeproTech, Inc., New Jersey) per ml at 32°C.

Transfection. BJAB, 293, 293T, U2OS, OT11, and OT13 cells were transfected by electroporation with a Bio-Rad Gene Pulser II electroporator. A total of 10 million cells harvested in exponential phase were collected and washed in phosphate-buffered saline and then resuspended in 100 μl of RPMI or DMEM with DNA for transfection. Resuspended cells were transferred to a 0.4 cm cuvette and electrotransfected at 950 μF and 220 V. The electroporated cells were then transferred to 10 ml of complete medium, followed by incubation at 37°C and 5% CO2. Transfections were harvested after 24 h and assayed for activity.

Luciferase assay. HEK293 or U2OS cells were collected at 70% confluency. A total of 10 million cells were resuspended, along with plasmid DNA, in 400 μl medium and electrotransfected by electroporation with the Bio-Rad Gene Pulser II at 210 V and 975 μF. Transfected cells were transferred to 100-mm plates in 10 ml of DMEM with 10% fetal bovine serum. The plates were incubated at 37°C with 5% CO2 for 20 h. BJAB cells were collected at 5 × 10⁶ cells/ml. A total of 10 million cells were transfected at 220 V and 975 μF. At 20 h, cells were harvested and washed once with phosphate-buffered saline (Invitrogen-Gibco, Inc.). The cells were subsequently lysed with 200 μl Reporter Lysis Buffer (Promega, Inc.). A total of 40 μl of the lysate was mixed with 100 μl of luciferase assay reagent. Luminescence was measured for 10 s by the Opticomp I luminometer (MGM Instruments, Inc.). The lysates were also tested at various dilutions to ensure that luciferase activity was within the linear range of the assay. The results shown represent experiments performed in triplicate.

Electrophoresis mobility shift assays (EMSAs). A probe containing the furthest downstream RBP-Jκ binding site was designed (5′-GATCTACTATCCTCCACACATC-3′ [underlining indicates RBP-Jκ sequence], annealed, and labeled). 32P-labeled probes were synthesized via Klenow fill-in reaction and purified with Select-D with G-25 columns (Shelton/IIBI, Inc.). Radioactive probes were diluted in water to a final concentration of 80,000 cpm/μl. DNA binding reactions were performed in a manner similar to that described previously (34). Proteins from nuclear extracts or in vitro translation products were mixed with 1 μg poly(dI-dC) (Sigma) in 1× DNA binding buffer for 5 min at room temperature. A total of 1 μl of labeled probe was added to each reaction mixture, and the tubes were incubated at room temperature for 15 min. DNA-protein complexes were resolved by nondenaturing 6% polyacrylamide gel electrophoresis (PAGE). The gel was run in 0.5× Tris-borate-EDTA buffer at a constant voltage of 150 V. Following electrophoresis, the gel was transferred to Whatman paper and dried for 1 h at 80°C. Dried gels were exposed to BioMax MS autoradiography film at 20°C and 80°C with a intensifying screen. To determine the specificity of the complex of RBP-Jκ and probe, a 200-fold molar excess of unlabeled probe was added to the protein-DNA (dil-cm) mixture before incubation at room temperature; similarly, a 200-fold molar excess of unlabeled nosppecific cold competitor probe was added in a separate reaction mixture. Antibodies specific to RBP-Jκ and nonspecific antibody were used as controls to show supershift complexes.

Induction of KSHV lytic replication and infection. To prepare KSHV virus particles, we collected more than 600 million exponentially growing BCBL1 cells for induction. Cells were induced by using trisaccharide phenol acetaldehyde (TFA) at a concentration of 20 ng/ml of medium and butyrate at a concentration of 1.5 mM and were further incubated for 5 days at 37°C with 5% CO2. The supernatant was then collected and filtered with a 0.45-μm filter, and viral particles were spun down at 15,000 rpm for 2 h. Then the concentrated virus was collected for further use. For KSHV infection, 293 cells grown to 60 to 80% confluency in 100-mm-diameter tissue culture dishes and infected with same amount of concentrated virus induced from same number of cells. Since no protocol for detecting the infectious titer of HHV8-containing supernatants currently exists, fresh inocula prepared for different experiments were standardized by ensuring that identical numbers of passage-matched BCBL cells were likewise treated for virus induction. At different time points, unbound KSHV was removed by being washed.

