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Latency-Associated Nuclear Antigen (LANA) of Kaposi's Sarcoma-Associated Herpesvirus Interacts with Origin Recognition Complexes at the LANA Binding Sequence within the Terminal Repeats

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Kaposi's sarcoma-associated herpesvirus (KSHV) DNA persists in latently infected cells as an episome via tethering to the host chromosomes. The latency-associated nuclear antigen (LANA) of KSHV binds to the *cis*-acting elements in the terminal repeat (TR) region of the genome through its carboxy terminus. Previous studies have demonstrated that LANA is important for episome maintenance and replication of the TR-containing plasmids. Here we report that LANA associates with origin recognition complexes (ORCs) when bound to its 17-bp LANA binding cognate sequence (LBS). Chromatin immunoprecipitation of multiple regions across the entire genome from two KSHV-infected cell lines, BC-3 and BCBL-1, revealed that the ORCs predominantly associated with the chromatin structure at the TR as well as two regions within the long unique region of the genome. Coimmunoprecipitation of ORCs with LANA-specific antibodies shows that ORCs can bind and form complexes with LANA in cells. This association was further supported by in vitro binding studies which showed that ORCs associate with LANA predominantly through the carboxy-terminal DNA binding region. KSHV-positive BC-3 and BCBL-1 cells arrested in G_1/S phase showed colocalization of LANA with ORCs. Furthermore, replication of The TR-containing plasmid required both the N- and C termini of LANA in 293 and DG75 cells. Interestingly, our studies did not detect cellular ORCs associated with packaged viral DNA as an analysis of purified virions did not reveal the presence of ORCs, minichromosome maintenance proteins, or LANA.

Kaposi's sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus 8, primarily infects endothelial and B cells, persisting episomally in these latently infected cells (7, 44). Latency is characterized by the expression of a small subset of viral genes (12, 25, 26, 43). The latency-associated nuclear antigen (LANA) has been detected in almost all KSHV-infected cells by immunofluorescence and is characterized by a punctate nuclear pattern (13, 24, 34, 41, 43). cDNA library screening with serum from a human herpesvirus 8-positive patient identified LANA as the product of viral gene open reading frame 73 (ORF73) (25, 26, 43). LANA is expressed from a latently controlled 5.32-kb transcript that also encodes the viral cyclin (v-Cyc) and v-FLIP (12). The 5.32-kb latent transcript is spliced to form a 1.7-kb transcript that encodes v-Cyc and v-FLIP (12, 26). The LANA transcript encodes a protein which contains 1,162 amino acids and has a theoretical molecular mass of 135 kDa but runs on sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) with much higher molecular size (220 to 230 kDa) when analyzed by Western blotting (14, 26, 43).

The viral genome consists of an approximately 145-kbp unique coding region flanked by multiple terminal repeats of 801 bp with high GC content (47). The ability of LANA to support persistence of the KSHV genome is crucial for the establishment of latency and is essential for long-term maintenance of the KSHV-derived Z6 cosmid (3, 9). The colocalization of viral genome and LANA strongly suggested that LANA tethers the viral genome to the host chromosomes (3, 9). The above reports showed colocalization of LANA with the viral genome in the nucleus and demonstrated its role in episomal persistence as well as a potential role in partitioning of the viral genome during mitosis. LANA tethers the viral genome through binding to the 13-bp LANA binding sequence (LBS) in the terminal repeats (TRs) which were identified by overlapping probes (4, 10). Garber et al. identified another binding site LBS2 in the TR and LBS1/2 are located at nucleotides 571 and 610 (15, 16). LANA binds to these sites (LBS1/2) in a cooperative fashion and contributes to *ori* activity as determined by short-term replication assays (15).

During long-term persistence viral DNA replicates in a synchronized fashion and segregates to the dividing cells. LANA binds to the LBS through its carboxy-terminal DNA binding domain and the N terminus is important for tethering to the nucleosomes, in particular histone H1, and probably with other cellular proteins, which includes MeCP2 and DEK (9, 30, 50). The role of LANA in episome maintenance was addressed by recombinant KSHV cloned in a bacterial artificial chromosome (BAC36ΔLANA) disrupted for LANA expression. The viral episome was not maintained and the cells became virus

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free after 2 weeks of selection (61). More recent studies by Goldfrey and colleagues used the short hairpin RNA approach to knock down the expression of the oncogenic latent gene cluster, including LANA (17). This resulted in a reduced copy number of KSHV episomes per cell (17). LANA was also shown to bind to histone H1 but not core histones and tethers the viral episomes to the host chromatin (9). A deletion in the chromosome binding domain, amino acids 5 to 22 of the N-terminal region of LANA, abolished episomal maintenance but was restored by replacing the mutation with the histone H1 protein (50).

The DNA binding region of LANA was mapped to amino acid residues 996 to 1139 within the carboxy terminus (28). Studies showed that LANA amino acids 1007 to 1021 are important for DNA binding and episome maintenance and deletions within this region ablated both LANA1 oligomerization and DNA binding (28, 51). Plasmids containing a single copy of a TR element have been shown to replicate in LANA-expressing cells (18, 21, 22, 37). Mapping of the minimal replicator element was attempted and led to the identification of a 71-bp-long region in the TR comprising LANA binding sites 1 and 2 and a 29- to 32-bp-long GC-rich region adjacent to LBS1/2 which were essential for replication of the TR elements (22).

The above report compared the functional *ori* region of KSHV with that of Epstein-Barr virus and concluded that these two viruses differ to some extent in sequence homology but retain structural similarities. For example, the EBNA1 binding site (dyad symmetry) has four binding sites with high and low affinities similar to LANA1/2 (22). Thus, LANA and EBNA1 may share similar functions in terms of recruitment of cellular proteins at the *ori* site. However, this has not been experimentally demonstrated and requires further investigation. Recently, it was shown that the KSHV genome forms chromatin structures similar to cellular chromatin and the latent replication origin within the TR is bound by the LANA-associated proteins CBP, double-bromodomain-containing protein 2 (BRD2), as well as Origin Recognition Complex 2 protein (ORC2) (53). This region was enriched in hyperacetylated histones H3 and H4 relative to other regions of the latent genome (53).

In this report we demonstrate that LANA can form complex with ORCs when bound to its cognate sequence and that binding of LANA to ORCs is though the carboxy terminus. Chromatin immunoprecipitation assays demonstrated that the association of cellular replication machinery proteins ORC2 and MCM3 can occur at a number of locations along the KSHV genome, suggesting the presence of multiple regions capable of initiating replication.

MATERIALS AND METHODS

Cells and plasmids. BC-3 and BCBL-1 are KSHV-positive primary effusion lymphoma (PEL) cell lines, BJAB and DG75 are KSHV-negative cell lines cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin (5 U/ml and 5 μg/ml, respectively). Human embryonic kidney (HEK) 293 and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin (5 U/ml and 5 μg/ml, respectively). TR was cloned at the NotI site of pBS SKII+ (Stratagene) (pBSTR) and the puromycin resistance cassette-containing pBS SKII+ (pBSpuroTR) was analyzed for the presence of TR by restriction digestion and sequence analysis. The LANA expression vector having a *myc* tag at its carboxy terminus was described previously (27). The amino (amino acids 1 to 435) and carboxy (amino acids 762 to 1162) termini of LANA were cloned into the *myc*-tagged vector (pA3M) by PCR amplification. Hemagglutinin (HA)-tagged ORC1, -2, -4 and -5 were generated by PCR amplification using the respective template in pGEX-6p ORC1, -2, -3 and -4 (56) (generous gift from Ariga Hiroyoshi, Hokkaido University, Japan). cDNAs for ORC3 and ORC6 were provided by Bruce Stillman (Cold Spring Harbor Laboratory).

DNA affinity column. Oligonucleotides containing 17-bp core LANA binding sequence (LBS, italics and underlined) along with additional 3 and 6 -bp flanking sequence (italics) (**GATCC***GCCTCCCGCCCGGGCATGGGGCCGCG***GGATC**) having overlapping BamHI sites (bold) and its complementary strands were synthesized at IDT (Indianapolis, IN). Oligonucleotides were annealed and multimerized after polynucleotide kinase treatment. Multimerized LANA binding sequence containing oligonucleotides were then ligated to CNBr-activated Sepharose beads (Sigma, St. Louis, MO) according to the manufacturer's instructions. The unbound reactors were inactivated and the Sepharose was extensively washed with the binding buffer containing HEPES-HCl (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 10 μg of poly(dI-dC) (Amersham Bioscience)/ml, and 10% glycerol, and the approximately 300-ml bed volume of Sepharose was packed into an Econocolumn (Bio-Rad).

Nuclear extracts from BC-3 (KSHV infected) and BJAB (KSHV negative) cells were prepared as described earlier (59). Nuclear extracts from both BC-3 and BJAB cells containing 5 mg of total protein were diluted in binding buffer containing 25 mM HEPES-HCl, pH 7.9, 12.5 mM $MgCl_2$/1 mM dithiothreitol, 20% (vol/vol) glycerol, and 0.1% (vol/vol) Nonidet P-40. The nuclear extracts were added with 0.1% BSA, 0.4 mg of single-stranded DNA/ml, and 10 μg of poly(dI-dC)/ml. The columns were equilibrated with a binding buffer containing HEPES-HCl (pH 7.9), (12.5 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, 25% glycerol, 10 μg poly(dI-dC)/ml, and 0.1% BSA).

