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Aline C. Habison
Harvard Medical School

Chantal Beauchemin
Harvard Medical School

J. Pedro Simas
University of Lisbon, Portugal

Edward J. Usherwood
Dartmouth College

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Aline C. Habison,[a] Chantal Beauchemin,[a] J. Pedro Simas,[b] Edward J. Usherwood,[c] and Kenneth M. Kaye[a]

Channing Laboratory and Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA[a]; Instituto de Microbiologia e Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal[b]; and Department of Microbiology and Immunology, Dartmouth Medical School, Lebanon, New Hampshire, USA[c]

Murine gammaherpesvirus 68 (MHV68) ORF73 (mLANA) has sequence homology to Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA). LANA acts on the KSHV terminal repeat (TR) elements to mediate KSHV episome maintenance. Disruption of mLANA expression severely reduces the ability of MHV68 to establish latent infection in mice, consistent with the possibility that mLANA mediates episome persistence. Here we assess the roles of mLANA and MHV68 TR (mTR) elements in episome persistence. mTR-associated DNA persisted as an episome in latently MHV68-infected tumor cells, demonstrating that the mTR elements can serve as a *cis*-acting element for MHV68 episome maintenance. In some cases, both control vector and mTR-associated DNAs integrated into MHV68 episomal genomes. Therefore, we also assessed the roles of mTRs as well as mLANA in the absence of infection. DNA containing both mLANA and mTRs in *cis* persisted as an episome in murine A20 or MEF cells. In contrast, mTR DNA never persisted as an episome in the absence of mLANA. mLANA levels were increased when mLANA was expressed from its native promoters, and episome maintenance was more efficient with higher mLANA levels. Increased numbers of mTRs conferred more efficient episome maintenance, since DNA containing mLANA and eight mTR elements persisted more efficiently in A20 cells than did DNA with mLANA and two or four mTRs. Similar to KSHV LANA, mLANA broadly associated with mitotic chromosomes but relocalized to concentrated dots in the presence of episomes. Therefore, mLANA acts on mTR elements to mediate MHV68 episome persistence.

Murine gammaherpesvirus 68 (MHV68 or murid herpesvirus 4) is a gamma-2 herpesvirus that was isolated from a naturally infected rodent, the bank vole (*Clethrionomys glareolus*) (7, 8). Similar to the case with other gamma-2 herpesviruses, following viral replication at the primary site of inoculation, latent, persistent MHV68 infection occurs. MHV68 predominantly latently infects B cells, but latent infection also occurs in epithelial cells, macrophages, and dendritic cells (22, 23, 46, 49, 59, 67). The establishment of latent infection in mice causes an infectious mononucleosis-like syndrome with lymphocyte activation (20).

MHV68 is genetically similar to other gammaherpesviruses, including the human Kaposi's sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8 [HHV-8]) (a gamma-2 herpesvirus) and Epstein-Barr virus (EBV) (a gamma-1 herpesvirus), both of which are associated with human malignancies (26, 39, 53, 57). For example, the ~118-kb unique sequence of MHV68 features a GC content of 46% and contains ~1.2-kb terminal repeat (TR) elements that are 78% GC rich, while the KSHV genome contains an ~140-kb unique sequence with a GC content of 54% and 0.8-kb TR elements that are 85% GC rich (54, 64). Moreover, the ~80 open reading frames (ORFs) carried by the MHV68 genome are largely colinear with those of the genomes of KSHV, EBV, and other gammaherpesviruses, such as herpesvirus saimiri (HVS), a New World monkey virus (1). MHV68 infection of mice is often used as a tractable small-animal model for gammaherpesviruses and is particularly relevant for KSHV (24, 56, 58).

During latency, the gammaherpesvirus genome persists as a circularized, extrachromosomal episome (plasmid) (16, 17, 61). Therefore, in order to persist in proliferating cells, the episomal genomes must replicate and segregate to daughter nuclei. In latently infected cells, only a limited subset of genes is expressed (46, 57). Among them, ORF73 is known to be responsible for viral episome maintenance in at least two other gamma-2 herpesviruses, i.e., HVS and KSHV (3, 4, 10, 13, 63).