Purification of KSHV virions. A total of 500 million BCBL cells were induced with 20 ng of TPA/ml and 1.5 mM sodium butyrate (Sigma) for 5 days. The medium was then collected and clarified by centrifugation at 2,000 rpm for 15 min to remove cells and cell debris. Then, the medium was filtered through 0.45-μm pore-size filters. Virions were pelleted at 20,000 rpm for 2 h and resuspended in 1× PBS. Then, the concentrated virus particles were centrifuged through a 30 to 60% sucrose step gradient at 20,000 rpm for 2 h. The virus band at the gradient junction was collected.

Trypsin treatment of purified virions. Sucrose-gradient-purified virions were treated with trypsin (1×) (HyClone) in a 100-μl volume at 37°C for 1 h. Trypsin digests were terminated by the addition of phenylmethylsulfonyl fluoride to a 0.5 mM concentration and a 1/100 volume of protease inhibitors (Sigma). The reaction mixture was separated in two fractions, supernatant and pellet, by centrifugation at 20,000 rpm for 1 h for further analysis.

Detergent treatment of purified virions. Sucrose-gradient-purified virions were treated with 1% Triton X-100 for 30 min at 37°C. The reaction mixture was separated into two fractions and pellets were determined nucleocytoplasmic, by centrifugation at 20,000 rpm for 1 h for further analysis.

Real-time reverse transcription-PCR. Quantitative real-time PCR (qPCR) was used to make a relative quantitative comparison of RTA and LANAS levels over the course time of infection or induction of KSHV. At various time points post-infection or postinduction, cells were harvested and total RNA was collected using Trizol reagent (Invitrogen, Inc.) following the manufacturer’s instructions. DNase was then used making a Supernscript II RT kit (Invitrogen, Inc.), following the manufacturer’s instructions. The primers used for real-time PCR were as follows: for RTA, 5′-TATCCAGGAGAAGGCTCTCT-3′ and 5′-GGTAAAAAGGGGATAGTGC-3′; for LANA, 5′-TAGAGGAGTGGGAGGAGCA-3′ and 5′-CGTGAAGATTATGGGCTCT-3′ and for β-actin, 5′-GCTGCTGCT CGACAAGGCCT-3′ and 5′-CAAACATGTGCTGTTCC-3′. The cDNA was amplified using 10 μl of the DyNaMO SYBR Green qPCR kit (MJ Research, Inc.), 1 mM each primer and 2 μl of cDNA product in a total volume of 20 μl. Forty-five cycles, each of 1 min at 94°C, 1 min at 56°C, 30 s at 72°C, were followed by 7 min at 72°C and were performed with an MJ Research Opticon II thermocycler. Each cycle was followed by two plate readings with the first done at 72°C and the second done at 85°C. A melting curve analysis was performed to verify the specificity of the products. The values for the relative quantitation were calculated by the ΔΔCt method with each sample tested in triplicate.

Immunofluorescence. Immunofluorescent assays were performed essentially as described previously (34, 35, 39). Briefly, fixed cells were blocked in the appropriate serum and then incubated with the specific primary antibody for LANA and RTA for 1 h. Cells were washed and then further incubated with the appropriate secondary antibody conjugated to fluorescein isothiocyanate or Texas red at 1:1,000 dilutions in phosphate-buffered saline for 1 h. Slides were washed, visualized with an Olympus X710 inverted fluorescence microscope, and photographed with a digital PixelFly camera and software (Cooke, Inc., Warren, MI).

