View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Carolina Digital Repository

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 16, Issue of April 16, pp. 16822–16831, 2004
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

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

Received for publication, November 24, 2003, and in revised form, January 20, 2004
Published, JBC Papers in Press, January 24, 2004, DOI 10.1074/jbc.M312801200

Joseph H. Jeong‡, Joshua Orvis, Jong Wook Kim, Curtis P. McMurtrey, Rolf Renne§, and Dirk P. Dittmer¶‖

From the University of Oklahoma Health Sciences Center, Department of Microbiology and Immunology, Oklahoma City, Oklahoma 73104 §Case Western Reserve University, Cleveland, Ohio 44106 and ‖University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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/Sp1 site 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)[1] 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 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

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dana-Farber Cancer Institute, Harvard University, Boston, MA 02115.

¶ To whom correspondence should be addressed: University of Oklahoma Health Sciences Center, Dept. of Microbiology and Immunology, 940 Stanton L. Young Blvd., Oklahoma City, OK 73104. Tel.: 405-271-2133 (ext. 46645); Fax: 405-271-3117; E-mail: dirk-dittmer@ouhsc.edu.

[1] The abbreviations used are: KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; PEL, primary effusion lymphoma; EBV, Epstein-Barr virus; LANA, latency-associated nuclear antigen; ORF, open reading frame; TR, terminal repeat(s); CREB, cAMP-response element-binding protein; LANAp, LANA promoter; DPE, downstream promoter element; UTR, untranslated region; aa, amino acid(s); INR, initiator element.

This is an Open Access article under the CC BY license.

shown that these proteins were expressed by differentially spliced, multicistronic mRNAs. The latent messages all were regulated by the same *cis*-regulatory region, which is called the LANA promoter (LANAp) (19, 45, 46). The *cis*-minimal regulatory region of LANA is sufficient for the high level LANAp activity in cell lines of different origin: B cell-derived, endothelium-derived, and epithelium-derived. We and others previously identified DNA fragments upstream of the LANA ORF, which exhibited promoter activity in reporter-based assays (14, 19, 45). Fig. 1*A* summarizes the location of all LANA promoter mutants reported previously or generated for this study and summarizes their promoter activity in HEK293 cells. Similar results were observed in SLK (a KS-derived endothelial cell line) and B cell tumor lines (BJAB and BCBL-1). A set of mutants (pGL3-1 to pGL3-5) were constructed by Sarid *et al.* (14) and kindly provided to us by the authors. All constructs exhibited basal activity independently of the presence of other KSHV proteins. However, our studies also showed that LANAp was regulated positively by LANA and negatively by the p53 tumor suppressor protein (47). Thus, so far these two are the only known *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 members of the Sp1/Krueppel family of transcription factors. LANAp is the first viral promoter that is a member of the class of eukaryotic promoters that contain both a DPE element and a TATA element but lack a defined initiation site.

MATERIALS AND METHODS

Plasmids—All nucleotide sequence positions are based on the BC-1 KSHV isolate as sequenced by Russo *et al.* (82). Plasmids pDD41, pDD83, pDD104, pDD124, pDD125, pDD163, pDD274, and pDD267 were described previously (47, 49). The plasmids pGL3–1 (pDD463), pGL3–2 (pDD464), pGL3–3 (pDD465), pGL3–4 (pDD466), and pGL3–5 (pDD467) were a gift from Yuan Chang (University of Pittsburg, OH) (14). These were originally PCR-amplified from the BC-1-derived KSHV isolate, whereas all other plasmids in this paper were cloned from a KS-derived KSHV λ-library (13). All plasmids were sequenced to confirm sequence and orientation and showed no deviation from the KSHV sequence deposited by Russo *et al.* (82). The 5′-UTR region up to the NcoI site (nucleotides 127,296 to 127,609) in pDD124, pDD125, and pDD168 was PCR-amplified from the BCBL-1 KSHV isolate. The NcoI/NheI fragment of pDD83 was modified to blunt ends using DNA polymerase I, large (Klenow) fragment (New England BioLabs, Beverly, MA). The blunted fragment was inserted into the SmaI site of pBluescript II KS (+/−) (Stratagene, La Jolla, CA) to yield pDD750. The plasmids pDD751 and pDD753 were generated by inserting the SacI/KpnI fragment of pDD750 into the SacI/KpnI sites of pGL3 basic (Promega, Madison, WI) and inserting the XhoI/SacI fragment of pDD750 into the XhoI/SacI sites of pGL3 basic, respectively. Plasmids expressing LANA mutants, LANA1 ΔC50 (pDD770), LANA1 NLS ΔDED (pDD771), LANA1 NLS LZ-C (pDD772), and LANA1 Δ1002–1062 (pDD774) (courtesy of Joonho Choe, Korea Advanced Institute of Science and Technology, Korea), were described previously (70, 83). Plasmids with larger deletions of LANA, LANA-A (pDD720), LANA-AB (pDD721), LANA-BC (pDD722), and LANA-C (pDD7230) were as described by Garber *et al.* (29).

For the construction of the target plasmid for transposon mutagenesis, the NotI restriction site in pDD83 was destroyed by digestion with NotI, Klenow-mediated fill in and blunt religation. This yielded pDD365. pDD365 gives results identical to pDD83, confirming that removal of the NotI site and vector sequences distal to the LANAp insert had no effect on LANAp-dependent reporter gene transcription. Afterward, transposon mutagenesis was performed on pDD365 using the EZ::TN In-Frame Linker Insertion Kit (Epicenter Technologies). This method relies on the ability of a modified 1000-bp Tn5 transposon element-coupled to transposase to insert itself randomly into target DNA *in vitro* (81, 84). Transposon events are identified by kanamycin resistance. DNAs from positive clones were subsequently digested with NotI to release the Kan^R marker and religated to regenerate a NotI site and a 57-bp insertion in the clone. 400 mutants were randomly selected for plasmid purification and analysis. Since the size of the LANAp fragment is 552 bp compared with ~2500 bp (pGL3 basic vector minus origin and minus Amp^R region), we expected one in five clones to contain an insertion in the LANAp region. Unfortunately, of 400 Tn5 mutants, we isolated only 12 mutants with an insertion in the 552-bp KSHV LANAp sequence. This is less than the expected ratio and suggests integration bias on the part of the Tn5 *in vitro* transposon reaction. Transposon insertions in plasmids pDD700–pDD711 were confirmed by sequencing. In order to screen the TN5 mutants, we eliminated a NotI site in the vector backbone, yielding pDD365. PstI linkers (New England BioLabs, Beverly, MA) were ligated into the SmaI and PvuII sites of pDD83 to yield pDD461 and pDD413, respectively. A map of all reporter constructs is shown in Fig. 1. For the PCR-directed site mutagenesis, pDD83 served as the wild-type template. Mutants containing two base pair substitutions in the putative transcription factor binding sites of LANAp were generated using the GeneTailor™ site-directed mutagenesis system (Invitrogen). The two base pair substitutions in pDD780, pDD782, pDD783, and pDD784 are shown in Fig. 2*A* and were confirmed by sequencing. To eliminate the possibility that inadvertent second-site mutations in the luciferase ORF might be responsible for the loss of luciferase activity, we recloned the LANAp region containing 2-bp substitutions that result in loss of function into pGL3 basic. The "recloned" plasmids showed similar promoter activity compared with the parent plasmids, demonstrating that loss of function was due to the internal mutation (data not shown).