Diluted nuclear extract was combined with sonicated calf thymus DNA (0.4 mg/ml), poured into the column, and allowed to stand for 30 min at 4°C. The nuclear extracts were passed through the affinity resin by gravity flow (15 ml/hour). The resin was washed three times with binding buffer to remove any nonspecific protein associating with the Sepharose beads. The column was then washed with binding buffer containing 500 mM NaCl and 1 M NaCl in order to elute the proteins specifically associated to LANA when bound to its cognate sequence. BJAB nuclear extract was used as a control protein in the above affinity column. The eluted fractions were aliquotted and frozen at −80°C.

Western blot analysis of the elute. An aliquot of each fraction was resolved on SDS-PAGE followed by detection of ORC1 to ORC6 using specific antibodies. ORC1, ORC2, ORC4, and ORC5 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and ORC3 and ORC6 antibodies were generous gifts from Bruce Stillman (Cold Spring Harbor Laboratory). LANA was detected in these elutes using rabbit anti-LANA antibody (Cocalico Inc., Reamstown, PA). The signals were detected using the Odyssey infra-red scanning technology (Li-Cor, Lincoln, NE) using Alexa Fluor 680 and Alexa Fluor 800 (Molecular Probes, Carlsbad, CA, and Rockland, Gilbertsville, PA, respectively).

Electrophoretic mobility shift assay. A single copy of the LANA binding sequence annealed to its antisense strand was used in the electrophoretic mobility shift assay. The probe was filled in and labeled with α-^{32}P-labeled dCTP, dATP, dGTP, dTTP, and Klenow (New England Biolabs, Beverly, MA). The labeled probes were purified by Nuc Trap probe purification column (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions. The total incorporation of [α-^{32}P]dCTP was measured, and approximately 50,000 cpm of probe was used per reaction. Proteins used in the binding assay were nuclear extracts of 293 cells expressing the carboxy terminus of LANA (pA3M, LANA amino acids 762 to 1162), ORC2 (pCDNA3.1HA-ORC2), pA3M, and pCDNA3.1HA vectors alone.

Nuclear extracts (5.0 μg total protein) was used for binding to the LBS probe in electrophoretic mobility shift assay (EMSA) reactions. Unlabeled competitor (100-fold) was added 5 min prior to the addition of radiolabeled probes; 1.0 μg of goat anti-ORC2 was used to supershift the complex. The protein-DNA binding reaction mixture was incubated at 25°C for 15 minutes. The bound complex was loaded onto a 6.0% polyacrylamide gel containing 0.5× TBE (0.045 M Tris Borate, pH 8.2/1 mM EDTA). The gel was resolved in 0.5× TBE for 4 h at 150 V, dried, and autoradiographed using PhosphoImager plate (Molecular Dynamics, Inc.).

Chromatin immunoprecipitation assay. KSHV-positive BC-3 and BCBL-1 cells were growth arrested at G_1/S phase by treating them with thymidine followed by cross-linking with 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by adding 125 mM glycine to the culture medium. Cells were washed twice with unlabeled phosphate-buffered saline (PBS) and

TABLE 1. Primers used for amplification of KSHV genomic regions

Region and amplicon size (bp)	Sequence (5′–3′) and coordinates[a]	
	Sense strand	Antisense strand
TR, 90	GGGGGACCCCGGGCAGCGAG, 424–443	GGCTCCCCCAAACAGGCTCA, 496–515
K1, 205	AAATTTGTGCCCTGGAGTGA, 179–198	GTTGTGCACGCCATGTAATC, 365–384
ORF11, 199	GGCACAACAACAGCTACACG, 16679–16698	GATGATGAAGAGGGCGTTTC, 16859–16878
ORF17, 211	GCCCAGAAGCTTTTCATTTG, 32970–32989	TTAAACGCACCAAGGTAGGC, 33162–33181
ORF28, 217	CCTCCCGTGATTGGTCTTAT, 49066–49085	ATTGTACGGTAGGGCTCCTG, 49264–49283
ORF43, 206	CTGAGATGAGCTCCGAGGAC, 66492–66511	ACGTTGAAAAATCGGAGTGG, 66679–66698
ORF57, 201	GGGTGGTTTGATGAGAAGGA, 83077–83096	TGACCTCGCCAAGAAGGTTA, 83259–83278
ORF61, 200	GCTCGCATTCCTGACTCTGT, 98041–98060	GACCTTCGGGTGGTTAGACA, 98222–98241
K13, 202	ATTAGGGCATCCACGTCAGT, 122257–122276	GCGGGCACAATGAGTTATTT, 122440–122459
K15, 216	GCTTGAGACAAGGCCCATTA, 136055–136074	TCAGCCATTCTATCACTTGGTG, 136250–136271

[a] Designed based on the nucleotide sequence of BC-1 PEL cell line (47), accession number NC_003409.

resuspended in cell lysis buffer containing 5 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), KOH, pH 8.0, 85 mM KCl, 0.5%NP-40 and protease inhibitors and incubated on ice for 10 min. Cells were dounced for efficient lysis followed by centrifugation at 5,000 rpm for 5 min at 4°C. Nuclei were resuspended in nuclei lysis buffer, 50 mM Tris, pH 8.0/10 mM EDTA/1% SDS containing protease inhibitors, and incubated for 10 min.

Chromatin were sonicated to an average length of 700 bp and cell debris were removed by centrifugation at high speed for 15 min at 4°C. Supernatant containing the sonicated chromatin was diluted fivefold with chromatin immunoprecipitation dilution buffer containing 0.01% SDS/1.0% Triton X-100/1.2 mM EDTA, 16.7 mM Tris, pH 8.1/167 mM NaCl including protease inhibitors. Samples were precleared with salmon sperm DNA/protein A/Sepharose slurry for 30 min at 4°C with rotation. Supernatants were collected after brief centrifugation; 10% of the total supernatant was saved for input control and the remaining 90% was divided into three fractions: (i) control antibody (Sigma, Inc.), (ii) rat monoclonal anti-ORC2 (Santa Cruz, CA), and (iii) anti-MCM3 antibody (Abcam Inc.).

Immune complex was precipitated using salmon sperm DNA/protein A/protein G slurry. Beads were then washed consecutively with low-salt buffer containing 0.1% SDS/1.0% Triton X-100/2 mM EDTA–20 mM Tris, pH 8.1/150 mM NaCl; high-salt buffer containing 0.1% SDS/1.0% Triton X-100/2 mM EDTA–20 mM Tris, pH 8.1/500 mM NaCl; LiCl wash buffer containing 0.25 M LiCl/1.0%NP-40/1% deoxycholate–1 mM EDTA–10 mM Tris, pH 8.0; and twice in Tris-EDTA. The complex was eluted using elution buffer containing 1%SDS/0.1 M $NaHCO_3$ and reverse cross-linked by adding 0.3 M NaCl at 65°C for 4 to 5 h. Eluted DNA was precipitated and treated with proteinase K at 45°C for 2 h and was subjected to purification.

The primers (listed in Table 1) equally spaced on the KSHV genome were chosen to effectively cover the entire genome and used for amplification from chromatin-purified DNA. Amplified bands were quantified using KODAK 1D 3.6 image quantitation software (Kodak Gel Logic, Rochester, NY).

Coimmunoprecipitation of LANA and ORCs. ORC1 to ORC6 were cloned in pCDNA3.1 HA by PCR amplification of the respective template (56). The clones were analyzed for integrity by restriction digestion and sequence analysis. pA3M LANA (Myc-tagged) and pCDNA3.1 HA ORC1 to ORC6 were transfected separately into 293T cells. Cells were harvested 36 h posttransfection and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, pH 8.0) with protease inhibitors (1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml aprotinin).

Lysates were centrifuged to remove cell debris and precleared using control antibody. Precleared lysates were then incubated with anti-Myc antibody (Myc ascites) overnight at 4°C with rotation followed by incubation with protein A+G-Sepharose beads at 4°C for 1 h. The resulting immunoprecipitates were collected by centrifugation at 2,000 × *g* for 3 min at 4°C and the pellets were washed four times with 1 ml of ice-unlabeled RIPA butter and resuspended in 30 μl of 2× SDS protein sample buffer (62.5 mM Tris, pH 6.8, 40 mM dithiothreitol, 2% SDS, 0.025% bromophenol blue, and 10% glycerol). The proteins were resolved on SDS-PAGE using 8 to 10% acrylamide, transferred to nitrocellulose membranes, and subjected to immunodetection of ORCs using specific HA antibody. The membrane was stripped for the detection of LANA in immunoprecipitation.

Immunolocalization of LANA and ORCs. BC-3 and BCBL-1 cells were growth arrested in G_1/S phase by colchicine treatment and spread on glass slides. Cells were fixed in acetone/methanol (1:1) for 30 min at −20°C, air dried and incubated with 20% normal goat serum in 1× PBS to block the nonspecific binding sites. Slides were then incubated with rabbit anti-LANA polyclonal serum at room temperature in a humidified chamber followed by washing three times for 5 min in PBS. Rat-monoclonal anti-ORC2 and goat polyclonal anti-ORC3 (Santa Cruz, CA) followed by washing three times in PBS. Rabbit anti-LANA was detected using Alexa Fluor 594 (Molecular Probes, Carlsbad, CA). ORC2 and ORC3 were detected by Alexa Fluor 488. Slides were then washed four times with 1× PBS, mounted with Paramount G and visualized using an Olympus confocal laser scanning microscope.

