Regulation and Autoregulation of the Promoter for the Latency-associated Nuclear Antigen of Kaposi’s Sarcoma-associated Herpesvirus*  

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Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 has been established as the etiological agent of Kaposi’s sarcoma and certain AIDS-associated lymphomas. KSHV establishes latent infection in these tumors, invariably expressing high levels of the viral latency-associated nuclear antigen (LANA) protein. LANA is necessary and sufficient to maintain the KSHV episome. It also modulates viral and cellular transcription and has been implicated directly in oncogenesis because of its ability to bind to the p53 and pRB tumor suppressor proteins. Previously, we identified the LANA promoter (LANAp) and showed that it was positively regulated by LANA itself. Here, we present a detailed mutational analysis and define cis-acting elements and trans-acting factors for the core LANAp. We found that a downstream promoter element, TATA box, and GC box/Spt1 site located at −29 are all individually required for activity. This architecture places LANAp into the small and unusual group of eukaryotic promoters that contain both the downstream promoter element and TATA element but lack a defined initiation site. Furthermore, we demonstrate that LANA regulates its own promoter via its C-terminal domain and does bind to a defined site within the core promoter.

With the demonstration of herpesvirus-like DNA sequences in Kaposi’s sarcoma (KS) biopsy samples but not other tissue (1) and subsequent sequence analysis, Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 was uncovered as an etiological agent of KS. Later, two lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman’s disease (1–4), were shown to also contain KSHV. Since then, exhaustive epidemiological studies have confirmed the role of KSHV in these cancers. KSHV is of the first γ-2 herpesvirus (rhadinovirus) to infect hominoids (5–7). Characteristically, γ-herpesviruses, which also include lymphocryptoviruses such as Epstein-Barr virus (EBV), infect lymphocytes (8, 9). Consistent with this classification, KSHV establishes life-long latent infection in B cells (10–12), which eventually progress to lymphomas in immunocompromised patients. 

Like other herpesviruses, KSHV can enter two different life cycles: lytic replication or latency. During lytic replication, all viral proteins are expressed and viral particles are released from the host cell, eventually resulting in the destruction of the host cell. During latent infection, only a few viral genes are expressed, and these are required for the establishment and maintenance of latency (13–18). In KS, KSHV persists latently in the majority of tumor cells, with fewer than 5% of cells undergoing spontaneous lytic replication (19–22). All stages of KS as well as multicentric Castleman’s disease and PEL, express high levels of the latent proteins, in particular the latency-associated nuclear antigen (LANA) (19, 23–25). The LANA (open reading frame (ORF) 73, LNA) protein is the predominant target of anti-KSHV antibodies in infected individuals. Since KSHV does not encode an ORF with homology to either EBV EBNA-1 or EBNA-2, it has been surmised that KSHV LANA has subsumed the functions of the various EBNA proteins. Although LANA shows no homology at the sequence level, its phenotype and structural features are reminiscent of the EBV EBNA-1 protein, such as an extended central region of repeats. Like EBNA-1, LANA is localized to the nucleus and associated with chromatin. Two nuclear localization signals are located one each in the C-terminal region and N-terminal region (26, 27). The C terminus of LANA is required for dimerization (26) and DNA binding. LANA specifically binds to two 17-bp motifs in the KSHV terminal repeats (TR). This interaction is necessary and sufficient for latent DNA replication (28–30). Chromatin binding is mediated primarily by the N terminus of the protein and tethers the KSHV episome to cellular chromosomes, thereby ensuring proper segregation during host cell division (31–37). In addition to its role in viral episome maintenance, LANA modulates host cell behavior to create a suitable environment for latent KSHV persistence. LANA can cooperate with activated ras in fibroblast transformation assays (38) and activates the human telomerase promoter (39). Biochemical studies show that LANA binds to p53, Rb (38, 40), and a growing number of cellular transcription factors: Ring3, mSin3, ATF/CREB2, and CREB-binding protein (41–44).

