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Kaposi's Sarcoma-Associated Herpesvirus Reactivation Is Regulated by Interaction of Latency-Associated Nuclear Antigen with Recombination Signal Sequence-Binding Protein Jκ, the Major Downstream Effector of the Notch Signaling Pathway

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Kaposi's sarcoma-associated herpesvirus (KSHV) is the major biological cofactor contributing to development of Kaposi's sarcoma. KSHV establishes a latent infection in human B cells expressing the latency-associated nuclear antigen (LANA), a critical factor in the regulation of viral latency. LANA controls KSHV latent infection through repression of RTA, an activator of many lytic promoters. RTA activates the expression of several lytic viral genes by interacting with recombination signal sequence-binding protein Jκ (RBP-Jκ), a transcriptional repressor and the target of the Notch signaling pathway. The recognition that a number of KSHV lytic gene promoters, including RTA, contain RBP-Jκ binding sites raised the possibility that RBP-Jκ-mediated repression may be central to the establishment of latency. Here, we tested this hypothesis by examining the regulation of RTA by LANA through binding to RBP-Jκ. This study demonstrates that LANA physically associates with RBP-Jκ in vitro and in KSHV-infected cells, with the complex formed capable of binding to RBP-Jκ cognate sequences. RBP-Jκ binding sites within the RTA promoter have been found to be critical for LANA-mediated repression. Our study describes a novel mechanism through which LANA maintains KSHV latency by targeting a major downstream effector of the Notch signaling pathway.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with a number of human malignancies, including Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (8, 12, 13, 60, 63, 64). Similar to other herpesviruses, KSHV is a large double-stranded DNA virus which displays two alternative genetic life cycle programs upon infection of host cells (49). 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 (ORF) 73, viral cyclin 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 (20, 60, 71).

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 the death of the host cell (71). Latently infected cells can be induced to enter the lytic cycle under certain cellular conditions (18, 28). Thus, the pool of latently infected cells represents a reservoir of viral persistence from which infectious virus can later be reactivated, with production of viral progeny which can spread to new target cells.

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 (21, 33). Occasionally, lytic reactivation of the virus may be critical, as viral cytokine homologues during this phase may function as paracrine factors in stimulating cell growth and proliferation (3, 4, 14, 65). 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 (9, 10). In addition, a number of studies have shown that the genes expressed during latency play a major role in tumorigenesis of KSHV-associated cancers (21, 24, 27, 29, 57).

Among the limited number of latent genes, the ORF73 gene, which encodes latency-associated nuclear antigen (LANA), is likely to be critical for the establishment of latent KSHV infection through maintenance of the viral episome (5, 6, 52). LANA is a large nuclear protein (222 to 234 kDa, based on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and has three distinct domains (Fig. 1A): a proline-rich N-terminal region with a putative nuclear localization signal, a long glutamic acid-rich internal repeat domain, and a carboxy-terminal domain including a putative nuclear localization signal (16, 34, 56). In latently infected cells LANA helps maintain the viral episome by tethering the episome to cellular mitotic chromosomes via direct interactions with histone H1 and possibly other cellular proteins, including methyl-CpG binding protein and DEK (17, 37). LANA has also been shown to modulate the transcriptional activity of the human immu-

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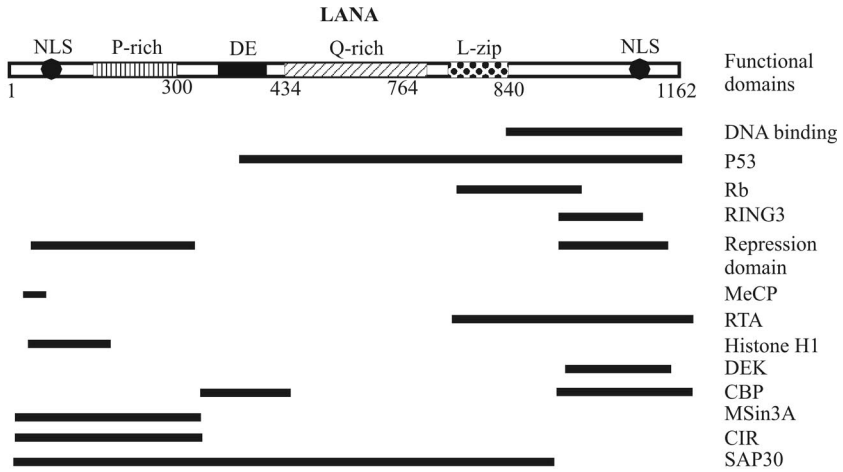

FIG. 1. Scheme showing the LANA protein. As shown, LANA is a 1,162-amino-acid protein (BC-1 strain). Numbers indicate the amino acids (aa). The putative domains include an N-terminal proline-rich domain (P-rich); aspartic acid and glutamic acid repeat region (DE); glutamine-rich domain (Q-rich); and leucine zipper (LZ). LANA also has two nuclear localization signals (NLS) in both the amino- and carboxy-terminal regions. The approximate position of each functional domain is shown by a black bar, corresponding to the function shown in the column on the right (17, 21, 37, 38, 41, 44, 53).

nodeficiency virus long terminal repeat promoter and to transactivate the LMP1 and Cp major latent promoters of Epstein-Barr virus which are required for transcription of essential Epstein-Barr virus latent genes (27, 29, 57). 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 (21, 23, 35).

Recently, studies in our laboratory showed that LANA is capable of maintaining viral latency through repression of the transcriptional activity of the RTA (replication and transcription activator) promoter (41). RTA is encoded by ORF50 of KSHV and is an immediate-early protein, and exogenous expression of RTA from a heterologous promoter or induction of endogenous RTA expression can initiate lytic reactivation of KSHV (47, 48). The mechanism by which LANA regulates the transcriptional activity of the RTA promoter is not understood. In this study, we show that RBP-Jκ binding sites within the RTA promoter are critical for LANA-mediated repression of RTA promoter. Additionally, we show that LANA physically associates with RBP-Jκ in vitro as well as in infected cells. This association forms a complex with RBP-Jκ which binds to the RBP-Jκ cognate DNA sequence within the RTA promoter, resulting in repression of the promoter as well as repression of RTA-mediated autoactivation of its promoter. Taken together, our results demonstrate that the association of LANA with RBP-Jκ is likely to be a critical component of the mechanism by which LANA regulates RTA activity. Targeting the downstream regulator of the cellular Notch signaling pathway once again is another stealthy approach by which gammaherpesviruses can usurp a cellular signaling pathway. This provides a novel example of a viral molecule's targeting the critical Notch cellular signaling pathway to control latency and reactivation from latency.

