

## Involvement of SSRP1 in Latent Replication of Kaposi's Sarcoma-Associated Herpesvirus<sup>∇</sup>

Jianhong Hu, Eugene Liu, and Rolf Renne\*

Department of Molecular Genetics and Microbiology and UF Shands Cancer Center, University of Florida, Gainesville, Florida 32610

Received 6 May 2009/Accepted 19 August 2009

**Kaposi's sarcoma-associated herpesvirus (also named human herpesvirus 8) is a  $\gamma$ -herpesvirus that undergoes both lytic and latent infection. During latent infection, two viral elements are required: latency-associated nuclear antigen (LANA), which functions as an origin binding protein, and the latent origin, which resides within the terminal repeats (TRs) of the viral genome. Previously, we identified two *cis*-elements within the TRs which are required for latent DNA replication: two LANA binding sites (LBS1 and LBS2 [LBS1/2]) and a GC-rich replication element (RE) upstream of LBS1/2. To further characterize the RE, we constructed a 71-bp minimal replicon (MR) and performed a detailed mutational analysis. Our data indicate that the first 8 nucleotides within the RE are critical for replication. Moreover, both the position and the distance between the RE and LBS1/2 can affect origin replication activity, suggesting that the RE may function as a loading pad for cellular proteins involved in replication. Using biotinylated DNA fragments of wild-type or mutant MRs as probes, we identified 30 proteins that preferentially bind to the origin. Among these proteins, structure-specific recognition protein 1 (SSRP1), a subunit of the FACT complex, and telomeric repeat binding factor 2 (TRF2) formed complexes with LANA at the MR region. Furthermore, the small interfering RNA-based knockdown of SSRP1, but not the dominant-negative-based knockdown of TRF2, significantly decreased the efficiency of LANA-dependent DNA replication. These results indicate that SSRP1 is a novel cellular protein involved in LANA-dependent DNA replication.**

Kaposi's sarcoma-associated herpesvirus (KSHV), also named human herpesvirus 8 (HHV-8), is associated with Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman's disease (MCD) (6, 7, 37, 45). During latent infection, viral gene expression is highly restricted and only a small subset of viral proteins encoded by the KSHV latency-associated region is expressed, including the latency-associated nuclear antigen (LANA), vCyclin, vFLIP, Kaposins, and 12 viral miRNA gene products (11, 19, 36, 38, 39).

LANA, encoded by *orf73*, plays an important role in the maintenance of KSHV latency. The disruption of LANA enhances KSHV lytic gene expression and infectious virion production (22a). LANA regulates the transcription of several viral and cellular genes and is required for latent DNA replication and segregation during S phase and mitosis (1, 2, 17, 27). The interaction of LANA with two major tumor suppressors, p53 and Rb, results in the inhibition of apoptosis and the dysregulation of the cell cycle (13, 35). LANA also targets the Wnt pathway by stabilizing  $\beta$ -catenin, thereby inducing cells into S phase (14). In addition, LANA interacts with a number of cellular proteins, including ATF4/CREB2, mSin3, HP1 $\alpha$ , CBP, RBP-J $\kappa$ , and RING3, to modulate viral and cellular gene expression (21, 22, 24–26, 34).

Ballestas et al. first demonstrated that LANA conveys episomal long-term maintenance of terminal repeat (TR)-containing cosmids (2). A chromosome binding sequence within the N terminus of LANA was shown to interact with histones

H2A/H2B and several chromatin-associated proteins (MeCP2 and Brd4), thereby tethering viral episomes to mitotic chromosomes (4, 20, 54). Similar tethering models have also been proposed for Epstein-Barr virus (EBV) and bovine papilloma virus (28, 53).

LANA also functions as an origin binding protein for KSHV latent DNA replication (17, 27). The C-terminal DNA binding domain of LANA binds to two sites within TRs which leads to the recruitment of host cell origin recognition complex (ORC) and minichromosome maintenance (MCM) proteins and, subsequently, to DNA replication (15, 27, 46, 48). While the C terminus alone is sufficient for the replication of TR-containing plasmids, the N-terminal chromosome binding sequence of LANA enhances the replication activity of the LANA C terminus, suggesting that both replication and maintenance are interrelated (3, 17). Latent KSHV DNA replication is dependent on the host replication machinery, and as a result, viral episomes, like host chromosomes, replicate once per cell cycle (49).

The KSHV latent origin resides within the TRs of the viral genome. Each 801-bp-long TR contains a high- and a low-affinity LANA binding site (LBS1 and LBS2 [LBS1/2], respectively), separated by 22 bp from center to center, and is functionally reminiscent of half of a dyad symmetry (DS) of EBV *oriP* (15, 16, 51). Multiple copies of LBS1/2 appear to function analogously to the family of repeats of EBV for viral genome segregation (43). In addition to LBS1/2, an adjacent highly GC-rich sequence, termed the replication element (RE), is required for replication. Deletion of the RE within a TR-containing plasmid completely abolishes origin activity (18, 49). These observations suggest that the RE may serve as a landing pad for cellular proteins involved in DNA replication.

\* Corresponding author. Mailing address: University of Florida, 1376 Mowry Rd., Gainesville, FL 32610-0232. Phone: (352) 273-8204. Fax: (352) 273-8299. E-mail: rrenne@ufl.edu.

<sup>∇</sup> Published ahead of print on 26 August 2009.

It has been well established by several laboratories using *in vitro* and *in vivo* assays that TR sequences and LANA, analogous to *oriP* in conjunction with EBNA-1 of EBV, recruit host cellular ORC and MCM proteins (8, 10, 27, 40, 46, 48). Furthermore, Stedman and colleagues demonstrated the presence of several chromatin-remodeling factors residing at TRs within latently infected primary effusion lymphoma. Additionally, the TRs are part of a highly structured nucleosome array, which undergoes reorganization in late G<sub>1</sub>/S phase when replication licensing and initiation occur (46). The overall goals of this study were to define the minimal KSHV latent replicator and to use this information to identify cellular proteins involved in LANA-dependent DNA replication. A strategy of targeted mutagenesis was employed that resulted in the delineation of a 71-bp minimal replicon (MR). Wild-type and mutant MRs were then used as probes in a proteomics approach to identify cellular proteins involved in LANA-dependent DNA replication. Thirty proteins were identified that bound preferentially to MRs compared to controls. Among these candidate proteins, novel proteins that bound to LANA at the origin were identified, and the functional significance of these interactions was evaluated using knockdown approaches.

#### MATERIALS AND METHODS

**Cell lines and plasmids.** Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics at 37°C under a 5% CO<sub>2</sub> atmosphere. BJAB Tet-on/LANA cells (1) were cultured in RPMI 1640 medium supplemented with 10% Tet-on fetal calf serum.

pcDNA3.1/ORF73 and pCRII-TR were described before (17). The wild-type MR, pCRII-MR, eight MR mutant plasmids, pCRII-ER-LBS1/2, pCRII-LBS1/2-RE, pCRII-RE(+5)-LBS1/2, and pCRII-RE(+10)-LBS1/2 were constructed by inserting the upper and lower oligonucleotides into the HindIII and NotI sites of pCRII after the phosphorylation and annealing of the corresponding oligonucleotides. The structure-specific recognition protein 1 (SSRP1) expression plasmid pcDNA3-2xFLAG-SSRP1 was a generous gift from Hua Lu (Oregon Health & Science University). The dominant-negative TRF2 (DN-TRF2) expression vector was kindly provided by Paul Lieberman (Wistar Institute).

**Short-term replication assay.** Short-term replication assays were performed as previously described (17). Briefly, 8 µg of each mutant TR construct was cotransfected with 2 µg of pcDNA3/ORF73 or carrier DNA into 293 cells using TransIt-293 transfection reagent (Mirus). Seventy-two hours after transfection, extrachromosomal DNA was recovered by Hirt extraction. Ten percent of the episomal DNA was linearized with HindIII as input, and 90% of the extracted DNA was double digested with DpnI and HindIII. Newly synthesized, DpnI-resistant DNA was detected by Southern blotting and quantified using radiographic densitometry. The replication efficiency of each plasmid was calculated by comparing the density of the replicated band with that of its input.

**Western blot analysis.** A total of 1 × 10<sup>5</sup> cells were lysed in 100 µl Laemmli sample buffer. Cell lysates were boiled for 5 min before loading. Ten microliters of each cell lysate was separated on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were blocked for 2 h in Tween 20-Tris buffered saline containing 5% fat-free dry milk. Primary antibodies against specific proteins were diluted according to the manufacturer's instructions and hybridized with membrane at 4°C overnight. After washing was completed, 1:5,000-diluted corresponding secondary antibodies conjugated with peroxidase were incubated with the membrane for 1 h at room temperature. After a final washing, the blots were developed with ECL substrates (Millipore) and exposed to films.

**Affinity chromatography and protein identification.** The purification of the nuclear extracts and nuclear pellet extracts and affinity chromatography was performed as described previously (9) with modifications. A total of 7 × 10<sup>9</sup> BJAB Tet-on LANA cells were induced with 1 µg/ml doxycycline for 4 days. The cells were lysed with 26 ml buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]) on ice. The nuclei were homogenized and lysed in 21 ml buffer C (20

mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 1 mM PMSF). After centrifugation was performed, the soluble fraction (nuclear extract) was collected and dialyzed against D150 buffer (10 mM HEPES [pH 7.9], 150 mM NaCl, 20% glycerol, 0.4 mM EDTA, 0.05% NP-40, 1 mM PMSF, 10 mM 2-mercaptoethanol, protease inhibitor cocktail) at 4°C overnight. The insoluble fraction (nuclear pellet) was suspended in 21 ml nuclear pellet solubilization buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM DTT, protease inhibitor cocktail, 1% Triton X-100, 0.1% Na deoxycholate), homogenized, and sonicated. The supernatant fraction (solubilized nuclear pellet) was collected after centrifugation. The nuclear extract was mixed with solubilized nuclear pellet at a 1:1 ratio (11 mg/ml) and preincubated with 0.4 mg/ml salmon sperm DNA at 4°C for 1.5 h prior to affinity chromatography. Biotinylated fragments were PCR amplified with 5'-biotinylated forward primers and regular reverse primers. The forward and reverse primers for the MR fragment and the fragment of the RE mutant with an 8-bp sequence substitution at positions 1 to 8 (RE<sub>m1-8</sub>-LBS1/2) are 5'-CCCAGGCTTTACTTTATGC-3' and 5'-CTCTCGCTATTACGCCAG-3'. The forward and reverse primers for the ampicillin fragment are 5'-GGATCTCAACACGGTAAA-3' and 5'-TCACGCTCGTGGTTGGTA-3'. Fifty micrograms of biotinylated PCR fragments were coupled with streptavidin-conjugated magnetic beads (Invitrogen) at room temperature for 45 min and then incubated with 10 ml nuclear extract mixture at 4°C overnight. After being washed three times with 10 ml D150 buffer, the beads were sequentially eluted in 100 µl D300 buffer and 100 µl D1000 buffer (both the same as D150 except for 300 mM KCl or 1,000 mM KCl, respectively) for 15 min. Eluates were combined and adjusted with D0 buffer (the same as D150 except for no KCl) to make the KCl concentration 150 mM. The affinity-purified proteins were subjected to another round of purification. The affinity-purified proteins were separated on a 4 to 20% gradient SDS-PAGE gel and stained with colloidal blue. Gel slices were cut out and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry as previously described (ICBR at the University of Florida). Small-scale affinity purification with 5 µg biotinylated DNA fragments and 1 ml nuclear extract mixture was performed to confirm the mass spectrometry result. Purified proteins were eluted in Laemmli sample buffer and subjected to Western blotting analysis.