KSHV ORF73 encodes the latency-associated nuclear antigen (LANA) (54) (Fig. 1A). LANA is a 1,162-amino-acid viral protein that mediates KSHV episome persistence (3, 4). C-terminal LANA binds specific sequence in the KSHV TR element to mediate its replication, and this binding is essential for episome maintenance (4, 15, 21, 27, 28, 32, 33, 41, 45). In addition, N- and C-terminal LANA regions associate with mitotic chromosomes (5, 36, 37, 42, 44, 51, 69). N-terminal LANA attaches to mitotic chromosomes through binding of core histones H2A and H2B (6). Through simultaneous binding of KSHV DNA and chromosomes, LANA tethers the viral episome to mitotic host chromosomes to ensure efficient episome segregation to daughter nuclei during cellular division. Therefore, KSHV LANA allows genome persistence by mediating both viral DNA replication and segregation of episomes to progeny nuclei.

MHV68 ORF73 (mLANA) is homologous to KSHV LANA, particularly in the C-terminal domain (Fig. 1A). mLANA is comprised of 314 amino acids, which is considerably smaller than the 1,162-amino-acid KSHV LANA (64). Most of the difference in size is due to the absence of internal acidic and glutamine-rich repeat elements in mLANA. Recombinant MHV68 disrupted for

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Address correspondence to Kenneth M. Kaye, kkaye@rics.bwh.harvard.edu.

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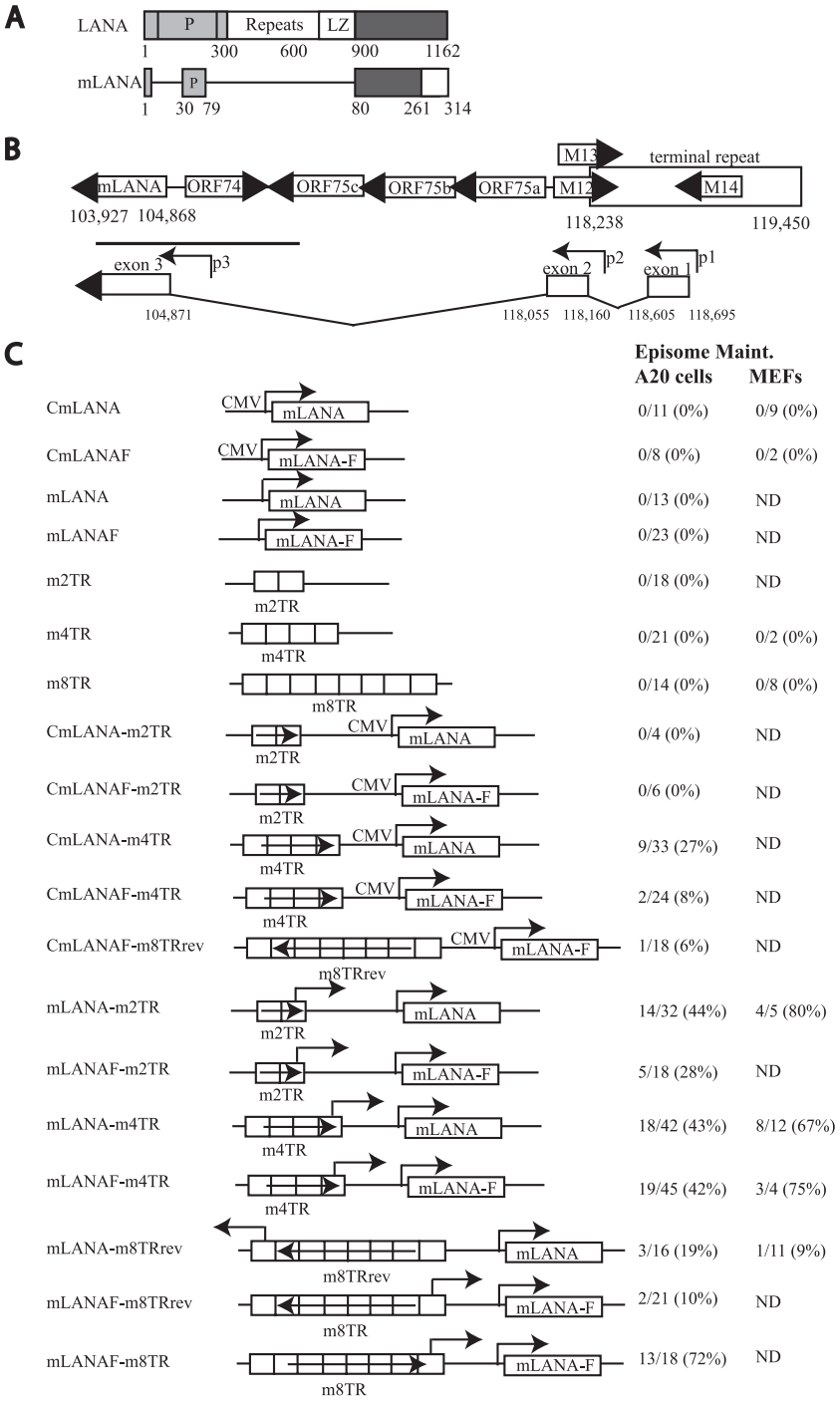