Tissue Culture and Transfection—HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (0.05 μg/ml), and streptomycin (5 units/ml) (all from Invitrogen) at 37 °C under 5% CO_2. At day 1, cells were seeded at 5×10^4 cells/ml/well (6-well plate) to reach 50% confluence after 24 h. At day 2, 4000 ng of total DNA together with 1000 ng pDD173 (pCDNA3.1-hislacZ; Invitrogen) was suspended in 150 μl of Dulbecco's modified Eagle's medium (no serum, no antibiotics) and 7.5 μl of Superfect (Qiagen, Valencia, CA). The transfection mixture was incubated for 30 min at room temperature, and the total volume was adjusted to 1.5 ml with complete medium. Cells were washed once with Dulbecco's modified Eagle's medium, and 0.5 ml of transfection mixture was added per triplicate well. Cells were incubated overnight at 37 °C under 5% CO_2, and the medium was exchanged with complete medium. All transfections were repeated at least three times each in triplicate.

Luciferase Activity—Firefly luciferase activity was measured 48 h after transfection using the Dual-Luciferase reporter assay system (Promega) in a Turner TD20/20 luminometer according to the manufacturer's instructions. β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside as described (47) or the Galacto-Light Plus kit (Tropix, Bedford, MA) according to the manufacturer's recommendations.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from BCBL-1 cells and diluted with dilution buffer at 1:1 ratio (12 mM HEPES (pH 7.5), 50 mM KCl, 6 mM $MgCl_2$, 5 μM $ZnSO_4$, 2 mM dithiothreitol, 0.1% Nonidet P-40, and 50% glycerol). Annealed double-stranded oligonucleotides (50 ng) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The binding reaction was performed at room temperature for 30 min in a mixture containing 2 μl of the diluted nuclear extract (~10 μg), 1 μg of poly(dI-dC), 2 μl of 1 M NaCl, 3 μl of 5× binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 20% glycerol), and 2 μl of labeled oligonucleotide. For the competition experiments, unlabeled double-stranded oligonucleotides were added in up to a 100-fold molar excess in the initial reaction mixture. For supershift analysis, after the incubation for 10 min at room temperature, 3 μl of a polyclonal rabbit antibody against LANA (a gift from Jae U. Jung, Harvard University) or a polyclonal goat anti-Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the binding reaction mixture. DNA-protein complexes were analyzed by electrophoresis through 4.7% polyacrylamide gel at 200 V at 4 °C for 2 h.