**Immunoprecipitation.** The 293 cell lysates were assayed by immunoprecipitation 48 h after transfection. One 10-cm plate of transfected 293 cells was washed once with ice-cold phosphate-buffered saline (PBS) and lysed directly with 1 ml ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EDTA [pH 8.0], supplemented with 1 mM PMSF and protease inhibitors). Cell lysates were scraped off the plates, transferred into centrifuge tubes, and incubated at 4°C for 15 min with rotation. The cell lysates were centrifuged at 14,000 × g for 15 min to remove cell debris. The supernatant was transferred immediately to a fresh centrifuge tube and precleared with 75 µl protein A/G beads at 4°C for 2 h. Five percent of the total cell lysates was saved as input. The remaining lysates were incubated with 5 µg appropriate antibodies and 100 µl protein A/G beads at 4°C overnight with rotation. The beads were collected by pulse centrifugation and washed five times with 1 ml ice-cold radioimmunoprecipitation assay buffer. The immune complexes were eluted in 60 µl of 2× Laemmli loading buffer (62.5 mM Tris [pH 6.8], 40 mM DTT, 2% SDS, 0.025% bromophenol blue, and 10% glycerol) and subjected to Western blotting analysis.

**ChIP-real-time PCR.** Transfected 293 cells were assayed by chromatin immunoprecipitation (ChIP) 48 h posttransfection. The cells were cross-linked by directly adding formaldehyde to the growth medium to a final concentration of 1% at room temperature for 10 min. Cross-linking was stopped with 0.125 M glycine at room temperature for 5 min. The cells were washed twice in ice-cold PBS with protease inhibitors and lysed in ice-cold swelling buffer (5 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid); pH 8.0], 85 mM KCl, 0.5% NP-40) with protease inhibitors. The nuclei were spun down and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1]). The chromatin was sheared to about 500-bp fragments with three sets of 10-s pulses using a sonic dismembrator (Fisher Scientific) set to 40% of maximum power. Eight percent of each sample was saved and used for input DNA, and the remainder was diluted to 2 ml with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM Tris-HCl [pH 8.1], 167 mM NaCl, and protease inhibitor cocktail). The chromatin was precleared for 30 min at 4°C with 75 µl protein A/G agarose beads and 0.4 mg/ml salmon sperm DNA. Five micrograms of appropriate antibodies and 100 µl protein A/G beads were added to each sample and incubated with rotation at 4°C overnight. The immune complexes were collected by pulse centrifugation and washed once with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.1], 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.1], 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate,

1 mM EDTA, 10 mM Tris [pH 8.1]) and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The immune complexes were eluted twice in freshly prepared elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>) at room temperature for 15 min each, and the eluates were combined. The chromatin was reverse cross-linked by adding 20  $\mu$ l of 5 M NaCl and heated at 65°C for 4 h. The samples were treated with 10  $\mu$ M EDTA (pH 8.0), 40  $\mu$ M Tris-HCl (pH 6.5), and 40  $\mu$ g/ml proteinase K for 1 h at 37°C. DNA was recovered by phenol-chloroform extraction and ethanol precipitation. Twenty micrograms glycogen was added as a DNA carrier. DNA pellets were washed once with 70% ethanol and resuspended in 50  $\mu$ l H<sub>2</sub>O. Two microliters of each sample was used as the template for real-time quantitative PCR using SYBR green master mix (Bio-Rad). Forward primer 5'-CCCAGGCTTTACACTTTATGC-3' and reverse primer 5'-CTCTT CGTATTACGCCAG-3' were utilized to amplify fragments spanning the MR or REM<sub>1-8</sub>-LBS1/2 from pCRII-MR or pCRII-REM<sub>1-8</sub>-LBS1/2, respectively. The negative-control fragment was amplified with primers 5'-AATCTGGTCACCC TGAGCT-3' and 5'-GGCTGCGCTTAAGGACATT-3' specific for the immunoglobulin G (IgG) Fc gene. The relative amount of DNA immunoprecipitated in the ChIP sample was normalized on the input DNA.

**Synchronization of cells and cell cycle analysis.** A total of  $3 \times 10^6$  cells were double treated with thymidine for G<sub>1</sub>/S-phase arrest. A total of 2 mM thymidine was added directly to the cell culture medium for 19 h. The cells were washed three times with PBS and then incubated with fresh medium for 10 h. A second block with 2 mM thymidine was performed for 15 h. For S-phase arrest, the double thymidine-arrested cells were washed three times with PBS and released into S phase in complete medium for 4 h. To obtain M-phase-arrested cells, the cells were treated with 100 ng/ml nocodazole for 14 h. The arrested cells were fixed with ice-cold 70% ethanol for 30 min, stained with staining buffer (0.1% Triton X-100, 0.2 mg/ml RNase A, 20  $\mu$ g/ml propidium iodide in PBS) at room temperature for 45 min, and analyzed by flow cytometry.

**siRNA inhibition of SSRP1.** A total of 100 nM scrambled small interfering RNA (siRNA) or siRNA against SSRP1 (Dharmacon) was transfected into 293 cells with Lipofectamine 2000 (Invitrogen) when the cell density was about 30 to 50% according to the manufacturer's protocol. Western blotting was performed 96 h posttransfection to determine the knockdown efficiency. For replication assays in SSRP1 siRNA-transfected cells,  $1.5 \times 10^6$  293 cells were first transfected with 100 nM siRNA with Lipofectamine 2000. At 24 h posttransfection, the cells were transfected with pCRII-TR in the presence or absence of LANA plasmid. The cells were harvested for the replication assay 72 h after the second transfection.

## RESULTS

**The first 8 nucleotides (nt) within the RE are required for latent origin activity.** Two *cis*-regulatory elements found within the terminal repeat, LBS1/2 and an upstream GC-rich RE, are absolutely required for LANA-dependent DNA replication. Additionally, a deletion analysis previously determined that at minimum, 32 bp of the RE are essential for latent origin activity (18, 49).

To further study the contribution of RE to DNA replication, we first constructed a 71-bp MR-containing plasmid, pCRII-MR, which contains a 32-bp RE and LBS1/2 without additional flanking sequences from the KSHV genome. Short-term replication assays were performed to determine the replication activity of pCRII-MR in comparison to a full-length TR-containing plasmid (pCRII-TR). For each assay, 10% of the Hirt-extracted DNA was linearized with HindIII (input), while the remaining 90% of DNA was digested with HindIII and DpnI to detect *de novo*-synthesized DNA. The replication efficiency for each plasmid was determined by comparing the intensity of the replicated DNA band with that of the corresponding input band from two or three independent assays. As shown in Fig. 1, both the MR-containing and full-length TR-containing plasmids replicated with similar efficiency (5% of the total input) in the presence of LANA (Fig. 1A, compare lanes 1 and 3 with lanes 2 and 4).

In order to determine which sequences within the RE are important for function, we constructed four RE mutants:

REM<sub>1-8</sub>-LBS1/2, REM<sub>9-16</sub>-LBS1/2, REM<sub>17-24</sub>-LBS1/2, and REM<sub>25-32</sub>-LBS1/2. Each RE mutant has an 8-bp sequence substitution at positions 1 to 8, 9 to 16, 17 to 24, and 25 to 32, respectively (Fig. 1B). In the mutants, 50% GC-content sequences instead of high GC-content sequences were substituted, assuming that the DNA sequence itself and not GC content determines origin activity. This assumption is based on previous data showing that upstream GC-rich sequences could not replace the RE element in the context of TRs to support DNA replication (18). As shown in Fig. 1C, the replication efficiency decreased for all four mutants compared to that of the wild-type MR. Interestingly, replication was inhibited to a greater extent when sequences located more distally from LBS1/2 were mutated than sequences directly adjacent to LBS1/2 (Fig. 1C, compare lanes 9 to 12).

Replication activity for REM<sub>1-8</sub>-LBS1/2 and REM<sub>9-16</sub>-LBS1/2 dropped to 4.8% and 9.6% of that of wild-type MR activity, suggesting that sequences within the first 16 nt of the RE are critical for function. To further define critical bases within the RE, we constructed four additional mutants, REM<sub>1-4</sub>-LBS1/2, REM<sub>5-8</sub>-LBS1/2, REM<sub>9-12</sub>-LBS1/2, and REM<sub>13-16</sub>-LBS1/2, each with a 4-nt substitution (Fig. 1B). Short-term replication assays with these four mutants showed that the mutation of nt 13 to 16 does not change the origin activity (Fig. 1D, lane 12). REM<sub>9-12</sub>-LBS1/2 exhibited a moderate reduction in replication (41%) (Fig. 1D, lane 11), while the mutation of nt 1 to 4 and nt 5 to 8 decreased the replication efficiency to 13.2% and 8.7%, respectively (Fig. 1D, lanes 9 and 10). These data identify the first 8 nt within the RE as the most important for origin activity.

**The orientation, position, and spacing of the RE relative to LBS1/2 are critical for origin function.** We hypothesize that the RE may interact with cellular proteins, thereby contributing to the initiation of DNA replication. If so, both the orientation and the position of the RE relative to LBS1/2 should be important for origin activity. To test this hypothesis, we constructed a mutant origin ER-LBS1/2, in which the orientation of the RE was reversed upstream of LBS1/2, and mutant LBS1/2-RE, in which the RE was cloned in the wild-type orientation downstream of LBS1/2. As shown in Fig. 2A, neither of these mutants replicated (Fig. 2A, lanes 7 and 8), suggesting that cellular proteins occupying the RE might cross talk with LANA bound to LBS1/2. To further investigate this interpretation, two more mutants were constructed in which the distance between the RE and the LBS1/2 was altered. Mutant RE(+5)-LBS1/2 contains a 5-nt insertion between the RE and LBS1/2, positioning the RE half a DNA helix away from LBS1/2 and, importantly, on the opposite face of the DNA; the mutant RE(+10)-LBS1/2 has a 10-nt insertion, and the RE is one full helical turn away from LBS1/2.