FIG 1 Schematic diagrams. (A) Comparison of KSHV LANA and mLANA. Homologous regions are shown with similar shading. Unshaded regions lack homology. The C-terminal domains (darkly shaded) share the highest level of homology. C-terminal KSHV LANA mediates self-association, DNA binding, and chromosome association. N-terminal KSHV LANA (residues 1 to 32) mediates chromosome association through interaction with histones H2A and H2B and includes a nuclear localization signal. Amino acid residues are indicated. P, proline-rich region; LZ, predicted leucine zipper. (B) Schematic diagram of mLANA transcription from the MHV68 genome. MHV68 ORFs are indicated. The three potential LANA promoters, p1, p2, and p3, are indicated. Genomic nucleotide positions are indicated for the terminal repeat, the mLANA ORF, and exons 1, 2, and 3 (2, 12). When expressed from an upstream mTR element, multiple exon 1 copies are present in transcripts. A second exon (not shown) in the mTR (located at positions 119,373 to 119,195) can be driven by promoter p2 from an upstream mTR element, and a single copy of this exon is then spliced to exon 1 copies (12, 34). The line drawn above exon 3 and promoter 3 and extending into ORF75c indicates the sequence upstream of mLANA included in the native promoter constructs used in this work. (C) Schematic diagrams of mLANA and mTR constructs used in this work. Promoters are indicated. One arrow is used in the mTRs to represent the two potential promoters present in each of the mTRs (see panel A and Discussion for details). Arrows within the mTR elements indicate the direction relative to mLANA. In the native genomic MHV68 orientation, the arrow points toward mLANA. Summaries of numbers of G418-resistant cell lines positive for episomes over the total number tested, with percentages in parentheses, are shown at right for A20 and MEF cells. ND, not determined.

mLANA expression is highly compromised in the ability to establish efficient latent infection and persistence in mice (25, 48, 50). These data are consistent with the possibility that mLANA may mediate episome persistence of MHV68 DNA. In addition, since LANA acts on the KSHV TRs to mediate episome persistence, it is possible that mLANA may act on the MHV68 TR (mTR) elements to mediate episome maintenance.

In this work, we evaluated whether mLANA acts on the mTR elements to mediate episome persistence of MHV68 DNA. We found that a plasmid containing mTR elements can persist as an episome in an MHV68-infected mouse tumor cell line. Furthermore, plasmids containing mLANA and mTR elements in *cis* were capable of persisting as episomes in uninfected cells. These findings indicate that mLANA acts on the MHV68 TR elements to maintain viral episomes, analogous to the effect of LANA on the KSHV TRs.

MATERIALS AND METHODS

Cell lines. A20 murine B lymphoma cells (40) were cultured in RPMI medium supplemented with 10% Fetalplex (Gemini) or bovine growth serum (BGS) (HyClone), beta mercaptoethanol, sodium pyruvate, Glutamax (Invitrogen), and 15 μg/ml gentamicin. Baby hamster kidney (BHK) cells and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% BGS, beta mercaptoethanol, and 15 μg/ml gentamicin. S11 cells (61) were maintained in RPMI medium supplemented with 20% BGS supplemented with beta mercaptoethanol.