We and others have identified a cluster of three latently expressed proteins (v-FLIP, v-cyclin, and LANA) and have...
shown that these proteins were expressed by differentially spliced, multicistronic mRNAs. The latent messages were regulated by the same trans-acting factors that regulate LANAp. An analysis of the methylation status in the LANA promoter revealed the complete absence of methylation in LANAp in PEL and KS infection (48). This phenotype is consistent with the constitutive transcriptional activity of LANAp and sets LANAp apart from other KSHV promoters that are rapidly methylated and silenced upon infection (48). In transgenic mice, LANAp was active in CD19+ B cells but not in CD3+ T cells, demonstrating that the cis-acting elements in LANAp are sufficient for appropriate lineage specificity (49) and that LANAp, in the absence of other viral genes, is sufficient to drive latent gene expression.

Here, we present a detailed mutational analysis of LANAp to define cis-acting elements and trans-regulatory factors. We demonstrate that LANA promoter activity depends on a downstream promoter element (DPE) and TATA box as well as cis-regulated by the same transcription factor binding sites of LANAp were generated using the GeneTailor™ site-directed mutagenesis system (Invitrogen). The two transacting elements in LANAp are sufficient for appropriate transcriptional activity of LANAp and sets LANAp apart from other KSHV promoters that are rapidly methylated and silenced upon infection (48). In transgenic mice, LANAp was active in CD19+ B cells but not in CD3+ T cells, demonstrating that the cis-acting elements in LANAp are sufficient for appropriate lineage specificity (49) and that LANAp, in the absence of other viral genes, is sufficient to drive latent gene expression.

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Western Blot Analysis—HEK293 cells were transfected with 10 μg of total DNA using 20 μl of Superfect (Qiagen, Valencia, CA) per 10-cm dish. 48 h after the transfection, whole cell extracts were prepared in 300 μl of sample buffer (80 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% sucrose, and 0.004% bromphenol blue) with boiling for 5 min. Lysates were electrophoretically separated on Bio-Rad 4–15% gradient-
RESULTS

Sequences Downstream of the Transcription Start Site Contribute to Core Promoter Activity—The side-by-side comparison of the deletion clones from Sarid et al. (14) with those from Jeong et al. (47) allowed us to further define LANAp. Confirming previous observations, both sets of clones exhibited significant constitutive promoter activity as long as sequences up to −88 (127,968) were included. However, the deletion clones starting at +11 (127,869) showed a generally lower level of activity compared with the clones starting at +271 (127,609). Of the two deletion mutants that extended to −279 (128,159) and differed only by their proximal site at +271 (127,609) or +11 (127,869), the clone starting at +271 exhibited ≥10-fold promoter activity, whereas the clone starting at +11 exhibited only ≥3-fold promoter activity above vector control (Fig. 1A).

A second difference between the +271 to −279 reporter construct (pDD83) and the +11 to −279 reporter construct (pGL3−3) was that in the latter the KSHV promoter region was cloned into the multiple cloning site of pGL3 basic (Promega), whereas pDD83 starts at the luciferase start codon and replaces the 5′-UTR and translation-initiation sequence originally present in pGL3 basic with KSHV sequences (Fig. 1B). To investigate the influence of this difference, we cloned the region +271 to −279 into the multiple cloning sites of pGL3 basic as shown in Fig. 1B. This yielded pDD753 retained the 5′-UTR of pGL3 basic and differed from pGL3−3 by 291 nucleotides downstream of LANA promoter start site. As shown in Fig. 1D, transfection assays in HEK293 cells with pDD465 (pGL3−3) and pDD753 showed significant differences in promoter activity, demonstrating that sequences between +11 and +271 contribute to LANA promoter activity. Similar results were obtained in the K562-derived SLK, the B cell-derived BJAB and the PEL-derived BCBL-1 cell lines (data not shown).