As shown in Fig. 2B, both mutants were completely deficient in DNA replication (Fig. 2A, lane 7, and B, lane 8). The fact that the orientation, relative position, and distance of the RE and LBS1/2 are critical for origin function strongly suggests that the RE may function as a binding site for cellular protein(s) which form a complex with LANA when bound to LBS1/2.

**Identification of cellular proteins interacting with the KSHV minimal replicator occupied by LANA.** Data from several groups demonstrated that LANA interacts with ORC and

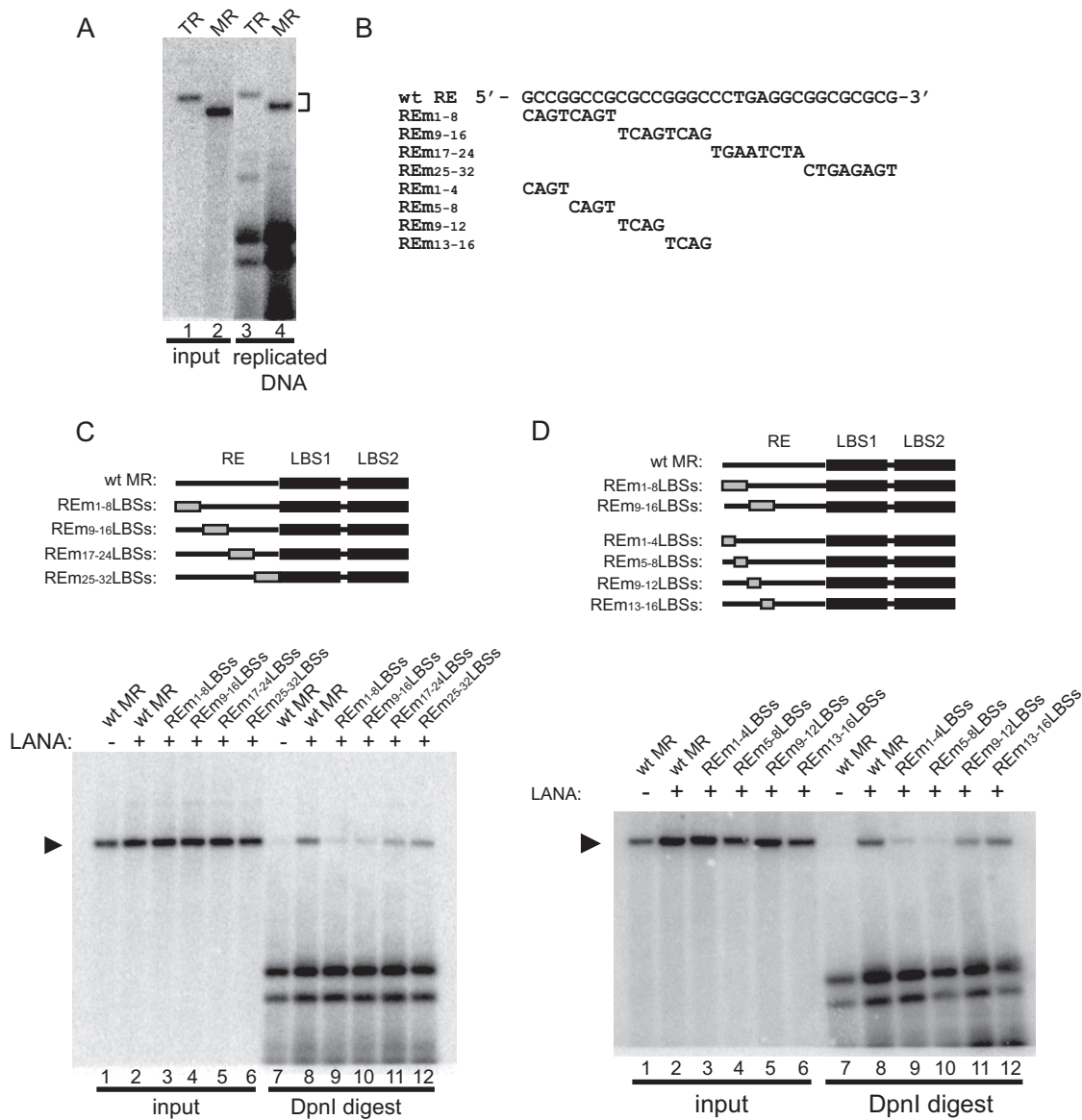


FIG. 1. The first 8 nt within the RE are pivotal for KSHV latent origin function. Replication assays were performed as previously described. Briefly, 8  $\mu$ g of each origin-containing construct was cotransfected with 2  $\mu$ g of pcDNA3/orf73 or carrier DNA into 293 cells. Episomal DNA was recovered by Hirt extraction 72 h posttransfection. Ten percent of the episomal DNA was digested with HindIII (input), while 90% was double digested with HindIII and DpnI for 16 h (DpnI digest). DNA were electrophoretically separated in an agarose gel, immobilized on nylon membranes, and hybridized with radiolabeled probes using pCRII-TR as the template. The replication efficiency of each plasmid was calculated by comparing the intensity of the replicated DNA band with that of its input from independent experiments. The replication activity of each mutant was compared with that of the wild-type MR, which was set at 100%. (A) The MR replicates with efficiency similar to that of the full-length TR. (B) Sequences of the wild-type RE and converted sequences within RE mutants, respectively. (C) The first 8 nt in the RE are critical for origin activity. The RE<sub>m1-8</sub>-LBS1/2, RE<sub>m9-16</sub>-LBS1/2, RE<sub>m17-24</sub>-LBS1/2, and RE<sub>m25-32</sub>-LBS1/2 mutants replicate with efficiencies of 4.8%, 9.6%, 27% and 60%, respectively, compared to that of the wild-type MR. (D) Fine mapping of the first 8 nt to determine the sequences important for LANA-dependent DNA replication. The RE<sub>m1-4</sub>-LBS1/2, RE<sub>m5-8</sub>-LBS1/2, RE<sub>m9-12</sub>-LBS1/2, and RE<sub>m13-16</sub>-LBS1/2 mutants replicate with efficiencies of 13.2%, 8.7%, 41%, and 100%, respectively, compared to that of the wild-type MR. The bracket and arrowheads indicate the positions of the origin-containing plasmids. LBSs, LBS1/2.

MCM proteins in vivo and in vitro, thereby recruiting the host cellular DNA replication machinery (27, 46, 48). However, whether direct interactions between cellular proteins and the origin occur has not been determined. Additionally, while Si and colleagues enriched cellular proteins that interact with TR sequences but not with control DNA and identified more than 95 proteins interacting with TRs, they did not evaluate whether

these proteins contribute to DNA replication (41). To address these questions, we employed a proteomics approach with the goal of identifying cellular proteins that interact specifically with the minimal replicator bound by LANA.

As an affinity probe, the 71-bp-long MR, flanked by 160-bp pCRII vector sequences on both sides, was amplified by PCR using a biotin-labeled forward primer. As a control, RE<sub>m1-8</sub>-

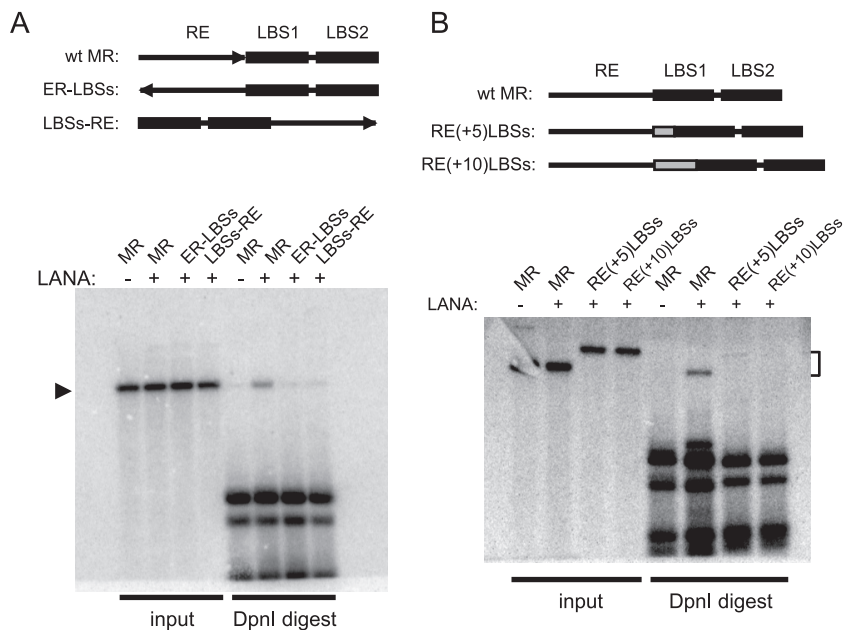


FIG. 2. The orientation and position of the RE element relative to LBS1/2 (LBSs) play an important role for origin function. The wild-type MR or mutants were constructed by inserting synthetic oligonucleotides into pCRII plasmids. Replication assays were performed as described in Materials and Methods. (A) Origin activity was diminished when the RE was flipped or moved downstream of LBS1/2. (B) Spacing changing between the RE and LBS1/2 abrogated the origin activity. The bracket and arrowhead indicate the positions of the origin-containing plasmids. The top panels show the representative structure of the wild-type MR and mutants; the bottom panels show the results of the replication assays with the wild-type MR and mutants.