MATERIALS AND METHODS

Constructs, antibodies, and cell lines. The pRpluc reporter plasmid which contains a 3-kb upstream KSHV Rta promoter sequence is a kind gift from Ren Sun (University of California, Los Angeles) (19). A series of truncation plasmids of pRpluc were made to remove RBP-Jκ binding sites in sequence (Fig. 2C). The truncated promoters were named pRplucΔ2570, pRplucΔ2039, pRplucΔ1490, pRplucΔ1327, and pRplucΔ550. To construct pRplucΔ2570, in which one RBP-Jκ binding site was deleted, pRpluc was digested with ScaI and BglII and the target fragment was purified from the agarose gel and inserted into pGL2-Basic digested with SmaI and BglII. For pRplucΔ2039, in which two RBP-Jκ binding sites were removed, pRpluc was digested with NheI and PstI. The ends of the fragments were blunted and the largest fragment purified from the agarose gel was self-ligated. To generate pRplucΔ1490, in which three RBP-Jκ binding sites were removed, pRpluc was digested with NheI and SacII and the ends were blunted. Similarly, the largest fragment purified from the agarose gel was self-ligated. Both pRplucΔ1327 and pRplucΔ550 are deleted for all RBP-Jκ binding sites. For pRplucΔ1327, pRpluc was digested with XbaI and the fragment was blunted. This product was digested with BglII, and the target fragment was purified from agarose gel. This product was cloned into pGL2-Basic digested with SmaI and BglII. The pRplucΔ550 construct was prepared by digesting pRpluc with NheI and StuI and then blunted and self-ligated. Expression vectors, including pA3M-LANA, pA3M-LANA1-435, pA3M-LANA1-756, pA3M-LANA762-1162, pCDNA3-LANA, pCR3.1-RTA, and pA3M-RBP-Jκ, were described previously (41).

RBP-Jκ rabbit polyclonal antibody was provided by Elliott Kieff (Harvard Medical School, Boston, Mass.). Human polyclonal serum which recognizes LANA was provided by Gary Nabel (Vaccine Institute, National Institutes of Health, Bethesda, Md.). Myc mouse monoclonal immunoglobulin G was produced at the University of Michigan Hybridoma Core Facility with the hybridoma cell line 9E10.

Human embryonic kidney fibroblast 293 and 293T cells, transformed with E1A and T antigens, respectively, were obtained from Jon Aster (Brigham and Women's Hospital, Boston, Mass.). BJAB is an Epstein-Barr virus-negative B-cell line isolated from Burkitt's lymphoma and were provided by Elliott Kieff (Harvard Medical School). BCBL1 and BC3 are KSHV-positive body cavity-based lymphoma-derived cell lines obtained from the American Type Culture Collection. JSC-1 cells are also KSHV latently infected B lymphoma cells provided by Richard F. Ambinder (11). Human embryonic kidney fibroblast 293 and 293T cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 25 U of penicillin per ml, and 25 μg of streptomycin per ml. BJAB, BCBL1, BC3, and JSC-1 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 U of penicillin per ml, and 25 μg of streptomycin per ml.

Transfection. BJAB, 293, and 293T cells were transfected by electroporation with a Bio-Rad Gene Pulser II electroporator; 10 million cells harvested in exponential phase were collected and washed in phosphate-buffered saline and then resuspended in 400 μl of RPMI or Dulbecco's modified Eagle's medium with DNA for transfection. Resuspended cells were transferred to a 0.4-cm cuvette and electroporated at 975 μF and 220 V. The electroporated cells were then transferred to 10 ml of complete medium, followed by incubation at 37°C and 5% CO_2. Transfections were harvested after 24 h and assayed for activity.

Luciferase assay. HEK293 cells were collected at 70% confluency; 10 million cells were resuspended, along with plasmid DNA, in 400 μl of medium. The cells were transfected 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 Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The plates were incubated at 37°C with 5% CO_2 for 20 h. BJAB cells were collected at 5×10^5 cells/ml; 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 of reporter lysis buffer (Promega, Inc.); 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.

Immunoprecipitation and Western blotting. 293T or BJAB cells were transfected with pA3M-LANA and/or pCR3.1-Rta and incubated for 24 h. The cells were then lysed in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin [1 μg/ml], and pepstatin [1 μg/ml]) for 1 h on ice with brief vortexing every 15 min. A portion of the lysate was removed for use as the control. The lysates were precleared by 1 h of incubation with control antibody conjugated to protein A beads. Anti-Myc or anti-Rta antibodies were incubated with the lysates overnight at 4°C. Immunoprecipitates were collected by rotating with protein A-Sepharose beads for 1 h and washed four times in radioimmunoprecipitation assay buffer. The protein was then heated in sodium dodecyl sulfate/β-mercaptoethanol lysis buffer and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Western blot analyses were done with antibodies specific for the detection of LANA-Myc or RTA (41). For BC3 or BCBL1 cells, a total of 10^8 cells were used following the same protocol (41).

Preparation of GST fusion proteins and in vitro binding assays. *Escherichia coli* DH5α cells were transformed with the plasmid constructs for each fusion protein, and single colonies were picked and grown overnight in 2 ml of Luria-Bertani (LB) medium. Five hundred milliliters of LB medium was inoculated at a dilution of 1:200 and allowed to shake at 37°C until the mid-exponential growth phase. Cells were then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h with continuous shaking at 37°C. Cells were harvested and sonicated, and the proteins were solubilized in the presence of protease inhibitors. Solubilized proteins were incubated with glutathione *S*-transferase (GST)-Sepharose beads for 6 h or overnight at 4°C with rotation and then collected by centrifugation and washed three times in NETN buffer with protease inhibitors. GST fusion proteins bound to beads were used for binding assays and stored in NETN buffer with protease inhibitors and 1 mM phenylmethylsulfonyl fluoride at 4°C.

To determine binding of the GST-Jκ fusion to full-length and different truncation mutants of LANA, proteins were translated in vitro with Express[S^{35}] protein labeling mix from Perkin Elmer with the in vitro transcription and translation (TNT) system (Promega Inc.) as per the manufacturer's instructions. Labeled LANA protein was incubated with equivalent amounts of GST-Jκ fusion protein bound to beads and rotated at 4°C for 4 h, followed by four washes in 1 ml of NETN with protease inhibitors. Bound proteins were eluted from the beads in sodium dodecyl sulfate lysis buffer by heating at 95°C for 10 min and then fractionated by SDS–10% polyacrylamide gel electrophoresis. Dried gels were analyzed with a PhosphorImager (Molecular Dynamics Inc.), and signals were quantified with ImageQuant software.

Immunofluorescence. Immunofluorescent analyses were performed essentially as described previously (35, 36, 41). Briefly, fixed cells were blocked in the appropriate serum and then incubated with the specific primary antibody for LANA, Myc, and RBP-Jκ 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 and visualized with an Olympus XI70 inverted fluorescence microscope (Olympus Inc.) and photographed with a digital PixelFly camera and software (Cooke, Inc.).

