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Brd2/RING3 Interacts with a Chromatin-Binding Domain in the Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen 1 (LANA-1) That Is Required for Multiple Functions of LANA-1

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Latency-associated nuclear antigen 1 (LANA-1) of Kaposi's sarcoma-associated herpesvirus (KSHV) mediates the episomal replication of the KSHV genome, as well as transcriptional regulation, in latently infected cells. Interaction of LANA-1 with cellular chromatin is required for both these functions. An N-terminal heterochromatin-binding site in LANA-1 is essential for the replication and maintenance of latent episomes, as well as transcriptional regulation. We have recently described a C-terminal domain in LANA-1 that modulates the interaction with cellular interphase chromatin or elements of the nuclear matrix. Here, we used a series of LANA-1 deletion mutants to investigate the relationship between the different functions of LANA-1 and its interaction with the host chromatin-binding protein Brd2/RING3. Our findings suggest that the C-terminal chromatin-binding domain in LANA-1 is required for multiple LANA-1 functions, including the ability to bind to and replicate viral episomal DNA, to modulate transcription, and to interact with Brd2/RING3. Similar to the recently described tethering of bovine papillomavirus E2 protein to host chromatin via Brd4/MCAP, Brd2/RING3, another member of the Brd family of chromatin-binding proteins, therefore interacts with a chromatin-binding region of another viral latent nuclear protein and could play a role in its multiple functions.

Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8, is an indispensable infectious cause of Kaposi's sarcoma (KS), and is also linked to primary effusion lymphoma and multicentric Castleman's disease (63). KSHV infection is characterized by a long latency period during which only a few genes are expressed (43, 48, 59, 68). Among them is open reading frame 73, whose product, the latency-associated nuclear antigen 1 (LANA-1), can be detected in the nucleus of virtually all KSHV-infected cells by immunofluorescence or immunohistochemistry (13, 28, 48).

LANA-1 is a multifunctional protein with an apparent molecular mass of approximately 220 to 232 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). LANA-1 is required for the replication and long-term persistence of the KSHV episomal genome in dividing cells (3, 4, 19, 23, 25). LANA-1 binds to mitotic chromosomes via its amino (N)-terminal region (amino acids [aa] 5 to 22) and to a 16-bp imperfect palindrome within the origin of replication (*oriP*) located in the KSHV terminal repeat (TR) through its carboxy (C)-terminal region (amino acids 996 to 1139) (19, 29, 45, 64). Binding of LANA-1 to mitotic heterochromatin via its N-terminal chromatin-binding domain is essential for viral replication (31, 56). In addition, we have recently shown that a domain in the C-terminal region of LANA-1, bordered by aa 1143, affects the association of LANA-1 with components of interphase nuclear heterochromatin and the nuclear matrix (64). The binding of the C-terminal domain of LANA-1 to viral DNA and its ability to form oligomers in solution (29, 55) are reminiscent of other viral DNA-binding proteins such as Epstein-Barr virus nuclear antigen 1 (EBNA-1), the E1/E2 complex of papillomavirus, and the simian virus 40 large T antigen (7, 11, 14, 38, 61, 66).

To mediate replication, these proteins must promote the assembly of an initiation-of-replication complex by recruiting cellular factors at or near their binding sites. One example of such association is the interaction of EBNA-1 and LANA-1 with the origin recognition complex (54, 60), a complex of six subunits involved in heterochromatin and chromosome condensation that allows assembly of prereplication complexes in specific sites of the genome (37, 42, 44). E2 and EBNA-1 also activate transcription when bound to their respective *oriP*s, and this activity has been proposed to be required for initiation of DNA replication (49, 50, 58). In contrast, LANA-1 represses downstream promoters when bound to the TR, suggesting the presence of an enhancer region within the TR that might facilitate the formation of the initiation-of-replication complex (20). Whether the ability of LANA-1 to repress transcription and to replicate a TR-containing plasmid when bound to the TR are coupled processes is unknown at present.

LANA-1 is a pleiotropic transcriptional regulator capable of activating, as well as repressing, heterologous viral and cellular

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promoters (19, 51, 64); it interacts with proteins or protein complexes such as CREB, CBP, AP1, mSIN3, or Sp1 (2, 31, 33, 36, 62). LANA-1 also interacts with p53, retinoblastoma protein (pRb), and glycogen synthase kinase 3β (GSK-3β); inhibits the activation of p53-dependent promoters; induces the activation of E2 promoter-binding factor (E2F)-dependent genes; and promotes entry into the S phase of the cell cycle (16, 17, 47).

It has been proposed that binding of LANA-1 to mitotic chromosomes via its N-terminal end occurs through interaction with cellular proteins such as methyl-CpG-binding protein 2 (MeCP2) (30). MeCP2 is the first reported member of a family of proteins that bind methylated CpG (32). MeCP2 silences gene expression through recruitment of the histone deacetylase machinery (6, 27, 41), through facilitation of methylation of histone H3 (18), and through condensation of chromatin (21). Additional components of the nuclear chromatin interacting with LANA-1 include DEK-1, reported to bind to its C-terminal domain (30), heterochromatin protein 1 (34, 53, 60), and histone H1 (9).

LANA-1 also recruits RING3/Brd2, a member of the *Drosophila* female sterile gene (*fsh*)/BET/Brd family of proteins involved in E2F-mediated activation of cell cycle regulatory genes, to chromatin (24, 40, 46). RING3/Brd2 mediates phosphorylation of Ser/Thr residues within the C-terminal domain of LANA-1 in in vitro assays (46). BET proteins form a family of transcriptional regulators characterized by the presence of one or two N-terminal bromodomains required for chromatin binding and an extraterminal (ET) domain involved in protein-protein interaction (15). Members of this family have been shown to play roles in cell cycle control. RING3/Brd2 and E2F interact in protein complexes isolated from nuclear extracts, and this interaction has been hypothesized to promote G_1-S transition (12, 24). A murine homologue of RING3/Brd2 has been shown to be part of the murine mediator complex (26). Another BET protein, Brd4/MCAP, plays a role in S-phase entry (39) and seems to be required for E2-mediated bovine papillomavirus (BPV) episome persistence and transformation (5, 8, 67). These observations suggest that the BET homologue RING3/Brd2 could be one of the proteins that LANA-1 utilizes to adhere to nuclear chromatin, modulate transcription, or promote replication-segregation of KSHV genomes.

In view of the role of the C-terminal domain of LANA-1 in heterochromatin interactions (64), replication of viral episomes (25), and transcriptional activation and repression (20, 22, 55), we have in this study characterized a series of LANA-1 deletion mutants for their ability to mediate individual functions of LANA-1 and to interact with two nuclear chromatin-associated proteins, RING3/Brd2 and MeCP2. We show that only mutants that retain functional activity are still able to interact with Brd2/RING3. The results indicate that Brd2/RING3 binding to LANA-1 may be important for the interactions of the C-terminal domain of LANA-1 with nuclear heterochromatin and its functional activity. This would suggest interesting parallels to the recently described role of Brd4/MCAP, a homologue of Brd2/RING3, in heterochromatin binding and the function of BPV E2.