In vitro binding of ORCs to LANA amino and carboxy termini. ORC1 to ORC6 were expressed in vitro by the coupled in vitro transcription/translation system (TNT) of Promega Inc. (Madison, WI) according to the manufacturer's instructions using [^{35}S]methionine/cysteine (Perkin Elmer Inc., Boston, MA) in the TNT reaction mixture. LANA glutathione *S*-transferase (GST) fusion proteins (approximately 10 μg for each reaction), LANA N (amino acids 1 to 340) and LANA C (amino acids 940 to 1162) expressed in *Escherichia coli* were used for in vitro bindings. In vitro-translated proteins were precleared with glutathione-Sepharose beads in binding buffer (1× phosphate-buffered saline, 0.1% NP-40, 0.5 mM dithiothreitol, 10% glycerol, 1 mM phenylmethyl sulfonyl fluoride, 2 μg of aprotinin per ml) for 30 min. Precleared labeled protein were then incubated with either GST or GST-LANA fusion proteins in binding buffer. Binding was performed overnight at 4°C with constant rotation followed by collecting the beads through centrifugation. The beads were washed three times with 1 ml of binding buffer followed by resuspending them in SDS lysis buffer and resolving them on SDS-PAGE. The bound fraction was analyzed after drying the gel and exposing to a phosphorImager plate (Molecular Dynamics, Inc.).

HA-tagged ORC1 to ORC6 expressed in 293T cells were also subjected to binding to GST-LANA:N and GST-LANA:C. 293T cells expressing HA-tagged ORC1 to ORC6 (pCDNA3.1 HA ORC1 to ORC6) were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, pH 8.0) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and centrifuged at 15,000 rpm and 4°C to remove cell debris. LANA-GST fusion proteins LANA:N and LANA:C (approximately 10 μg for each reaction) were subjected to overnight binding in RIPA buffer. The bound complex was washed five times to remove loosely bound proteins and resolved on SDS-PAGE. ORC1 to ORC6 were detected using anti-HA antibody after transfer to nylon membranes.

BCBL-1 (KSHV-infected) cell lysate was used for precipitation of endogenous ORCs with GST-LANA:N and GST-LANA-C. Approximately 20 million cells were lysed in RIPA buffer and incubated with LANA-GST fusion proteins as described above. Endogenous ORCs associating with LANA-N and –C-GST fusion proteins were detected using specific antibodies as described earlier (Santa Cruz Biotechnology, Santa Cruz, CA).

Short-term replication assay. pBS containing three copies of the TR was cotransfected into HEK293 and DG75 cells in four sets: (i) without LANA expression, (ii) with LANA expression, (iii) with N-terminal LANA expression, and (iv) with C-terminal LANA expression vector. At 96 h posttransfection DNA was extracted using modified Hirt's procedure (20). Briefly, medium from the 100 mm plates was removed and cells were washed with 1× PBS followed by lysing the cells in plates with a 1:2 mixture of solution I and II (solution I: Tris, glucose, and EDTA; solution II: SDS and NaOH). Lysed cells were transferred to a tube and added with 1.5 volume of solution III (potassium acetate, 3 M) followed by incubation on ice for 10 min and centrifugation at 8,000 rpm for 10 min. The supernatant was further extracted with phenol using phenol-chloro-

form-isoamyl alcohol and DNA was precipitated using 0.6 volume of 2-propanol. The pellet was dried and dissolved in TE with RNase and incubated at 37°C for 30 min followed by proteinase K treatment. Proteins were removed by a second round of phenol extraction followed by precipitation of DNA. Extracted DNA was digested with either EcoRI (to linearize) or with EcoRI and DpnI with sufficient enzyme overnight. Digested DNA was resolved on a 0.8% agarose gel and transferred to a nylon membrane using capillary method. DpnI-resistant copies of TR plasmid were detected by hybridization with a ^{32}P-labeled TR probe using PhosphorImager plates (Molecular Dynamics Inc.).

BrdU labeling and immunoprecipitation of labeled DNA. BrdU (5-bromo-2-deoxyuridine, thymidine analog) was administered 72 h posttransfection in 293 and DG75 transfected cells in the above-described combination of plasmids and pulsed for 4 h to label the replicating DNA. DNA was extracted as described previously. Hirt DNA was digested with DpnI overnight followed by immunoprecipitation of BrdU-labeled DNA using anti-BrdU mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (19); 1 μl of the above immunoprecipitated DNA was used for the detection of BrdU-labeled DNA by PCR using primers Puro-S, (5′-GCCACATCGAGCGGGTCAC-3′, and Puro-AS, 5′-TCGGCGGTGACGGTGAAG-3′). The 420-bp product was resolved on 2% Nusieve/1% agarose gel.

Purification of KSHV virion. Virions were purified as described previously (33). In brief, approximately 500 million BCBL-1 cells were induced with 20 ng of tetradecanoyl phorbol acetate/ml and 1.5 mM sodium butyrate (Sigma, St. Louis, MO) for 5 days. Medium containing virion particles was centrifuged to remove cell debris followed by filtering through 0.45-μm syringe filters. Virions were pelleted at 20,000 rpm for 2 h and resuspended in 1× PBS followed by purification in sucrose gradients at 25,000 rpm. Virion proteins were fractionated by treating the purified virions with trypsin and Triton X-100. A fraction of uninduced and induced cell lysates were resolved on SDS-PAGE along with the protein extracted with trypsin, Triton X-100 fractions, and core virion proteins.

RESULTS

ORCs elutes at high NaCl-containing binding buffer from LANA binding sequence affinity column. It has been reproducibly shown that LANA mediates replication of TR-containing plasmids in various cell backgrounds, supporting the hypothesis that the TR has the origin of replication (5, 18, 21, 37). Recently, it was demonstrated that a single copy of TR and a 72-bp region of the TR which contains the LBS and an upstream region can support replication of plasmids (22, 37). Since LANA has not been shown to have any known enzymatic activity required for replication but supports replication, it is believed that LANA recruits cellular proteins for replication of the TR-containing plasmids.

In order to find LANA-interacting proteins involved in the replication process, we used the LANA binding sequence to generate a DNA affinity column for the identification of cellular proteins associated with LANA that are also involved in replication. Sequences between coordinates 567 to 592 corresponding to the TR (32, 45) and containing the LBS sequence were synthesized along with the antisense strand to generate BamHI overhangs. Oligonucleotides were annealed and treated with polynucleotide kinase to add a phosphate group at the 5′ end. Polynucleotide kinase-treated double-stranded DNA was then subjected to multimerization by ligation with T4 DNA ligase (Fig. 1A).

Multimerized LBS DNA was ligated covalently to CNBr-activated Sepharose beads. Ligation efficiency, which was calculated based on the unbound DNA, was 70%. Nuclear extracts from BC-3 and BJAB were used order to find proteins interacting with LANA. BC-3 nuclear extract is expected to contain LANA but not the BJAB nuclear extract. To avoid nonspecific interactions we used relatively high concentrations of BSA (0.1%), and poly(dI-dC) (10 μg/ml) in the samples and

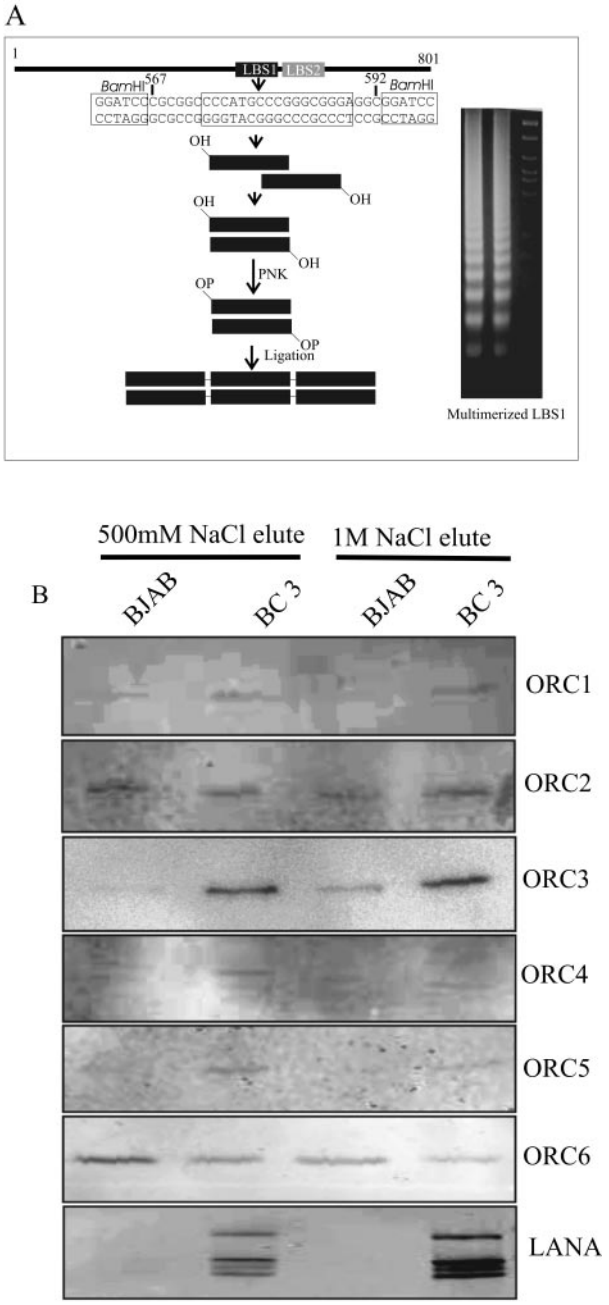

FIG. 1. DNA affinity column of LANA binding sequence. A: Core LANA binding sequence (LBS, italics and underlined) along with additional 3- and 6-bp flanking sequence (italics) (**GATCC*GCCTCCCGCC CGGGCATGGG***GCCGCG**GGATC**) having overlapping BamHI sites (bold) and the complementary strands were synthesized. Oligonucleotides were annealed and purified on native polyacrylamide gel electrophoresis. The double-stranded DNA strand containing LBS was treated with polynucleotide kinase (PNK) to add a phosphate group followed by multimerization using T4 DNA ligase. The multimerized LBS unit was ligated covalently to the CNBr-activated Sepharose beads and packed into the Econo Column (Bio-Rad Laboratories). Nuclear extracts from KSHV-positive BC-3 and KSHV-negative BJAB cells in binding buffer were added to the column and allowed to bind for 30 min at 4.0°C. Fractions of bound proteins were eluted with binding buffer containing NaCl. B: Fraction of proteins eluted in binding buffer containing 500 mM and 1 M NaCl were resolved on SDS-PAGE and probed with antibodies specific for ORC1 to ORC6.

elution buffers. The protein profiles eluted from the affinity column with increasing NaCl concentration were quite different between the BC3 (a KSHV-infected cell line) and BJAB (a KSHV-negative, Epstein-Barr virus-negative cell line) cell extracts (data not shown).