RESULTS

RTA is associated with KSHV viral particles. KSHV can quickly establish latency postinfection. However, little is known about the mechanism of establishment of latency. Recently, it was shown that immediately after infection there was a burst of lytic viral gene expression, including that of RTA, but the full lytic replication program was not induced (36). This lytic gene expression was followed by a sharp decline in expression and detection of an increase in latent transcripts (36). We previously showed that LANA, a key latency-associated protein, can down-regulate RTA expression through repression of the transcriptional activity of the RTA promoter (39). These observa-
sions led us to hypothesize that there is an interplay between these two viral proteins, which may result in the decline of lytic gene expression postinfection and the establishment of a latent infection. RTA is expressed at relatively high levels during lytic replication. Therefore, it was possible that the virus can package this protein into virions during lytic reactivation, and as such RTA may play a role in up-regulating LANA expression before full lytic replication can proceed during the early stages of infection.

A total of 500 million BCBL1 cells were induced with TPA at 20 ng/ml. Five days postinduction, the supernatants were collected and filtered with a 0.45-μm filter. Crude virions in the supernatant were collected by ultracentrifugation at 15,000 rpm for 2 h, washed once with PBS, and then pelleted again for analysis. Immunoblotting for LANA showed that there were no detectable levels of LANA detected in all fractions, including the mock-treated virion particles (Fig. 2A, lanes 4, 5, and 6). However, LANA signals were clearly detected in cell lysates of uninduced and TPA-induced BCBL1 cells (Fig. 2A, lanes 2 and 3). As expected, LANA was not found in cell lysate of BJAB cells, a KSHV-negative B-lymphoma cell line (Fig. 2A, lane 1). Treatment with Triton X-100 denatures the double lipid layers of the viral envelope and exposes the tegument proteins. Therefore, proteins located inside or outside of the envelope can be detected if trypsin is used in combination. Interestingly, the immediate early transactivator RTA was consistently detected in mock-treated virus particles after the same membrane is stripped and reprobed with RTA-specific monoclonal antibody (Fig. 2A, lane 6). Specifically, RTA was detected in the trypsin but not preparations treated with trypsin plus Triton X-100. As controls, KSHV ORF45, a known tegument protein, was detected in mock- and trypsin-treated virions (Fig. 2A), and KSHV gB, a known glycoprotein located in the envelope of the virus, was detected in mock-treated virions (Fig. 2A).

To further verify RTA is associated with the KSHV virions instead of nonspecifically adhering to aggregated virions, we utilized a sucrose gradient to purify the virions, followed by treatment with trypsin and Triton X-100, solubilization, fractionation, and Western blot analysis. As shown in Fig. 2B, there was no detectable levels of LANA detected in the different fractions. However, LANA was detected in both uninduced and induced BCBL1 cell lysates. In contrast, RTA was detected in the virion pellet treated with trypsin and Triton X-100 (Fig. 2B). A darker-exposure RTA signal was also weakly seen in the Triton X-100-treated soluble fraction (data not shown). As expected, ORF45 was also detected in same fraction (Fig. 2B). This suggested that RTA is associated with KSHV virions and is most likely present as a component of the tegument.