Transposon-mediated Insertion Mutagenesis—To identify additional cis-acting elements in the LANA region, we subjected our reporter plasmid pDD365 (+271 to −279) to in vitro transposon mutagenesis using the EZ::TN in-frame insertion mutation kit (Epicenter Technologies). The side-by-side comparison of the transposition initiation site of LANAp; *, alternative transcriptional initiation site mapped by Sarid et al. (14). S.E. is indicated by error bars.
control, suggesting that the transposons did not disrupt an essential site. We also investigated whether any of these insertion mutants had lost the ability to be transactivated by LANA (data not shown). None of them did, suggesting that LANA's transactivation of LANAp uses other sites in the promoter. Based on our deletion analysis, we conclude that essential LANAp elements are located between +11 and +331 but not distal to +88 relative to the transcription start site. The loss-of-function transposon mutant at +43 prompted us to investigate the core LANA promoter in detail.

Core Promoter Function Depends on TATA Box, DPE, and Sp1 Elements—To investigate specific transcriptional factors that would be important for core LANAp activity, we introduced targeted mutations into predicted cis-acting elements in the LANAp (pDD83). Fig. 2A shows a detailed sequence analysis and insertion mutagenesis. In general, eukaryotic promoters are considered either TATA-containing or TATA-less. In TATA-containing promoters, the TATA box is located ~30 nucleotides upstream of the transcription initiation site (50, 51). The LANA promoter did not contain a TATA box consensus site 30 bp upstream of the latent LANA mRNA start site (indicated by 1 in Fig. 2A) as previously determined (19). TATA-less promoters invariably contain a DPE located at about 30 nucleotides downstream of the transcription initiation site. The DPE consensus sequence is 5’-(A/G)(A/T)(C/T)(G/A/C) (52). We identified such a sequence in the LANA promoter at position +32 (127,848) (i.e. within the required distance of the LANA start site at 127,880). Alternatively, Sarid et al. (14) identified additional, distal transcription initiator sites at +19 (127,899, indicated by a star in Fig. 2A) and +69 (127,949) relative to the start site at +1. The transcription initiation site at +19 is located 34 bp downstream of a TATA consensus motif. Therefore, we investigated two hypotheses. (a) Is the DPE required for LANAp activity and/or (b) is the TATA element required for LANAp activity?

The activity of DPE-containing promoters is dramatically decreased if the exact spacing between the DPE and the transcription initiation site is altered (50). To examine the possibility that LANAp was dependent on the DPE, we introduced a 6-bp PstI linker insertion between the DPE and the transcription initiation site (pDD413). As shown in Fig. 2, B–E, this insertion mutation completely abrogated LANAp activity in every cell line tested. To determine the importance of the DPE directly, we introduced a targeted mutation (CG to AT) into the DPE element (pDD788). This point mutation also destroyed LANAp activity (Fig. 2, B–E), which demonstrates that LANAp activity was dependent on the DPE.

To examine the possibility that LANAp activity was dependent on the TATA box, we performed mutagenesis of the TATA box. Mutation (TA to GC) of the putative TATA box abolished LANAp activity (pDD784 in Fig. 2, B–E). A 6-bp linker inser-
tion (pDD461) between +1 and the putative TATA box also abolished activity. A larger 57-bp transposon insertion (pDD711) between the start site and putative TATA box totally demolished LANAp activity, suggesting that the TATA box is also required for LANAp activity. These data strongly suggest that the activity of LANAp was simultaneously dependent on a TATA box as well as DPE.

Sequence analysis revealed two putative GC box/Sp1 binding sites at positions −29 and −46 in LANAp. To examine the contribution of these sites to LANAp activity, we introduced 2-bp substitution mutations into the sites. As shown in Fig. 2, B–E, the mutation introduced into the first GC box/Sp1 binding site (Sp1B at −29 and −30) completely abrogated LANAp activity (pDD780), whereas two independent mutations in the second GC box/Sp1 binding site (Sp1C at −46 and −47 or at −50 and −51) reduced the promoter activity by 50% (pDD782 and pDD783). These data demonstrate that the GC box/Sp1 site at −29 was essential for LANAp activity, whereas the GC box/Sp1 site at position −46 augmented promoter activity. In summary, we identified three cis-acting elements (TATA, DPE, and GC box/Sp1) within the core LANAp that were independently required for activity.