Electrophoresis mobility shift assays. A probe containing the furthest-downstream RBP-Jκ consensus binding site (19, 60) was designed (5′-GATCATTTC C*GTGGGAA*GACGAT-3′), annealed, and labeled. ^{32}P-labeled probes were synthesized via the Klenow fill-in reaction and purified with Select-D with G-25 columns (Shelton/IBI 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 (35). Proteins from nuclear extracts or in vitro translation products were mixed with 1 μg of poly(dI-dC) (Sigma) in 1× DNA binding buffer for 5 min at room temperature; 1 μl of labeled probe was added to each reaction, and the tubes were incubated at room temperature for 15 min. DNA-protein complexes were resolved via nondenaturing 6% 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 (Kodak Inc.) for 24 to 48 h at −70°C with an 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-poly(dI-dC) mix before incubation at room temperature, and similarly, a 200-fold molar excess of unlabeled nonspecific unlabeled competitor probe was added in a separate reaction. Antibodies specific to RBP-Jκ and nonspecific antibody were used as controls to show supershifted complexes.

RESULTS

RBP-Jκ binding sites within the ORF50 promoter are critical for LANA-mediated regulation of RTA expression. In our previous study, we demonstrated that LANA can repress the RTA promoter (41). In this study we wanted to determine the mechanism by which LANA regulates this promoter. Typically, proteins regulating the transcription activity of specific target genes can bind with high affinity to their own responsive elements within the promoter or by indirectly binding to another specific cellular transcriptional factor involved in direct binding to cognate DNA sequences within the promoter of the target gene (73).

Initial investigation of the Rta promoter did not find LANA-specific sequence motifs based on previous studies which identified the LANA binding sites. Their studies also did not find any other target sequences similar to that of the LANA promoter (24, 32). Studies of the Epstein-Barr virus transcriptional activator EBNA2 showed that EBNA2 did not bind directly to DNA but is rather indirectly targeted to its responsive promoter through direct interaction with the cellular DNA binding protein RBP-Jκ (26, 75). Although LANA shows no homology at the sequence level, its phenotype and structural features are reminiscent of the Epstein-Barr virus EBNA-1 and EBNA-2 proteins, and one such example is its extended central region of repeats (66). In addition, another study showed that RTA can bind to RBP-Jκ to reactivate expression of KSHV lytic promoters (42). This led us to hypothesize that LANA may also regulate the RTA promoter through RBP-Jκ binding sites, since there are four RBP-Jκ sites within the Rta promoter.

To address this hypothesis, a series of truncated versions of the RTA promoter were generated. RBP-Jκ binding sites were removed sequentially until all RBP-Jκ binding sites were deleted to determine the contribution of each site in the regulation of RTA expression (Fig. 2). These reporter constructs were transfected into HEK293 cells along with the expression vector pA3M-LANA, a Myc-tagged LANA expression vector (17). As expected, repression of the promoter activity was observed when reporter constructs containing the full-length Rta promoter with the four RBP-Jκ binding site were cotransfected with the LANA expression vector (Fig. 2A and 2C). This

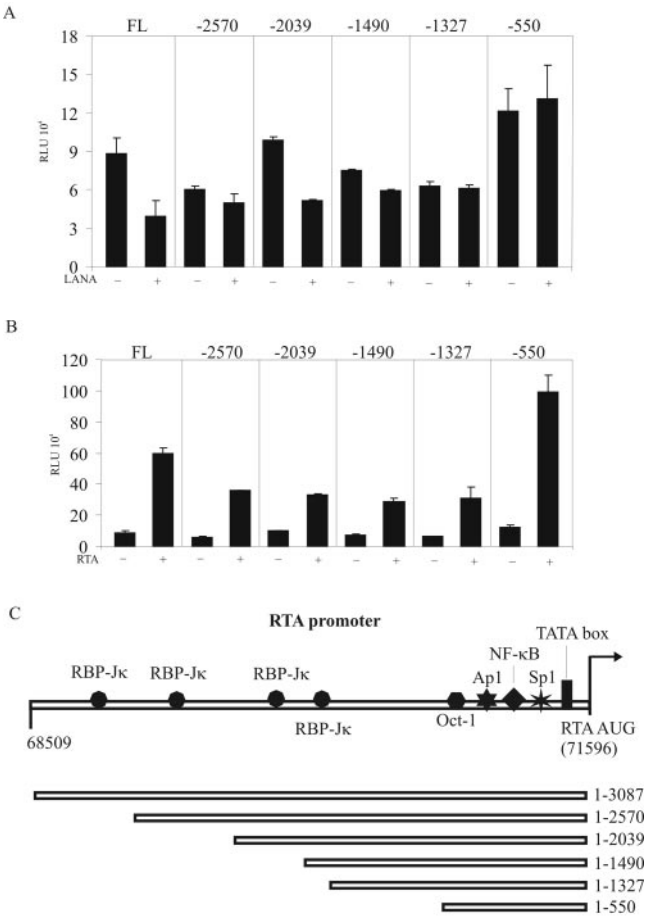

FIG. 2. Transcriptional activity of LANA (A) and RTA (B) on ORF50 promoters in cells. The reporter plasmid pRpluc contains a 3-kb sequence upstream of the translational initiation site of the *rta* gene that drives the expression of firefly luciferase (19). A series of truncated promoters named pRplucΔ2570, pRplucΔ2039, pRplucΔ1490, pRplucΔ1327, and pRplucΔ550 were made for deletion of RBP-Jκ binding sites (C). A fixed amount (5 μg) of the reporter plasmids was transfected or cotransfected into human embryonic kidney 293 cells (A) with 5 μg of pA3M-LANA or 5 μg of pCR3.1-RTA. Promoter activity was expressed as the relative light units (RLU) relative to the reporter-alone control. The means and standard deviations from three independent transfections are shown.

repression was not observed when cotransfections were performed with reporter constructs deleted for most of the RBP-Jκ binding sites (Fig. 2A and 2C).

LANA was able to repress pRplucΔ2570 as well as pRplucΔ1490 (one and three RBP-Jκ sites, respectively). However, this repression was approximately 20% compared to the greater than 50% seen with the full-length promoter (Fig. 2A). This observed decrease in the level of repression suggests that this region of the promoter which is deleted and contains RBP-Jκ sites is critical for repression mediated by LANA. Interestingly, LANA repressed pRplucΔ2039 at a level similar to the full-length promoter and the level of activation in the absence of LANA was also similar to the full-length promoter. This was distinct from the level of activation seen with truncations pRplucΔ2570 and pRplucΔ1490, which was about 40% of that of the full-length promoter (Fig. 2A and 2C). This suggests that regulatory sequences in addition to the RBP-Jκ sites which may contribute at least in part to some of the activation seen with full-length promoter are located between −3087 and −2039. However, the data strongly suggest that the RBP-Jκ sites do contribute to the regulation of Rta expression by LANA but this effect occurs primarily in the context of the full-length promoter with the four RBP-Jκ sites intact (Fig. 2A and 2C).

As anticipated, the two reporters lacking RBP-Jκ binding sites, pRplucΔ1327 and pRplucΔ550, showed no repression by LANA (Fig. 2A and 2C). In addition, pRplucΔ550 showed an increase in activation by approximately 30%. This may be a result of the loss of potential negative regulatory elements, which include the RBP-Jκ sites upstream of −550.