LBS1/2, deficient in replication (Fig. 1C), was amplified from pCRII-RE<sub>m1-8</sub>-LBS1/2 using identical primers. An additional control, a fragment with a length similar to that of RE, was amplified from the ampicillin resistance gene (*amp*). Figure 3A shows an outline of the experimental procedure. Briefly, DNA fragments were immobilized and incubated with extracts from BJAB/Tet-on/ORF73 cells expressing LANA. Nuclear extracts as well as solubilized nuclear pellet extracts were prepared as

previously described (9). Combined nuclear extracts were pre-cleared with salmon sperm DNA and subjected to two rounds of affinity purification using immobilized MR, RE<sub>m1-8</sub>-LBS1/2, or *amp* fragments. The first round of purified materials was eluted with low-salt buffer, followed by high-salt buffer. The second round of affinity-purified proteins was eluted with Laemmli buffer and analyzed by SDS-PAGE. Several proteins appeared only in the MR affinity purification but not in con-

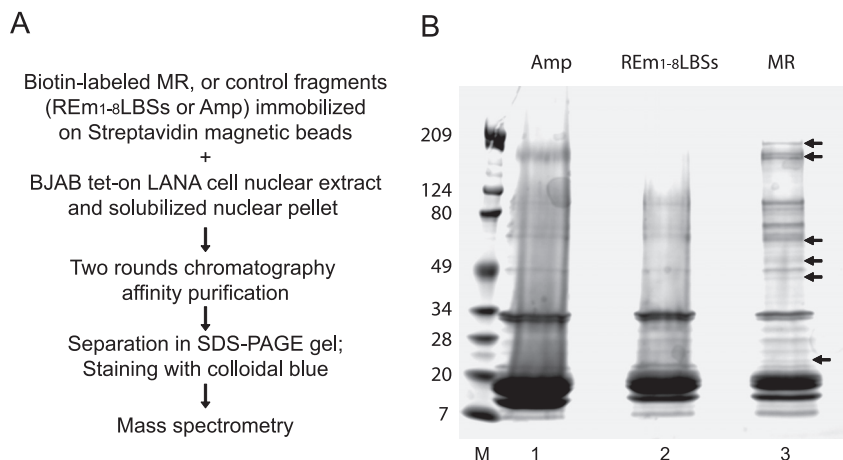


FIG. 3. Proteomics approach to study RE binding proteins. (A) Flow chart for experimental design. Biotinylated PCR fragments containing the MR, RE<sub>m1-8</sub>-LBS1/2, or Amp were immobilized on magnetic streptavidin beads. Nuclear extract and solubilized nuclear pellet extract mixture from BJAB Tet-on/ORF73 cells induced with doxycycline were subjected to two rounds of DNA affinity purification with the immobilized DNA. The second round of purified proteins was resolved on a 4 to 20% gradient SDS-PAGE gel. Gels in each lane were sliced and subjected for mass spectrometry analysis. (B) Colloidal blue staining of DNA affinity chromatography-purified proteins. Unique bands representing proteins specifically bound to REs are indicated with arrows. M, protein size marker. The molecular weight (in thousands) for each band of the marker is labeled as shown. LBSs, LBS1/2.

TABLE 1. Proteins identified in DNA affinity chromatography which bind only to the MR or have higher binding affinity to the MR than the mutants

Name <sup>a</sup>	Accession no.	Predicted molecular mass (kDa)	No. of peptides identified in MR pulldown	Function(s)
<b>DNA topoisomerase II<math>\beta</math></b>	IPI00217709	183	46	Control and alter the topologic states of DNA during transcription and replication; DNA damage response
<b>DNA topoisomerase II<math>\alpha</math></b>	IPI00218753	179	37	Control and alter the topologic states of DNA during transcription and replication
Structural maintenance of chromosome 1	IPI00291939	143	5	Mitotic spindle organization and biogenesis; DNA repair
Structural maintenance of chromosome 3	IPI00219420	142	5	Mitotic spindle organization and biogenesis
AF5q31 protein	IPI00004344	127	4	Transcription regulation
<b>Scaffold attachment factor B</b>	IPI00300631	103	5	Nuclear matrix component; transcription regulation
M-phase phosphoprotein 8	IPI00030408	97	3	N/A <sup>b</sup>
Gamma interferon-inducible protein Ifi-16	IPI00003443	88	9	Transcription regulation; DNA damage signaling; cell cycle checkpoint
Structure-specific recognition protein 1	IPI00005154	81	5	Transcription regulation; chromatin remodeling; DNA replication
<b>DEAD box polypeptide 17 isoform p82</b>	IPI00023785	80	8	RNA metabolism
Heterogeneous nuclear ribonucleoprotein M isoform a	IPI00171903	78	4	RNA metabolism
<b>Splicing factor proline/glutamine-rich (SFPQ)</b>	IPI00010740	76	3	RNA metabolism; DNA repair
Lamina-associated polypeptide 2 $\alpha$	IPI00216230	75	33	Nuclear architecture
Interleukin enhancer-binding factor 3	IPI00219330	75	6	Transcription regulation
ALB protein	IPI00022434	72	3	Carrier protein
Probable RNA-dependent helicase p68	IPI00017617	69	9	RNA processing
Nucleolar protein Nop56	IPI00411937	66	3	RNA processing
Telomeric repeat binding factor 2	IPI00024214	56	6	Telomere maintenance; DNA replication
Serine/threonine-protein kinase VRK3	IPI00009291	53	11	Protein binding
DERPC	IPI00171540	51	5	DNA replication
Elongation factor 1- $\alpha$ 2	IPI00014424	50	2	Protein synthesis
<b>Heterogeneous nuclear ribonucleoprotein H1</b>	IPI00013881	49	2	RNA processing
KH domain-containing, RNA binding, signal transduction-associated protein 1 (Sam68)	IPI00008575	48	8	RNA processing
Gamma interferon-inducible protein Ifi-16 isoform 4	IPI00384836	47	10	Transcription regulation
JKTBP2	IPI00011274	46	3	RNA processing
SRP55-1 of splicing factor	IPI00012345	40	2	RNA processing
Pre-mRNA cleavage factor I 25-kDa subunit	IPI00012995	26	2	RNA processing
FUS-interacting serine-arginine-rich protein 1	IPI00009071	22	2	RNA processing
CGG triplet repeat binding protein 1	IPI00295585	19	12	Transcription regulation

<sup>a</sup> Proteins in bold are six proteins that have previously been described as either LANA-interacting proteins or proteins which bind to the full-length TRs (41).

<sup>b</sup> N/A, not applicable.

trols when REM<sub>1-8</sub>-LBS1/2 or the Amp fragment was used as an affinity probe (Fig. 3B, compare lane 3 with lanes 1 and 2), indicating that these proteins may interact with the RE. To identify all proteins that bound differentially to the MR and control fragments, each lane was cut into five slices representing molecular-weight ranges, and peptides in each slice were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (ICBR Protein Chemistry Core, University of Florida).

Mass spectrometry data were analyzed with Scaffold software. Proteins having at least two peptides and 95% sequence accuracy were scored positive. Additionally, proteins with discrepancies in molecular weight were eliminated from the list. A total of 114 proteins with molecular masses ranging from 13 to 360 kDa, including LANA, were identified. Importantly, 30 proteins bound to the MR only, or with a higher affinity to the MR than to either REM<sub>1-8</sub>-LBS1/2 or

Amp control fragments. The number of identified peptides for each protein isolated from the MR pulldown is listed in Table 1. These proteins can be grouped into several functional categories, including cell cycle regulation, transcription regulation, translation regulation, DNA repair, chromatin remodeling, and DNA replication (Table 1). Seven proteins—including DNA topoisomerase II $\alpha$  and - $\beta$ , scaffold attachment factor B, DEAD box polypeptide 17, the 71-kDa heat-shock cognate protein, heterogeneous nuclear ribonucleoprotein H1, and splicing factor proline/glutamine-rich (SFPQ)—have previously been described as either LANA-interacting proteins or proteins which bind to the full-length TRs (Table 1) (41). We confirmed the presence of several identified proteins in the original pulldowns by Western blotting (data not shown). In this study, we chose to further concentrate on SSRP1 and TRF2, proteins with known roles in DNA replication (9, 47).

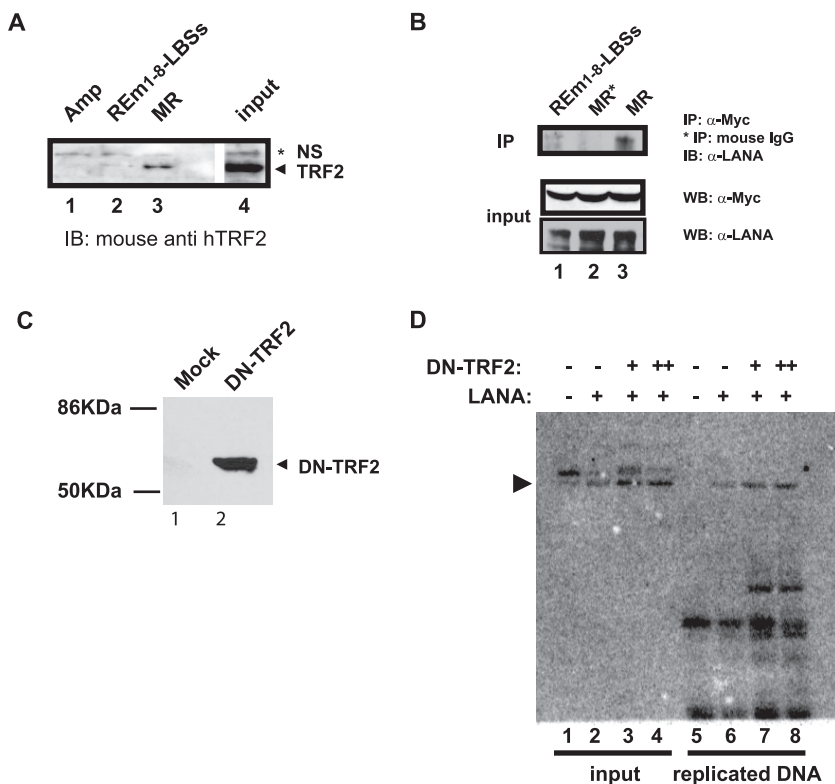


FIG. 4. Analysis of the TRF2 interaction with LANA and role in KSHV DNA replication. (A) LANA interacts with TRF2 in an *in vitro* pulldown assay. BJAB LANA Tet-on nuclear extracts and solubilized nuclear pellets were subject to affinity purification with either the wild-type MR or the REM<sub>1-8</sub>-LBS1/2 mutant. The isolated proteins were analyzed by Western blotting with antibody specifically against human TRF2. A total of 0.5 ml nuclear protein mixture was loaded as the input (lane 4). NS, nonspecific signal. (B) LANA interacts with TRF2 *in vivo* in cotransfected cells. 293 cells were cotransfected with LANA and Myc-TRF2 expression vectors in conjunction with REM<sub>1-8</sub>-LBS1/2 (lane 1) or MR (lanes 2 and 3) plasmid. The nuclear extracts were immunoprecipitated with either monoclonal antibody against Myc (lanes 1 and 3) or control mouse IgG (lane 2). The immunoprecipitated proteins were separated on an 8% SDS-PAGE gel and immunoblotted with rabbit polyclonal antibody against LANA. (C) Expression of DN-TRF2. 293 cells were mock transfected or transfected with FLAG-DN-TRF2. Cell lysate was hybridized with antibody against FLAG. (D) DN-TRF2 did not interfere with LANA-dependent DNA replication. 293 cells were transfected with 8 mg TR-containing plasmid, 2 mg LANA expression vector in the absence of DN-TRF2 (lanes 2 and 6), and 2 mg (lanes 3 and 7) or 5 mg (lanes 4 and 8) DN-TRF2 expression vector. The short-term DNA replication assay was performed as described. The position of the TR plasmid is shown with an arrowhead LBSs, LBS1/2.