MATERIALS AND METHODS

Plasmids. MeCP2 was amplified by reverse transcription with an oligo(dT) primer using RNA extracted from HeLa cells, followed by nested PCR using primers MeCP5′nest (CTTGCTTCTGTAGACCAGCTCC) and MeCP3′nest (GCTTTGCAATCCGCTC CGTG) for first-round PCR and MeCPNFLAG (AGACTCGAGATGGACTACAAGGACGACGACGACAAGGTAGCTGGG ATGTTAGGGCTC) and MeCPC (AGAGGATCCTCAGCTAACTCTCTC GGTCAC) for the second round. Following sequence confirmation of the amplified product, FLAG-MeCp2 was inserted into pcDNA3.1 using the restriction sites for XhoI and BamHI. A 2.4-kb coding sequence for full-length human BRD2/RING3 (801 aa) was generated by PCR from the plasmid template BRD2/RING3 pcDNA3 9E10 (46) with primers RING3-GFPC1-FOR (ACT AGATCTCTGCAAAACGTGACTCCCCAC) and RING3-GFPC1REV (AGA CCCGGGATTAGCCTGAGTCTGAATCACTG). The amplified product was cloned into the EGFPC1 (for enhanced green fluorescent protein [EGFP] C1; Clontech) vector using the restriction sites BglII and SmaI to generate the EGFP-RING3 fusion construct. LANA-1 deletion constructs used in this study are shown in Fig. 1. All LANA constructs used in this study were within the pcDNA3.1 vector background. The numbering of amino acids in LANA-1 corresponds to the prototype KSHV BC-1 sequence (52). Full-length LANA-1 and C-terminal truncation constructs L-1107, L-1128, L-1143, and L-1160 have been described previously (64). The C-terminal region of constructs L-1133, L-1139, and L-1153 were amplified using primers CTCGATGCGGCCGCTTATTGT AGGTTTCCTGCC and GS28B (CTCGATGCGGCCGCTTATTGTAGGTTT CCTGCC), GS28C (CTCGATGCGGCCGCTTAAAACTTAACTATGGAA GATTG), and GS29B (CTCGATGCGGCCGCTTATGGCTGGGTTAAT GGCAGG), respectively, and cloned into the LANA expression vector as described in reference 64. To produce the LANA-1 construct lacking the internal acidic region (L-Δ331-933) the primers 73AC1 (GGGGTACCAGATTTCCCG AGGATGG), 73Ac2 (GGAATTCATCATCCTTATTGTCATTGTC), 73AC3 (GGAATTCGAAGAGCCCATAATCTTG), and 73AC4 (CCGCTCGAGTGT CATTTCCTGTGGAGAGT) were used. To produce L-Δ275-933, the DNA amplified with primers 73AC3 and 73AC4 was cloned downstream the N-terminal region of the dLANA-1 (aa 1 to 275) construct described in reference 64. In a similar manner, the LANA region amplified with primers 73AC1 and 73AC2 was cloned upstream of the C-terminal region of the dLANA-1 (aa 972 to 1162) construct described in reference 64. All constructs were sequenced to ensure that no errors occurred during the cloning procedure. The TR-containing plasmids, pBluescript-1TR and pBluescript-2TR, contain one or two copies, respectively, of the KSHV TR inserted into pBluescript II backbone. pBluescript-1TR was a gift from Vincent Lacoste. To produce pBluescript-2TR, the TR fragment was excised from pBluescript-1TR using NotI, and an excess of TR fragment was ligated into NotI-digested pBluescript II (Stratagene). The TR was cloned into pGL3basic (Promega) using the KpnI and SacI restriction sites to produce pGL3basic-TR.

Cell lines. The human epithelial cell line HEK 293 and HEK 293 cells expressing the simian virus 40 large T antigen (HEK 293-T) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (300 μg/ml), streptomycin (50 μg/ml), and penicillin (50 IU/ml) (D10 medium).

Transient transfection and luciferase reporter assays. All transfection experiments were carried out using FuGENE 6 (Roche) according to the manufacturer's instructions. Cells were plated in six-well plates, transfected 16 to 24 h later, and harvested 40 h after transfection in lysis buffer containing leupeptin, benzamidin, aprotinin, pepstatin A, and phenylmethylsulfonyl fluoride (PMSF).

For luciferase reporter assays, 3×10^5 293 cells were seeded per well of a six-well tray and transfected with 50 ng of the pGL3basic-TR plasmid or the vector containing the cyclin E promoter plus LANA-1-expressing constructs (or mock). Control cells were transfected with pGL3basic-TR plasmid to obtain the basal activity of the TR. The experiments were performed in triplicate.

To measure luciferase activity, transfected cells were washed with cold phosphate-buffered saline (PBS) and harvested in luciferase reporter lysis buffer (Promega) 48 h after transfection. Luciferase activity was measured as recommended by the manufacturer's manual with a luminometer (Lumat LB9501; Berthold). The relative activity obtained with each construct, and error bars representing standard deviations are depicted.

Short-term replication assay. Short-term replication assays were performed as previously described (19). Briefly, 10^6 293 cells were cotransfected with pBluescript-1TR, pBluescript-2TR, or control pBluescript plus LANA-1-expression constructs or pcDNA3.1. At 16 h after transfection, the cells were trypsinized, washed three times with PBS, plated in new flasks, and allowed to grow for a further 48 h. At that time, the cells were harvested in 700 μl of lysis buffer

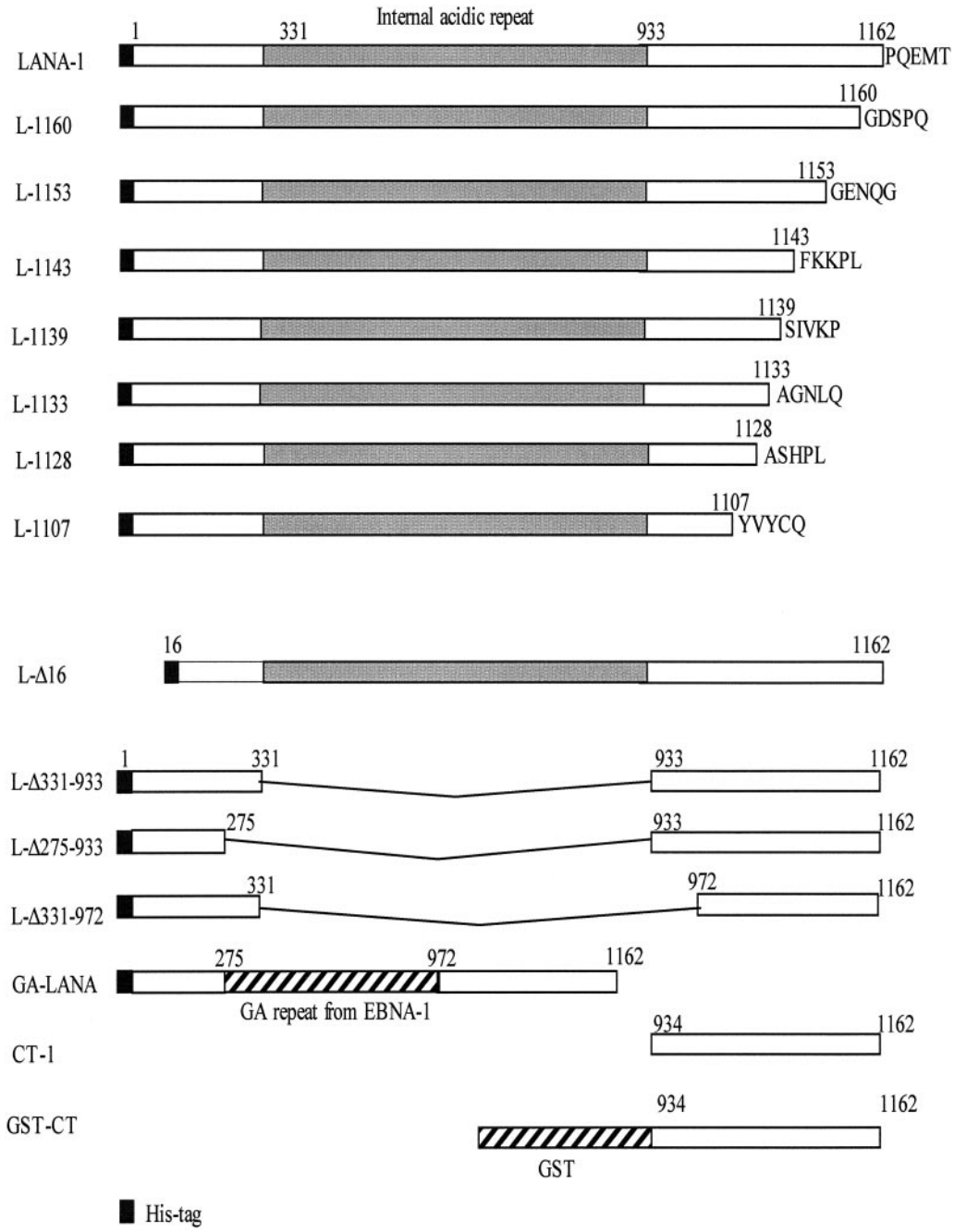

FIG. 1. Schematic representation of LANA-1 constructs used in this study. The internal acidic repeat is shown in gray. The numbering of LANA-1 protein corresponds to that of Russo et al. (52).