Previous studies have shown that RTA can regulate KSHV lytic genes through regulation of activities involving the RBP-Jκ binding sites (42, 43, 68, 74, 76). We therefore wanted to determine if RTA regulates its own promoter through the RBP-Jκ sites. Similarly, reporter assays were performed with an Rta expression construct. The results of these assays showed that RTA is consistently able to activate its own promoter and notably that RTA induces the greatest activation on pRplucΔ550, which lacks all Jκ sites (Fig. 2B and 2C). These results strongly suggest that the ability of RTA to activate its native promoter is likely to be independent of the RBP-Jκ binding sites, although the coordinated repression by RBP-Jκ is likely to contribute to the overall level of activation (Fig. 2B). In this assay, we also show that deletion of the RBP-Jκ sites resulted in loss of approximately 50% of the transactivation activity mediated by RTA and that this was similar when comparing a promoter with one RBP-Jκ site to that of one with four RBP-Jκ sites deleted (Fig. 2B). Therefore, the RBP-Jκ sites may be contributing to regulation of the RTA promoter through interaction with complexes that form at each of these sites. Therefore, loss of one of those sites can potentially affect the overall regulation contributed by all four RBP-Jκ sites.

RBP-Jκ interacts with LANA in vitro as well as in KSHV-infected cells. In order to address if LANA interacts directly with RBP-Jκ we performed in vitro binding assays. Full-length as well as carboxy-terminal and amino-terminal constructs of LANA were in vitro translated with [^{35}S]methionine label. The labeled proteins were then incubated with beads bound to GST alone or beads bound to GST-Jκ.

The results of the binding assay indicate that full-length LANA bound strongly to GST-Jκ (Fig. 3A). In addition, a carboxy-terminal truncation of LANA, which consists of amino acids 762 to 1162, also bound to RBP-Jκ (Fig. 3A). However, an amino-terminal truncation of LANA showed little or no specific binding affinity with LANA at a level which was detectable in our binding assay (Fig. 3A). These data suggest that the carboxy terminus of LANA is the primary region responsible for binding to RBP-Jκ. This region is also known to be involved in direct binding to DNA, specifically a 20-bp region of the KSHV terminal repeats (17).

To further verify the site of RBP-Jκ binding to LANA, we performed a reverse binding experiment with two LANA-GST truncation constructs, one consisting of amino acids 341 to 939 of LANA and the other consisting of the carboxy-terminal amino acids 990 to 1162. RBP-Jκ was in vitro translated with [^{35}S]methionine labeling and incubated with GST and one of

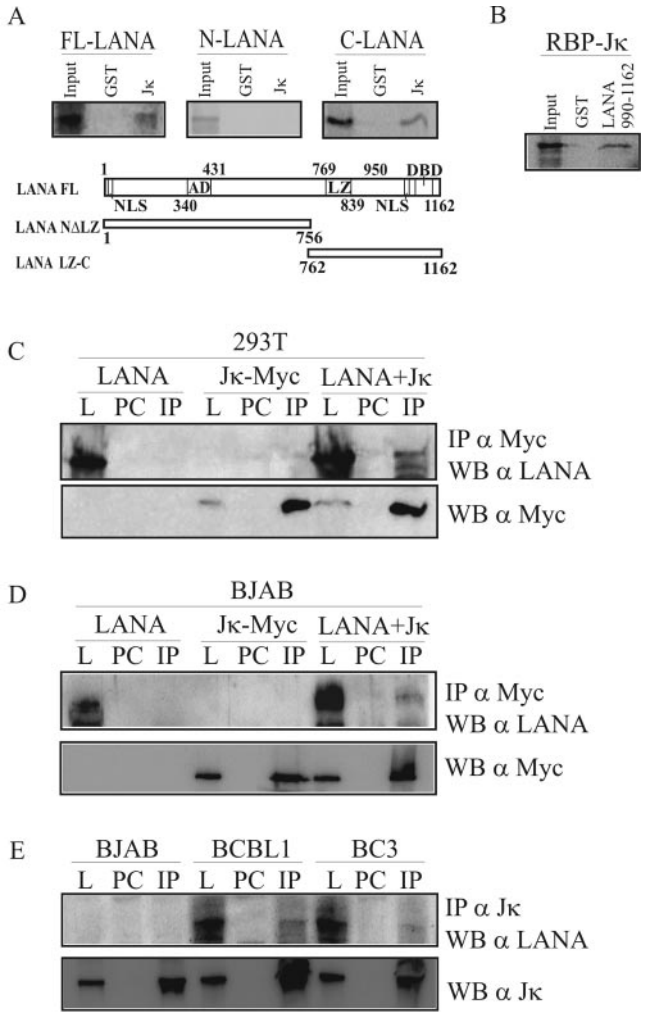

FIG. 3. LANA interacts with RBP-Jκ in vitro as well as in vivo. (A) Full-length LANA as well as LANA clones with truncations in the carboxy- and amino-terminal regions were in vitro transcribed and translated. The ^{35}S-labeled products were incubated with GST or GST-Jκ. The pulldown products were electrophoresed on SDS–8% PAGE gel, dried, and exposed to a Phosphoimager. Input controls of 10% for LANA were run as well. (B) Reverse binding assay. ^{35}S-labeled RBP-Jκ was incubated with truncated GST-LANA, and the pulldown products were electrophoresed on an SDS–10% PAGE gel, dried, and exposed to a Phosphoimager. A schematic for the LANA clones used is given in the middle of the figure. Immunoprecipitation analysis with mouse anti-Myc antibody showed that RBP-Jκ was directly immunoprecipitated with LANA in 293T (C) and BJAB (D) cells. For each experiment, 30 million cells were cotransfected with pA3M-RBP-Jκ and pcDNA3-LANA expression vectors or transfected with these vectors, respectively; 24 h posttransfection, cell lysates were uses for immunoprecipitation analysis. Immunoprecipitation analysis with polyclonal rabbit RBP-Jκ antiserum showed that endogenous LANA was directly immunoprecipitated with RBP-Jκ in BC3 and BCBL1 cells (E). For each experiment, 100 million cells were harvested, and cell lysates were used for immunoprecipitation analysis. NLS, nuclear localization signal; AD, acidic domain; LZ, leucine zipper; DBD, DNA binding domain. L, lysate; PC, preclear; IP, immunoprecipitate.

the GST-LANA constructs. The results demonstrated that RBP-Jκ binds to the carboxy terminus of LANA (Fig. 3B) and that the extreme C terminus is required for binding. These 172 carboxy-terminal-most amino acids (amino acids 990 to 1162) of LANA are responsible for direct interaction with RBP-Jκ (Fig. 3B). The construct comprising the central region of LANA showed no detectable binding (data not shown).