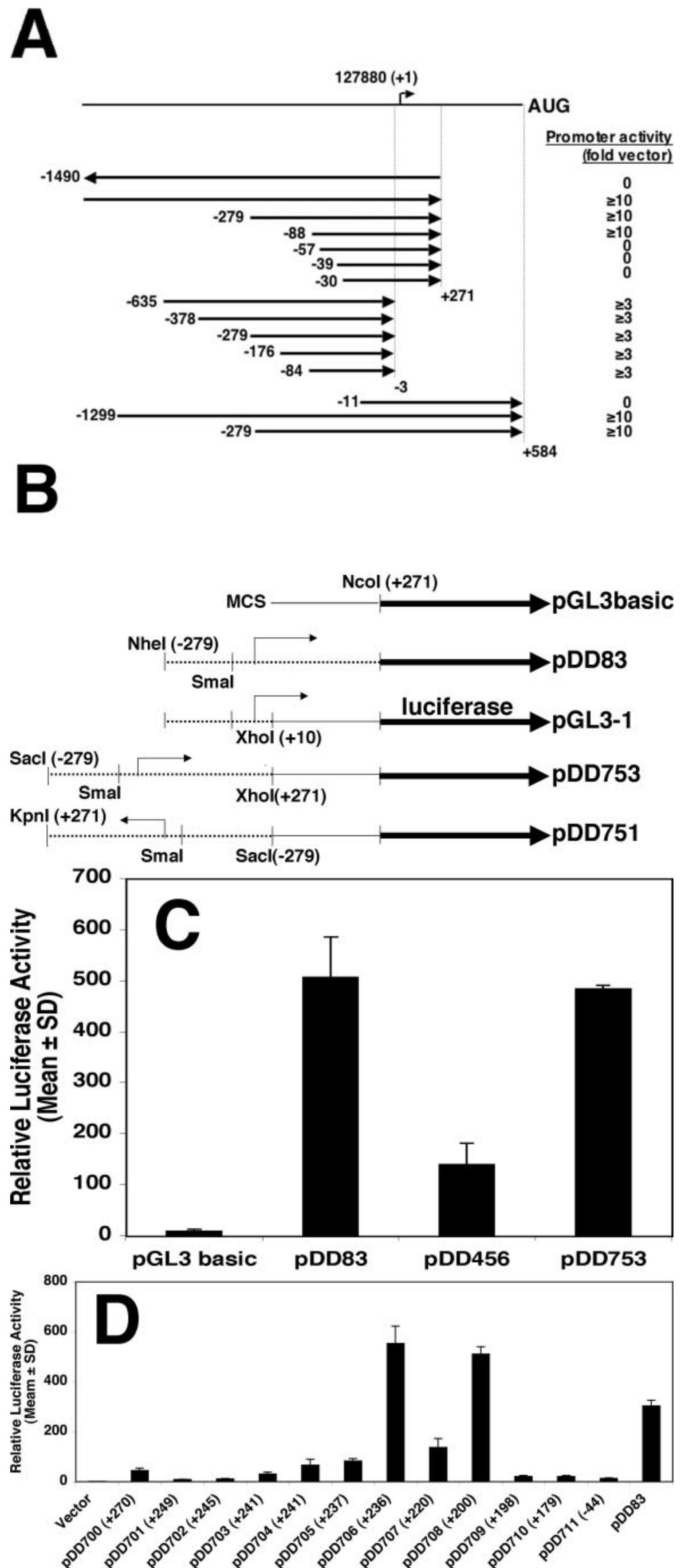

FIG. 1. **Mutational analysis of LANAp.** *A*, summary of reporter plasmids with deletion mutants in LANAp. *≥10* or *0*, relative promoter activity is higher than 10-fold, or less than 10-fold compared with the basal promoter activity of control vector (pGL3 basic). *B*, diagram of the reporter plasmids used in transient transfection assay to investigate the contribution of sequences downstream of +1 transcriptional initiation site to LANAp activity. *MCS*, multiple cloning site. *C*, promoter activity of mutations in LANAp, which affect sequences downstream of the start site. HEK293 cells were transfected with the indicated plasmids. Data represent the mean relative luciferase activity after 48 h of triplicate experiments normalized by co-transfected β-galactosidase activity. S.D. is indicated by *error bars*. *D*, analysis of transposon-generated insertion mutants in LANAp. HEK293 cells were transfected with the indicated plasmids representing insertion mutants in LANAp. Each insertion site is indicated relative to the transcriptional initiation site (127,880 as +1). *Bars* represent the mean relative luciferase activity in triplicate experiments. Transfection efficiency was normalized by co-transfected β-galactosidase activity. S.D. is indicated by *error bars*.

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-

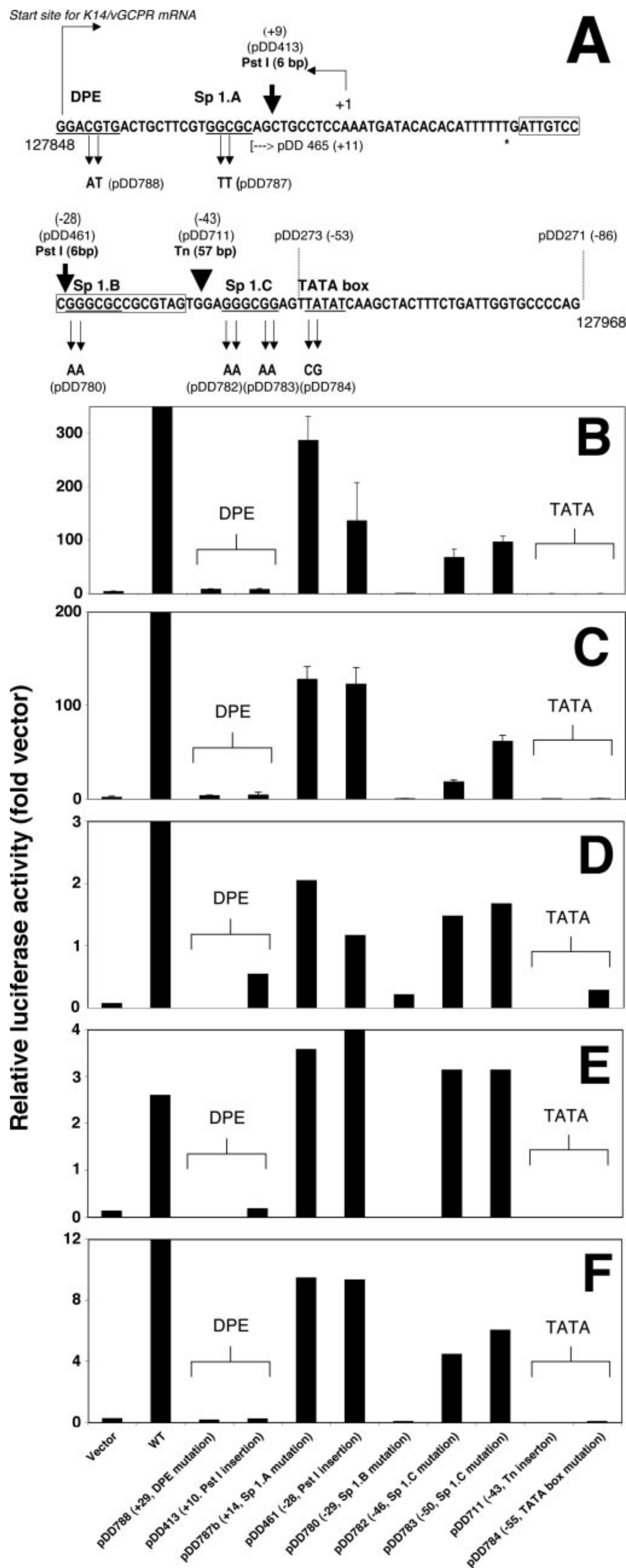

FIG. 2. **Analysis of minimal *cis*-acting elements in LANAp.** *A*, site-directed mutagenesis of putative transcription factors binding sites. 2-bp mutations were introduced into the TATA box and the two putative Sp1 binding sites. Two PstI linker insertion mutations (+9 and −28) and one transposon-mediated insertion mutation (−46) were also introduced in *cis*-minimal regulatory region of LANAp. *B*, luciferase activity in HEK293 cells after transfection with the indicated plasmids. *C*, luciferase activity in SLK cells after transfection with the indicated plasmids. *D*, luciferase activity in BCBL-1 cells after transfection with the indicated plasmids. *E*, luciferase activity in BC-3 cells after transfection with the indicated plasmids. *F*, luciferase activity in BJAB cells after transfection with the indicated plasmids. Data represent the mean -fold induction after 48 h of triplicate experiments. *+1*, transcriptional initiation site of LANAp; *, alternative transcriptional initiation site mapped by Sarid *et al.* (14). S.E. is indicated by *error bars*.

ready gels. Proteins were transferred to Hybond™-P membranes (Amersham Biosciences) in transfer buffer (25 mM Tris-HCl, pH 8.3, 12 mM glycine, and 10% methanol), and the membranes were blocked with 5% milk overnight at 4 °C. The membranes were washed three times with phosphate-buffered saline (0.05% Tween 20) for 10 min each time. The membranes were incubated with a polyclonal rabbit antibody (1:5000 dilution) against LANA (a gift from Jae U. Jung) in Blotto HD (28 mM Tris-HCl, 22 mM Tris base, 1.4 mM $CaCl_2$, 80 mM NaCl, 2% Nonidet P-40, and 0.2% SDS) containing 0.2% bovine serum albumin for 1 h and then washed three times with Blotto HD (0.2% bovine serum albumin). The blots were incubated with horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:5000 dilution; Santa Cruz Biotechnology) in Blotto HD (0.2% bovine serum albumin) for 45 min and subsequently washed three times with Blotto HD. After final washing with phosphate-buffered saline for 10 min, the blots were developed with the Western blotting detection system (Amersham Biosciences).

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. 1*A*).