(10 mM Tris-HCl, 10 mM EDTA, 0.6% SDS), and chromosomal DNA was precipitated overnight with 0.85 M NaCl. An aliquot was collected from the supernatant to determine the expression levels of the different LANA constructs by immunoblotting. Plasmid DNA was purified from the supernatant using phenol:chlorophorm, precipitated with ethanol, and resuspended in 20 μl of H_2O containing 100-μg/ml RNase A. A total of 90% of the DNA was digested with KpnI and 180 U of DpnI during 48 h, while the other 10% was digested with KpnI to measure input DNA. The digested DNA was precipitated with ethanol, resuspended in 20 μl H_2O, and analyzed by Southern blotting with a 801-bp TR fragment or the complete pBluescript-1TR plasmid as an alkaline phosphatase-labeled probe (Amersham Pharmacia).

Electrophoresis mobility shift assay. Oligonucleotides containing both LBS (LBS A, 5′ AGGCGGCGCGCGGCCCCATGCCCGGGCGGGAGGCGCCGC AGGCCCCGGCGGCGTCCCCTT 3′; and LBS B, 5′ AAGGGGACGCCG CCGGGGCCTGCGGCGCCTCCCGCCCGGGCATGGGGCCGCGCG CCGCCT 3′) were annealed and labeled with [α-^{32}P]dCTP using Klenow. Sephadex 50 spin columns were used to purify the labeled probe from nonincorporated nucleotides. LANA-1 proteins were in vitro transcribed-translated with rabbit reticulocytes (TNT Promega). Expression of LANA proteins was confirmed by immunoblotting. LANA proteins were incubated with 20,000 cpm of ^{32}P-labeled probe in a buffer containing 30 mM Tris HCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol, and 2 μg poly(dI-dC).

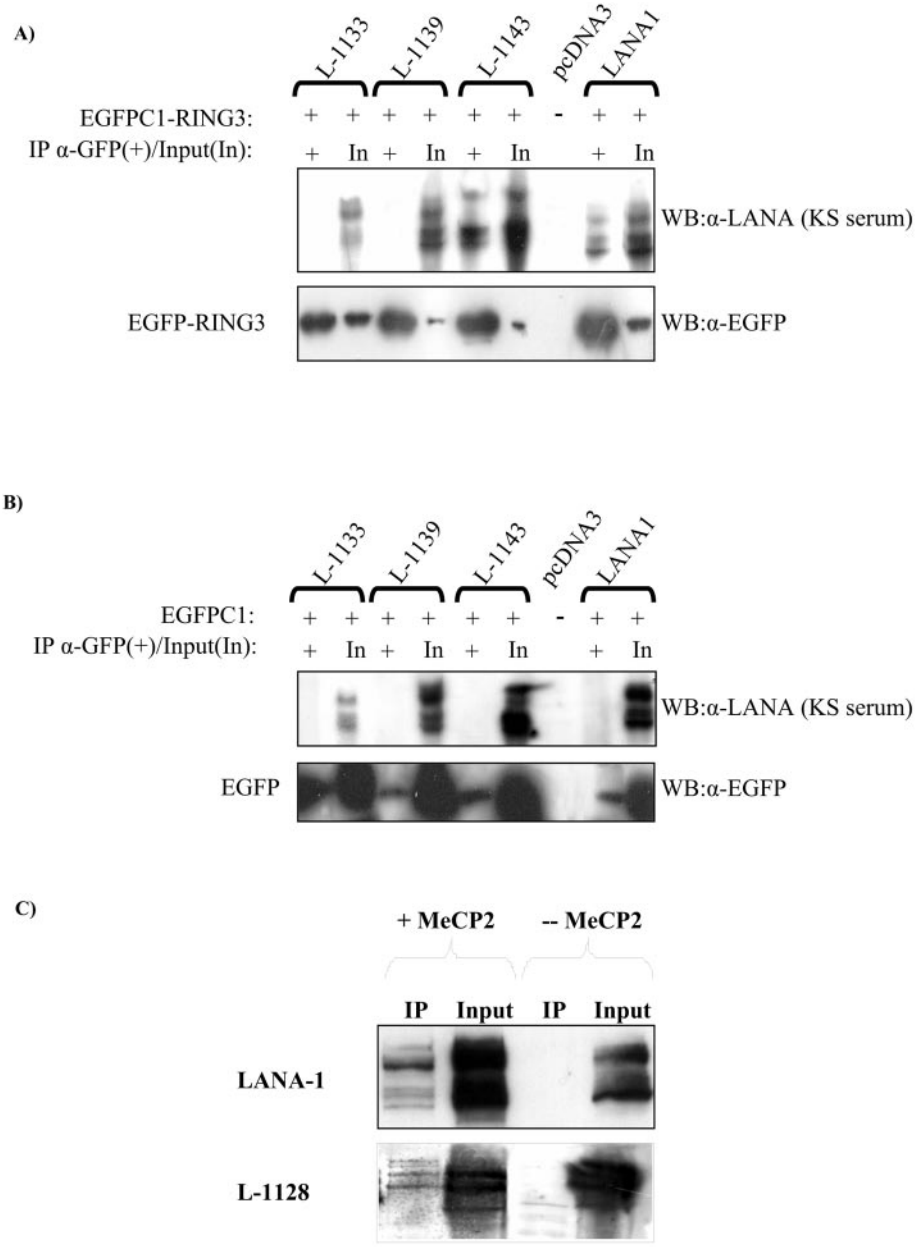

FIG. 2. Binding of LANA-1 to RING3/Brd2. (A) IP assay (see Materials and Methods) showing binding of LANA-1 and L-1143 to RING3/Brd2. L-1139 was not observed to bind to RING3/Brd2. (B) Control experiment showing that the control EGFP protein did not coimmunoprecipitate any of the LANA-1 constructs tested. (C) MeCP2 and LANA-1 interaction. Binding of LANA-1 to FLAG-MeCP2 was observed following IP of MeCP2 with an anti-FLAG antibody and detection of LANA-1 constructs by immunoblotting with KSHV-positive human serum.

The samples were separated by electrophoresis on a native 3% polyacrylamide gel in 1× Tris-borate-EDTA. Dried gels were exposed to Kodak film and developed.

GST pulldown. Glutathione *S*-transferase (GST) and GST-C terminus (CT) LANA fusion proteins were produced in *Escherichia coli* and bound to GST beads. Expression and binding of GST and GST-LANA fusion proteins to GST beads was assessed by SDS-PAGE and Coomassie staining. Beads loaded with equal amounts of protein were incubated with cell lysates (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, leupeptin, benzamidin, aprotinin, pepstatin A, and PMSF) of cells transfected with expression vectors for LANA-1 or LANA-1 mutants (see above) for 1 h at room temperature. Following three washes in PBS containing 0.1% Triton X-100, the beads were boiled in SDS-PAGE loading buffer containing 2% SDS and 1% β-mercaptoethanol to elute the bound proteins. Binding of LANA-1 proteins was detected by immunoblotting.