Surprisingly, the profiles eluted were quite different for some of the ORCs (Fig. 1). The content of ORCs expressed in these two cell lines was comparable but the protein eluted at 500 mM and 1 M NaCl showed different amounts of BC-3 compared to that seen in BJAB cells, suggesting that LANA has various affinities for different ORCs. It is interesting that ORCs can directly bind to the LBS sequence though with a somewhat weaker affinity (Fig. 1B). The majority of bound LANA was eluted at 1 M NaCl confirming the specificity of LANA binding to its cognate sequence. The results suggested that both ORC2 and ORC6 can bind to the LBS independently of LANA but ORC1, ORC4, and ORC5 are bound for the most part in the presence of LANA. Interestingly, ORC3 bound greater than around fivefold in the presence of LANA (Fig. 1B).

ORC2 associates with the LANA carboxy terminus bound to its cognate sequence in electrophoretic mobility shift assay. In order to confirm the binding of ORCs to LANA when complexed with its binding sequence in the TR, we performed electrophoretic mobility shift assays using a single copy of LBS as a probe. Nuclear extracts prepared from 293 cells expressing Myc-tagged LANA carboxy terminus (amino acids 762 to 1162) or pA3M vector only was used for binding to radiolabeled LBS probe. Nuclear extract containing LANA-C showed a specific shift in the mobility of the LBS probe (Fig. 2, lane 3, asterisk) which was abolished in the presence of a 100-fold excess of specific unlabeled competitor. Similar amounts of nonspecific unlabeled competitor did not show any effect on the mobility, suggesting that the interaction with LANA was specific, which was similar to previous report (10).

Addition of nuclear extract containing ORC2 altered the mobility of the probe:LANA-C complex (Fig. 2, lane 6, open circle) most probably due to the interaction of ORC2 with LANA bound to the LBS probe. Anti-ORC2 antibody when added to the complex supershifted the mobility of the bound complex suggesting that above shift was due to binding of LANA to ORC2 (Fig. 2, lane 7, two open circles). Lane 3 also has higher order complex similar to lane 6, which is more likely due to the binding of endogenous ORC2 to LANA. Nuclear extracts prepared from vector control cells did not change the mobility of specific bands (data not shown).

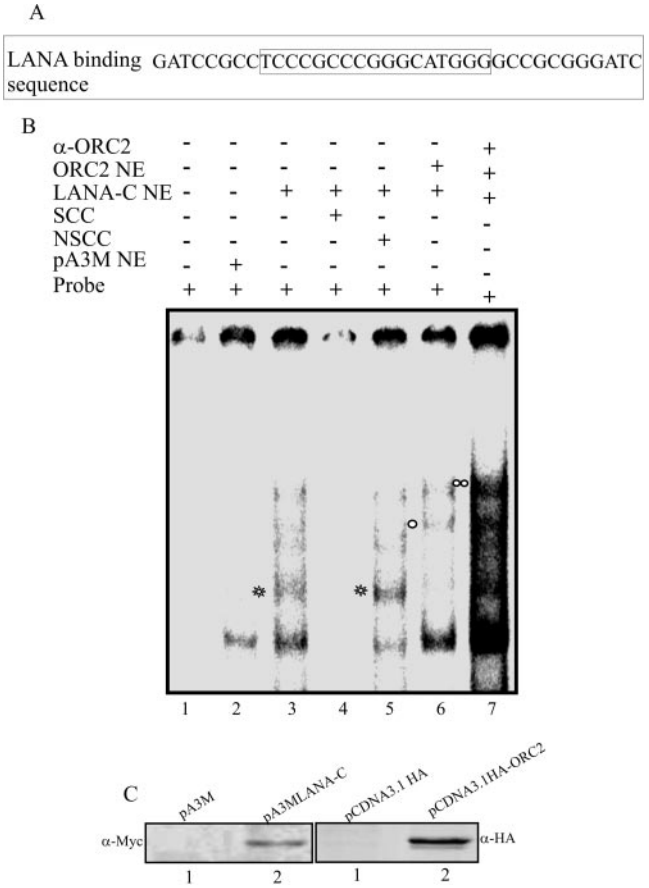

FIG. 2. Electrophoretic mobility shift assay. A, Single copy of LANA binding sequence used for the affinity column was end-labeled with ^{32}P and used as the probe. B, Binding of LBS with the carboxy terminus of LANA (LANA-C) as well as ORC2. Lane 1 is probe only (not seen in the gel as run faster). Lane 2 is probe with pA3M vector nuclear extracts. Lane 3 is probe with nuclear extract containing pA3M LANA-C (amino acids 762 to 1162) showing retarded mobility of the probe (asterisks) which was abolished by adding an excess (100-fold) of unlabeled specific probe (lane 4). The shift in mobility was unaffected with similar amounts (100-fold) of nonspecific unlabeled competitor (lane 5). Lane 6 contained probe with nuclear extracts of pA3M LANA amino acids 762 to 1162 (LANA-C) and pCDNA3.1 HA-tagged ORC2-expressing cells showed a change in the mobility of the probe due to the binding of ORC2 to LANA-C (open circle). Adding anti-ORC2 antibody changed the mobility of the entire complex, supershifting it (lane 7, two open circles). C, Western blots to detect proteins.

ORC2 and MCM3 bind to TR as well as regions in the LUR of the KSHV genome. Chromatin immunoprecipitation was performed on the entire KSHV genome of two PELs, BCBL-1 and BC-3; 10 regions were chosen which span the entire genome of KSHV and primers from these regions were used for PCR amplification from chromatins immunoprecipitated with anti-ORC2, anti-MCM3, and anti-LANA antibodies (see Table 1). Equal amounts of templates were used to amplify the respective regions and the amplicon was quantified for the relative amounts of DNA fragment immunoprecipitated in the chromatin immunoprecipitation assay (Fig. 3).

Amplification of the TR region using a specific primer confirmed that ORC2, MCM3, and LANA immunoprecipitated chromatin present at TR which is in agreement with the previous report (53). However, in addition to the TR region we found that at-least five other regions in the LUR can be immunoprecipitated using ORC2 and MCM3 antibodies but to a more limited extent with LANA antibody. These regions associates to various degrees of intensity with ORC2 and MCM3 at regions which include the K1, K13, ORF43, and ORF57 and possibly regions within these open reading frames (ORFs) (Fig. 3).

LANA has been shown to regulate ORF50 by transcription assays (33). Thus, it is highly probable that other regions including the regulatory regions flanking ORF50 may also have this activity but is not under the scope of this work. This study presents data suggesting that the replication protein complexes can associate with chromatin at multiple regions within the

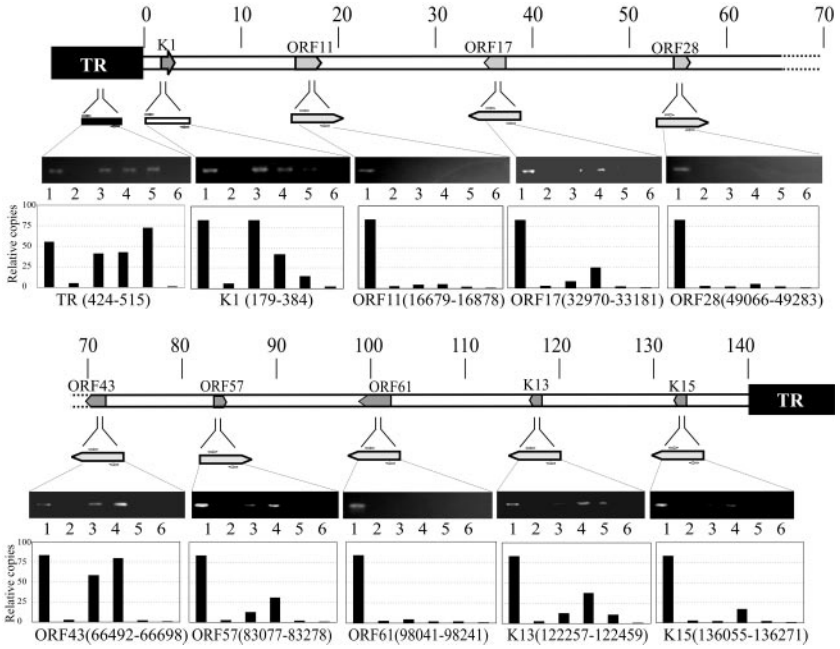

FIG. 3. Chromatin immunoprecipitation from PELs. G_1/S growth-arrested BC-3 and BCBL-1 cells were cross-linked with 1% formaldehyde followed by washing with PBS. Cell nuclei were released from these cells followed by sonication of chromatin to approximately 700 bp. Sonicated chromatins were diluted with chromatin immunoprecipitation dilution buffer. Chromatins were immunoprecipitated using antibodies specific for ORC2, MCM3, and LANA. The primer sets mentioned in Table 1 spanning the entire genome were used to amplify the regions of the KSHV genome. Amplified bands were quantified and the relative amounts were calculated. Lane 1, DNA purified from 10% of the total chromatin; lane 2, DNA from chromatin immunoprecipitated with control immunoglobulin G antibody (Sigma, Inc.); lane 3, DNA from chromatin immunoprecipitated with rat monoclonal anti-ORC2 antibody; lane 4, DNA present in chromatin immunoprecipitated with anti-MCM3 antibody; and lane 5, DNA from chromatin immunoprecipitated with rabbit anti-LANA antibody. Lane 6, water as a template for negative control of amplification.