Autoregulation of the LANAp by LANA Protein—We previously reported that LANA transactivates its own promoter (47, 53) but had been unable to demonstrate full-length LANA binding to the LANAp +271 to −279 fragment directly (29). This may have been due to technical difficulties of LANA-based electrophoretic mobility shift assay, but it left open the alternative possibility that LANA, like adenovirus E1A or EBV EBNA-2, regulated transcription via protein-protein interactions and did not contact DNA directly (54, 55). To map the domain of LANA that was required to activate LANAp, we transfected different deletion mutants of the LANA protein together with a reporter plasmid containing the LANAp (Fig. 3A). As depicted in Fig. 3B, two mutants, each containing different C-terminal deletions, both lost their ability to transactivate the LANAp. In contrast, mutants that contain central domain deletions but retain their intact C-terminal domain transactivated the LANAp similarly to wild-type LANA. These data suggest that the central domain of LANA is dispensable for the transactivation of its own promoter. We obtained essentially similar data using a second set of mutants (29) with larger deletions of the central domain (data not shown).

To confirm expression of these mutants, we performed Western blot analysis using polyclonal rabbit antiserum against LANA (Fig. 3C). All mutants were expressed upon transient transfection and migrated at the expected size. The wild type LANA in pDD105 contained shorter repeats in central domain
LANA Protein Binds to an Essential GC Box/Sp1 Motif in Its Own Promoter—To investigate the binding of cellular transcription factors to the essential GC box/Sp1 binding site at −29 of the LANA promoter, we performed an electrophoretic mobility shift assay. Seven protein-DNA complexes (complexes a, b, c, d, e, and f) were observed in BCBL-1 cells using an oligonucleotide containing the predicted GC box/Sp1 binding site (−29) in LANA (Fig. 4). Increasing amounts of the unlabeled probe diminished the complexes in a dose-dependent fashion. Since complexes a and b changed size upon competition, they probably represent multimeric complexes (Fig. 4, lanes 2–4). A 100-fold molar excess of an unrelated sequence (NF-xB) did not compete for binding (Fig. 4, lane 5). Competition experiments with increased amounts of an unlabeled Sp1 consensus oligonucleotide did not compete these complexes (Fig. 4, lanes 6–8). The addition of anti-LANA antisera yielded a supershifted band (Fig. 4, lane 9), indicating that some of these complexes contained LANA. As a positive control, a Sp1 consensus oligonucleotide was incubated with BCBl-1 extract (Fig. 4, lanes 10–19). Here, increasing amounts of the unlabeled Sp1 binding site diminished the complexes in a dose-dependent fashion (Fig. 4, lanes 15–17). Increasing amounts of unlabeled LANA probe oligonucleotide competed for some of the lower migrating complexes but not the main Sp1 complex (Fig. 4, lanes 11–13), consistent with the limited sequence similarity of both sites. A 100-fold molar excess of an unrelated sequence (NF-xB) did not compete with any complex (Fig. 4, lane 14). The addition of anti-LANA antisera did not change the banding pattern (Fig. 4, lane 18), but the addition of an anti-Sp1 specific antisera did (Fig. 4, lane 19).

The LANA binding motif in the TR is a 20-bp GC-rich imperfect palindromic palindrome (5′-cccactgcggcggccggagg-3′), which also fits a minimal GC box/Sp1 consensus sequence 5′-GGCC/AG. Hence, we tested whether a highly purified LANA C-terminal fragment that was known to bind to the TR (28, 29) was able to bind the GC box/Sp1 element in LANA. Shown in Fig. 5 is an electrophoretic mobility shift assay using as probe either the GC box/Sp1 element in LANA (LANAp) or a consensus Sp1 element (Sp1). The same multiple complexes as in Fig. 4 were visible in BCBL-1 extracts (Fig. 5, lane 2 in both panels). They were competed by a 100-fold excess of unlabeled probe (Fig. 5, lane 3 in both panels), but not a 100-fold excess of an irrelevant NF-xB probe (Fig. 5, lane 4 in both panels). Purified LANA C terminus shifts LANAp (Fig. 5A, lane 6) but not Sp1 probe (Fig. 5B, lane 6). In contrast, purified human Sp1 shifted a Sp1 consensus probe (Fig. 5B, lane 5) but not the
Fig. 7. Inhibition of the LANAp by ORF 50. A, luciferase activity in HEK293 cells after cotransfection with an ORF 50 expression vector or an empty expression vector together with reporter plasmids containing different LANAp regions. −279 RS, a reporter plasmid (pDD465 in Fig. 1) containing LANAp region between −20 and −279. B, luciferase activity in HEK293 cells after cotransfection with an ORF 50 expression vector or an empty expression vector together with a reporter plasmid containing vGPCR promoter (pDD163). vGPCR, viral G protein-coupled receptors. Bars represent the mean relative luciferase activity after 48 h of triplicate experiments. The -fold promoter activity with the expression of ORF 50 relative to the promoter activity without ORF 50 expression is indicated (A and B).