**RTA transactivates the major LANA promoter in human cells.** Since RTA is associated with KSHV virions and is likely to be located in tegument, it is possible that RTA present in the virions may immediately influence viral gene expression upon entry of the virion particles. Therefore, it is reasonable to suggest that RTA can regulate LANA expression. Previous studies showed that a small number of lytic genes including RTA are transiently expressed early after de novo infection of KSHV. This abortive lytic gene expression is terminated with the supervention of the latency program (36). LANA is accumulated at high levels during establishment and maintenance of latency, and it is a master control for latency through repression of RTA expression (39). Therefore, we hypothesize that RTA may transactivate the LANA promoter, resulting in expression of LANA during early stages of infection. This accumulation of LANA resulting from RTA expression will then repress expression of RTA closing the regulatory loop. We sought to investigate if RTA can operate as a transcriptional modulator of the LANA promoter. A total of 800 bp of the LANA promoter region was cloned into pGL2-basic to create reporter plasmid pGLLANAp. The pGLLANAp was transfected into U2OS cells (Fig. 3A), BJAB cells (Fig. 3B), or...
HEK 293 cells (Fig. 3C), all negative for KSHV. The expression construct pCR3.1-RTA was transfected along with the luciferase reporter construct driven by the 800-bp LANA promoter region. Cotransfection of the RTA expression vector with the pGLLAna reporter construct consistently resulted in activation of the reporter activity relative to reporter construct alone (Fig. 3A to C). Moreover, increasing the concentration of the RTA expression construct in this reporter assay resulted in activation of the LANA promoter in a dose-dependent manner in each cell line (Fig. 3). These results indicate that the activation of the LANA promoter in the U2OS, HEK 293, and BJAB cell lines was directly proportional to the quantity of RTA expressed as demonstrated by the observed dose-response relationship. To further support and confirm whether or not the LANA promoter was also responsive to natively synthesized RTA by KSHV, we took advantage of an established RTA-inducible cell line (48). In this cell line, a tetracycline regulatory element was cloned into the upstream of the RTA gene in the KSHV genome, so that tetracycline can induce RTA expression followed by induction of lytic replication (48). In this experiment, pGLLAna was transfected into BCBL1-RTAi 12 h posttransfection; tetracycline was added to the cultures at time points 0 h, 12 h, 24 h, 36 h, 48 h, and 72 h postinduction; cells were harvested; and then cell lysates were made for a luciferase assay. As expected, tetracycline induced RTA expression over time (Fig. 4A). Importantly, the increased levels of RTA also resulted in transactivation of the LANA reporter construct in a dose-dependent manner (Fig. 4A). In the control cell line where RTA expression was not induced by tetracycline, the LANA promoter activity showed
little or no change in activity, suggesting that activation of LANA promoter in BCBL1-RTAi cells is dependent on induction of RTA in the BCBL1 background (Fig. 4B).

**RTA induces LANA expression.** The above experiments showed that RTA activates the LANA luciferase promoter reporter construct, suggesting that RTA may also induce LANA from its native promoter. Therefore, we wanted to determine if RTA can up-regulate LANA expression in the context of the viral genomic DNA. The L54 phage DNA contains the LANA open reading frame and its native regulatory sequences (54). In this experiment, we transfected 293T cells with a fixed amount of L54 DNA and with increasing amounts of the RTA expression vector. Our results showed that LANA expression was at relatively low levels in the absence of RTA. However, when increasing amounts of RTA were introduced, there was an obvious increase in the levels of LANA protein detected by Western blotting (Fig. 5A). Arbitrary counts showed a gradual increase in LANA signal, which correlated with the increased levels of RTA detected (Fig. 5A). These results suggest that the native regulatory element upstream of LANA is responsive to RTA expression.

To determine if LANA expression up-regulated in response to RTA is at the transcriptional level, real-time qPCR was performed to examine the effect of RTA expression on the transcription of LANA. In RTA-inducible BCBL1 cells, tetracycline was used to induce RTA, and total RNA was collected at 0, 12, 24, 48, and 72 h postinduction. The results of the PCR analysis in this inducible background reflected the same trend observed for the L54 DNA experiment above (Fig. 5B and C). An initial large spike in RTA mRNA levels was observed after 12 h, after which RTA transcript levels began to level off from a Ct value of about 30 to approximately 12. A delayed increase in LANA was observed beginning sometime between the 24-h and 48-h time points. The observed reduction in Ct value was consistent and reproducible in multiple assays. The delayed effect on LANA transcript levels is likely due to a lag between increased transcription of RTA and the overall change in LANA transcripts, with endogenous LANA expression in the BCBL1 cell background. Nevertheless, the results of the transcription analysis do provide additional supporting data that the native regulatory element of LANA is responsive to RTA.