To corroborate the in vitro binding data above, immunoprecipitation experiments were carried out to determine if the interaction of LANA with RBP-Jκ seen in vitro also occurs in human cells. Myc-tagged RBP-Jκ and untagged LANA expression plasmids (pA3M-RBP-Jκ and pCDNA3-LANA, respectively) were cotransfected into HEK293 cells or BJAB cells by electroporation. Twenty-four hours posttransfection, cells were harvested and lysed. The RBP-Jκ protein was immunoprecipitated with mouse monoclonal antibody 9E10 against the Myc epitope. The bound complexes coimmunoprecipitating with RBP-Jκ were then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was probed for the presence of LANA coprecipitating with RBP-Jκ with a human polyclonal antiserum with specific reactivity to LANA.

As anticipated, LANA coimmunoprecipitated with RBP-Jκ in 293T and BJAB cells, respectively (Fig. 3C and 3D), while control immunoprecipitates of BJAB and HEK293T cells transfected with the RBP-Jκ or LANA expression plasmid alone did not show any signal for LANA (Fig. 3C and 3D). However, control blots show that RBP-Jκ was expressed in the transfected lane (Fig. 3C and 3D, lower panels). These results indicate that LANA and RBP-Jκ can associate with each other in human B cells and epithelial cells when expressed from a heterologous promoter in a transient transfection system.

The association in cells as determined above does not provide evidence of whether or not they associate in KSHV-infected cells. To determine association between endogenous LANA and RBP-Jκ, immunoprecipitation experiments were carried out in the naturally infected KSHV-positive cell lines BCBL1 and BC3. The KSHV-negative Burkitt's lymphoma cell line BJAB was used as a negative control. The cell lines were amplified in culture, and exponentially growing cells were harvested and lysed, and the lysate containing complexes of RBP-Jκ was immunoprecipitated with an RBP-Jκ-specific rabbit polyclonal antiserum. All recovered complexes were fractionated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was further probed for the presence of LANA bound to RBP-Jκ with a human polyclonal serum which recognizes LANA.

The results show that LANA immunoprecipitated with RBP-Jκ in both KSHV-infected cell lines BC3 and BCBL1 at similar levels (Fig. 3E). However, the control immunoprecipitates of KSHV-negative BJAB cells did not show any LANA signal, indicating that the signal was specific for the two KSHV-positive cell lines (Fig. 3E). Control blots to determine if RBP-Jκ was immunoprecipitated showed that antibody to RBP-Jκ efficiently immunoprecipitated RBP-Jκ in KSHV-positive and -negative cells (Fig. 3E).

LANA colocalizes to the same nuclear compartments as RBP-Jκ in human epithelial cells, B cells, and KSHV-positive pleural effusion lymphoma cell lines. To determine if the association of LANA and RBP-Jκ occurred in similar compartments in the nucleus or if there was a change in LANA or RBP-Jκ localization when they were coexpressed in cells, we preformed immunofluorescence analysis. First, we transiently cotransfected 293T and BJAB cells with RBP-Jκ and LANA

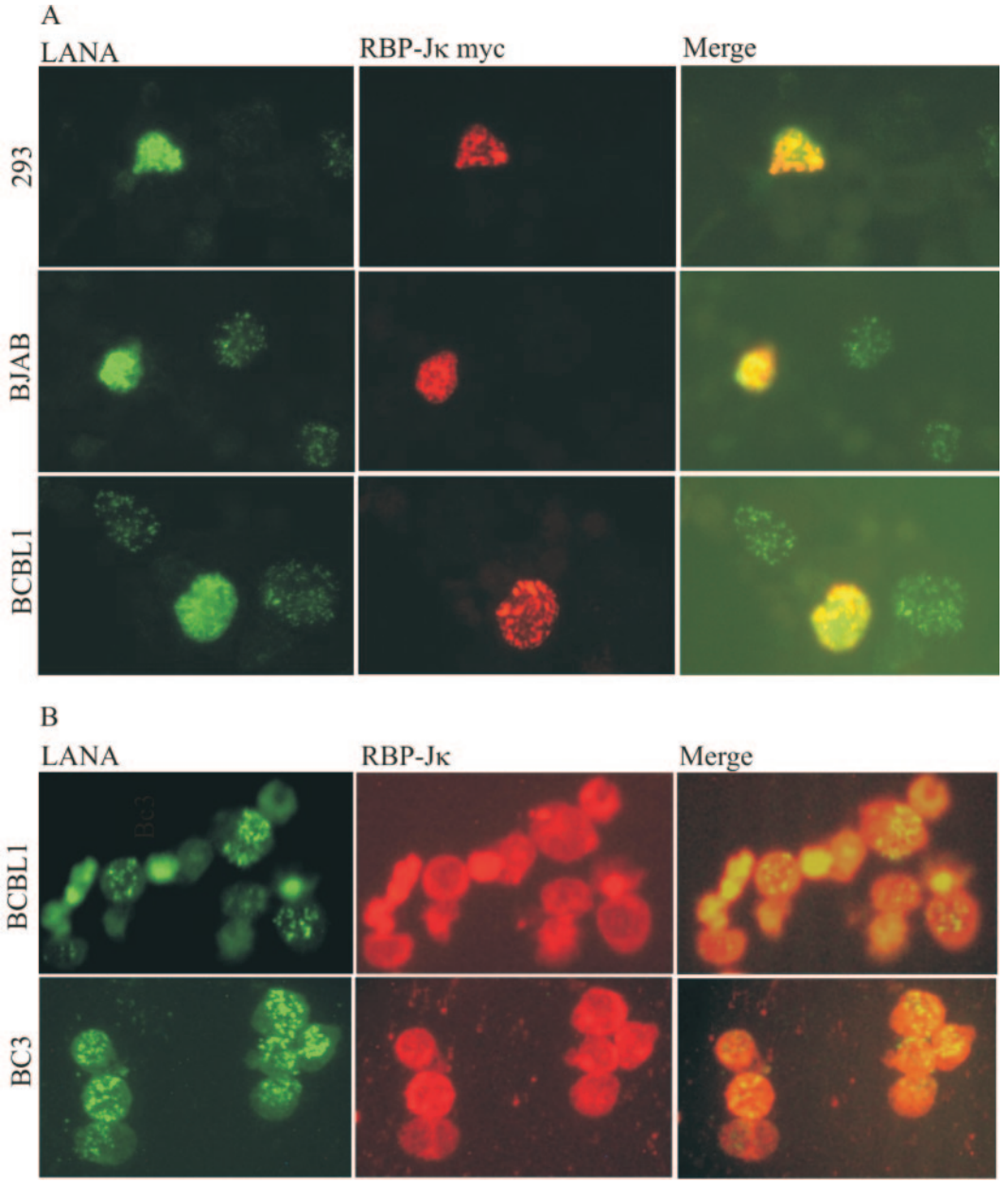

FIG. 4. Immunofluorescence analysis showed that RBP-Jκ was localized to the same nuclear compartment as LANA in different cells. (A) BJAB and 293T cells were cotransfected with the pA3M-RBP-Jκ and pcDNA3-LANA expression vectors, and BCBL1 cells were transfected with pA3M-RBP-Jκ; 24 h posttransfection, cells were harvested for immunofluorescence analysis. BCBL1and BC3 cells were also used for immunofluorescence analysis to show endogenous LANA and RBP-Jκ colocalization (B). Cells were harvested when they looked healthy. LANA and RBP-Jκ were expressed in almost every cell.

expression constructs; 24 h posttransfection the cells were harvested, fixed, and probed with mouse monoclonal antibody against the Myc epitope tagged to RBP-Jκ and human antiserum with specificity for LANA. Fluorescein isothiocyanate and Texas Red conjugated to the appropriate secondary antibodies were used for visualization of the proteins. The results showed that LANA and RBP-Jκ protein colocalized to the same nuclear compartments in 293T and BJAB cells when cotransfected from heterologous promoters (Fig. 4A).