LUR of the KSHV genome similarly to that proposed for Epstein-Barr virus showing that multiple regions in the Epstein-Barr virus genome can initiate replication as demonstrated by single molecule analysis of the replicated DNA (SMARD) (40). Further experiments are required and are ongoing to demonstrate that other regions within the LUR of KSHV can support replication.

Components of the ORCs immunoprecipitate with LANA. LANA is the KSHV functional homolog of EBNA1, which has been shown to interact with cellular replication machinery for the replication of Epstein-Barr virus genome (8, 11, 49) but has not been experimentally studied as to specific functions related to replication of KSHV TR plasmids or the KSHV genome. Previous in vitro binding studies investigated association with four components of the ORC (ORC1, ORC2, ORC4, and ORC5) indicated that LANA binding to ORCs (37). Here we performed a comprehensive study for mapping the interaction of ORCs with LANA in vivo in KSHV-positive cells as well as in vitro.

Cell lysates prepared from 293T cells transfected with Myc-tagged LANA and pCDNA3.1HA ORC1 to ORC6 were incubated with anti-Myc antibody after preclearing with matched immunoglobulin G control antibody. The immune complexes were precipitated using a mixture of protein A+G beads. HA-tagged ORC1-ORC6 transfected individually were also similarly processed. The results of the experiments showed that LANA can coimmunoprecipitate components of the human ORCs when expressed in human epithelial cells (Fig. 4). This suggests that LANA may form a large multi subunit complex which includes LANA and ORCs 1 to 6.

Components of ORCs colocalize to LANA in PELs. To further support the potential interactions of ORC components

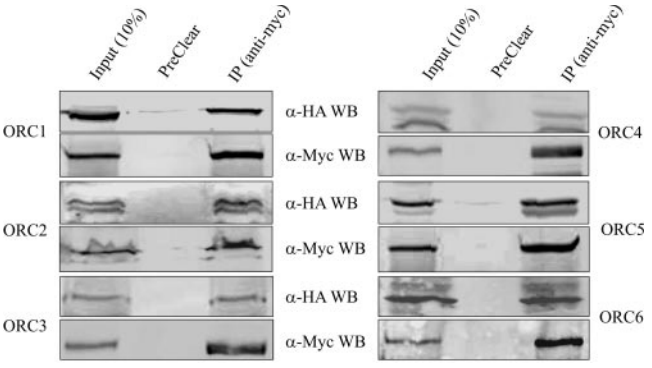

FIG. 4. Origin recognition complexes coimmunoprecipitate with LANA. pA3M LANA (Myc-tagged) and pCDNA3.1 ORC1-ORC6 (HA-tagged) were cotransfected into HEK293T cells; 36 h posttransfection, cells were harvested and lysed in RIPA buffer. Immunoprecipitates (IP) of anti-Myc antibody were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and immunodetected using antibodies specific for ORC1 to ORC6. Lane 1, input (10%) of the total lysate used for immunoprecipitation; lane 2, immunoprecipitation using control immunoglobulin G antibody; and lane 3, immunoprecipitation of anti-Myc antibody. Membranes were striped and reprobed with anti-Myc antibody for the immunoprecipitation of LANA in each set of immunoprecipitations.

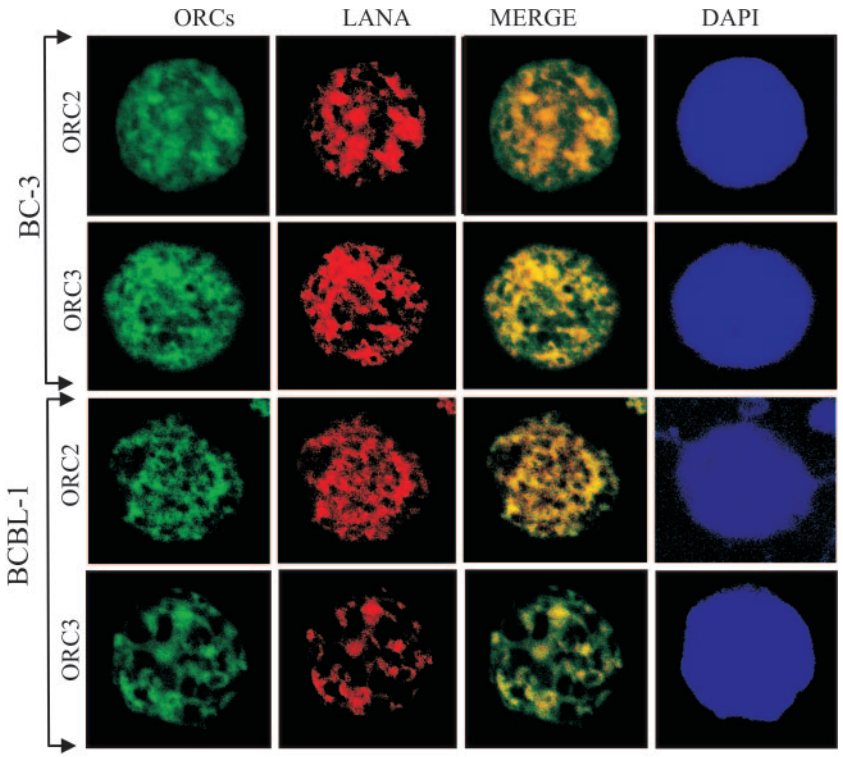

FIG. 5. Origin recognition complexes colocalize with LANA in PELs. G_1/S growth-arrested BC-3 and BCBL-1 cells were fixed in methanol/acetone and ORCs were detected using specific antibodies after blocking with 20% BSA. ORC2 and ORC3, which are ubiquitously expressed in human cells, were detected using rat monoclonal anti-ORC2 and goat polyclonal anti-ORC3 antibodies followed by detection using Alexa Fluor 488 (green) anti-rat and anti-goat antibodies for ORC2 and ORC3 proteins, respectively. LANA, which shows a punctate pattern in KSHV-infected cells, was detected using rabbit anti-LANA antibody followed by anti-rabbit Alexa Fluor 594 (red). The merge panel shows that most of the ORC2 and ORC3 colocalizes with LANA (yellow). The 4′,6′-diamidino-2-phenylindole (DAPI) panel shows both proteins are nuclear, as expected.

with LANA, we investigated the localization of LANA and ORC2 and ORC3 in G_1/S phase arrested cells by immunofluorescence assay. Localization of LANA using rabbit anti-LANA antibody visualized by Alexa Fluor 594 showed the characteristic punctate pattern of LANA in the nuclei of both BC-3 and BCBL-1 cells as reported previously (3, 9). ORC components which are expressed ubiquitously were detected by rat anti-ORC2 and goat anti-ORC3, respectively and detected by Alexa Fluor 488 secondary antibody showed nuclear staining and that specific components which contained LANA also showed localization of ORC2 and ORC3 (Fig. 5). These results suggest that LANA is present in compartments similar to that of cellular ORCs in KSHV-positive cells. The specificity of the signals was confirmed by incubating the cells with control immunoglobulin G and Alexa Fluor secondary antibodies which showed no specific signals (data not shown). Thus, these colocalization data strongly suggests that LANA binds to ORC components in vivo and most probably recruits as a pre-replicative complex at the replication initiation site.

Carboxy terminus of LANA binds to ORCs. LANA has distinct amino- and carboxy-terminal domains and these domains have well-defined roles in terms of tethering the viral genome to the host chromosomes and binding to the TR DNA. The amino terminus of LANA tethers to the nucleosomes through association with a number of cellular proteins (9, 30, 31, 35, 42). To determine the ORC interacting domain of LANA, we performed binding assay with GST fusion proteins of LANA:N and C with in vitro translated ORC1-ORC6. The binding data demonstrated that the recruitment of ORCs to the replication site is through a direct interaction with the carboxy terminus of LANA as all six ORC proteins showed strong affinity to the carboxy terminus of LANA (Fig. 6A). Among the ORC components, ORC1 and ORC5 showed strongest affinity to LANA followed by ORC6 and ORC2 (Fig. 6A). ORC3 and ORC4 showed relatively weaker affinity for LANA (Fig. 6A). In addition to binding to the carboxy terminus of LANA, ORC1 also has some affinity for the amino terminus of LANA, suggesting at least two major contact points. The specificity of binding was confirmed by using in vitro-translated luciferase protein which did not bind to either the amino or carboxy terminus of the LANA (Fig. 6A).