**Association of TRF2 with LANA and MR *in vitro* and *in vivo*.** Previously for EBV, telomeric repeat binding proteins 1 and 2 (TRF1 and TRF2) have been shown to bind to nanomer repeats within the DS element of *oriP*. The mutation of the nanomer sequences, the overexpression of TRF1, or the expression of a DN-TRF2 inhibited *oriP* activity. TRF1 and -2 may also contribute to episome segregation during mitosis (9). Interestingly, MR sequences do not show any homology to nanomer sequence motifs within *oriP*.

To confirm the presence of TRF2 within the affinity-purified extracts from the MR, we performed Western blot analysis. TRF2 was detectable in pulldown extracts from the MR (Fig. 4A, lane 3) but not from REM<sub>1-8</sub>-LBS1/2 or Amp control fragments (Fig. 4A, lanes 1 and 2). Next, we performed immunoprecipitations in 293 cells transfected with myc-tagged human TRF2, LANA, and either wild-type or mutant MR-containing plasmids. As shown in Fig. 4B, lane 3, TRF2 was only coimmunoprecipitated with LANA when wild-type MR was cotransfected but not with mutant MR REM<sub>1-8</sub>-LBS1/2. Together, both *in vitro* and *in vivo* interaction data and the fact that MR does not contain telomere repeat sequences would

suggest that TRF2 is recruited to the MR via an interaction with an as-yet-undetermined MR binding protein. To investigate whether TRF2 contributes to LANA-dependent replication, we asked if DN-TRF2, previously shown to inhibit EBV EBNA-1-dependent DNA replication (9), would affect LANA-dependent replication. The transfection of two different amounts of DN-TRF2 expression vector, leading to robust DN-TRF2 expression (Fig. 4C, lane 2), did not affect replication (Fig. 4D, lanes 7 and 8). As was suggested by Deng et al., TRF2 may also contribute to episomal maintenance and segregation, a step which is not measured by short-term replication assays (9). These data confirm TRF2 recruitment to the MR, although it was nonessential for the replication of a minimal origin.

**Association of SSRP1 with LANA *in vitro* and *in vivo*.** SSRP1 is a subunit of the chromatin structure modulator FACT (facilitating chromatin transcription) known to disrupt nucleosome structure and allow RNA and DNA polymerases to access the DNA. FACT plays a role in transcription initiation, elongation, and, importantly for this study, DNA replication (5, 31, 47).

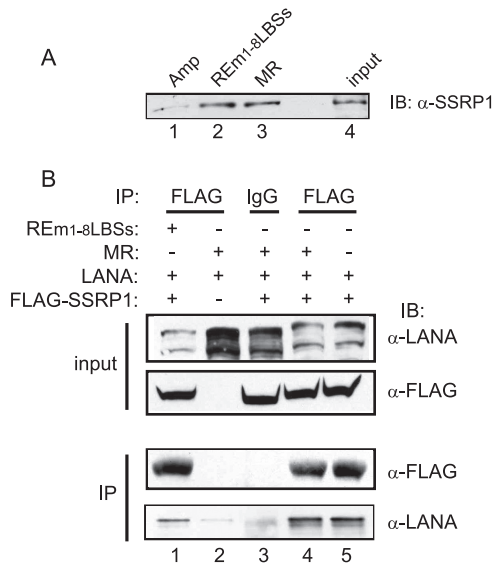


FIG. 5. Complex formation of SSRP1 with LANA/origin in vitro and in vivo. (A) SSRP1 was pulled down by the wild-type and mutant MR but not the *amp* control fragment when LANA was present. Affinity chromatography was performed as described in Materials and Methods. A small amount of pulldown lysates was separated on an 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The blot was immunoblotted with antibody specifically against SSRP1. Lanes 1 to 3 showed the lysates affinity purified with the *amp* fragment, mutant REM<sub>1-8</sub>-LBS1/2, or the wild-type MR fragment. Lane 4 showed 5% of the extract as input. (B) SSRP1 forms a complex with LANA/origin through protein-protein interaction. 293 cells were transfected with LANA expression vector and mutant REM<sub>1-8</sub>-LBS1/2 (lane 1), MR (lanes 2 to 4), or no origin (lane 5) in the presence (lanes 1, 3, 4, and 5) or absence (lane 2) of FLAG-tagged SSRP1. Cells were harvested 48 h posttransfection. Cells were lysed and immunoprecipitated with FLAG antibody (lanes 1, 2, 4, and 5) or mock antibody (lane 3) as a negative control. Immune complexes were separated on an 8% SDS-PAGE gel and immunoblotted with LANA antibody or FLAG antibody (bottom panels). The expression of LANA and FLAG was tested with 3% total cell lysate with antibodies specifically against LANA or FLAG (top two panels, shown as input). A faint background band was observed with the FLAG antibody (lane 2) compared to that with the control with no antibody (lane 3). LBSs, LBS1/2.

To confirm the specificity of SSRP1 binding to the MR, affinity-purified extracts from the MR and controls were analyzed by Western blotting. Surprisingly, robust amounts of SSRP1 were detectable in pulldown extracts not only from the MR but also from the REM<sub>1-8</sub>-LBS1/2 control fragment (Fig. 5A, lanes 2 and 3). Importantly, we did not observe the enrichment of SSRP1 using the *amp* fragment as an affinity probe.

Next, we investigated the association of SSRP1, LANA, and MR in vivo. 293 cells were cotransfected with 2 $\times$  FLAG-SSRP1 and LANA expression plasmids in the presence of vector plasmid, the wild-type MR, or mutant MR (REM<sub>1-8</sub>-LBS1/2) plasmids. At 48 h posttransfection, cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted for LANA. Consistent with the results of the in vitro pulldown assay, LANA was immunoprecipitated with FLAG antibody when either REM<sub>1-8</sub>-LBS1/2 or MR DNA was present (Fig. 5B, lanes 1 and 4). Moreover, LANA was also detectable in IPs in the absence of origin-containing plasmid

(Fig. 5B, lane 5), suggesting that SSRP1 can interact with LANA directly and independent of MR sequences. However, the fact that SSRP1 was more frequently identified in the MR pulldown may suggest that RE contributes to SSRP1 recruitment, potentially by binding to an unknown cellular protein.

**SSRP1 is recruited to the KSHV latent origin in a cell cycle-specific fashion.** SSRP-1, as part of the FACT chromatin remodeling complex, provides access to DNA for both Pol II-dependent transcription and DNA replication (31, 47). Interestingly, LANA also functions in transcriptional regulation and latent DNA replication. Mechanistically, FACT aids both processes by binding to H2A-H2B dimers and dissociating core histones, removing, as a result, a single nucleosome from DNA (5). Additionally, SSRP1 binds to and recruits MCM helicases to facilitate DNA replication (47).

We performed a ChIP assay to test whether LANA recruits SSRP1 to the KSHV latent origin. 293 cells were cotransfected with 2 $\times$  FLAG-SSRP1, LANA expression plasmids, and wild-type MR or mutant REM<sub>1-8</sub>-LBS1/2 plasmid, and ChIP was performed 48 h posttransfection. In agreement with the above-described IP data, wild-type MR and mutant REM<sub>1-8</sub>-LBS1/2 origin were enriched by FLAG antibody when both LANA and 2 $\times$  FLAG-SSRP1 were expressed (Fig. 6A, bars 1 and 3). The IgG antibody control yielded no detectable amounts of MR fragment (Fig. 6A, bar 2). A genomic fragment of the IgG Fc gene, which is known not to have origin activity and is not transcribed in 293 cells, was amplified as an additional negative control. SSRP1 was not enriched at this region (Fig. 6A, bar 4), further validating that the binding of SSRP1 to the origin in the presence of LANA is specific. These data further suggest that SSRP1 forms a complex with LANA at the KSHV latent origin and may contribute to latent KSHV replication.

The initiation of DNA replication in metazoan cells is tightly controlled by cell cycle-dependent formation and the licensing of prereplication complexes (pre-RCs) (for a review, see reference 12). Eukaryotic origins are bound by the ORC with low sequence specificity. Additionally, cellular proteins, including MCMs, are recruited to ORC with the aid of cyclin-dependent kinases and form pre-RCs at the G<sub>1</sub>/S cell cycle transition. After the initiation of DNA replication, pre-RCs disassemble, ensuring that each origin initiates only once per cell cycle. Similarly, the latent replication of KSHV episomes was demonstrated to be initiated once per cell cycle (48). Based on these observations, we asked whether the complex formation of SSRP1, LANA, and the KSHV origin is cell cycle dependent.

293 cells were transfected with 2 $\times$  FLAG-SSRP1, LANA expression plasmids, and wild-type MR or mutant REM<sub>1-8</sub>-LBS1/2 plasmid. At 48 h posttransfection, the cells were arrested in G<sub>1</sub>/S phase by double-thymidine treatment. S phase was induced by releasing cells for 4 h into fresh medium. Cells in M phase were enriched by nocodazole treatment as described in Materials and Methods. Cell cycle profiles were analyzed by flow cytometry and confirmed synchronization (data not shown). Cells at different cell cycle phases were analyzed by ChIP for the association of SSRP1 with the KSHV latent origin.