**Increase in levels of LANA transcripts correlates with the induction of RTA transcript production.** To further explore the role of LANA and RTA in the establishment of latency postinfection, we used real-time qPCR to examine RTA and
LANA transcriptional levels from initial infection to the establishment of latency 24 h postinfection in infection of a KSHV-negative cell line, 293. Consistent with the finding that RTA can autoactivate its own promoter (15), RTA mRNA was detected as early as 30 min postinfection (Fig. 6A). Following expression of RTA, the detection of LANA mRNA was observed 1 h postinfection and correlated with increasing levels of RTA during the initial 5 to 6 h of infection (Fig. 6A). The expression levels for RTA peaked at 5 h followed by a steady decline in levels up to 24 h (Fig. 6A). The LANA levels were increased to $10^6$ to $10^7$ copies by 12 h and gradually increased to approximately $10^8$ copies by 24 h postinfection (Fig. 6B). RTA mRNA levels were maintained at the same low level, likely due to the small subset of KSHV-infected cells which spontaneously undergoes lytic replication (Fig. 6A). At the same time, LANA mRNA levels increased steadily by 8 h, reflecting the establishment of KSHV latency by expressed LANA and the autoregulation of LANA on its own promoter (Fig. 6B) (31). The rapid increase in mRNA levels of these transcripts is likely due to RTA activating its own promoter prior to activating LANA expression. This results in an initial surge of infected cells which express RTA and simultaneously results in increased LANA levels to negatively regulate RTA transcription and establishment of latency.

**The RBP-Jκ binding site within the LANA promoter is critical for RTA-mediated transactivation.** The experiments above showed that RTA up-regulates LANA expression. To elucidate the potential mechanism of regulation by RTA, we analyzed the LANA promoter sequence using the TFsearch program (26). A number of potential transcriptional factor binding sites were identified within this promoter region (29–31). Interestingly, a single RBP-Jκ binding site was located within the region that contributes to the core promoter activity of the LANA promoter (29–31). As shown previously, RTA can activate its downstream genes such as ORF57 through interactions with RBP-Jκ (40). It is also possible that RTA regulates the LANA promoter through the RBP-Jκ binding site. To test this hypothesis, we mutated the RBP-Jκ site of the LANA promoter and then cloned the mutant type promoter into pGL2-basic to get reporter plasmid pGLLANApmt. In a luciferase assay, pGLLANAp and pGLLANApmt were transfected into cells in equivalent amounts and cotransfected with increasing amounts of the RTA expression construct. As shown, the activation level of mutant-type promoter by RTA was dra-
The results of the EMSA assay showed that a specific RBP-Jκ shift was observed with the probe in the KSHV-negative 293 cells and RBP-Jκ transfected 293 cell nuclear extracts (Fig. 8A, lanes 2 and 4), as well as in the presence of in vitro-translated RBP-Jκ (Fig. 8B, lane 2). The specificity of these bands was demonstrated by its disappearance in the presence of a specific cold competitor probe (Fig. 8A, lane 3, and B, lane 3) and supershift formed by using RBP-Jκ antibody (Fig. 8A, lane 5, and B, lane 4). In the presence of RTA and RBP-Jκ coexpressed in 293 cells and with in vitro-translated RTA and RBP-Jκ, an additional shift was observed, respectively (Fig. 8A, lanes 8 and 11, and B, lanes 7 and 10). The specificity of the shifts was further verified by their supershifting in the presence of specific antibody (Fig. 8A, lanes 9 and 10, and B, lanes 8 and 9), while no effect was observed when 293 cells were transfected only with the RTA expression vector. This was probably due to the complexes formed by RTA and endogenous RBP-Jκ. The results of these experiments therefore suggest that RBP-Jκ can bind to the consensus binding site within the LANA promoter and that RTA is able to form a complex with RBP-Jκ bound to its specific sequence. This then results in derepression of the promoter.

Expression of LANA is significantly delayed in OT11 cells postinfection by KSHV. Previous studies showed that mouse-derived OT11 and OT13 cells are permissive to infection by KSHV (41). These cell lines provide an ideal system to address the question of whether or not there is any functional significance to the role of RBP-Jκ involved in latency establishment postinfection. To address this, OT11 and OT13 cells were infected with KSHV virions induced from BCBL1 cells. At time points of 4 h, 12 h, 24 h, and 48 h postinfection, cells were harvested for immunoﬂuorescence analysis to determine the expression of RTA and LANA.
In OT13 cells, both RTA and LANA were detected by 4 h postinfection (Fig. 9A). RTA signals became faint from 24 h and it was difficult to see RTA signals by 48 h postinfection. However, LANA expression steadily intensified from 12 h postinfection and was significantly stronger by 48 h (Fig. 9A). These results supported the real-time PCR data above, suggesting that RTA induces LANA expression and that the accumulated LANA contributes to the repression of RTA very quickly within 24 h upon infection.