Immunoprecipitation. 293-T cells were cotransfected with 1 μg of LANA constructs (or mock) plus 1 μg of RING3/Brd2 or MeCP2 (or mock) constructs. Cells were lysed in 300 μl of TBST buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, leupeptin, benzamidin, aprotinin, pepstatin A, and PMSF) 48 h after transfection. Cell lysates were precleared with protein G-Sepharose beads (for MeCP2) and protein A-Sepharose beads (for RING3) (Amersham Bioscience). An aliquot of cleared lysate was used as input control, and the rest (260 μl) was incubated overnight with anti-FLAG-coupled protein G-Sepharose beads (for MeCP2) or with anti-GFP-coupled A-Sepharose beads (for RING3). Following five washing steps with lysate buffer (20 mM Tris

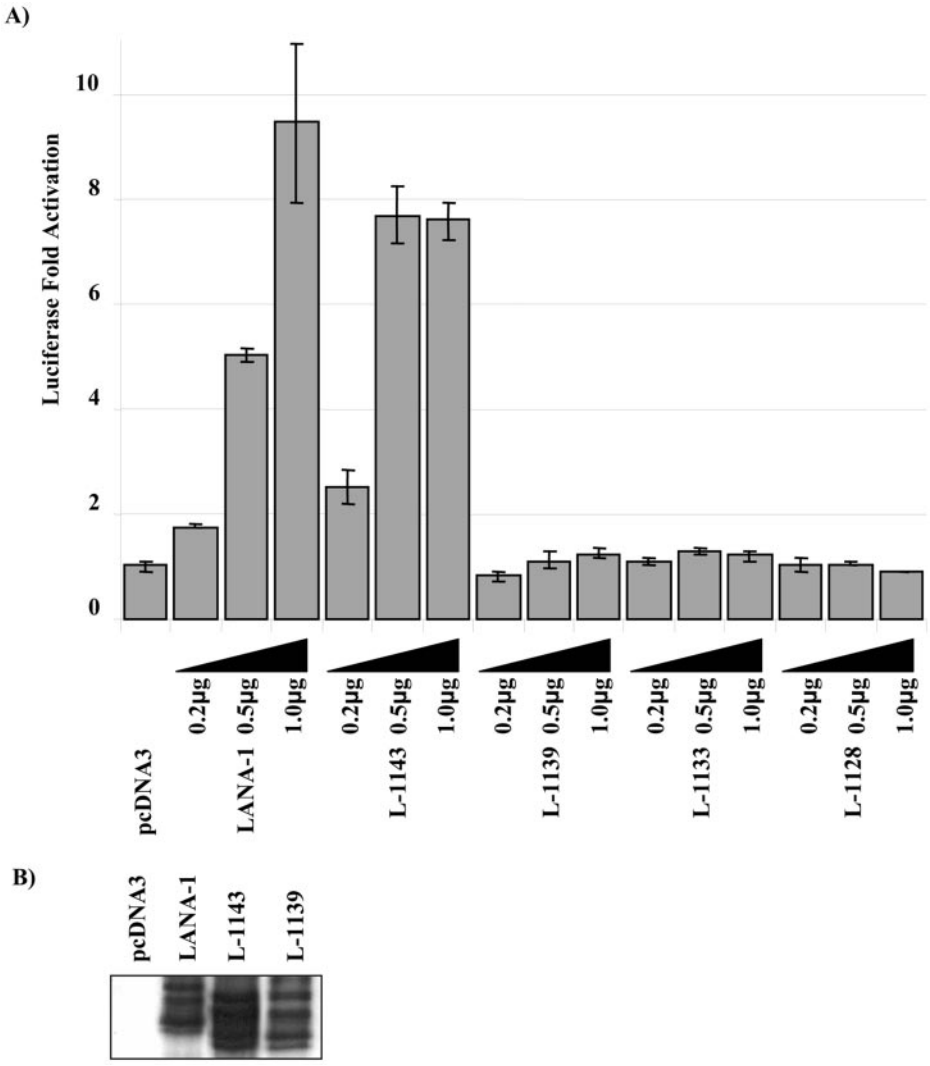

FIG. 3. Deletion of the last 23 aa of LANA-1 impaired the activation of the cyclin E promoter. (A) Luciferase reporter assay showing the effect of the different LANA-1 C-terminal truncations on the activation of the cyclin E promoter. 293 cells were cotransfected with a cyclin E promoter-containing luciferase construct and increasing concentrations of LANA-1-expressing constructs. At 48 h after transfection, cells were lysed and luciferase activity was measured. (B) Detection of LANA-1, L-1143, and L-1139 by immunoblotting. Lysates of cotransfected 293 cells were separated by SDS-PAGE, and the expression of LANA-1 proteins was detected with KSHV-positive human serum. (C) Luciferase reporter assay showing the effect of the LANA-1 internal deletions on the activation of the cyclin E promoter.

[pH 7.5], 150 mM NaCl, 0.5% Tween 20), the beads were boiled in SDS-PAGE loading buffer, run in 10% polyacrylamide gels, and detected by immunoblotting with KS patient serum.

Immunoblots. Western blots were performed as previously described (57). Briefly, protein extracts were separated on SDS-PAGE gels with 8%, 10%, or 12% polyacrylamide. Then, the proteins were transferred onto nitrocellulose membranes (Amersham) using a Mini-Trans blot unit (Bio-Rad) during 90 min at 200 mA. The membranes were blocked for 2 h in PBS-M (PBS containing 5% Marvel and 0.1% Tween) and incubated for 1 h at room temperature with the primary antibody diluted in PBS-M. After a washing step, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody diluted in PBS-M. Following another washing step, the presence of the proteins was detected with a chemiluminescence (ECL) kit (PerkinElmer Life Sciences) and exposed to Kodak film. LANA-1 constructs were detected with KSHV-positive patient sera (1:500) and a rabbit anti-human antibody (1:1,000; 2% rabbit serum). EGFP-RING3/Brd2 was detected with an anti-GFP monoclonal antibody (1:3,000; BD) and MeCP2 was detected with an anti-FLAG monoclonal antibody (1:2,000; Sigma).

RESULTS

Deletion of the carboxy-terminal 23 amino acids abrogates binding to RING3/Brd2. We previously showed that deletion of amino acids 1128 to 1162 abrogates binding of LANA-1 to heterochromatin or elements of the nuclear matrix in interphase cells (64). We therefore investigated which of the previously reported cellular chromatin-associated proteins could be responsible for this interaction and thereby contribute to the functional properties of LANA-1. As shown in Fig. 2A, deletion of the last 23 amino acids of LANA-1 resulted in lack of binding to RING3/Brd2 in an immunoprecipitation (IP) assay (compare constructs L-1133 and L-1139 with L-1143 and LANA-1). The control protein EGFPC1 did not immunoprecipitate any of the LANA-1 constructs (Fig. 2B). Thus, the