Virus infection and purification. BHK21 cells at ~75% confluence in five 500-cm^2 cell culture dishes were infected with MHV68 at a multiplicity of infection of 0.001 in DMEM containing 2% Fetalplex (12 ml/dish) for 1 h at 37°C. Fifty milliliters of DMEM containing 10% Fetalplex was then added, and cells were incubated for 3 days, at which time plaques were evident. Cells were trypsinized, collected by centrifugation, and incubated in 1.5 ml RSB buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$) per plate, supplemented with 0.5% NP-40, for 10 min at 4°C. Centrifugation was then performed to remove nuclei, and the supernatant was collected. Centrifugation of the supernatant in microcentrifuge tubes was performed at 20,800 × *g* for 2 h at 4°C. Pellets containing virus were resuspended in 400 μl NTE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) by sonication. SDS and additional EDTA were then added to final concentrations of 2.5% SDS and 10 mM EDTA, and incubation was performed for 5 min at 37°C. Virus DNA was purified using DNAzol (Invitrogen) according to the manufacturer's instructions.

Plasmids. MHV-68 TR (mTR) elements were cloned from purified MHV68 DNA. First, virus DNA was digested with Tsp509I, which digests frequently in the unique sequence but not in the mTRs. Subsequently, partial NotI digestion was performed. mTR elements each have one NotI site. Fragments containing two, four, and eight TR copies were gel purified and ligated into the NotI site of the pRepCK vector (4) to generate m2TR, m4TR, and m8TR, respectively (Fig. 1C).

Plasmids expressing mLANA from a cytomegalovirus (CMV) promoter were constructed. Linker NS contains NsiI and StuI sites and was generated by annealing the sequences NSfwd (TC GAG ATG CAT GCT CGA TAC AGC AGG CCT AAG G) and NSrev (GA TCC CTT AGG CCT GCT GTA TCG AGC ATG CAT C). Linker NS was then inserted into pRepCK, m2TR, m4TR, and m8TR, after digestion of each with XhoI and BamHI, to generate pRepCK-NS, m2TR-NS, m4TR-NS, and m8TR-NS, respectively. To generate pCMVFmLANA, mLANA was amplified from MHV68 DNA by using the primers mLANAfwd (CGC GGA TCC ATG CCC ACA TCC CCA CCG) and mLANArev1 (TCG ATA TCT TAT GTC TGA GAC CCT TGT CC), which add a BamHI site upstream of mLANA and an EcoRV site immediately downstream of mLANA. The PCR product was digested with BamHI and EcoRV and inserted into the BamHI and EcoRV sites of pCMV-3Tag-6 (Stratagene), resulting in pCMVFmLANA, which contains a 3× FLAG tag upstream of mORF73. The 3× FLAG tag was then removed by digestion with BamHI and NotI, and the sites were blunted and ligated, resulting in pCMVmLANA. pCMVmLANA was digested with NsiI and PsiI, releasing the CMVmLANA expression cassette, and this was inserted into the NsiI and StuI sites of pRepCK vector-NS, m2TR-NS, and m4TR-NS to generate CmLANA, CmLANA-m2TR, and CmLANA-m4TR, respectively. These constructs contain mLANA without an epitope tag driven by the CMV promoter and containing 0, 2, or 4 mTRs.

Constructs containing 3× C-terminal FLAG-tagged mLANA driven by a CMV promoter were generated. pCMVmLANAF was constructed in the same fashion as pCMVFmLANA, except that the PCR amplification of mLANA was performed with primers mLANAfwd and mLANArev2 (TCG ATA TCT GTC TGA GAC CCT TGT CC). mLANArev2 omits the native mLANA stop codon and contains an EcoRV site at its 5′ end. The N-terminal 3× FLAG tag was removed with BamHI and NotI as described above. The 3× FLAG tag was PCR amplified from pCMV-3Tag-6 with oligonucleotides FLAG fwd (CGG ATA TCG CGG TGG CGG CCG CC) and FLAG rev (CCG GCT CGA GTT AGA ATT CCT GCA GCC CGG G), which add a stop codon immediately downstream of the 3× FLAG tag and EcoRV and XhoI sites at the 5′ and 3′ ends, respectively. The PCR product was digested with EcoRV and XhoI and inserted into the EcoRV and XhoI sites (of the vector polylinker) in pCMVmLANA, lacking the native stop codon, to generate pCMVmLANAF, which contains a C-terminal 3× FLAG epitope-tagged mLANA sequence driven by the CMV promoter. The CMV mLANA FLAG expression cassette was released by digestion with NsiI and PsiI and inserted into the NsiI and StuI sites of pRepCK-NS, m2TR-NS, m4TR-NS, and m8TR-NS to generate CmLANAF, CmLANAF-m2TR, CmLANAF-m4TR, and CmLANAF-m8TRrev, respectively (Fig. 1C).