The binding of LANA to ORCs was further corroborated by expressing these proteins in human cells and incubating the cell lysates from 293T cells expressing HA-tagged ORC1 to ORC6 with LANA GST fusion proteins (Fig. 6B). Protein bound to the amino and carboxy termini of GST LANA was resolved and detected by HA antibody. Western blot data of the above binding showed that majority of ORC components bound to the carboxy terminus of LANA (Fig. 6B). Here again we found that the amino terminus of LANA bounds to ORC1 but also to some extent to ORC2 and ORC5 (Fig. 6B). Therefore these results confirm that the replication machinery is primarily

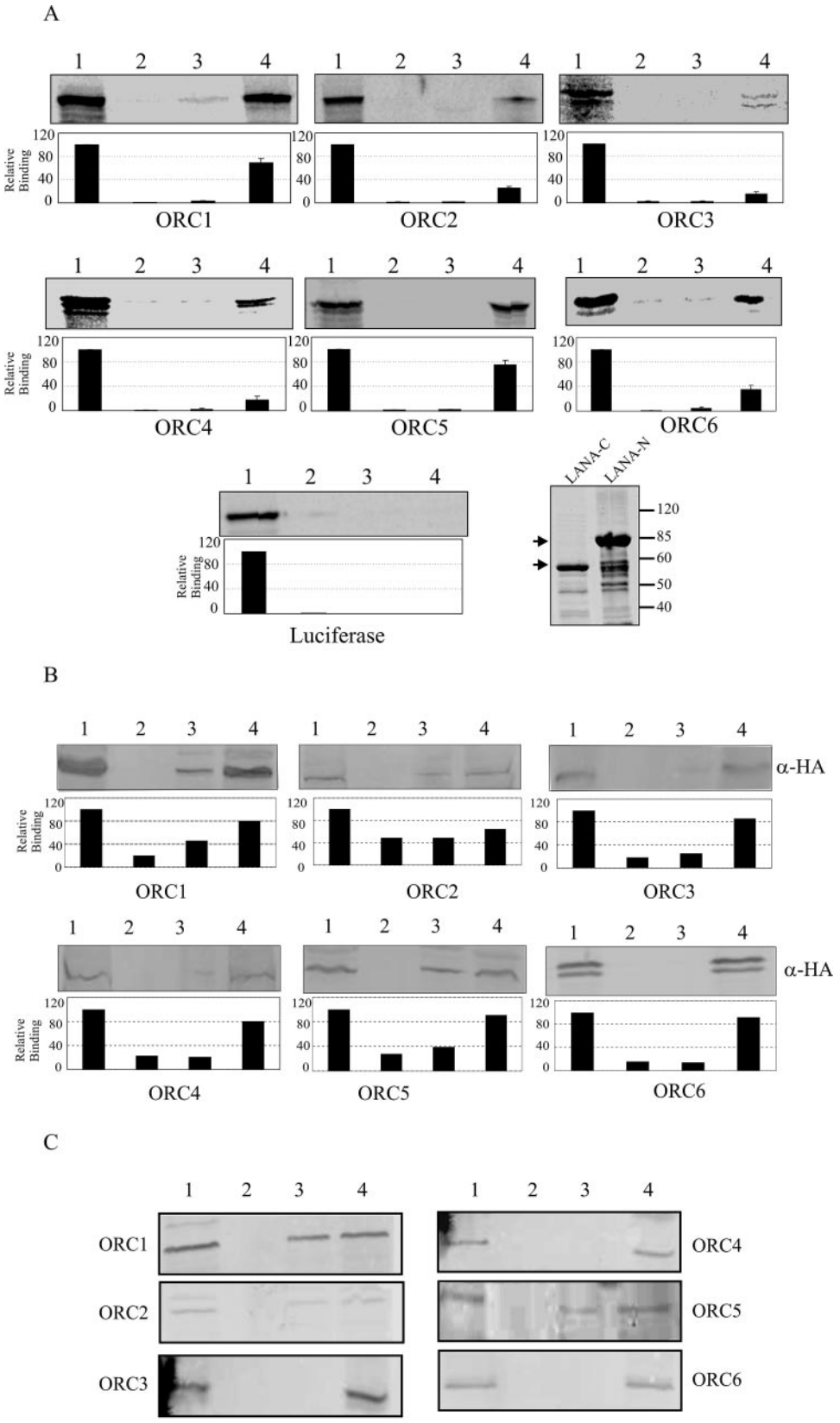

FIG. 6. Carboxy terminus of LANA binds to ORCs in vitro. A; [^{35}S]methionine-labeled in vitro translated ORC1 to ORC6 were incubated with either GST or GST fusion proteins with the amino and carboxy termini of LANA. Bound proteins were resolved on SDS-PAGE followed by detection by autoradiography. Lanes 1, input of in vitro translated ORC1 to ORC6; lanes 2, ORCs bound to GST protein; lanes 3 and 4, intensity of ORC1 to ORC6 bands associated with the amino and carboxy termini of LANA, respectively. Bands were quantified using Image Quant

recruited through the carboxy terminus of the LANA, which is also known to function as the DNA binding domain (10, 51).

To further support the above binding data, we determined whether the endogenous level of ORCs associated with LANA in KSHV-positive cells. Nuclear extracts of KSHV-positive BCBL-1 cells were incubated with GST fusion proteins of the amino and carboxy termini of LANA. The results again supported our observation that the carboxy terminus of LANA binds to ORCs and is thus capable of recruiting ORC components for replication (Fig. 6C). Moreover, ORC1 and ORC5 associated with the amino terminus of LANA with a relatively high affinity but ORC2 associated less in this particular assay (Fig. 6C).

KSHV TR-containing plasmids require both amino and carboxy termini of LANA for efficient replication. There is a strong correlation between replication of KSHV TR-containing plasmids and LANA expression (18, 21, 37). Our binding data suggested that the carboxy terminus of LANA is the primary binding site for the ORC complexes (see above). Therefore we wanted to determine if the carboxy terminus of LANA can support replication. A plasmid containing a single copy of the KSHV TR, pBSpuroTR, was transfected into DG75 (Fig. 7A, top panel) as well as 293 (Fig. 7B, bottom panel) cells and pulsed with BrdU to detect incorporation into newly synthesized DNA. Low-molecular-weight DNA isolated by a modified Hirt's procedure from LANA-expressing cells (+LANA), without LANA-expressing cells (−LANA) and two LANA truncation-expressing cells (LANA-N and LANA-C) with the TR plasmid were immunoprecipitated using anti-BrdU antibody.

The amplification of vector backbone from BrdU-precipitated DNA was the confirmation of incorporation of BrdU. PCR amplification of the directed site on the replicated plasmid shows that the TR plasmid replicated when LANA was present in *trans* (+LANA) but not in the absence of LANA expression (−LANA) (Fig. 7). Quantitations of amplicons are presented at the bottom of each gel showing presence of relative copies of BrdU incorporated DNA. BrdU incorporation data suggests that neither amino nor carboxy terminus of LANA is capable of supporting replication of the TR-containing plasmids (Fig. 7). The vector backbone control lacking TR did not show any specific incorporation of BrdU as expected (Fig. 7A, Vector).

We further confirmed the replication of a single copy of the TR-containing plasmid (pBSTR) in the DpnI sensitivity assay. 293 and DG75 cells were transfected with the TR and either vector pA3M, pA3M LANA, pA3M LANA-N, or pA3M LANA-C. Expression of LANA as well as its truncation mutants was detected using 9E10 Myc hybridoma (Fig. 7C). Low-molecular-weight DNA isolated 96 h posttransfection by a modified Hirt procedure was digested with either EcoRI (to linearize) or with EcoRI and DpnI followed by detection using the radiolabeled TR probe.

Southern blot analysis of resolved DNA detected DpnI-resistant copies of the input plasmids in LANA-expressing 293 as well as DG75 cells (Fig. 7B, open circles). However, neither the amino nor carboxy terminus of LANA was capable of supporting efficient replication, indicating that binding of ORCs to LBS with LANA-C was not sufficient for replication. These data suggests a critical role for the amino terminus of LANA in stabilizing the replication complex. Further experiments are warranted to identify additional proteins within the complex and dynamics of their association and dissociation for efficient replication and the role of amino terminus in stabilizing the replication complex.

ORCs and MCMs are not detected in association with KSHV virion DNA. The KSHV virion has been previously shown to carry some cellular as well as viral proteins in order to quickly establish latency following a short burst of lytic replication (29, 33). The replication transcriptional activator (RTA) has been shown to be present in the virion core, which is likely to be important in turning on the early lytic cycle (33). Therefore we wanted to determine whether components of the cellular replication machinery may also be packaged along with double-stranded DNA of KSHV. We screened for the presence of ORC2 and ORC3 in the purified virion by Western blot analysis with specific antibodies against these ORCs.

Our results indicate that ORCs were not present at detectable levels with the specific antibodies used in this assay (Fig. 8). Similarly, the minichromosome maintenance proteins (MCMs) were shown to function as helicases during replication were not convincingly detected in the KSHV virion (Fig. 8). As expected, our control, RTA, was detected in the virion core but not LANA which further supports our previous report (33).