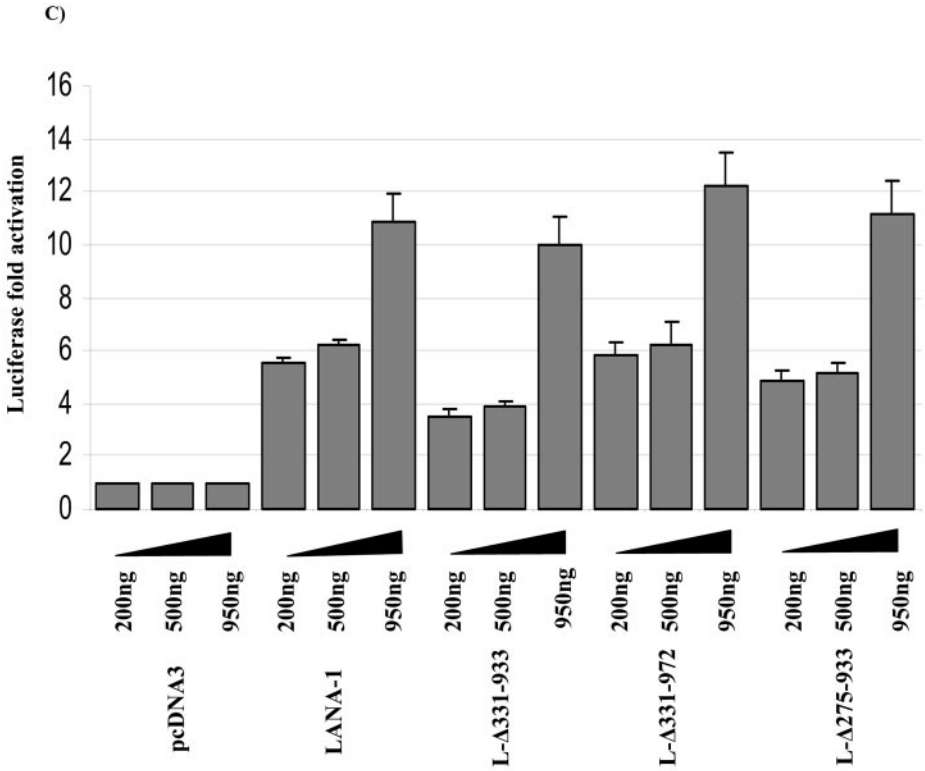

FIG. 3—*Continued.*

same deletion that eliminated binding to interphase heterochromatin or nuclear matrix (64) also led to a loss of binding to RING3/Brd2. In contrast, binding of LANA-1 to MeCP2, which has been reported to mediate the interaction of the N-terminal 16 aa of LANA-1 with mitotic chromosomes (30), was not affected by deleting aa 1128 to 1163 of LANA-1 (Fig. 2C).

Importance of the carboxy-terminal heterochromatin-binding domain for LANA-1 function. The C-terminal domain of LANA-1 binds to LANA-binding site 1 (LBS-1) and LBS-2 in the terminal repeat region of the viral genome and is required for mediating episomal latent replication, activation of heterologous promoters, and formation of LANA-1 dimers. We sought to correlate the interaction of LANA-1 with interphase chromatin and RING3/Brd2 with its ability to carry out these functions, using the set of carboxy-terminal and internal deletion mutants depicted in Fig. 1.

LANA-1 has previously been shown to activate E2F-dependent promoters like the cyclin E promoter (47) and to induce E2F-dependent cellular transcripts (1, 47, 65). Deletion of the last 23 amino acids of LANA-1 resulted in a LANA-1 construct unable to activate the cyclin E promoter (Fig. 3A). Lack of cyclin E activation by L-1139 was not due to lower levels of protein expression (Fig. 3B). Thus, the loss of activation of the cyclin E promoter correlated with the lack of RING3/Brd2 binding (Fig. 2). The internal repeat region was not required for the activation of the cyclin E promoter, nor were residues 278 to 331 and 933 to 972 flanking the internal repeat (Fig. 3C). Amino acids 933 to 972 are part of a region previously reported to be involved in the binding of LANA-1 to pRB (47), which is thought to mediate the activation of E2F-dependent genes by LANA-1 (1). KSHV's origin of replication (*oriP*) is located within the KSHV TR region (19, 25). Binding of the LANA-1 carboxy-terminal domain to the TR represses downstream promoters (20). We cloned one KSHV TR unit upstream of a luciferase reporter gene lacking any constitutive promoter (pGL3basic) and found that the TR unit alone was able to direct expression of the luciferase gene severalfold above background values obtained with the promoterless luciferase vector (not shown). Full-length LANA-1 repressed this promoter/enhancer activity within the TR (Fig. 4). The C terminus of LANA-1 (aa 934 to 1162) repressed the TR more efficiently than full-length LANA-1 (Fig. 4A). A LANA-1 construct lacking the last 23 amino acids of LANA-1 showed a reduced ability to repress the promoter/enhancer activity found within the full-length TR (Fig. 4B). More extensive carboxy-terminal truncations of LANA-1, however, not only failed to repress the TR promoter/enhancer activity but had a moderately stimulating effect (Fig. 4B). Deletion of the internal repeat and flanking domains of LANA-1 affected the repression of TR promoter/enhancer activity only moderately (Fig. 4C). Taken together, our results suggest that the region from aa 972 to 1139 is sufficient to repress TR promoter/enhancer activity (Fig. 4).

LANA-1 has been shown to mediate replication and segregation of TR-containing plasmids (19, 23, 25, 35). Full-length LANA-1 or LANA-1 truncation mutants were cotransfected into 293 cells together with a TR-containing plasmid, and their ability to mediate replication and segregation of the TR-containing plasmid was assessed as previously described (19). As