Fig. 8. Model for the regulation of the minimal regulatory region of LANAp based on Kutach et al. (65). Pol II, polymerase II; TBP, TATA-binding protein.

LANAp probe (Fig. 5A, lanes 5). This demonstrates that LANA protein can bind to an essential GC box/Sp1 binding site in its own promoter but not a consensus Sp1 site and that the predicted GC box/Sp1 in the LANAp does not bind purified human Sp1. Whether this site can bind other Sp1/Krueppel family members is currently under investigation.

To test the relative affinities of the three related sites LANAp (5′-ATTGTCCGGCGCCGGTGG), TR (5′-CCCCAGCCCGGGCGGGAGG), and consensus Sp1 (5′-CCCTTGTT-GGGGCGGCGGCTAACAGCTGG) directly, we performed cross-competition experiments in BCBl-1 extracts. Equal amounts of LANAp, NF-κB, TR, and Sp1 probe were used to compete a labeled LANAp probe (Fig. 6A) or a TR probe (Fig. 6B) or a Sp1 probe (Fig. 6C). The LANAp site did not compete efficiently with the TR site (Fig. 6B), whereas the TR site competed efficiently with itself and with the LANAp site (Fig. 6A), suggesting that the TR site had a higher affinity for DNA binding activities in BCBl-1 sites than the LANAp. An unrelated probe (NF-κB) did not compete with any of the three probes. An Sp1 consensus oligonucleotide did not compete with either the LANAp or TR sites (Fig. 6C), reemphasizing that in BCBl-1 cells under the binding conditions used here these sites were different.

KSHV ORF 50/Rta Inhibits LANAp—As an immediate early lytic gene product, KSHV ORF 50/Rta plays a critical role in the induction of the viral lytic promoters (56–61). The ORF 50/Rta also transactivated the promoter of vGPCR/ORF 74, which partially overlaps with the LANA 5′-UTR (47, 62). This close proximity suggested the hypothesis that ORF 50 was involved in regulating the LANAp. Since LANA (and LANA-2) are the only KSHV mRNAs that are not induced in response to TPA or ORF 50/Rta (15), we hypothesized that perhaps ORF 50/Rta inhibited the LANAp directly. To test this hypothesis, we transfected an ORF 50/Rta expression vector or an empty expression vector together with reporter plasmids containing different deletion mutants of the LANAp into HEK293 cells. As shown in Fig. 7A, ORF 50/Rta expression reduced LANAp activity to as little as 12% of vector control. Repression by ORF 50/Rta did not map to any particular cis-regulatory region of the promoter, suggesting that ORF 50/Rta squelched the LANA promoter by competing for cellular factors. Fig. 7B confirmed ORF 50/Rta protein expression and functionality by testing its transactivation ability on the ORF 74/vGPCRp, as previously defined (47).

DISCUSSION

The results of side-by-side transient transfection studies comparing the deletion clones from Sarid et al. (14) with those from Jeong et al. (47) demonstrated that important regulatory elements in LANAp are present downstream of the mRNA start site (Fig. 1A). The comparison of isogenic reporter plasmids differing only by the presence or absence of 260 bp within the LANAp 5′-UTR revealed significant differences in promoter activity. This implied that sequences between +11 and +271 contribute to LANAp activity (Fig. 1D). We found at least one essential promoter element, the DPE, to be located in this region.