In contrast to asynchronous cells in which SSRP1 bound the MR and mutant REM<sub>1-8</sub>-LBS1/2 with similar affinity (Fig. 6A, bars 1 and 3), SSRP1 bound MR with significantly higher



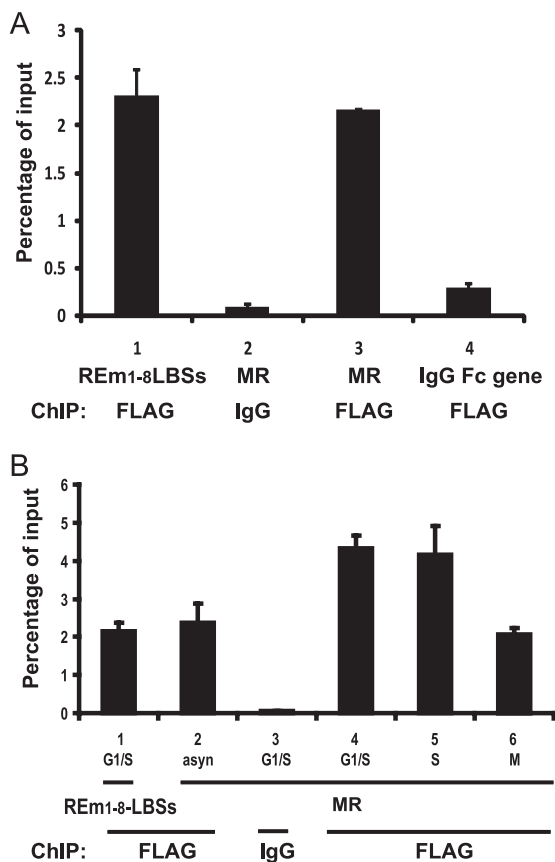


FIG. 6. SSRP1 forms a complex with KSHV latent origin in a cell cycle-dependent manner. (A) SSRP1 binds to the wild-type MR and mutant REm<sub>1-8</sub>-LBS1/2 when LANA is expressed. A total of  $3 \times 10^6$  293 cells were cotransfected with vector pcDNA3.1-LANA, pcDNA-2xFLAG-SSRP1 and pCII-REm<sub>1-8</sub>-LBS1/2, or pCII-MR. Cells were harvested 48 h posttransfection for the ChIP assay. SSRP1 IP significantly enriches for MR and REm<sub>1-8</sub>-LBS1/2 (lanes 1 and 3) but not for a genomic control (lane 4); IgG IP demonstrates the specificity of the SSRP1 antibody (lane 2). (B) SSRP1 binds to the MR in a cell cycle-dependent manner. 293 cells were cotransfected with pcDNA3.1-LANA, pcDNA-2xFLAG-SSRP1 and pCII-REm<sub>1-8</sub>-LBS1/2, or pCII-MR. At 48 h after transfection, cells were asynchronized (bar 2) or synchronized to G<sub>1</sub>/S phase (bars 1, 3, and 4), S phase (bar 5), or M phase (bar 6) with drug treatments. Cells were harvested for the ChIP assay to detect the binding of SSRP1 on the MR or REm<sub>1-8</sub>-LBS1/2. Relative amounts of immunoprecipitated DNA were normalized to input for each sample. LBSs, LBS1/2.

affinity than REm<sub>1-8</sub>-LBS1/2 during G<sub>1</sub>/S phase (Fig. 6B, compare bars 1 and 4). Moreover, the binding of SSRP1 to MR increased during both G<sub>1</sub>/S and S phase (Fig. 6B, bars 4 and 5) and decreased during M phase (Fig. 6B, bar 6) to levels observed in asynchronized cells (Fig. 6B, bar 2). The IgG control did not immunoprecipitate target fragments even in the G<sub>1</sub>/S-phase-synchronized cells (Fig. 6B, bar 3). Together, these data demonstrate that SSRP1 associates with MR in a LANA- and cell cycle-dependent fashion.

#### SSRP1 is required for LANA-dependent DNA replication.

To directly evaluate the role of SSRP1 in LANA-dependent DNA replication, we knocked down SSRP1 using siRNAs and performed short-term replication assays. To optimize the knockdown conditions, 293 cells were transfected with 100 nM

SSRP1 siRNA or scrambled siRNA. SSRP1 expression levels were analyzed at 48, 72, and 96 h post-siRNA transfection. The decrease in SSRP1 expression started to occur at 48 h post-transfection, while at 72 and 96 h, SSRP1 was reduced by more than 80% (data not shown). For the replication assay, 293 cells were transfected with 100 nM SSRP1 siRNA or the scrambled control. Twenty-four hours later, 8  $\mu$ g pCII-TR and 2  $\mu$ g pcDNA3.1/ORF73 or empty vector were cotransfected into the siRNA-transfected cells. The cells were harvested 72 h after the second transfection. Five percent of the cells was lysed in SDS-PAGE loading buffer and assayed by Western blotting with an SSRP1 antibody. As shown in Fig. 7A, at 96 h following siRNA treatment, SSRP1 expression was reduced to 20% (Fig. 7A, lane 4) compared to that of the mock (Fig. 7A, lanes 1 and 2) or scrambled siRNA-transfected cells (Fig. 7A, lane 3). The replication efficiency of pCII-TR dropped to about  $20.5\% \pm 2.1\%$  when SSRP1 was knocked down compared to that of the control with no siRNA (Fig. 7B, compare lane 8 with lane 6). The scrambled siRNA control had only a moderate effect on replication (Fig. 7B, compare lane 7 with lane 6). These data suggest that SSRP1 directly contributes to LANA-dependent replication.

Since SSRP1 is known to interact with MCM and contributes to eukaryotic DNA replication (47), we examined whether the siRNA-induced decrease in SSRP1 expression caused cell cycle perturbation, which may indirectly cause decreased replication of the TR plasmid. siRNA-transfected cells were fixed and stained with propidium iodide 96 h post-siRNA transfection. DNA content was analyzed by flow cytometry, which demonstrated that cell cycle profiles were identical between SSRP1 siRNA-transfected cells and either the scrambled or control with no siRNA (Fig. 7C). Together, these results demonstrate that SSRP1 directly contributes to LANA-dependent DNA replication of the KSHV latent origin.

## DISCUSSION

This study aimed to identify novel cellular proteins involved in KSHV latent DNA replication which, with the exception of LANA, solely depends on the host cellular DNA replication machinery. Since LANA and TR sequences have been reported to interact with a relatively large number of proteins and TR sequences also have enhancer activity potentially regulating K1 expression, our approach to identify novel origin/LANA-interacting proteins was based on first identifying the minimally active origin within the TRs. As we reported previously, the 32-bp-long highly GC-rich RE adjacent to LBS1/2 is required for replication, a finding which recently has been confirmed by Verma and colleagues, who also demonstrated that TR-containing plasmids replicate in synchrony with host chromosomes once per cell cycle (18, 49). Here we show that nt 1 to 8 within the RE are critically important for RE function and furthermore demonstrate that both the relative orientation and spacing between the RE and LBS1/2 are critical for *ori* function. These data suggest a model in which host cellular proteins interact with RE sequences and LANA bound to LBS1/2 during a step critical to DNA replication. The fact that the KSHV minimal origin requires the RE as a *cis*-regulatory element in addition to LBS1/2 is different from the EBV min-

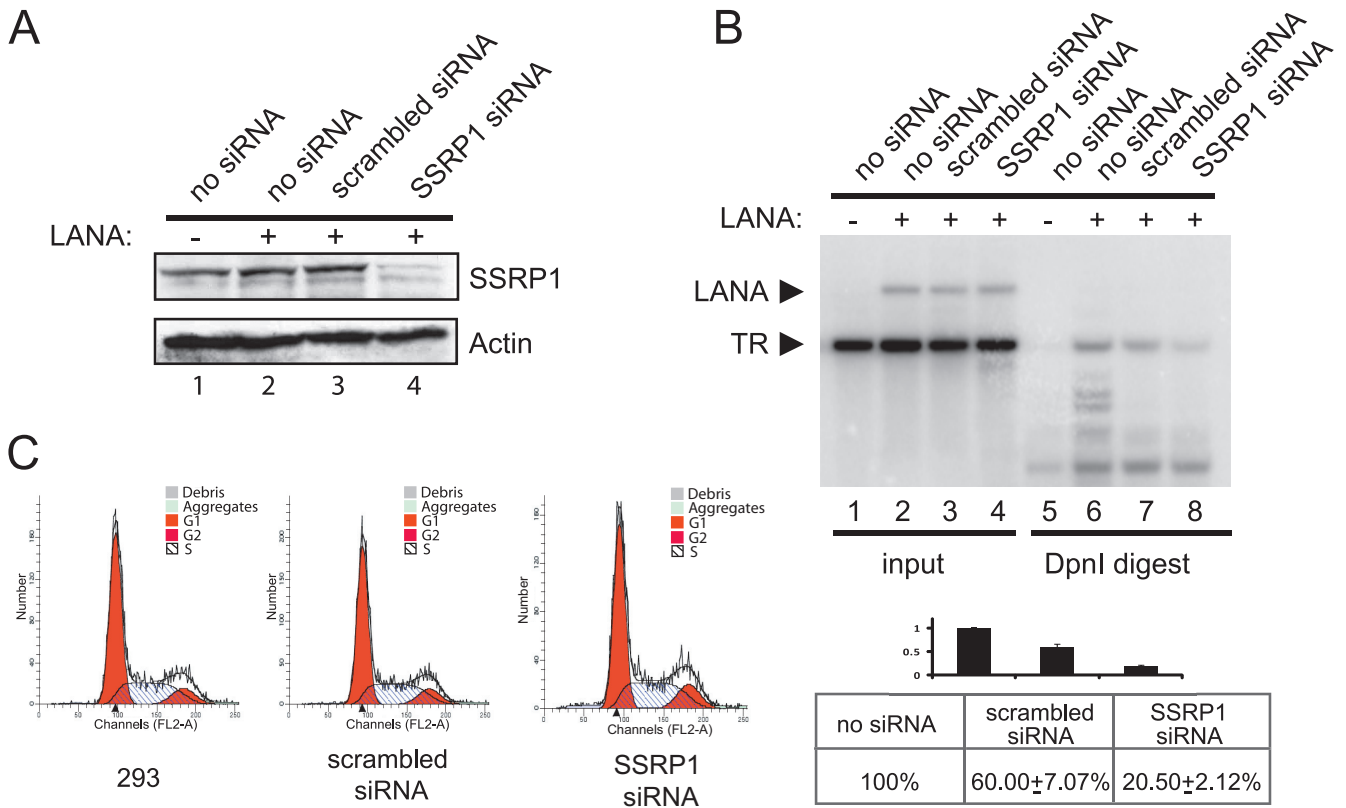


FIG. 7. SSRP1 is involved in KSHV latent origin replication. A total of  $1.5 \times 10^6$  293 cells were transfected with 100 nM scrambled or SSRP1 siRNA with Lipofectamine 2000. At 24 h posttransfection, cells were transfected again with 8  $\mu$ g TR-containing plasmid pCII-TR and 2  $\mu$ g empty pcDNA3.1 (panels A and B, lanes 1) or pcDNA3.1/LANA (panels A and B, lanes 2, 3, and 4). At 72 h after the second transfection, cells were harvested. One of 20 of the total cells was collected for Western blotting to detect the expression of SSRP1. The remaining cells were assayed for the replication assay as described in Materials and Methods. (A) Western blot analysis indicated efficient SSRP1 knockdown by siRNA at the time when replicating DNA was extracted. Lanes 1 and 2, no siRNA transfection; lane 3, scrambled siRNA-transfected samples; lane 4, SSRP1 siRNA-transfected cells. (B) The knockdown of SSRP1 inhibits the replication efficiency of the TR plasmid. The top panel shows a representative image of the results from three independent replication assays. Lanes 1 to 4 (same samples as for panel A) contain 10% of the Hirt-extracted DNA digested with HindIII; lanes 5 to 8 have 90% of the Hirt-extracted DNA double digested with HindIII and DpnI, corresponding to lanes 1 to 4, respectively. Arrowheads indicate the positions of linearized pcDNA3.1/LANA and pCII-TR. The bottom panel shows the quantification and statistical analysis of the results from three independent assays. (C) Cell profiling with mock-, scrambled siRNA-, or SSRP1 siRNA-transfected cells did not show any differences. DNA content was analyzed by flow cytometry after cells were fixed and stained with propidium iodide.

imal origin, where two EBNA-1 binding sites are sufficient for EBNA-1-dependent replication (52).