We also transfected BCBL1 with Jκ-Myc to determine if endogenous LANA in a predominantly latently KSHV-infected cell line would colocalize with exogenously expressed RBP-Jκ or recruit RBP-Jκ to similar compartments in the nucleus. As expected, LANA and RBP-Jκ-Myc can also colocalized in similar nuclear compartments in these latently infected cells (Fig. 4A). These results suggest that LANA endogenously expressed in KSHV-positive cells can recruit RBP-Jκ to similar compartments in the nucleus of infected cells.

RBP-Jκ has previously been shown to localize to both the cytoplasm and nucleus. However, when both LANA and RBP-Jκ were cotransfected from heterologous promoters, the RBP-Jκ signal appears to be predominantly nuclear to specific

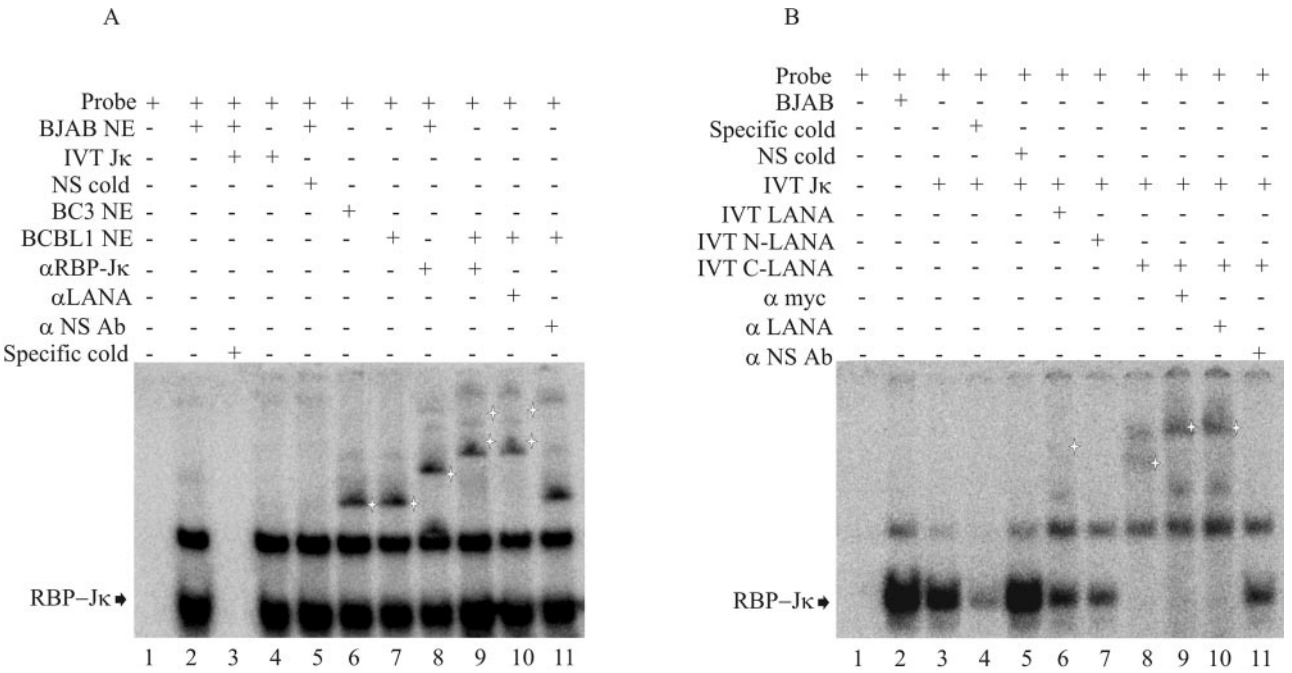

FIG. 5. LANA/RBP-Jκ DNA binding complex. The probe consisted of an RBP-Jκ consensus binding sequence and the flanking bases from −1469 to −1451 of the Rta promoter. This probe is the furthest downstream of the four RBP-Jκ consensus sites in the promoter from −3087 to −1. The arrow indicates the position of the RBP-Jκ/DNA complex. The asterisk indicates the position of the supershifted complex in the presence of BCBL1 cell lysate or the in vitro translated carboxy-terminal truncation of LANA with specific antibodies. NS, nonspecific; IVT, in vitro translated.

compartments (Fig. 4A). Therefore, LANA may be contributing directly or indirectly to nuclear translocation of RBP-Jκ. This may provide interesting insights as to the functional role of the interaction.

One question that needed addressing is whether or not the pool of RBP-Jκ was sequestered by LANA in pleural effusion lymphoma cells positive for KSHV. To determine if endogenous LANA and RBP-Jκ colocalize in similar compartments in latently KSHV infected BCBL1 and BC3 cells, cells were fixed on slides and probed with rabbit polyclonal antibody against RBP-Jκ and human antiserum specific for LANA. The antibody-protein complexes were visualized with Texas Red- and fluorescein isothiocyanate-conjugated secondary antibodies, respectively. The results clearly demonstrated that endogenous LANA and RBP-Jκ are colocalized in similar nuclear compartments in BCBL1 as well as BC3 cells. However, diffuse RBP-Jκ signals were also observed in the nucleus as well as in the cytoplasm of the cells, suggesting that RBP-Jκ may be involved in multiple cellular processes throughout the cell involving events in the cytoplasm as well as the nucleus (Fig. 4B). Surprisingly, similar amounts of RBP-Jκ signals were observed in the cytoplasm of both BCBL1 and BC3 KSHV-infected cells. Therefore, the function of RBP-Jκ has been adapted to that necessary for long-term survival of KSHV-positive pleural effusion lymphoma cells.

RBP-Jκ interacting with LANA can form complexes bound to its cognate sequence in vitro. The results of the luciferase reporter assays above indicate that the presence of potential RBP-Jκ binding sites within the Rta promoter are important for LANA's ability to repress the promoter. Therefore, to address whether LANA represses the Rta promoter through the formation of an RBP-Jκ/LANA complex bound to the *cis*-acting DNA element, an electrophoretic mobility shift assay was performed. A probe was designed that contained the furthest downstream RBP-Jκ consensus binding sequence as well as the six adjacent bases both 5′ and 3′ to the cognate sequence (60). This particular binding site was chosen for the probe as its deletion was shown to have the greatest effect on the ability of LANA to repress the RTA promoter in the assay above.