DISCUSSION

The KSHV genome persists as an episome in infected cells by tethering to the host chromosomes (3, 9). LANA has been shown to be the key molecule for episomal persistence and replication of the viral genome. During latent infection LANA tethers the episome to host chromatin via interactions with histone H1 and possibly with other cellular proteins, including MeCP2 and DEK (9, 30, 35, 48). Regions of the KSHV genome lacking ORF73 coding sequence failed to persist long term in culture (3). KSHV cloned in a bacterial artificial chromosome (BAC36) disrupted for LANA expression (BAC36ΔLANA) failed to maintain viral episome and the infected cells became virus free after 2 weeks of selection (61). Additionally, knocking down LANA expression using short hairpin RNA technology targeted against the onco-

(Molecular Dynamics, Inc.) software and the relative binding of ORC1 to ORC6 with LANA N and LANA C are plotted after normalizing with the input lane. Bacterially expressed amino and carboxy terminus-GST fusion proteins of LANA used in the above binding assays are shown in the inset. B; Binding of ORC1 to ORC6 expressed in human cells (HEK293T cells). pCDNA3.1 HA ORC1-ORC6 (HA-tagged) was transfected into 293T cells. Nuclear extracts prepared from these cells were incubated with the amino and carboxy termini of LANA-GST fusion proteins and the bound fraction was resolved and transferred onto nitrocellulose and detected using anti-HA antibody. C; the amino and carboxy termini of LANA interact with endogenous ORCs. Nuclear extracts of BCBL-1 cells were incubated with either GST alone or LANA-N and LANA-C–GST fusion proteins. Bound proteins were resolved, transferred to nitrocellulose membranes, and \ immunodetected using ORC-specific antibodies.

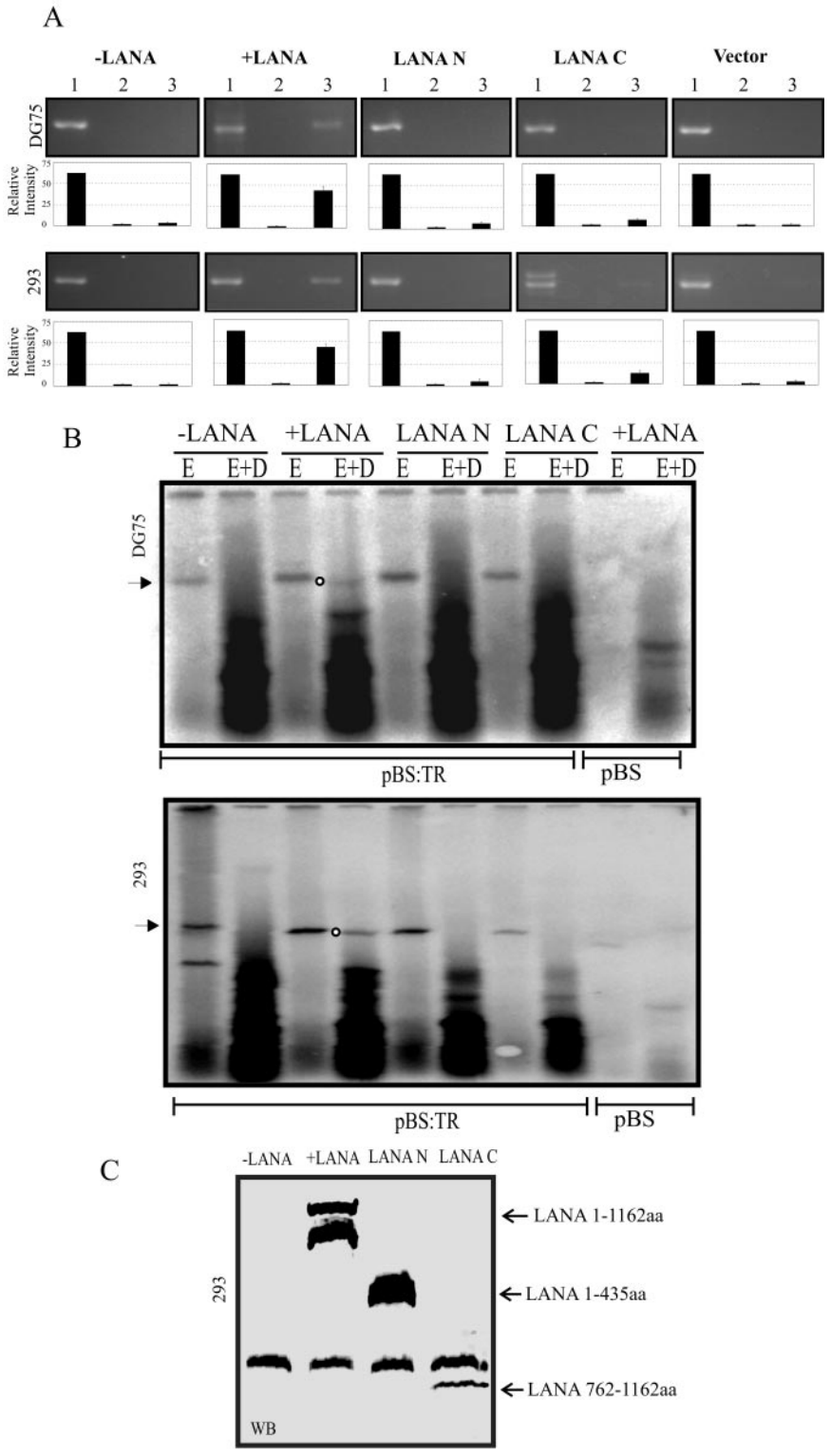

FIG. 7. Full-length LANA is required for replication of the TR-containing plasmids. A, Incorporation of BrdU in newly synthesized TR-containing plasmid DNA. pBSpurothe TR containing a single copy of the KSHV TR was cotransfected into 293 and DG75 cells with either empty vector pA3M (−LANA panel) or full-length LANA (+LANA panel), LANA amino acids 1 to 435 (LANA N panel), or LANA amino acids 762 to 1162 (LANA C panel). Vector panel, transfection of pBS vector backbone lacking the TR unit in the presence of the LANA expression vector. Transfected cells were pulsed with 50 μM of BrdU followed by isolation of low-molecular-weight DNA using a modified Hirt procedure. Isolated DNA was digested overnight with DpnI followed by immunoprecipitation of BrdU-labeled DNA using mouse anti-BrdU monoclonal antibody

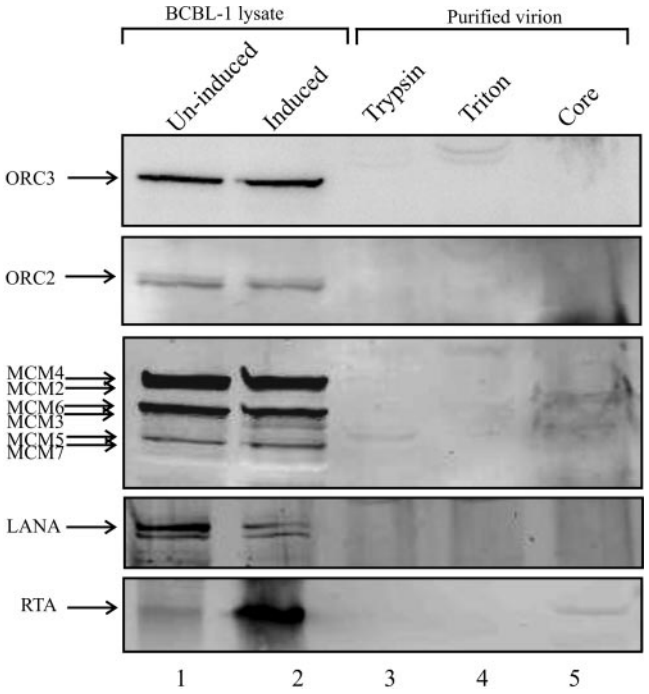

FIG. 8. ORCs and MCMs were not detectable in purified KSHV virions. Virions produced from 500 million BCBL-1 cells were concentrated and purified on a sucrose gradient and treated with trypsin followed by Triton X-100 to extract KSHV virion proteins (lanes 3 and 4, respectively). The remainder of the pellet containing virion core proteins (lane 5) was also resolved along with cell lysates from uninduced (lane 1) as well as tetradecanoyl phorbol acetate-induced (lane 2) BCBL-1 cells. Proteins were immunodetected using specific antibodies. ORC2 and ORC3 were detected using rat monoclonal anti-ORC2 and goat polyclonal anti-ORC3 antibodies. Pan-MCM antibody was used to detect MCM2 to MCM7. LANA was detected using anti-LANA rabbit serum and RTA was detected with mouse monoclonal antibody.

genic latent gene cluster including LANA reduced expression of LANA as well as the viral copy number in the PELs (17).

These data suggested that LANA is important for replication of the TR-containing plasmids. Another gammaherpesvirus which infects humans, Epstein-Barr virus, is also maintained as an episome by *cis*- and *trans*-acting elements and replicates once per cell cycle (1, 23, 38, 39). EBNA1 acts in *trans* to support the replication of the genome and segregation of the viral genome to daughter cells after mitosis (2, 23, 38, 39). Studies have established that EBNA-1 is the only viral *trans*-acting element required for stable persistence of the viral genome, although it is not required for initial rounds of replication (1). EBNA1 and LANA share functional homology despite differences in the amino acid sequence. Recently, a 3D-PSSM algorithm, which uses primary sequence information to predict the secondary structures of proteins, showed that the DNA binding domain of LANA shares structural similarity with the carboxy terminus of EBNA1 (18).