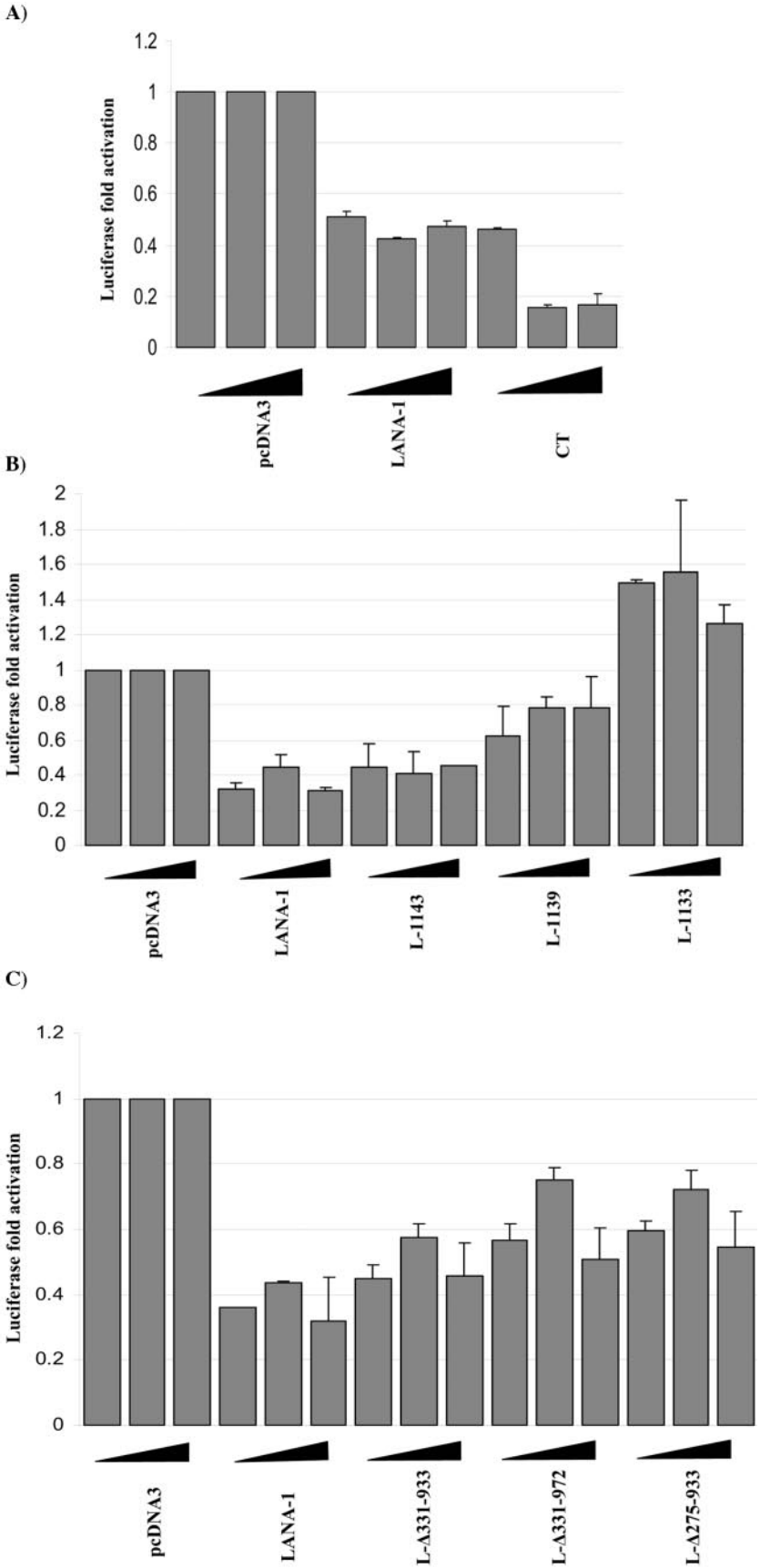

FIG. 4. Luciferase reporter assay showing the effect of (A) LANA-1 and LANA-1 C terminus, (B) LANA-1 C-terminal truncation mutants, and (C) LANA-1 internal deletions on the repression of the KSHV TR promoter/enhancer region. 293 cells were cotransfected with pGL3basicTR and increasing concentrations of LANA-1-expressing constructs. At 48 h after transfection, cells were lysed and luciferase activity was measured.

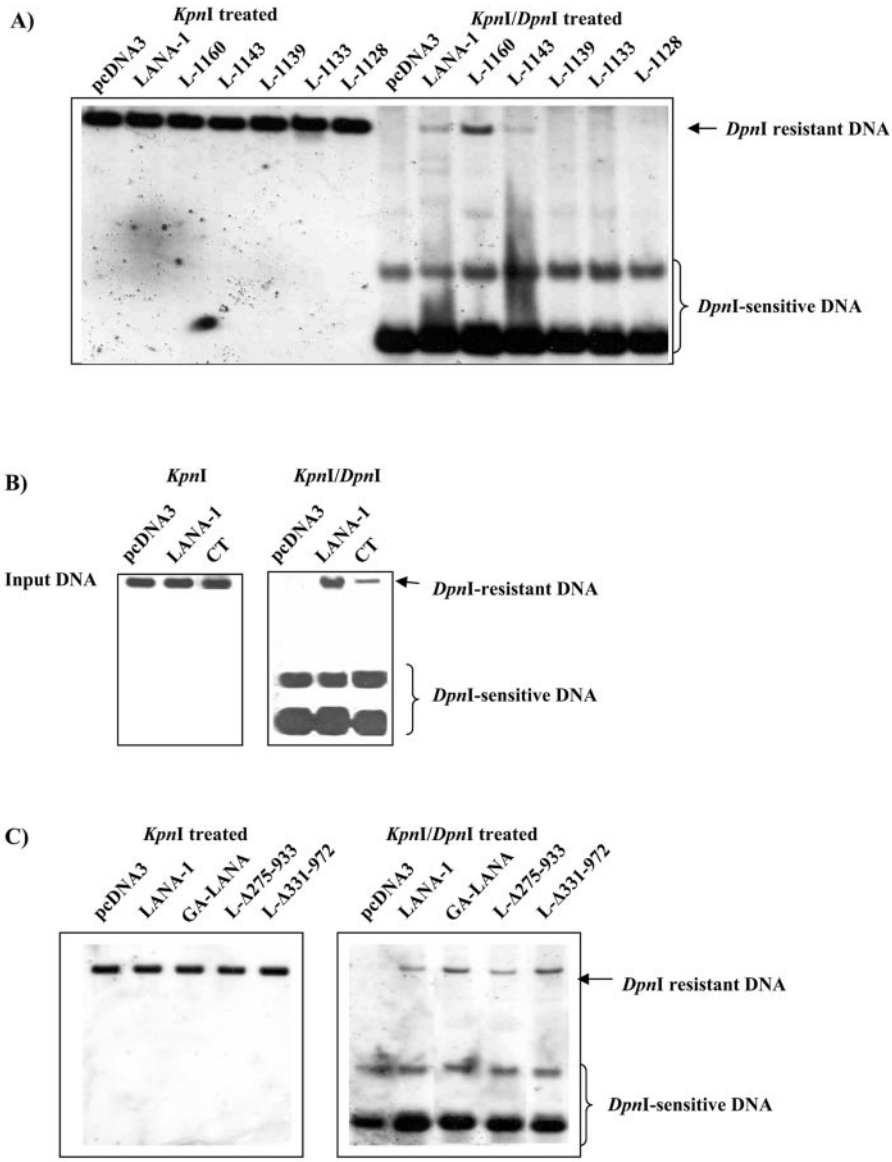

FIG. 5. Short-time replication assay (see Materials and Methods). (A) Deletion of amino acids 1139 to 1143 resulted in LANA-1 constructs unable to replicate a TR-containing plasmid. (B) The C terminus of LANA-1 was able to replicate a TR-containing plasmid, although at a reduced level compared to that of full-length LANA-1. (C) Deletion of LANA-1 internal repeat and flanking regions did not affect LANA-1-mediated replication of a TR-containing plasmid.

shown in Fig. 5, a full-length LANA-1, L-1160, and L-1143 were able to mediate replication of a TR-containing plasmid, while this property was lost upon deletion of amino acids 1139 to 1162. Similar amounts of DNA were loaded for each sample, as shown by both the input DNA (KpnI-digested DNA) and by the DpnI-digested DNA. Similar levels of LANA-1 protein expression were detected (not shown). We were able to show similar results when a plasmid containing two copies of the TR was used in the short-term replication assay (not shown). The LANA-1 CT (amino acids 934 to 1162) was able to mediate replication of a TR-containing plasmid, albeit less efficiently (approximately 50% less efficiently) than full-length LANA-1 (Fig. 5B). Deletion of the internal repeat and flanking regions did not affect replication of a TR-containing plasmid (Fig. 5C). The substitutions of LANA-1 internal repeat by the GA repeat of EBNA-1 did not affect LANA-1-mediated replication (Fig. 5C).

Through its carboxy-terminal region, LANA-1 binds to two small motifs of 16 nucleotides termed LBS-1 and LBS-2 within the KSHV TR (19). Binding to LBS-1 and LBS-2 is required for the replication and segregation of TR-containing plasmids and for the repression of a reporter construct containing TR elements upstream of a promoter. We tested the ability of our deletion constructs to bind to LBS-1 and LBS-2 within the TR. LANA-1 constructs were transcribed, translated in vitro, and tested for their ability to bind a double-stranded DNA sequence encompassing LBS-1 and LBS-2 (LBS DNA) (Fig. 6). Binding of LANA-1 to LBS DNA was observed by the pres-