The results of the electrophoretic mobility shift assay show that a specific RBP-Jκ shift was observed with the probe in the KSHV-negative BJAB cell nuclear extracts (Fig. 5A, lanes 2 and 5, and B, lane 2) as well as in the presence of in vitro translated RBP-Jκ (Fig. 5A, lane 4, and B, lane 3). The specificity of these bands was demonstrated by their disappearance in the presence of specific competitor probe (Fig. 5A, lane 3, and B, lane 4) compared to nonspecific competitor probe (Fig. 5A, lane 5, and B, lane 5). In the presence of in vitro translated LANA and LANA expressed in KSHV-positive BC3 and BCBL1 cells, an additional shift was observed (asterisk, Fig. 5A, lanes 6 and 7, and B, lane 6).

The specificity of these bands was demonstrated by the use of in vitro-translated carboxy- and amino-terminal truncations of LANA. An additional shift was observed in the presence of the RBP-Jκ binding carboxy-terminal end of LANA compared to the amino-terminal truncation, which is unable to bind RBP-Jκ (Fig. 5B, lanes 7 and 8). The specificity of the shifts was further verified by their supershifting in the presence of specific antibody (Fig. 5A, lanes 8 to 10, and B, lanes 9 and 10), while no effect was observed in the presence of nonspecific

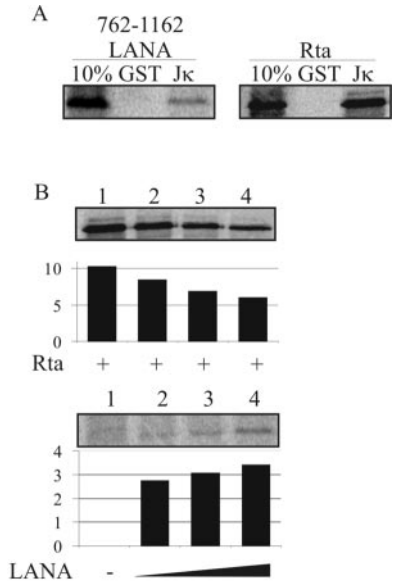

FIG. 6. LANA-Rta competition for RBP-Jκ binding. (A) The 990 to 1162 truncation of LANA and Rta were in vitro transcribed and translated. ^{35}S-labeled products were incubated with GST-RBP-Jκ. Pulldown products were electrophoresed on SDS–8% PAGE gels, dried, and exposed to a PhosphorImager. Input controls of 10% for LANA and for Rta were run as well. (B) Competition in binding to GST-RBP-Jk of in vitro translated Rta and the 990 to 1162 truncation of LANA. The amount of Rta remained constant for all lanes, while the amount of 990 to 1162 LANA was titrated from none in lane 1 to three times the amount of Rta in lane 3. The pulldown products were treated by the method mentioned above.

antibody (Fig. 5A, lane 11, and B, lane 11). The doublet supershifts observed when BCBL1 nuclear extracts were used may be due to the dissociation of other unidentified components of the complex. The data therefore suggest that RBP-Jκ can bind to the consensus binding site within the Rta promoter and that LANA is able to form a complex with RBP-Jκ bound to its specific sequence.

LANA competes with RTA for binding to RBP-Jκ in vitro. The reporter assays above indicate that the ability of Rta to regulate its own promoter is not likely through its interaction with RBP-Jκ. We therefore hypothesized that LANA may play a role in regulating the ability of Rta to activate responsive promoters through RBP-Jκ by competing for direct binding to RBP-Jκ (42). The carboxy terminus of LANA known to bind Rta as well as full-length Rta were in vitro translated with [^{35}S]methionine labeling. The in vitro-translated products were incubated with GST-RBP-Jκ to determine the relative binding affinity of each of the proteins to RBP-Jκ in this assay. The results indicate that Rta binds to RBP-Jκ with greater binding affinity for RBP-Jκ than LANA (Fig. 6A). The binding affinity of LANA was approximately threefold less than that seen for RTA.

To address whether LANA can compete with Rta for this binding, a titration experiment was performed in which the amount of Rta remained constant while the amount of LANA was titrated in increasing amounts to Rta bound to RBP-Jκ. The results of this experiment showed that LANA can compete with and disrupt Rta from binding to RBP-Jκ with about 50% loss of binding with arbitrary counts on the PhosphoImager (Fig. 6B). It should, however, be noted that the quantitative increase in LANA binding was somewhat less than the observed decrease in Rta binding (Fig. 6B, right panel). This suggests that LANA may disrupt as well as compete with Rta for binding to RBP-Jκ. Thus, as increasing amounts of LANA are added, the signal for RTA binding to RBP-Jκ is reduced.

DISCUSSION

The control of latency and reactivation of gammaherpesviruses is a complex process involving a number of critical players. In Epstein-Barr virus, the well-known master regulator BZLF1 controls reactivation from latency, leading to successful production of viral progeny. In KSHV, the BZLF1 homolog b-ZIP (K8) protein is functionally distinct and functions as a transcriptional repressor rather than an activator which induces reactivation of KSHV from latency (30, 47, 67). Rta encoded by KSHV is the predominant regulator master switch of KSHV controlling reactivation from latency (25, 30, 47, 48, 70). Recent studies have suggested that LANA, the major latent nuclear protein, is involved in regulating reactivation from latency by repressing the Rta promoter and downregulating the ability of Rta to autoactivate its own promoter (41). This has interesting potential as a new mechanism by which this virus can control its reactivation by utilizing a molecule that is expressed in all latently infected cells.

Typically, latent gene expression is required for targeting cellular processes, including cell cycle, apoptosis, and signaling. LANA has been shown to contribute to the oncogenic process through its functional interaction with major cellular regulators such as retinoblastoma protein, p53, and the glycogen synthase kinase-3, regulating the Wnt signaling pathway (21, 22, 54). These interactions have strongly supported LANA's role as a critical player mediating KSHV-driven oncogenesis. This new role in regulating reactivation was somewhat surprising but logical. However, the mechanism by which LANA regulates the Rta promoter was not known. This report for the first time nicely establishes a mechanistic link of LANA to a major cellular signaling pathway, the Notch signaling pathway. This once again shows the unique ability of tumor viruses to target essential cellular signaling events for regulating their replication and long-term persistence in the infected cell.

These studies establish a functional interaction between LANA and the major downstream effector of the Notch signaling pathway RBP-Jκ, a member of the CSL (CBF1, Suppressor of Hairless, and Lag) family of proteins (40, 50). These molecules are transcriptional repressors effecting numerous downstream targets of the Notch signaling pathway, which is responsible for determining the fate of cells in the development of higher eukaryotes (1). RBP-Jκ binds to a specific cognate sequence and functions as a repressor of these cellular genes, which include the hairy enhancer of split (HES-1) family of genes (31). Studies showing that viral molecules are capable of targeting the Notch signaling pathway include showing that the Epstein-Barr virus essential activator EBNA2 binds to RBP-Jκ, activating viral major latent promoters and cellular promoters (61, 62).