Additionally, unlike simian virus 40 large T antigen, papillomavirus E1, and origin binding proteins of herpes simplex virus type 1, LANA and EBNA1 sequence analysis did not reveal the presence of any motif associated with enzymatic activities such as helicase or ATPase, which are essential for replication (6, 52, 60). Recent data from various groups suggest that Epstein-Barr virus-encoded EBNA1 recruits ORCs at the *ori*P site (8, 11, 49). Based on the hypothesis that LANA is essential for replication and it binds to its cognate sequence in TRs, we attempted to identify the replication complex proteins associated with LANA when it is complexed with LBS. We focused our research on the components of ORCs because these proteins bind to DNA and serve as a launching pad for the assembly of replication machinery (54, 55). Our observation clearly demonstrates that ORCs can bind specifically to the LANA/DNA complex. Components of ORCs also came down in the 500 mM NaCl elute of BJAB nuclear extracts, suggesting that the ORCs may not require a specific nucleotide sequence (57). However, LANA-dependent binding of ORCs forms a distinct complex in KSHV-infected cells. ORC1 and ORC5 which were not detected in elutes of BJAB have the strongest binding affinity to LANA at both the amino and carboxy termini. Another possibility could be that ORCs bind to DNA in the immediate vicinity of LANA and stabilize the replication complex and are currently under investigation.

Electrophoretic mobility shift assay on monomers of the LBS used in the affinity column with LANA demonstrated a specific shift in mobility which was modulated by addition of ORC2 and corroborated the data showing binding of LANA with ORC2. Binding of ORC2 LANA can also be extrapolated to the association of other ORCs as these ORC components are known to bind to each other, forming a large multiprotein complex (58). We have demonstrated that ORC2 directly binds to LANA when it is bound to LBS DNA by electrophoretic mobility shift assay.

A previous report has mapped the DNA binding domain of LANA to residues 996 to 1139 of the carboxy terminus (28). Deletion mutation of this region identified a 15-amino-acid-residue segment (amino acids 1007 to 1021) as critical for DNA binding, replication, and episome persistence (28). Another report attempting to map the ORC1 and ORC2 interacting domain of LANA performed using GST fusion protein

(lane 3) in every panel. Lane 1, input (10%) of Hirt DNA used for immunoprecipitation. Lane 2, immunoprecipitation of BrdU-labeled DNA using control immunoglobulin G. Immunoprecipitated DNA was used for the amplification of vector-specific primers. Densitometric analysis of amplicons using Kodak 1D software is shown at the bottom of each panel. B, Southern detection of replicated copies of the TR-containing plasmids. pBSTR containing a single copy of the TR unit was cotransfected into 293 and DG75 cells with either empty vector pA3M (−LANA panel) or full-length LANA (+LANA panel), LANA amino acids 1 to 435 (LANA N panel), LANA amino acids 762 to 1162 (LANA C panel), and pBS vector with the LANA expression vector. Low-molecular-weight DNA was isolated and subjected to digestion with EcoRI (to linearize) or EcoRI and DpnI. Digested DNA was resolved on a 0.8% agarose gel, transferred to a Gene Screen membrane followed by detection of replicated copies using the ^{32}P-labeled TR-specific probe. C, Western blot showing the expression of LANA (amino acids 1 to 1162), LANA N (LANA amino acids 1 to 435), and LANA C (LANA amino acids 7622 to 1162) using anti-Myc antibody.

in vitro binding assays suggested that the region lacking residues 1062 to 1112 has no detectable binding to ORC1 and ORC2 (37). Taking together our electrophoretic mobility shift assay data along with the previously published data suggests that ORCs can bind to LANA in close proximity to the DNA binding domain and thus LANA may serve as stabilizing molecule by binding to DNA as well as ORCs. The complex of LANA and DNA might be facilitating the recruitment of ORCs to make direct contact with DNA, thus serving as a launching pad for the binding of other replication protein complexes for efficient replication. Therefore LANA may be helping to stabilize the ORC-DNA interaction and probably additional components of the replication machinery.

Recent studies have demonstrated that the KSHV genome persists as a highly ordered chromatin structure which favors the existing hypothesis that higher-order structures are critical for DNA replication and repair, chromosome condensation, and segregation (46). Here we have demonstrated that components of the replication machinery, ORC2 and MCM3, can associate with the chromatin structure of other regions besides the TR, suggesting the presence of additional potential sites of initiation of replication besides the TR region. These regions showed amplification in chromatin immunoprecipitation at the extreme left end of the LUR which could be due to its location near to the TR. Other potential regions immunoprecipitated in ORC2 and MCM3 chromatin immunoprecipitations are located in proximity to ORF43 and ORF57 of the KSHV genome, which flank ORF50, shown to be regulated by LANA (33). We are investigating the presence of other potential sites which can initiate/support replication.

This comprehensive study of LANA binding to ORCs strongly suggests a direct association between these proteins. Mapping the domain of LANA important for ORC binding showed that the carboxy terminus, which is also shown to be the DNA binding domain, is the primary domain for interaction with the replication machinery. GST pulldown assays done from in vitro-translated ORC1 to ORC6 as well as human cells expressing ORCs strongly suggest that ORCs are recruited to the repliosomes by the carboxy terminus of LANA. Precipitation of endogenous ORC complexes from PEL cells supports the fact that these proteins strongly interact in vitro as well as in vivo. Localization of LANA in BCBL-1 and BC-3 also showed the punctate pattern characteristic of its localization in KSHV-positive cells detected in G_1/S-arrested cells (3, 9). Immunolocalization of ORC2 and ORC3 in these cells supports the binding data that these two proteins are located within similar cellular compartments and associate in vivo. It has been reported that LANA binds to TR and colocalizes to DNA containing TR (9). The association of ORCs with LANA is likely to occur in close association with TR DNA. Experiments are warranted to demonstrate the association of LANA, ORCs, and TR in vivo with changes in cell cycle to further comment on the changes in the dynamics at the *ori* site within the TR.

Our data suggests that the carboxy terminus of LANA is sufficient for binding to ORC components and therefore we evaluated its potential to support replication of the TR-containing plasmids. Previous efforts to find out the replicative potential of the carboxy terminus of LANA have given mixed reports (21, 36). Hu et al. reported that LANA-C can partially support replication of TR plasmids in 293T cells, however; Lim et al. did not observe detectable replication (21, 36). Since all the replication was done only in epithelial/endothelial cells, we performed replication assays in epithelial (293) as well as B cells (DG75). We used the BrdU incorporation assay which is based on incorporation of the halogenated nucleotide BrdU (analog of thymidine) in the newly synthesized DNA followed by immunoprecipitation of labeled DNA to determine the ability of specific domains of LANA that support replication.

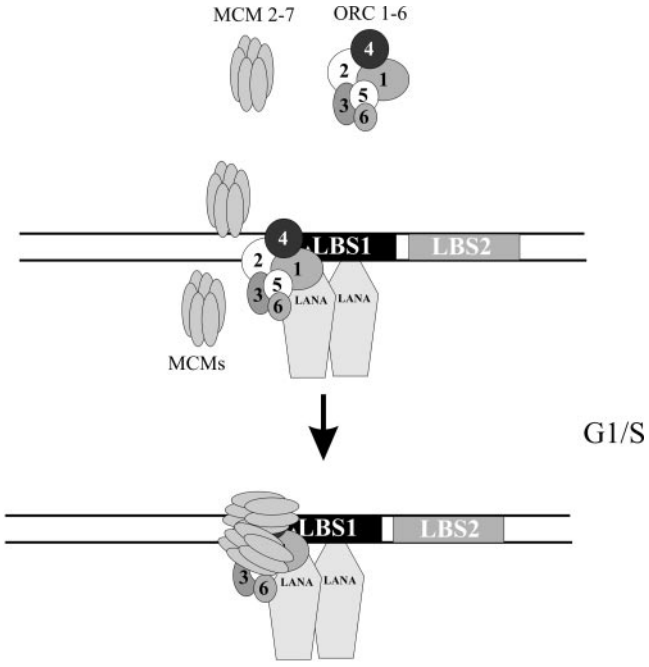

FIG. 9. Model of KSHV latent replication with recruitment of ORCs at the TR. LANA binds to its cognate sequence in TR through its carboxy terminus. The carboxy terminus of LANA also interacts with ORCs and binds to either the LBS core sequence or the flanking sequence. As LANA is important for tethering the viral genome to the host chromosomes, it is believed that LANA remains bound to the LBS throughout the cell cycle and most probably the ORC components also remain bound to the complex and thus could serve as a launching pad for the binding of other components of the cellular replication machinery, including MCMs. LANA may also be important for stabilizing the replication complex, which facilitates initiation of replication of the KSHV genome at the TR.

Our short-term replication data indicate that the amino and carboxy termini of LANA are ineffective in supporting efficient replication. This is likely due to the fact that ORCs and the associated replication components form aggregates at the TR and that the amino terminus of LANA may be important for stabilizing the entire replication complex. Experiments are under way to identify the specific TR region essential for supporting short-term replication. Recently, it has been reported that the AT region of the TR is dispensable for replication (22). Region containing LBS1/2 and the 39-bp GC-rich sequence upstream to these sequence lacking Sp1 binding sites are sufficient for replication (22). However, the exact site of initiation is yet to be mapped.

An analysis of virion proteins from purified virus did not show the presence of ORC2, ORC3 or MCMs. We hypothesized that viral double-stranded DNA embedded in the core of

the virion may be bound to these replication protein complexes and they may get transmitted along with associated viral DNA. However, these replication-associated proteins are not likely to be associated in abundance (detectable level) with packaged viral DNA, indicating that they may assembling and disassembling just prior to replication of viral DNA after infection and prior to packaging of DNA, respectively.

A current model of DNA replication at the KSHV TR based on this report as well as previously published reports would be that ORCs remain bound to DNA in close association with LANA and serves as a launching pad for the binding of other cellular replication proteins (Fig. 9). The MCMs bind to these complexes in a cell cycle-dependent manner and initiate replication at or near the LBS. Therefore, LANA plays an important role in recruitment and stabilization of the replication machinery for initiation of replication at the TR elements of KSHV.

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