Generally eukaryotic promoters are composed of DNA sequences that direct accurate transcriptional initiation by the RNA polymerase II complex (63, 64). One or all of these sequence motifs can be recognized in most eukaryotic promoters: the TATA box, the initiator element (INR), and the DPE. In TATA-dependent promoters, both the TATA box, typically located about 30 bp upstream of the transcriptional initiation site and bound by the TATA-binding protein subunit of TFII D, and the INR are used to accurately position the basal transcription machinery, leading to a single defined mRNA initiation site (50, 65). In TATA-less promoters, the DPE in conjunction with the INR is used for the same purpose (50, 65). The DPE is typically located 28–32 bp downstream of the transcriptional

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initiation site, and the precise spacing between DPE and the INR site can be found in TATA-dependent and TATA-independent promoters. The INRs encompassing the transcription initiation site can be found in TATA-dependent and TATA-independent promoters, and TFIID has been found to contact the INR site. Although most experimental data are derived from Drosophila promoters, the INR or DPE and INR that have been demonstrated for other promoters for which no other experimental model currently exists.

We propose the arrangement shown in Fig. 2 to accommodate the results of our mutational analyses. Point mutants in the TATA, the DPE, or GC box/Sp1 sites independently abolished promoter activity. In the case of LANAp, the TATA box and DPE alone or in conjunction is not sufficient to stabilize the basal transcription machinery because of the aberrant spacing. In the LANAp, the distance between the DPE and TATA box is ~90 bp (as opposed to the 30-bp distance between TATA and INR or DPE and INR that has been demonstrated for other promoters). We surmise that this would prevent appropriate interactions between these cis-acting elements and their cognate DNA binding subunits in TFIIID and that a third DNA contact, in our case through a GC box/Sp1 site, is required to ensure proper spacing and transcription initiation. Since we have been unable to demonstrate direct binding of Sp1 (Fig. 5) and Sp3 (data not shown) to LANAp, we surmise that other Sp1/Kruppel family members supply this function and that in BCBL-1 cells, LANA itself is part of this complex (Fig. 4).

We found that LANA transactivated LANAp through aa 751–1161, which contains the putative leucine zipper and DNA binding region. By contrast, the central region containing the repeats was not required for LANAp transactivation (Fig. 4). The requirements for autoactivation of LANAp differ from the requirements for activation of the EBV LMP-1 promoter, since Groves et al. (69) showed that a LANA mutant containing amino acids 267–971 but lacking the C-terminal region was able to activate this promoter (69). At this point, we do not know which domains within the C-terminal DNA binding domain are required for LANAp transactivation. We cannot exclude the possibility that more than one domain of LANA may be needed for transactivation, since, for instance, Lim et al. (70) previously demonstrated that the N-terminal 90 amino acids were required for the C-terminal region-mediated DNA binding and dimerization function of LANA (70). In addition, amino acids 1129–1143 were shown to be required for the transactivation of the EBV Cp by LANA (71). Clearly, there are multiple mechanisms through which LANA can regulate target promoters, and these involve multiple regions of the LANA protein. Since KSHV latent persistence is dependent on LANA protein and LANA protein positively regulates its own expression, studies of this feedback circuitry such as presented here may identify novel targets for antiviral drugs, with the potential to clear latent infection.

LANAp overlaps with a lytic gene promoter, K14/vGCR (ORF 74) promoter, which is transactivated by an immediate early lytic gene product, ORF 50/Rta. The ORF 50/Rta transactivator interacts with many host cellular transcription regulators, such as cCAAT/enhancer-binding protein α (72, 73), Sp1 (74), octamer-binding protein (Oct-1) (75), STAT5 (76), CREB-binding protein (77), SWI/SNF (78), TRAP/Mediator (78), MGC 2663 (79), and p53 (80). Hence, we set out to test the hypothesis that ORF 50/Rta can repress LANAp. Such an interaction would explain why LANAp is not induced upon KSHV reactivation (47). As shown in Fig. 6, Rta/ORF 50 repressed LANAp activity up to 10-fold. Because we could not localize a defined site through which this repression was mediated, we suggest that ORF 50 squelches LANAp core activity through protein-protein interactions.

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