The Notch family of proteins are single transmembrane receptor proteins that are activated in a ligand-dependent fash-

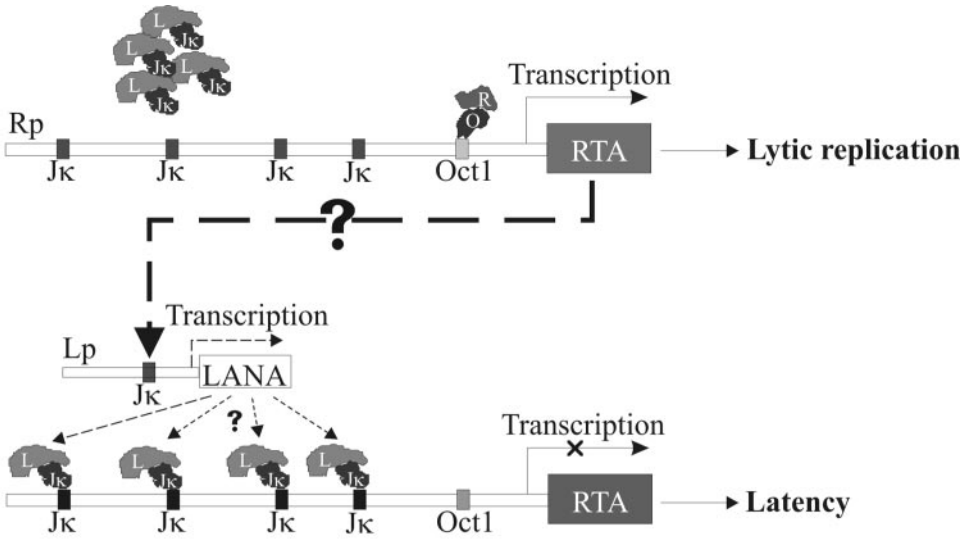

FIG. 7. Hypothetical model for LANA regulation of Rta expression. Rp, Rta promoter; L, LANA; Lp, LANA promoter; R, Rta.

ion (1). A number of these ligands have been identified, including Jagged and Senate, which bind to extracellular repetitive domains (55). This interaction results in the triggering of a series of events which occur in sequence. Ligand-mediated activation results in proteolytic cleavage of Notch1 at sites located in proximity to the transmembrane domain and involves the furinlike convertase and desintegrin metalloproteases encoded by the Kuzbanian gene (1, 55). This event releases the intracellular cytoplasmic tail, which contains the RBP-Jκ domain or RAM domain, containing a critical tryptophan motif 5′ to the ankyrin repeats (2, 72).

The interaction of intracellular activated Notch with RBP-Jκ requires the critical RAM domain but also involves the ankyrin repeats (2, 7). The functional domain of RBP-Jκ has been mapped predominantly to the central domain responsible for repression (15). Interaction of intracellular activated Notch with RBP-Jκ leads to activation of responsive promoters involving complexes with coactivator MAML-1 and acetyltransferases p300, pCAF, and GCN5 (39, 45, 51). LANA has little or no sequence homology with intracellular activated Notch but does have a number of tryptophan residues in the carboxy-terminal 200 amino acids that may be important for binding RBP-Jκ (60). Interestingly, we find that LANA can repress the Rta promoter and that this repression requires the RBP-Jκ binding sites. LANA also binds to RBP-Jκ and may recruit additional molecules involved in repression of promoters, including CBF1 interacting corepressor, silencing mediator of retinoid and thyroid hormone receptors, and histone deacetylases, corepressors, and deacetylases (38).

The facts that LANA is expressed in all latently infected KSHV cells and has been shown to have at least two domains involved in repression activities support our hypothesis that it may play a critical role in repressing exit from latency by regulating expression of the master regulator Rta (38). LANA binding to the downstream effector of the Notch signaling pathway also provides a novel mechanism by which KSHV can regulate reactivation (Fig. 7). Previous studies have also shown that Rta can bind to RBP-Jκ to relieve repression of lytic genes, which results in activation of the lytic promoters (38, 42, 43). This indicates that LANA and Rta may target this major cellular signaling pathway in a temporal manner independent of each other (Fig. 7). Although LANA can bind directly to DNA, we have not shown that LANA can bind specifically to any region of the Rta promoter. In fact there are no specific LANA binding sequences within the Rta promoter, based on sequence data from the BC-1 genome (60). Therefore, LANA can utilize this independent mechanism of targeting a major effector of the Notch cellular signaling pathway to control reactivation from latency by KSHV.

Studies of EBNA2, encoded by Epstein-Barr virus, provide another way in which tumor viruses target the Notch signaling pathway to activate promoters, including viral promoters that regulate expression of the viral oncogenes (26, 69). EBNA2 does not bind directly to DNA but forms complexes with RBP-Jκ at its cognate sequence to activate promoters (69). The binding site on EBNA2 includes a pair of tryptophans that are absolutely required for binding and Epstein-Barr virus transformation of B cells (46). Another Epstein-Barr virus molecule essential for transformation, EBNA3C, regulates this activation and functions similarly to LANA in this respect to modulate the activities for RBP-Jκ-responsive promoters (58, 59). EBNA3C disrupts RBP-Jκ binding to its cognate sequence and can bind directly to RBP-Jκ and competes with EBNA2 for binding to RBP-Jκ, modulating the level of activation (58).

Interestingly, LANA and EBNA proteins are expressed during latency and are critical components of the latent repertoire of genes required to maintain latency and transformation. Here we add another crucial aspect of the process, viral reactivation, which we now show to be regulated by targeting RBP-Jκ, resulting in repression of Rta expression as well as Rta autoactivation of its own promoter. This is likely to be the Achilles heel of reactivation control, as viral genomes incapable of repressing Rta expression will be constitutively active, resulting in production of viral progeny. This will lead to the death of the infected cell. However, RBP-Jκ interaction may be only part of the story, albeit a critical part of the control

mechanism for reactivation. To maintain latency, this process is now shown to be controlled by LANA's usurping the Notch signaling pathway. Continued studies will lead to understanding of the involvement of other viral or cellular genes in reactivation and latency establishment, which are likely to be regulated by LANA binding to RBP-Jκ and so results in repression of these particular genes.

Actually our more recent experiments indicated that Rta also can activate the LANA promoter in vitro (data not shown), and this implies that Rta may contribute to the establishment of latency by activation of LANA during primary KSHV lytic infection or spontaneous reactivation (Fig. 7). A feedback loop in which LANA and Rta regulate each other would be established if we can confirm the above results in vivo. These lines of investigation will elucidate additional targets for potential therapeutic approaches for regulating viral reactivation.

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