In the current study, we found that TRF2 associates with the minimal replicator in the presence of LANA, and confirmatory immunoprecipitations clearly showed that TRF2 associates with LANA only if bound to the wild-type MR (Fig. 4B). In contrast to its role in EBV latent origin replication, where TRF2 was shown to facilitate EBNA-1 function in *oriP* replication, the overexpression of DN-TRF2 had no effect on the LANA-dependent replication of TR-containing plasmids (Fig. 4D). Three nanomer repeats flanking and separating pairs of EBNA-1 binding sites resemble telomeric repeat sequences which were shown to be bound by TRF2 (9). In contrast, the RE, which is very GC rich, does not have any sequence homology to telomeric repeats, suggesting that TRF2 may interact with a yet-unknown protein which bridges the RE and LANA or that TRF2 has affinity to GC-rich DNA; both possibilities are currently under study.

Although TRF2 association at latent origins of KSHV and EBV seems to be conserved, TRF2 inhibition did not affect

KSHV latent origin replication, suggesting that TRF2 may play additional roles at the KSHV origin. In addition to telomere maintenance, TRF2 is also involved in DNA damage repair and relocalizes from telomeres to double-stranded break sites and, together with MRE11/Rad50/NBS and Ku70, contributes to the initial response to genotoxic stress and DNA damage (44, 55). It has therefore been suggested that TRF2 in such complexes may act as a sensor of DNA damage and regulate EBV *oriP* activity in the setting of genotoxic stress. A number of proteins involved in DNA damage repair (DNA-PK, Ku70, PARP-1, etc.) have also been shown to bind to TRs and were found to be associated with the minimal replicator and LANA (Table 1) (41). Thus, TRF2 may perform important additional functions related to replication control at the KSHV origin.

Cellular replication licensing factors have been shown to be recruited to  $\gamma$ -herpesvirus latent origins, where all subsequent steps required for origin licensing occur, guaranteeing that each origin fires once per cell cycle. For EBV, it has been elegantly demonstrated that EBNA-1 recruits ORC proteins and MCMs to DS of *oriP* (8, 10, 40). Since then, several

laboratories have demonstrated that LANA recruits host cellular licensing factors to the latent origin. Lim et al. demonstrated that ORC1 and ORC2 specifically interact with LANA via its C-terminal DNA binding domain, and Stedman et al. demonstrated using ChIP that LANA, CBP, BRD2, ORC, and MCM proteins are found in complexes at TR sequences (27, 46). The association of ORCs with LANA and TRs as well as their requirement for latent DNA replication was confirmed by Verma et al., who additionally identified two regions outside the TRs which showed enrichment for ORC binding using ChIP (48).

Surprisingly, we did not enrich for any ORC or MCM proteins in our LANA-MR complexes, although we isolated several previously identified TR-interacting proteins, among the 30 proteins identified by mass spectrometry (Table 1). While the functional association of ORCs and MCMs with origins is temporal, ORC proteins are chromatin associated throughout the cell cycle, and therefore, the amount of free ORC and MCM proteins might have been limited in our extracts of asynchronized cells. Analogously, Si et al. used full-length TRs for a similar approach and did not identify any ORC or MCM proteins (41). In contrast, Stedman et al. detected very small amounts of ORC2 but also no MCM and suggested that advanced replication licensing steps are not recapitulated under these affinity purification conditions utilizing naked DNA as the probe (46).

Our finding that SSRP1, a component of FACT, a chromatin remodeling complex, interacts with LANA and contributes to the efficiency of KSHV replication strongly suggests that chromatin remodeling is indeed important for latent KSHV replication. FACT is required for the efficient transcription of organized nucleosomes which in yeast is essential for survival (30). FACT is a heterodimer composed of a large Spt16 subunit and a small SSRP1 subunit through which concerted action reorganizes nucleosomal structure by removing and reassembling H2A-H2B histone dimers from DNA in a process which does not require ATP (42). In addition, FACT is required for transcription and DNA replication and provides a core activity that together with process-specific factors conveys chromatin loosening during transcription, replication, and DNA repair (5, 32). With respect to DNA replication, FACT complexes interact and cooperate with MCM in DNA unwinding, thereby directly contributing to the initiation of DNA replication (47). Like cellular chromosomes, KSHV episomes are histone-associated and organized into chromatin domains (46), which require remodeling and reorganization in order to allow access for proteins involved in replication, suggesting that FACT plays a similar role in KSHV as in cellular DNA replication.

Our findings that SSRP1 interacts with LANA independent of DNA binding, that SSRP1 is present at the origin during G<sub>1</sub>/S, and that its knockdown inhibits LANA-dependent plasmid replication all support the model that SSRP1 plays a critical role in KSHV DNA replication. The finding that the other functional component of FACT, Spt16, was not associated with SSRP1 at the origin raises some intriguing questions, as FACT loosens chromatin via interactions with histones that require both Spt16 and SSRP1 (5). Spt16 binds to the H2A-H2B dimers and mononucleosomes, whereas SSRP1 interacts with H3/H4 but not mononucleosomes. FACT activity in mod-

ulating cellular chromatin therefore requires Spt16. Barbera et al. demonstrated that the N-terminal domain of LANA, which tethers KSHV episomes to host chromatin, binds specifically to an acidic pocket within the H2A-H2B interface (4, 33). Based on these observations, it is tempting to speculate that LANA recruits SSRP1 to the minimal replicator and that the concerted binding of LANA to H2A-H2B replaces the requirement for the Spt16 FACT component; thus, LANA and SSRP1 would function as a KSHV replicon-specific chromatin remodeling factor. In this regard, it was previously demonstrated that a subset of promoters can be activated by SSRP1 independent of Spt16, suggesting the existence of SSRP1 complexes containing other binding partners (23). Substantiating such a model will require additional experiments, which are currently ongoing, to probe whether Spt16 is involved in LANA-dependent DNA replication.

A still-open question is how the activity of the KSHV *ori* is regulated within the context of an episome harboring 20 to 40 copies of TRs, each containing a potential origin. TR sequences are bound by LANA in latently infected cells, giving rise to the nuclear speckled patterns observed by immunofluorescence assays (2). Based on the strong interaction between LANA and SSRP1, many of these sites will likely also associate with SSRP1, specifically during G<sub>1</sub>/S. Assuming that the initiation of DNA replication on several sites within close proximity would be deleterious, this early step of SSRP1-dependent chromatin loosening may convert the entire TR region amenable to replication licensing. The initiation of DNA replication at different TRs may create a replication zone as demonstrated for EBV (29) and recently proposed for KSHV (50). The signal which specifies a firing TR *ori* will likely be an epigenetic modification such as the hyperacetylated nucleosome adjacent to LBS1/2 as demonstrated by Stedman et al. (46). Ongoing work will concentrate on the question of how LANA, SSRP1, and other potentially unknown players contribute to the licensing process. In addition to being involved in nucleosome disassembly, FACT complexes also play a role in histone reassembly during transcriptional elongation. Hence, the enrichment of FACT complexes on TR sequences may aid in the efficient unwinding of DNA through the very stable GC-rich TR sequences of rhadinoviruses and possibly in restoring nucleosomal structure after initiation. The fact that SSRP1, a component of the FACT complex, interacts with LANA and contributes to LANA-dependent DNA replication further underlines that  $\gamma$ -herpesvirus origins provide attractive model systems to study eukaryotic DNA replication.

#### ACKNOWLEDGMENTS

This work was supported by grants from National Institutes of Health (CA88763) and Start-up funding from the UF Shands Cancer Center to R.R.

We thank Rebecca Skalsky, Duke University, and Sankar Swaminathan, University of Florida, for helpful discussion, editing, and critical reading of the manuscript. We thank Hua Lu, Oregon Health & Science University, for providing plasmid pcDNA3.1-2xFLAG-SSRP1 and Titia de Lange at the Rockefeller University for providing the Myc-TRF2 expression vector. We also thank Paul Lieberman, Wistar Institute, for providing both the wild-type and DN-TRF2 expression constructs and Scott McClung at the ICBR Protein Chemistry Core at University of Florida for mass spectrometry analysis.