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 pGL3basic (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. 1*B*). 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. 1*B*. This yielded pDD753 retained the 5′-UTR of pGL3basic and differed from pGL3-3 by 291 nucleotides downstream of LANA promoter start site. As shown in Fig. 1*D*, 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 LANAp activity. Similar results were obtained in the KS-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 LANAp region, we subjected our reporter plasmid pDD365 (+271 to −279) to *in vitro* transposon mutagenesis using the EZ::TN in-frame linker insertion kit (Epicenter Technologies). Fig. 1*D* shows the relative reporter activity of each insertion mutant in HEK293 cells. Mutants pDD700 (+270), pDD701 (+249), pDD702 (+245), pDD703 (+241), pDD704 (+241), pDD705 (+237), pDD707 (+200), pDD709 (+198), pDD710 (+179), and pDD711 (−44) show decreased promoter activities compared with the wild-type control, pDD83. In contrast, mutants pDD706 and pDD708 show promoter activities higher than the wild-type

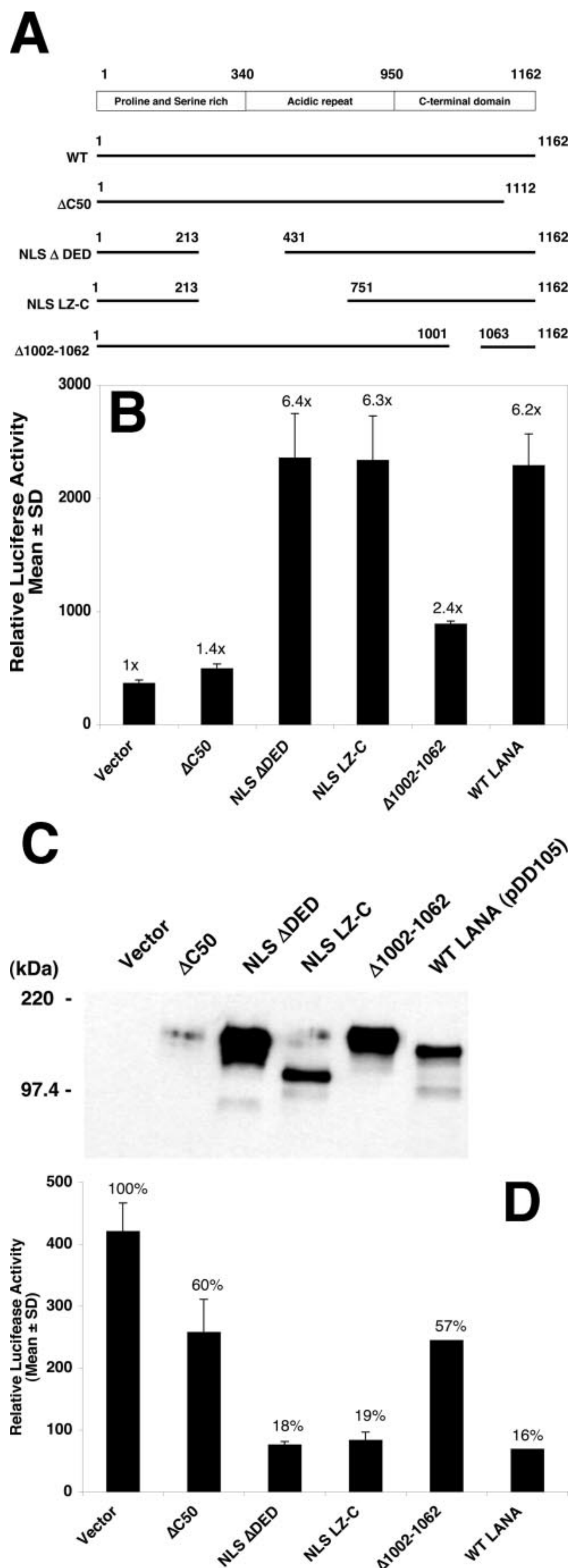

FIG. 3. **Mapping of LANA transactivation domain.** *A*, a schematic diagram of LANA expression vectors containing different LANA deletion mutants as described by Lim *et al.* (42). *B*, luciferase activity in HEK293 after transfection with the indicated LANA expression vectors or with an empty expression vector together with a reporter plasmid containing LANAp. *C*, Western blot analysis of LANA protein expression. HEK293 cells were transfected with 10 μg of total DNA/10-cm dish. Whole cell extracts, prepared 48 h after the transfection, were electrophoretically separated on 4–15% gradient SDS-PAGE gel. The expression of LANA mutants was detected using a polyclonal rabbit antibody (1:5000 dilution) against LANA. The wild type (*WT*) LANA in pDD105 contains shorter acid repeats in the central domain with a total length of 1003 aa, whereas the other mutants are constructed from wild type LANA containing longer repeats with a total length of 1162 aa. *D*, luciferase activity in HEK293 cells after transfection with the indicated LANA expression vectors or with an empty expression vector together with a reporter plasmid containing seven copies of the entire KSHV TR sequence in the pGL3 promoter. *Bars* represent the mean relative luciferase activity after 48 h of triplicate experiments, and transfection efficiency was normalized by co-transfected β-galactosidase activity. S.D. is indicated with *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 +271 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. 2*A* 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. 2*A*) 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)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. 2*A*) 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-