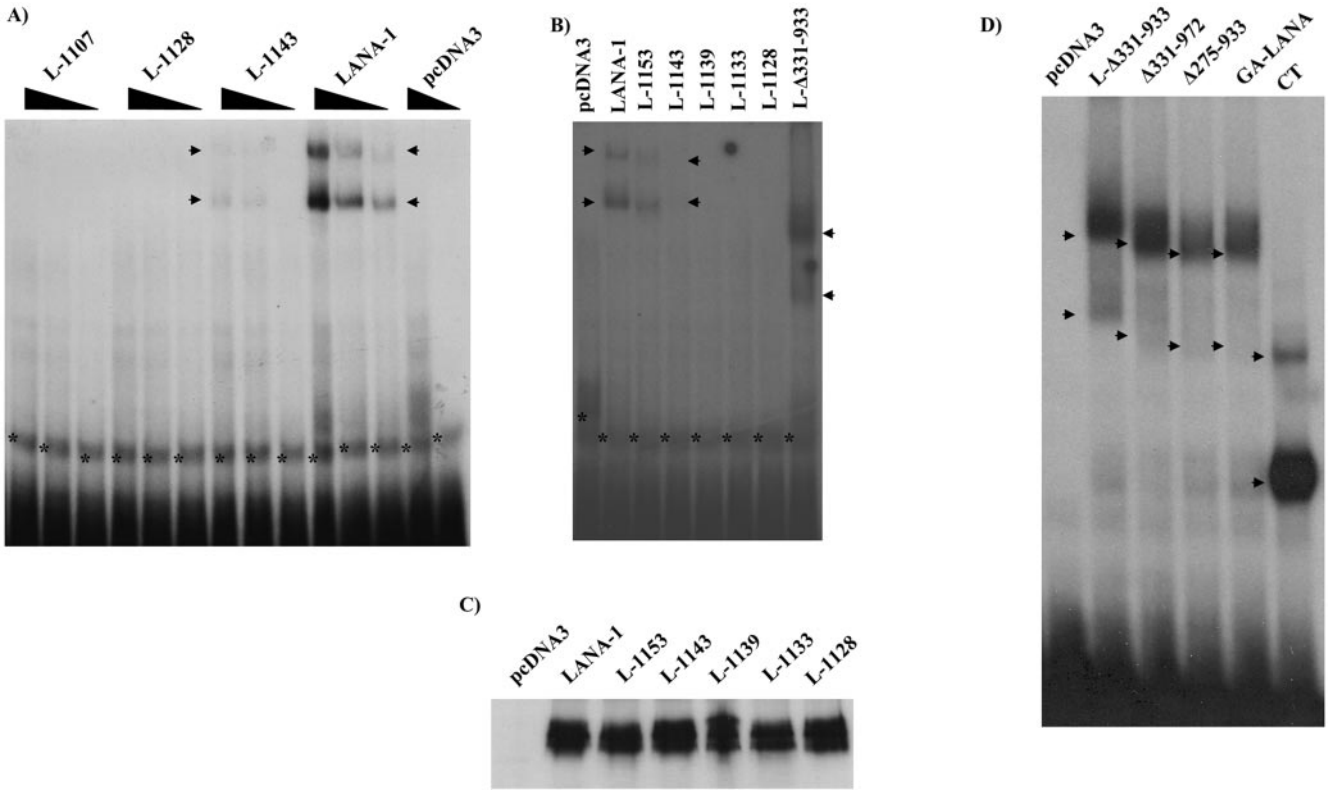

FIG. 6. (A, B, and D) Electrophoresis mobility shift assay showing binding of LANA-1, L-1159, L-1153, L-1143, L-1139, L-1133, L-1128, L-Δ331-933, L-Δ331-972, and L-Δ275-933 to a radiolabeled probe containing LBS-1 and -2. (C) The expression levels of in vitro-transcribed/translated LANA-1, L-1153, L-1143, L-1139, L-1133, and L-1128 were tested by immunoblotting. (A, B, and D) Arrowheads indicate the two bandshifted LANA-1-containing complexes of higher and lower mobility (see text). Asterisks (A and B) indicate an unspecific shifted band.

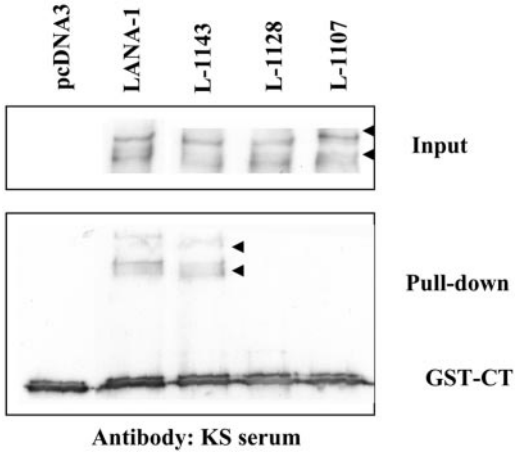

FIG. 7. Dimerization of LANA-1 deletion mutants. GST and GST-CT fusion protein were expressed in bacteria as explained in Materials and Methods. LANA-1 proteins were in vitro transcribed/translated, incubated with GST or GST-CT, and bound to Sepharose beads for 1 h. Following extensive washing steps, the beads were boiled in loading buffer containing β-mercaptoethanol, and the samples were separated by SDS-PAGE (see Materials and Methods). The detection of LANA-1 proteins and GST-CT was carried out with a KSHV-positive patient serum.

ence of the previously described double shift (Fig. 6A) (19). It has been suggested that the faster-migrating complex represents a dimer of LANA-1 bound to LBS-1, whereas the more slowly migrating complex represents a tetramer of LANA-1 bound to both LBS-1 and LBS-2 in a cooperative fashion (19). The dimeric LANA-1/LBS-1 complex is formed preferentially at lower LANA-1 concentrations, while higher LANA-1 concentrations promote formation of the tetrameric complex (Fig. 6) (19). L-1143 was less efficient in LBS binding than full-length LANA-1, with no complex formation at lower concentrations of L-1143 (Fig. 6A). L-1128, L-1133, and L-1139 did not bind LBS (Fig. 6A and B). This lack of binding was not due to low levels of protein expression (Fig. 6C). Lack of binding to LBS by L-1139 correlated with its lack of ability to replicate and/or segregate a TR-containing plasmid, to repress TR promoter/enhancer activity (Fig. 4 and 5), and also to interact with Brd2/RING3, but not MeCP2. Deletion of the LANA-1 internal repeat or sequences flanking the internal repeat did not affect binding to LBS (Fig. 6D). Interestingly, the more slowly migrating LANA-1/LBS-1–LBS-2 complex was much more pronounced than the faster band-shifted complex in the case of LΔ331-933, LΔ331-972, LΔ275-933, and GA-LANA, which all lack the internal repeat region of LANA-1 (Fig. 1), compared to the typical migrating pattern of LANA-1 (compare Fig. 6D with Fig. 6A and B). The carboxy-terminal domain of LANA-1 (CT) bound to LBS (Fig. 6D). In this case, and in contrast to LΔ331-933, LΔ331-972, LΔ275-

933, and GA-LANA, the faster-binding complex predominated over the slower-binding complex. Overall, our results show that binding of LANA-1 to TR occurred in the region between amino acids 973 and 1143. In addition, the amino-terminal region of LANA-1 (aa 1 to 275) may contribute to the formation of the tetrameric LANA-1/LBS-1–LBS-2 complex, which may be required for efficient episomal replication, given the inefficient replication mediated by the CT construct (Fig. 5B).

Constructs lacking the last 34 amino acids do not form oligomers in solution. The carboxy-terminal domain of LANA-1 (aa 957 to 1162) is able to form oligomers in solution (29, 55). Oligomerization of LANA-1 is believed to be required for the interaction of LANA-1 with LBS, since constructs that do not form oligomers are not able to bind to a probe encompassing LBS (29). As shown in Fig. 7, deletion of the last 34 amino acids of LANA-1 resulted in lack of LANA-1 oligomerization. In agreement with Komatsu et al. (29), we conclude that oligomerization of LANA-1 appears to be a prerequisite for the interaction of LANA-1 with LBS, as well as for episomal replication and transcriptional repression from a TR containing plasmid. Interestingly, the oligomerization domain of LANA-1 partially overlapped with the RING3/Brd2 interacting domain. These results could indicate that oligomerization of LANA-1 or other structural features destroyed in LANA-1 truncations shorter than L-1143 contribute to the ability to bind to RING3/Brd2. Oligomerization of LANA-1, however, does not seem to be required for the interaction with other chromatin-interacting proteins such as MeCP2 (Fig. 2C).