## REFERENCES

- An, F. Q., N. Compitello, E. Horwitz, M. Sramkoski, E. S. Knudsen, and R. Renne. 2005. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus modulates cellular gene expression and protects lymphoid cells from p16 INK4A-induced cell cycle arrest. *J. Biol. Chem.* **280**:3862–3874.
- Ballestas, M. E., P. A. Chatis, and K. M. Kaye. 1999. Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* **284**:641–644.
- Barbera, A. J., M. E. Ballestas, and K. M. Kaye. 2004. The Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 N terminus is essential for chromosome association, DNA replication, and episome persistence. *J. Virol.* **78**:294–301.
- Barbera, A. J., J. V. Chodaparambil, B. Kelley-Clarke, V. Joukov, J. C. Walter, K. Luger, and K. M. Kaye. 2006. The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. *Science* **311**:856–861.
- Belotserkovskaya, R., S. Oh, V. A. Bondarenko, G. Orphanides, V. M. Studitsky, and D. Reinberg. 2003. FACT facilitates transcription-dependent nucleosome alteration. *Science* **301**:1090–1093.
- Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* **332**:1186–1191.
- Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**:1865–1869.
- Chaudhuri, B., H. Xu, I. Todorov, A. Dutta, and J. L. Yates. 2001. Human DNA replication initiation factors, ORC and MCM, associate with oriP of Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA* **98**:10085–10089.
- Deng, Z., L. Lezina, C. J. Chen, S. Shtivelband, W. So, and P. M. Lieberman. 2002. Telomeric proteins regulate episomal maintenance of Epstein-Barr virus origin of plasmid replication. *Mol. Cell* **9**:493–503.
- Dhar, S. K., K. Yoshida, Y. Machida, P. Khaira, B. Chaudhuri, J. A. Wohlschlegel, M. Leffak, J. Yates, and A. Dutta. 2001. Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. *Cell* **106**:287–296.
- Dittmer, D., M. Lagunoff, R. Renne, K. Staskus, A. Haase, and D. Ganem. 1998. A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **72**:8309–8315.
- Dutta, A., and S. P. Bell. 1997. Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell Dev. Biol.* **13**:293–332.
- Friberg, J., Jr., W. Kong, M. O. Hottiger, and G. J. Nabel. 1999. p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* **402**:889–894.
- Fujimuro, M., F. Y. Wu, C. ApRhyas, H. Kajumbula, D. B. Young, G. S. Hayward, and S. D. Hayward. 2003. A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency. *Nat. Med.* **9**:300–306.
- Garber, A. C., J. Hu, and R. Renne. 2002. Latency-associated nuclear antigen (LANA) cooperatively binds to two sites within the terminal repeat, and both sites contribute to the ability of LANA to suppress transcription and to facilitate DNA replication. *J. Biol. Chem.* **277**:27401–27411.
- Harrison, S., K. Fisenne, and J. Hearing. 1994. Sequence requirements of the Epstein-Barr virus latent origin of DNA replication. *J. Virol.* **68**:1913–1925.
- Hu, J., A. C. Garber, and R. Renne. 2002. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells. *J. Virol.* **76**:11677–11687.
- Hu, J., and R. Renne. 2005. Characterization of the minimal replicator of Kaposi's sarcoma-associated herpesvirus latent origin. *J. Virol.* **79**:2637–2642.
- Kedes, D. H., M. Lagunoff, R. Renne, and D. Ganem. 1997. Identification of the gene encoding the major latency-associated nuclear antigen of the Kaposi's sarcoma-associated herpesvirus. *J. Clin. Investig.* **100**:2606–2610.
- Krithivas, A., M. Fujimuro, M. Weidner, D. B. Young, and S. D. Hayward. 2002. Protein interactions targeting the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus to cell chromosomes. *J. Virol.* **76**:11596–11604.
- Krithivas, A., D. B. Young, G. Liao, D. Greene, and S. D. Hayward. 2000. Human herpesvirus 8 LANA interacts with proteins of the mSin3 corepressor complex and negatively regulates Epstein-Barr virus gene expression in dually infected PEL cells. *J. Virol.* **74**:9637–9645.
- Lan, K., D. A. Koppers, and E. S. Robertson. 2005. Kaposi's sarcoma-associated herpesvirus reactivation is regulated by interaction of latency-associated nuclear antigen with recombination signal sequence-binding protein J $\kappa$ , the major downstream effector of the Notch signaling pathway. *J. Virol.* **79**:3468–3478.
- Li, Q., F. Zhou, F. Ye, and S. J. Gao. 2008. Genetic disruption of KSHV major latent nuclear antigen LANA enhances viral lytic transcriptional program. *Virology* **379**:234–244.
- Li, Y., S. X. Zeng, I. Landais, and H. Lu. 2007. Human SSRP1 has Spt16-dependent and -independent roles in gene transcription. *J. Biol. Chem.* **282**:6936–6945.
- Lim, C., Y. Gwack, S. Hwang, S. Kim, and J. Choe. 2001. The transcriptional activity of cAMP response element-binding protein-binding protein is modulated by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J. Biol. Chem.* **276**:31016–31022.
- Lim, C., D. Lee, T. Seo, C. Choi, and J. Choe. 2003. Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus functionally interacts with heterochromatin protein 1. *J. Biol. Chem.* **278**:7397–7405.
- Lim, C., H. Sohn, Y. Gwack, and J. Choe. 2000. Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) binds ATF4/CREB2 and inhibits its transcriptional activation activity. *J. Gen. Virol.* **81**:2645–2652.
- Lim, C., H. Sohn, D. Lee, Y. Gwack, and J. Choe. 2002. Functional dissection of latency-associated nuclear antigen 1 of Kaposi's sarcoma-associated herpesvirus involved in latent DNA replication and transcription of terminal repeats of the viral genome. *J. Virol.* **76**:10320–10331.
- Lin, A., S. Wang, T. Nguyen, K. Shire, and L. Frappier. 2008. The EBNA1 protein of Epstein-Barr virus functionally interacts with Brd4. *J. Virol.* **82**:12009–12019.
- Little, R. D., and C. L. Schildkraut. 1995. Initiation of latent DNA replication in the Epstein-Barr virus genome can occur at sites other than the genetically defined origin. *Mol. Cell. Biol.* **15**:2893–2903.
- Malone, E. A., C. D. Clark, A. Chiang, and F. Winston. 1991. Mutations in SPT16/CDC68 suppress *cis*- and *trans*-acting mutations that affect promoter function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**:5710–5717.
- Orphanides, G., G. LeRoy, C. H. Chang, D. S. Luse, and D. Reinberg. 1998. FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell* **92**:105–116.
- Orphanides, G., W. H. Wu, W. S. Lane, M. Hampsey, and D. Reinberg. 1999. The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. *Nature* **400**:284–288.
- Piolot, T., M. Tramier, M. Coppey, J. C. Nicolas, and V. Marechal. 2001. Close but distinct regions of human herpesvirus 8 latency-associated nuclear antigen 1 are responsible for nuclear targeting and binding to human mitotic chromosomes. *J. Virol.* **75**:3948–3959.
- Platt, G. M., G. R. Simpson, S. Mittnacht, and T. F. Schulz. 1999. Latent nuclear antigen of Kaposi's sarcoma-associated herpesvirus interacts with RING3, a homolog of the *Drosophila* female sterile homeotic (*fish*) gene. *J. Virol.* **73**:9789–9795.
- Radkov, S. A., P. Kellam, and C. Boshoff. 2000. The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat. Med.* **6**:1121–1127.
- Rainbow, L., G. M. Platt, G. R. Simpson, R. Sarid, S. J. Gao, H. Stoiber, C. S. Herrington, P. S. Moore, and T. F. Schulz. 1997. The 222- to 234-kilodalton latent nuclear protein (LNA) of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) is encoded by *orf73* and is a component of the latency-associated nuclear antigen. *J. Virol.* **71**:5915–5921.
- Renne, R., W. Zhong, B. Herndier, M. McGrath, N. Abbey, D. Kedes, and D. Ganem. 1996. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat. Med.* **2**:342–346.
- Samols, M. A., J. Hu, R. L. Skalsky, and R. Renne. 2005. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **79**:9301–9305.
- Sarid, R., J. S. Wieszorek, P. S. Moore, and Y. Chang. 1999. Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. *J. Virol.* **73**:1438–1446.
- Schepers, A., M. Ritz, K. Bousset, E. Kremmer, J. L. Yates, J. Harwood, J. F. Diffley, and W. Hammerschmidt. 2001. Human origin recognition complex binds to the region of the latent origin of DNA replication of Epstein-Barr virus. *EMBO J.* **20**:4588–4602.
- Si, H., S. C. Verma, and E. S. Robertson. 2006. Proteomic analysis of the Kaposi's sarcoma-associated herpesvirus terminal repeat element binding proteins. *J. Virol.* **80**:9017–9030.
- Singer, R. A., and G. C. Johnston. 2004. The FACT chromatin modulator: genetic and structure/function relationships. *Biochem. Cell Biol.* **82**:419–427.
- Skalsky, R. L., J. Hu, and R. Renne. 2007. Analysis of viral *cis* elements conferring Kaposi's sarcoma-associated herpesvirus episome partitioning and maintenance. *J. Virol.* **81**:9825–9837.
- Song, K., D. Jung, Y. Jung, S. G. Lee, and I. Lee. 2000. Interaction of human Ku70 with TRF2. *FEBS Lett.* **481**:81–85.
- Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, et al. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castlemann's disease. *Blood* **86**:1276–1280.
- Stedman, W., Z. Deng, F. Lu, and P. M. Lieberman. 2004. ORC, MCM, and histone hyperacetylation at the Kaposi's sarcoma-associated herpesvirus latent replication origin. *J. Virol.* **78**:12566–12575.
- Tan, B. C., C. T. Chien, S. Hirose, and S. C. Lee. 2006. Functional cooper-

- ation between FACT and MCM helicase facilitates initiation of chromatin DNA replication. *EMBO J.* **25**:3975–3985.
48. **Verma, S. C., T. Choudhuri, R. Kaul, and E. S. Robertson.** 2006. 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. *J. Virol.* **80**:2243–2256.
49. **Verma, S. C., T. Choudhuri, and E. S. Robertson.** 2007. The minimal replicator element of the Kaposi's sarcoma-associated herpesvirus terminal repeat supports replication in a semiconservative and cell-cycle-dependent manner. *J. Virol.* **81**:3402–3413.
50. **Verma, S. C., K. Lan, T. Choudhuri, M. A. Cotter, and E. S. Robertson.** 2007. An autonomous replicating element within the KSHV genome. *Cell Host Microbe* **2**:106–118.
51. **Wysokenski, D. A., and J. L. Yates.** 1989. Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within *oriP* of Epstein-Barr virus. *J. Virol.* **63**:2657–2666.
52. **Yates, J. L., S. M. Camiolo, and J. M. Bashaw.** 2000. The minimal replicator of Epstein-Barr virus *oriP*. *J. Virol.* **74**:4512–4522.
53. **You, J., J. L. Croyle, A. Nishimura, K. Ozato, and P. M. Howley.** 2004. Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell* **117**:349–360.
54. **You, J., V. Srinivasan, G. V. Denis, W. J. Harrington, Jr., M. E. Ballestas, K. M. Kaye, and P. M. Howley.** 2006. Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen interacts with bromodomain protein Brd4 on host mitotic chromosomes. *J. Virol.* **80**:8909–8919.
55. **Zhu, X. D., B. Kuster, M. Mann, J. H. Petrini, and T. de Lange.** 2000. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat. Genet.* **25**:347–352.