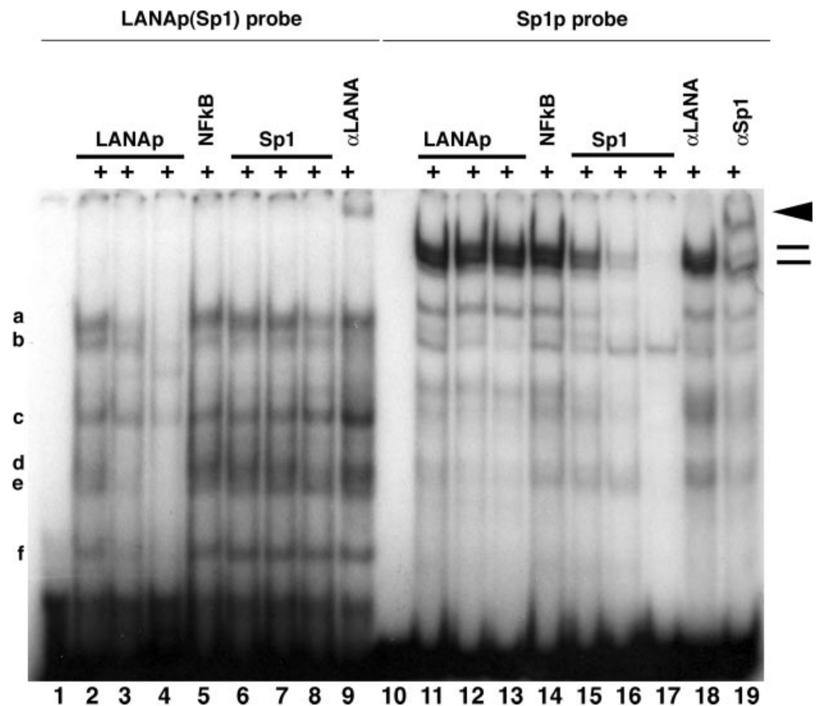

FIG. 4. **DNA binding activities for the essential GC box/Sp1 site in LANAp.** Nuclear extracts were prepared from BCBL-1 cells. Gel shift assay with ^{32}P-labeled oligonucleotide containing a consensus Sp1 binding site (5′-CCCTTG-GTGGGGGCGGGGCCTAAGCTGCG-3′). Competition experiments were performed by the addition of increasing concentrations of unlabeled DNA oligonucleotides, as indicated *above* the gel, and gel shift assay with ^{32}P-labeled LANAp oligonucleotide containing the second putative Sp1-binding-site (5′-ATTGTCCCGGGCG-CCGCGTAG-3′). DNA-protein complexes are labeled with the letters *A–G* on the *right*. *P*, free probe. The *first lane* is probe only.

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 (Sp1.B at −29 and −30) completely abrogated LANAp activity (pDD780), whereas two independent mutations in the second GC box/Sp1 binding site (Sp1.C 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. 3*A*). As depicted in Fig. 3*B*, 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

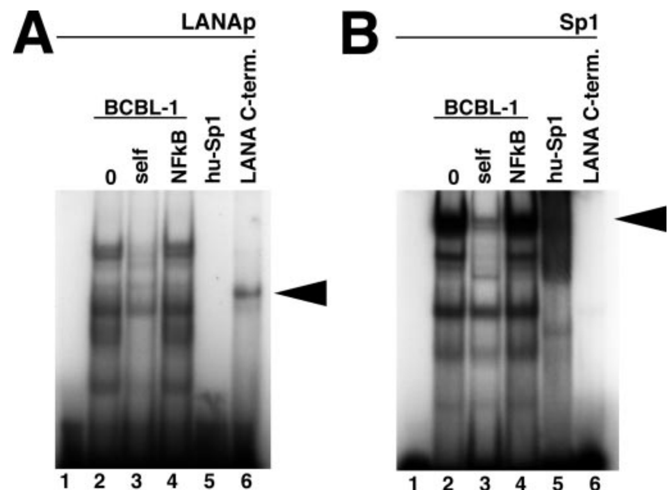

FIG. 5. **Purified LANA C terminus binds to LANAp.** Nuclear extracts were prepared from BCBL-1 cells. *A*, gel shift assay with ^{32}P-labeled LANAp oligonucleotide (5′-ATTGTCCCGGGCGCCGCG-TAG-3′). *Lane 1*, probe only; *lane 2*, probe with BCBl-1 nuclear extract; *lane 3*, probe with BCBL-1 nuclear extract and excess cold probe; *lane 4*, probe with BCBL-1 nuclear extract and excess NF-κB probe; *lane 5*, probe with purified human Sp1 protein; *lane 6*, probe with purified LANA C terminus protein. *B*, gel shift assay with ^{32}P-labeled Sp1 oligonucleotide (5′-CCCTTGGTGGGGGCGGGGCCTAAGCTGCG-3′). *Lane 1*, probe only; *lane 2*, probe with BCBl-1 nuclear extract; *lane 3*, probe with BCBL-1 nuclear extract and excess cold probe; *lane 4*, probe with BCBL-1 nuclear extract and excess NF-κB probe; *lane 5*, probe with purified human Sp1 protein; *lane 6*, probe with purified LANA C terminus protein.

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. 3*C*). 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

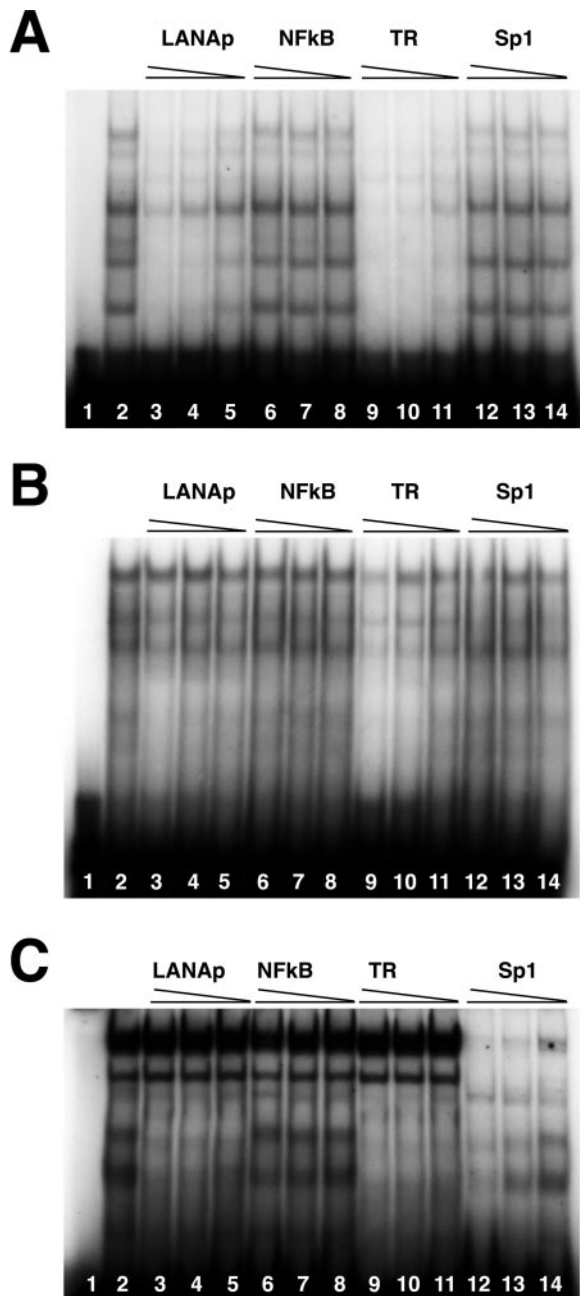

FIG. 6. **Cross-competition between the LANA binding site in the TR and in the LANAp.** Nuclear extracts were prepared from BCBL-1 cells. *A*, gel shift assay with ^{32}P-labeled LANAp oligonucleotide. *Lane 1*, probe only; *lane 2*, probe with BCBl-1 nuclear extract; *lanes 3–5*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled LANAp probe; *lanes 6–8*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled NF-κB probe; *lanes 9–11*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled TR probe; *lanes 12–14*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled Sp1 probe. *B*, gel shift assay with ^{32}P-labeled TR oligonucleotide. *Lane 1*, probe only; *lane 2*, probe with BCBl-1 nuclear extract; *lanes 3–5*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled LANAp probe; *lanes 6–8*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled NF-κB probe; *lanes 9–11*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled TR probe; *lanes 12–14*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled Sp1 probe. *C*, gel shift assay with ^{32}P-labeled Sp1 oligonucleotide, *Lane 1*, probe only; *lane 2*, probe with BCBl-1 nuclear extract; *lanes 3–5*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled LANAp probe; *lanes 6–8*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled NF-κB probe; *lanes 9–11*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled TR probe; *lanes 12–14*, probe with BCBL-1 nuclear extract and decreasing amounts of unlabeled Sp1 probe.

with a total length of 1003 aa, whereas the other mutants were constructed from wild type LANA containing longer repeats with a total length of 1162 aa.