In OT11 cells, RTA can be detected at 4 h postinfection; however, there were no detectable levels of LANA (Fig. 9B). By 12 h postinfection, very faint signals of LANA were detected (Fig. 9B). The LANA signal was still relatively weak by 24 h postinfection but was noticeably stronger by 48 h (Fig. 9B). Interestingly, some clear signal of RTA was detected at 24 h postinfection (Fig. 9B). This suggests that by 24 h postinfection, the levels of LANA were not sufficient to efficiently shut down RTA expression by negative feedback. Clearly, there were little or no detectable levels of LANA at 4 h postinfection, comparing to that of OT13 cells (compare Fig. 9A and B). Therefore, RBP-Jk is likely to be critical for activation of LANA expression at an early stage of infection. However, relatively stronger LANA levels were detected by 24 h and increased by 48 h, only after levels of RTA were effectively shut down. This implies that LANA is likely to also be regulated by other cellular transcriptional regulators. The reporter assays above demonstrated that the transcriptional activity of the LANA promoter was not completely shut down in the absence of RBP-Jk binding site, again suggesting that other transcriptional factors may also contribute to the regulation of LANA. Indeed, binding sites for transcriptional factors which include Oct1 are located within the LANA promoter (26). We are currently investigating this possibility that Oct1 can play a role in establishment of latency. Previous studies also showed that LANA can autoactivate its own promoter (31) and may explain at least in part the eventual accumulation of LANA in OT11.

**DISCUSSION**

ORF50 of KSHV which encodes RTA is an immediate early gene necessary and critical for initiation of full lytic reactivation (43–45). RTA has been shown to regulate and transactivate a number of downstream viral genes that function in lytic replication (20, 44, 59, 65). Moreover, it is widely accepted that the function of RTA is mainly associated with lytic replication of KSHV. Recent studies by Chandran and colleagues suggesting that KSHV can establish latency within 24 h after infection (36) drew our attention to additional studies by Jung and colleagues which also showed up-regulation of RTA followed by increased LANA transcripts by array analysis (48). Therefore, we hypothesized that RTA may contribute to latency establishment through activation of LANA expression during the early stages of infection.

Initial studies suggested that the RTA protein is likely to be associated with the virion particles and supports the possibility that there could be a requirement for RTA in establishment of KSHV latency. Previously, we showed that LANA can repress the RTA promoter (39). Upon infection, RTA may also activate the LANA promoter. Accumulated LANA then can autoactivate its own promoter as well as repress RTA expression through a negative feedback mechanism. Our reporter assays clearly showed that RTA can activate the LANA promoter in a dose-dependent manner. In addition, LANA transcription is shown to be responsive to induction of RTA by using RTA-inducible BCBL1 cells, which supports the hypothesis that LANA expression is downstream of RTA expression during de novo infection and establishment of latency and that the tran-
scription of LANA correlates with that of RTA during the very early stages of de novo infection. The results of these studies therefore suggest a new mechanism by which LANA can be induced by the lytic transactivator RTA, expanding the role of RTA in regulation in the KSHV life cycle and examining a possible new mechanism by which the lytic transactivator RTA also can contribute to establishment of KSHV latency.