DISCUSSION

In addition to the previously described amino-terminal region (aa 1 to 24) required for attaching LANA-1 to mitotic chromosomes (45, 65) and for replication of a TR containing plasmids in transient transfection assays (35) and the observation that key residues required for binding to interphase chromatin are located between Gly5 and Gly11 at the amino terminus of LANA-1 (65), we have recently described a region in the carboxy-terminal domain of LANA-1 that modulated the interaction of LANA-1 with interphase heterochromatin or elements of the nuclear matrix and the activation of a heterologous promoter. The carboxy-terminal border of this domain was mapped to a region between aa 1129 and 1143 (64).

Using a series of carboxy-terminal and internal deletion mutants of LANA-1, we show here that loss of the interaction with interphase heterochromatin or components of the nuclear matrix, as reported previously (64), correlates with the loss of binding to Brd2/RING3. In contrast, binding of the methyl CpG-binding protein MeCP2, which had been found to bind to the amino-terminal 16 aa of LANA-1 (30), did not correlate with the interaction with interphase heterochromatin, as expected.

Our previous GST-pulldown experiments with the carboxy-terminal domain of LANA-1 and corresponding truncation mutants had suggested binding sites for Brd2/RING3 between aa 1007 to 1055 and 1048 to 1162 of LANA-1 (46), while the coimmunoprecipitation assays shown here suggest that, in the context of nearly full-length LANA-1, carboxy-terminal truncation beyond aa 1143 leads to a loss of Brd2/RING3 binding (Fig. 2). This most likely suggests that the contact region between aa 1007 to 1055 plays an important role in the context of the correctly folded molecule or that deletion of LANA-1 beyond aa 1143 affects the overall conformation of LANA-1. Since deletion of the carboxy-terminal domain beyond aa 1143 destroyed several functions and properties of LANA-1, including dimerization (Fig. 7) and binding to TR DNA (Fig. 6), we favor the latter explanation. Our observation that the region of LANA-1 required for binding to Brd2/RING3 also affects its interaction with interphase heterochromatin or elements of the nuclear matrix (64) is compatible with the interpretation that, as recently suggested for the Brd homologue Brd4 and BPV E2 (67), Brd2 could play a role in tethering LANA-1 and the associated viral episomes to nuclear heterochromatin. In this case, the conserved ET domain of Brd2/RING3 would mediate this tethering (46), unlike in the case of Brd4 and BPV E2 where a region downstream of the ET domain is thought to be responsible (67). However, given the suspected impact of truncating LANA-1 beyond aa 1143 on its conformational structure, other interpretations cannot be excluded. Previous immunofluorescence studies showed a nearly quantitative relocalization of Brd2/RING3 from its usual widespread intranuclear distribution in uninfected cells into the LANA-1 and viral episome containing nuclear speckles in KSHV-infected cells (40). A recent study showed that Brd2/RING3 is associated with terminal repeat sequences in KSHV-infected primary effusion lymphoma cell lines and could play a role in maintaining the acetylated state of nucleosomes positioned in the vicinity of LBS-1 and LBS-2 (60). Together with the findings reported here, these two observations would suggest that a minimal region (aa 973 to 1143) in the carboxy-terminal domain of LANA-1 is sufficient for binding to LBS-1 and -2, for recruiting additional cellular factors such as Brd2/RING3, for mediating at least a basic level of replication, and for repressing promoter/enhancer activity located in the TR region.

Our observations and previously published data indicate that RING3/Brd2 is a good candidate to play a role in LANA-1-mediated functions. RING3/Brd2 interacts with the E2 promoter-binding factor (E2F) and is involved in the E2F-mediated activation of cell cycle regulatory genes (12, 24); it associates in the nuclei of HeLa cells with the cyclin-dependent kinase 8 and TRAP220 mediator subunits and the polymerase II large subunit (10). Finally, RING3/Brd2 homologues have been shown to be part of the mediator complex and to be required for certain BPV E2-mediated functions (5, 8, 26, 39, 67).

Amino acids 735 to 990 of LANA-1 interact with pRB, and this interaction is thought to be important for E2F-dependent gene expression (47). Our observation that deletion of aa 933 to 972 does not have an impact on the activation of E2F-dependent genes does not exclude a role of pRB in the activation of the cyclin E promoter studied here. The C-terminal region of LANA-1 also interacts with GSK-3β (17) and this interaction has been shown to be required for the stabilization of β-catenin, thereby promoting entry into the S phase of the cell cycle. Lack of binding to GSK-3β could also have an impact on the activation of the cyclin E promoter. It is therefore possible that LANA-1 activates E2F-dependent genes through several pathways and its suspected role in maintaining an open chromatin configuration by binding to acetylated histones may complement the effect of pRB and GSK-3β, also recruited by LANA-1, on the expression of E2F-dependent promoters.

Our data do not prove that the interaction with RING3/Brd2 is essential for LANA-1-mediated episomal replication, since constructs that do not bind RING3/Brd2 are also deficient for LBS binding. To address this question more directly, we are currently attempting to develop dominant negative mutants of Brd2/RING3 and to explore their ability to inhibit LANA-1-mediated episomal replication.

The minimal replication of a TR containing plasmid observed in this study with the carboxy-terminal domain (aa 934 to 1162) of LANA-1 (Fig. 5) has previously also been reported by some (25) but not other (23, 35) groups. Although capable of binding to LBS-1 and LBS-2 motifs in a band shift assay, LANA-1 aa 934 to 1162 appear to preferentially form a complex consisting of a dimeric protein bound to viral DNA (Fig. 6) (29). Adding the amino terminal residues 1 to 275 is sufficient to increase TR replication to wild-type levels (Fig. 5); this is accompanied by a preferential formation of a higher-order band-shifted complex, thought to consist of a tetrameric protein interacting with both LBS-1 and LBS-2 (Fig. 6) (19). It is thus conceivable that the increased replication seen with the LΔ275 −933 and GA-LANA (lacking aa 275 to 972) constructs relative to the construct expressing only aa 934 to 1162 is due to the formation of higher-order LANA-1/viral DNA complexes in the presence of the amino-terminal domain (aa 1 to 272) of LANA-1. However, Wong et al. (65) recently showed that a chromatin-binding motif located within aa 2 to 24 is required for the ability of LANA-1 to act as a transcriptional modulator, and Lim et al. (34) showed that this region is also required for the replication of episomal DNA. An alternative explanation would therefore be that the additional interaction with nuclear heterochromatin provided by the amino-terminal region of LANA-1 promotes viral DNA replication either by enabling the segregation of replicated episomes during mitosis or via an as-yet-unknown mechanism. Moreover, the carboxy-terminal domain (aa 934 to 1162) is a more potent repressor of the promoter/enhancer activity in the terminal repeat region than LANA-1, LΔ275-933, or GA-LANA (Fig. 4 and data not shown), suggesting that formation of the lower-order complex (Fig. 6) could be sufficient for silencing transcriptional activity through nucleosome silencing in the TR region (60).

In summary, our findings define a minimal region in the carboxy-terminal domain of LANA-1, which is required but not sufficient for the ability of LANA-1 to bind to and replicate episomal DNA, activate heterologous promoters, interact with interphase heterochromatin, and produce dimerization. The same region is indispensable for the binding of LANA-1 to a member of the Brd family, Brd2/RING3. Although other LANA-1-associated nuclear proteins are undoubtedly required for the multiple functions of LANA-1, this correlation is in keeping with the interpretation that recruitment of Brd2/RING3 plays a role in LANA-1-associated functions.

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