To confirm the functionality of the LANA mutants with regard to their known TR-specific DNA binding, we transfected the different LANA expression plasmids together with a reporter plasmid containing seven copies of the LANA binding site in the KSHV TR region downstream of a minimal SV40 promoter. Previously, Garber *et al.* (28) showed that purified LANA protein bound directly to the TR element and that in the context of this reporter construct, LANA binding resulted in strong transcriptional suppression of the adjacent heterologous SV40 promoter. As shown in Fig. 3*D*, plasmids expressing the intact C-terminal region of LANA showed the transcriptional suppression comparable with wild-type LANA, whereas the two C-terminal LANA mutants did not. This demonstrates that all LANA mutants were expressed, that all were capable of binding to the high affinity TR LANA binding site, and that the central domain was dispensable for LANA's transcriptional activation activity on LANAp.

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 LANAp, 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 LANAp (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-κB) 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 antiserum 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 LANAp oligonucletide 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-κB) did not compete with any complex (Fig. 4, *lane 14*). The addition of anti-LANA antiserum did not change the banding pattern (Fig. 4, *lane 18*), but the addition of an anti-Sp1 specific antiserum did (Fig. 4, *lane 19*).

The LANA binding motif in the TR is a 20-bp GC-rich imperfect palindrome (5′-ccccatgcccgggcgggagg) (29, 35), which also fits a minimal GC box/Sp1 consensus sequence 5′-GG(C/A)G. 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 LANAp. Shown in Fig. 5 is an electrophoretic mobility shift assay using as probe either the GC box/Sp1 element in LANAp (*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-κB probe (Fig. 5, *lane 4* in *both panels*). Purified LANA C terminus shifts LANAp (Fig. 5*A*, *lane 6*) but not Sp1 probe (Fig. 5*B*, *lane 6*). In contrast, purified human Sp1 shifted a Sp1 consensus probe (Fig. 5*B*, *lane 5*) but not the

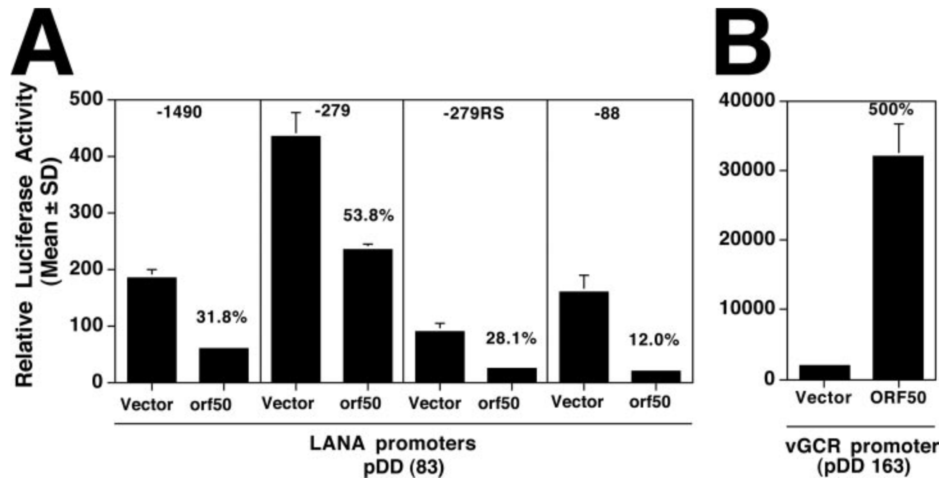

FIG. 7. **Inhibition of the LANAp by ORF 50.** *A*, luciferase activity in HEK293 after cotransfection with an ORF 50 expression vector or an empty expression vector together with reporter plasmids containing different LANAp regions. −279 RS, a reporter plasmid (pDD465 in Fig. 1) containing LANAp region 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*).

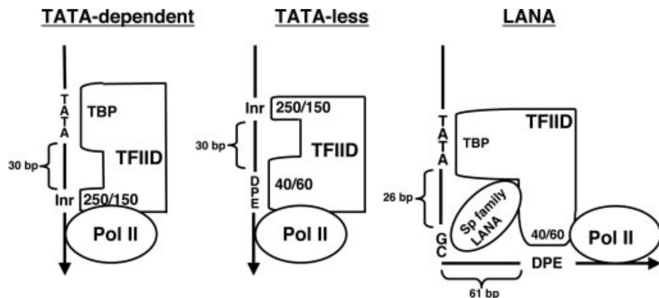

FIG. 8. **Model for the regulation of the minimal regulatory region of LANAp based on Kutach *et al.* (65).** *Pol II*, polymerase II; TBP, TATA-binding protein.

LANAp probe (Fig. 5*A*, *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′-ATTGTCCCGGGCGCCGCGTAG), TR (5′-CCCCA-TGCCCGGGCGGGAGG), and consensus Sp1 (5′-CCCTTGGT-GGGGGCGGGGCCTAAGCTGCG) 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. 6*A*) or a TR probe (Fig. 6*B*) or a Sp1 probe (Fig. 6*C*). The LANAp site did not compete efficiently with the TR site (Fig. 6*B*), whereas the TR site competed efficiently with itself and with the LANAp site (Fig. 6*A*), 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. 6*C*), 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),[2] 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. 7*A*, 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. 7*B* 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. 1*A*). The comparison of isogenic reporter plasmids differing only by the presence or absence of 260 bp within the LANA 5′-UTR revealed significant differences in promoter activity. This implied that sequences between +11 and +271 contribute to LANAp activity (Fig. 1*D*). 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 TFIID, 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

[2] Damania, B., Jeong, J. H., Bowser, B. S., DeWire, S. M., Staudt, M. R., and Dittmer, D. P. (2004) *J. Virol.*, in press.

initiation site, and the precise spacing between DPE and the INR is critical for the cooperative binding of holo-TFIID to a TATA-less core promoter (50, 63). Consistent with this notion, it has been shown that $TAF_{II}60$ and $TAF_{II}40$ of TFIID interact with the DPE to stabilize the transcription initiation complex (50). So far, all known DPE-containing promoters are TATA-less, and all TATA-containing promoters are DPE-less, although most experimental data are derived from *Drosophila* 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 through its $TAF_{II}150$ and $TAF_{II}250$ subunits (66).