In these studies, we also addressed the potential mechanism by which RTA activates LANA expression through functional interaction with RBP-Jκ. Recently, Liang et al. demonstrated that RTA can functionally interact RBP-Jκ to up-regulate its downstream lytic genes such as ORF57 (40, 41). RBP-Jκ is a sequence-specific DNA binding protein in the CSL/CBF-1 family that is normally thought to repress transcription in response to the Notch signaling pathway (40, 41). CSL family proteins recruit corepressor proteins via their interaction with the CSL central domain (amino acids 179 to 361 in RBP-Jκ) (27). These corepressors, in turn, recruit histone deacetylases that induce promoter silencing (38). Regulation of the CSL family members is mediated by Notch transmembrane receptors, which induce a proteolytic cleavage and release of the Notch intracellular domain (ICN) in response to interaction with ligand (64). Nuclear localization signal (NLS) sequences in the ICN mediate transport to the nucleus where the peptide interacts with RBP-Jκ. This interaction appears to involve the central repressor domain that is also the target for RTA interaction. RTA binding may also displace repressor proteins from RBP-Jκ or may be involved in the recruitment of the ICN-activation domain. Liang et al. also suggested that RTA acts as a functional homologue of Notch (ICN). Interestingly, EBV latency proteins EBNA-2 and EBNA-3 have also been shown to interact with RBP-Jκ (21, 52, 53). EBNA-2 does not bind to DNA but is essential for EBV-mediated B-cell proliferation. Interaction of EBNA-2 with the RBP-Jκ central repressor domain region is coincident with that mapped for RTA interaction. This also results in derepression of RBP-Jκ-responsive

FIG. 9. LANA expression is delayed in OT11 cells. OT13 (A) and OT11 (B) cells were infected by KSHV for 2 h and then washed once with PBS. Cells were incubated and harvested at 4 h, 12 h, 24 h, and 48 h and fixed with 1:1 methanol/acetone. Latent protein LANA and lytic protein RTA were detected by immunofluorescence assay.
promoters (61). However, EBNA-3 appears to function in the converse in regulating transactivation activity (52, 53). This study establishes a functional interaction between RTA and the major downstream effector of the Notch signaling pathway RBP-Jκ and shows the unique features of tumor viruses to target essential cellular signaling events for regulating their lytic replication and long term persistence in the infected cell.

LANA is a large nuclear protein (222 to 234 kDa, based on analysis by sodium dodecyl sulfate [SDS]-PAGE) and has three distinct domains: a proline-rich N-terminal region with a putative NLS, a long glutamic acid-rich internal repeat domain, and a carboxy-terminal domain including a putative NLS (12, 33, 50). In latently infected cells, LANA is critical for maintenance of the viral episome by tethering the episome to cellular mitotic chromosomes via direct interactions with histone H1 and possibly other cellular proteins, including MeCP and DEK (13, 37). Moreover, LANA has also been shown to modulate the transcriptional activity of the human immunodeficiency virus long terminal repeat promoter and to transactivate latent membrane protein 1 (LMP1) and Cp major latent promoters of EBV, which are required for transcription of essential EBV latent genes (22, 28, 51). Importantly, LANA also contributes to viral oncogenesis by promoting cell survival through targeting and alteration of p53 function, interaction with the retinoblastoma protein and glycogen synthase kinase 3β, and activation of the telomerase promoter (17, 18, 34). It has also been shown that LANA prolongs the life span of primary human umbilical vein endothelial cells (63). These studies demonstrate that LANA can function as a viral oncogene contributing to cell transformation. Since this study shows that LANA can be regulated by RTA, this may be an indirect mechanism by which RTA can regulate KSHV viral oncogenesis and raises another aspect of RTA role in KSHV pathogenesis which remains to be elucidated.

In summary, the mechanism by which KSHV can establish latency postinfection is still incomplete and needs further study. However, this study provides some clues to a possible mechanism by which the immediate early transactivator RTA may contribute to establishment of viral latency. Here, we establish a potential model which suggests a mechanism of feedback regulatory loop between RTA and LANA and implies that the interplay between RTA and LANA could be the master control for the switch between lytic replication and latency of KSHV utilizing the major cellular Notch signaling pathway through targeting of the RBP-Jκ effector protein (Fig. 10) (39a). Further studies are ongoing and should address the detailed mechanism involved in this process. Interestingly, a recent report showed that RTA of EBV, another human gammaherpesvirus, can induce major latent protein LMP1 (11). Along with this report, induction of latent genes by a lytic protein could be a highly conserved process in gammaherpesviruses for the establishment of latency in the infected cells. The immediate establishment of latency after infection is a critical component and consequence of the interaction between virus and host. Therefore, further studies will be focused on the identification of cellular molecules interacting with the viral regulatory molecules required for establishment of latency.

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