The LANAp is different in its core architecture. LANAp does not contain a recognizable INR, which is consistent with the multiple start sites that we and others previously described (19, 46). We found that by mutational analysis, both DPE and TATA box elements were independently required for LANAp function (Fig. 2). This arrangement is rarely found in eukaryotic promoters and is in contrast to the latent EBV EBNA Qp promoter (67, 68), which is TATA-independent. By sequence analysis, only 14% of eukaryotic promoters contain both a DPE and TATA box, whereas ~31% contain neither and 55% contain one or the other (65). Understanding LANAp, in addition to understanding KSHV latency, will yield general insights into the mechanism and significance of this unique group of promoters for which no other experimental model currently exists.

We propose the arrangement shown in Fig. 8 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 TFIID 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/Krueppel 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), STAT3 (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.

Acknowledgments—We thank members of the Dittmer laboratory and B. Damania for critical reading of the manuscript.

REFERENCES

1. Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994) *Science* **266,** 1865–1869
2. Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995) *N. Engl. J. Med.* **332,** 1186–1191
3. Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995) *Blood* **86,** 1276–1280
4. Gessain, A., Sudaka, A., Briere, J., Fouchard, N., Nicola, M. A., Rio, B., Arborio, M., Troussard, X., Audouin, J., Diebold, J., and de Thé, G. (1996) *Blood* **87,** 414–416
5. Hayward, G. S. (1999) *Semin. Cancer Biol.* **9,** 187–199
6. Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., McGeoch, D. J., and Chang, Y. (1996) *J. Virol.* **70,** 549–558
7. Neipel, F., Albrecht, J. C., and Fleckenstein, B. (1998) *J. Natl. Cancer Inst. Monogr.* **23,** 73–77
8. Boshoff, C., and Weiss, R. A. (1998) *Adv. Cancer Res.* **75,** 57–86
9. Damania, B., and Desrosiers, R. C. (2001) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356,** 535–543
10. Decker, L. L., Shankar, P., Khan, G., Freeman, R. B., Dezube, B. J., Lieberman, J., and Thorley-Lawson, D. A. (1996) *J. Exp. Med.* **184,** 283–288
11. Kliche, S., Kremmer, E., Hammerschmidt, W., Koszinowski, U., and Haas, J. (1998) *J. Virol.* **72,** 8143–8149
12. Mesri, E. A., Cesarman, E., Arvanitakis, L., Rafii, S., Moore, M. A., Posnett, D. N., Knowles, D. M., and Asch, A. S. (1996) *J. Exp. Med.* **183,** 2385–2390
13. Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6641–6646
14. Sarid, R., Flore, O., Bohenzky, R. A., Chang, Y., and Moore, P. S. (1998) *J. Virol.* **72,** 1005–1012
15. Fakhari, F. D., and Dittmer, D. P. (2002) *J. Virol.* **76,** 6213–6223
16. Paulose-Murphy, M., Ha, N. K., Xiang, C., Chen, Y., Gillim, L., Yarchoan, R., Meltzer, P., Bittner, M., Trent, J., and Zeichner, S. (2001) *J. Virol.* **75,** 4843–4853
17. Nakamura, H., Lu, M., Gwack, Y., Souvlis, J., Zeichner, S. L., and Jung, J. U. (2003) *J. Virol.* **77,** 4205–4220
18. Jenner, R. G., Alba, M. M., Boshoff, C., and Kellam, P. (2001) *J. Virol.* **75,** 891–902
19. Dittmer, D., Lagunoff, M., Renne, R., Staskus, K., Haase, A., and Ganem, D. (1998) *J. Virol.* **72,** 8309–8315
20. Chang, J., Renne, R., Dittmer, D., and Ganem, D. (2000) *Virology* **266,** 17–25
21. Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997) *J. Virol.* **71,** 715–719
22. Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., McGee, J. O., Weiss, R. A., and O'Leary, J. J. (1995) *Nat. Med.* **1,** 1274–1278
23. Parravicini, C., Chandran, B., Corbellino, M., Berti, E., Paulli, M., Moore, P. S., and Chang, Y. (2000) *Am. J. Pathol.* **156,** 743–749
24. Katano, H., Sato, Y., Kurata, T., Mori, S., and Sata, T. (2000) *Virology* **269,** 335–344
25. Dupin, N., Fisher, C., Kellam, P., Ariad, S., Tulliez, M., Franck, N., van Marck, E., Salmon, D., Gorin, I., Escande, J. P., Weiss, R. A., Alitalo, K., and Boshoff, C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4546–4551
26. Schwam, D. R., Luciano, R. L., Mahajan, S. S., Wong, L., and Wilson, A. C. (2000) *J. Virol.* **74,** 8532–8540
27. Piolot, T., Tramier, M., Coppey, M., Nicolas, J. C., and Marechal, V. (2001) *J. Virol.* **75,** 3948–3959
28. Garber, A. C., Hu, J., and Renne, R. (2002) *J. Biol. Chem.* **277,** 27401–27411
29. Garber, A. C., Shu, M. A., Hu, J., and Renne, R. (2001) *J. Virol.* **75,** 7882–7892
30. Hu, J., Garber, A. C., and Renne, R. (2002) *J. Virol.* **76,** 11677–11687
31. Kedes, D. H., Lagunoff, M., Renne, R., and Ganem, D. (1997) *J. Clin. Invest.* **100,** 2606–2610

32. Kellam, P., Boshoff, C., Whitby, D., Matthews, S., Weiss, R. A., and Talbot, S. J. (1997) *J. Hum. Virol.* **1,** 19–29
33. Rainbow, L., Platt, G. M., Simpson, G. R., Sarid, R., Gao, S. J., Stoiber, H., Herrington, C. S., Moore, P. S., and Schulz, T. F. (1997) *J. Virol.* **71,** 5915–5921
34. Moore, P. S., and Chang, Y. (1998) *J. Natl. Cancer Inst. Monogr.* **23,** 65–71
35. Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999) *Science* **284,** 641–644
36. Cotter, M. A., 2nd, and Robertson, E. S. (1999) *Virology* **264,** 254–264
37. Szekely, l., Kiss, C., Mattsson, K., Kashuba, E., Pokrovskaja, K., Juhasz, A., Holmvall, P., and Klein, G. (1999) *J. Gen. Virol.* **80,** 2889–2900
38. Radkov, S. A., Kellam, P., and Boshoff, C. (2000) *Nat. Med.* **6,** 1121–1127
39. Knight, J. S., Cotter, M. A., II, and Robertson, E. S. (2001) *J. Biol. Chem.* **276,** 22971–22978
40. Friborg, J., Jr., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999) *Nature* **402,** 889–894
41. Lim, C., Gwack, Y., Hwang, S., Kim, S., and Choe, J. (2001) *J. Biol. Chem.* **276,** 31016–31022
42. Lim, C., Sohn, H., Gwack, Y., and Choe, J. (2000) *J. Gen. Virol.* **81,** 2645–2652
43. Krithivas, A., Young, D. B., Liao, G., Greene, D., and Hayward, S. D. (2000) *J. Virol.* **74,** 9637–9645
44. Platt, G. M., Simpson, G. R., Mittnacht, S., and Schulz, T. F. (1999) *J. Virol.* **73,** 9789–9795
45. Talbot, S. J., Weiss, R. A., Kellam, P., and Boshoff, C. (1999) *Virology* **257,** 84–94
46. Sarid, R., Wiezorek, J. S., Moore, P. S., and Chang, Y. (1999) *J. Virol.* **73,** 1438–1446
47. Jeong, J., Papin, J., and Dittmer, D. (2001) *J. Virol.* **75,** 1798–1807
48. Chen, J., Ueda, K., Sakakibara, S., Okuno, T., Parravicini, C., Corbellino, M., and Yamanishi, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4119–4124
49. Jeong, J. H., Hines-Boykin, R., Ash, J. D., and Dittmer, D. P. (2002) *J. Virol.* **76,** 11024–11032
50. Burke, T. W., and Kadonaga, J. T. (1997) *Genes Dev.* **11,** 3020–3031
51. Burke, T. W., and Kadonaga, J. T. (1996) *Genes Dev.* **10,** 711–724
52. Smale, S. T., and Kadonaga, J. T. (2003) *Annu. Rev. Biochem.* **72,** 449–479
53. Renne, R., Barry, C., Dittmer, D., Compitello, N., Brown, P., and Ganem, D. (2001) *J. Virol.* **75,** 458–468
54. Lundblad, J. R., Kwok, R. P., Laurance, M. E., Harter, M. L., and Goodman, R. H. (1995) *Nature* **374,** 85–88
55. Mannervik, M., and Akusjarvi, G. (1997) *FEBS Lett.* **414,** 111–116
56. Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999) *J. Virol.* **73,** 9348–9361
57. Gradoville, L., Gerlach, J., Grogan, E., Shedd, D., Nikiforow, S., Metroka, C., and Miller, G. (2000) *J. Virol.* **74,** 6207–6212
58. Zhu, F. X., Cusano, T., and Yuan, Y. (1999) *J. Virol.* **73,** 5556–5567
59. Miller, G., Heston, L., Grogan, E., Gradoville, L., Rigsby, M., Sun, R., Shedd, D., Kushnaryov, V. M., Grossberg, S., and Chang, Y. (1997) *J. Virol.* **71,** 314–324
60. Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10866–10871
61. Liang, Y., and Ganem, D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8490–8495
62. Guo, H. G., Browning, P., Nicholas, J., Hayward, G. S., Tschachler, E., Jiang, Y. W., Sadowska, M., Raffeld, M., Colombini, S., Gallo, R. C., and Reitz, M. S., Jr. (1997) *Virology* **228,** 371–378
63. Butler, J. E., and Kadonaga, J. T. (2002) *Genes Dev.* **16,** 2583–2592
64. Kadonaga, J. T. (1990) *Curr. Opin. Cell Biol.* **2,** 496–501
65. Kutach, A. K., and Kadonaga, J. T. (2000) *Mol. Cell. Biol.* **20,** 4754–4764
66. Tora, L. (2002) *Genes Dev.* **16,** 673–675
67. Nonkwelo, C., Ruf, I. K., and Sample, J. (1997) *J. Virol.* **71,** 354–361
68. Nonkwelo, C., Skinner, J., Bell, A., Rickinson, A., and Sample, J. (1996) *J. Virol.* **70,** 623–627
69. Groves, A. K., Cotter, M. A., Subramanian, C., and Robertson, E. S. (2001) *J. Virol.* **75,** 9446–9457
70. Lim, C., Sohn, H., Lee, D., Gwack, Y., and Choe, J. (2002) *J. Virol.* **76,** 10320–10331
71. Viejo-Borbolla, A., Kati, E., Sheldon, J. A., Nathan, K., Mattsson, K., Szekely, L., and Schulz, T. F. (2003) *J. Virol.* **77,** 7093–7100
72. Wu, F. Y., Tang, Q. Q., Chen, H., ApRhys, C., Farrell, C., Chen, J., Fujimuro, M., Lane, M. D., and Hayward, G. S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 10683–10688
73. Wang, S. E., Wu, F. Y., Fujimuro, M., Zong, J., Hayward, S. D., and Hayward, G. S. (2003) *J. Virol.* **77,** 600–623
74. Zhang, L., Chiu, J., and Lin, J. C. (1998) *DNA Cell Biol.* **17,** 735–742
75. Sakakibara, S., Ueda, K., Chen, J., Okuno, T., and Yamanishi, K. (2001) *J. Virol.* **75,** 6894–6900
76. Gwack, Y., Hwang, S., Lim, C., Won, Y. S., Lee, C. H., and Choe, J. (2002) *J. Biol. Chem.* **277,** 6438–6442
77. Gwack, Y., Byun, H., Hwang, S., Lim, C., and Choe, J. (2001) *J. Virol.* **75,** 1909–1917
78. Gwack, Y., Baek, H. J., Nakamura, H., Lee, S. H., Meisterernst, M., Roeder, R. G., and Jung, J. U. (2003) *Mol. Cell. Biol.* **23,** 2055–2067
79. Wang, S., Liu, S., Wu, M. H., Geng, Y., and Wood, C. (2001) *J. Virol.* **75,** 11961–11973
80. Gwack, Y., Hwang, S., Byun, H., Lim, C., Kim, J. W., Choi, E. J., and Choe, J. (2001) *J. Virol.* **75,** 6245–6248
81. Goryshin, I. Y., Miller, J. A., Kil, Y. V., Lanzov, V. A., and Reznikoff, W. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10716–10721
82. Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14862–14867
83. Lim, C., Lee, D., Seo, T., Choi, C., and Choe, J. (2003) *J. Biol. Chem.* **278,** 7397–7405
84. Goryshin, I. Y., and Reznikoff, W. S. (1998) *J. Biol. Chem.* **273,** 7367–7374