Plasmids containing mLANA driven from its native promoter were generated. Oligonucleotides m73fwd and m73rev were used to amplify the mLANA sequence, including 2,037 bp upstream of the mLANA open reading frame, from MHV68 DNA.

Oligonucleotide m73fwd (CAGCTCGAGATCCAGACTTTGGAGCA TATGTTT) contains an XhoI site at the 5′ end, and oligonucleotide m73rev (CGCGGATCCTTATGTCTGAGACCCTTGTCC) contains a BamHI site at its 5′ end. The PCR product was digested with XhoI and BamHI, gel purified, and inserted into XhoI- and BamHI-digested pRepCK, m2TR, m4TR, and m8TR to generate mLANA, mLANA-m2TR, mLANA-m4TR, and mLANA-m8TRrev, respectively (Fig. 1C). To generate mLANA-m8TR, mLANA-2TR (Fig. 1C) was digested with NheI and XhoI to remove the mTR elements, and a linker containing the XhoI, NsiI, SacI, StuI, and NheI restriction sites was generated by annealing oligonucleotides NXfwd (CTAGGCTCGAGATGCATGAGCTCAGGCCTGCTA GCG) and NXrev (TCGACGCTAGCAGGCCTGAGCTCATGCATCTC GAGC) and inserted into the NheI and XhoI sites. The linker construction destroyed the original NheI and XhoI sites in the parent plasmid, and the inserted sites resulted in reversing the orientation of XhoI and NheI compared to the original vector. m8TR was digested with XhoI and NheI, removing the 8 mTR units, and these were inserted into the XhoI and NheI sites of the plasmid containing the linker to generate mLANA-m8TR, which contains the 8 mTR units in opposite orientation compared with mLANA-m8TRrev and results in the native genomic orientation of the mTRs relative to mLANA.

C-terminal mLANA FLAG epitope tags were inserted into constructs expressing mLANA from its native promoter. pCMVmLANAF (described above) was digested with PciI and PsiI, releasing a C-terminal portion of the mLANAF expression cassette, and this fragment was inserted into PciI- and HpaI-digested mLANA, mLANA-m2TR, mLANA-m4TR, mLANA-m8TRrev, and mLANA-m8TR to generate mLANAF, mLANAF-m2TR, mLANAF-m4TR, mLANAF-m8TRrev, and mLANAF-m8TR, respectively (Fig. 1C). All PCR-amplified coding sequences were confirmed by sequencing. Clones were expanded in Stbl2 (Invitrogen)

bacteria for large-scale DNA preparations due to the greater stability of the repeat elements in these bacteria.

Episome maintenance assays. S11 cells were maintained in log-phase growth by reseeding at 0.2×10^6 cells/ml for three consecutive days. A total of 10×10^6 cells were transfected by electroporation with 35 μg of m4TR, m8TR, or vector (pRepCK) in 400 μl, using a BTX Electrosquare Porator T820 electroporation system, pulsing the cells once at 200 V for 65 milliseconds. Immediately after transfection, cells were supplemented with 0.5 ml of medium in the cuvette and incubated at room temperature for 10 min. Cells were then transferred to T25 flasks in 5 ml medium. At 3 days posttransfection, cells were seeded at 10,000 or 1,000 cells per well in 96-well plates in medium containing G418 (400 μg/ml) (Gemini). G418-resistant cells were then expanded in preparation for detection of episomes.

A20 cells were kept in log-phase growth by reseeding at 0.3×10^6 cells/ml for three consecutive days. A total of 10×10^6 A20 cells were transfected with 35 μg vector (pRepCK), mLANA, CmLANA, mLANAF, CmLANAF, m2TR, m4TR, m8TR, m73-m2TR, mLANA-m4TR, mLANA-m8TR, mLANA-m8TRrev, mLANAF-m2TR, mLANAF-m4TR, mLANAF-m8TR, mLANAF-m8TRrev, CmLANA-m2TR, CmLANA-m4TR, CmLANAF-m2TR, CmLANAF-m4TR, or CmLANAF-m8TRrev in 400 μl by electroporation using a BTX Electrosquare Porator T820 electroporation system, pulsing the cells once at 225 or 250 V for 65 milliseconds. After transfection, cells were treated similarly to S11 cells, except that at 72 h posttransfection cells were seeded at 1,000, 100, or 10 cells per well in medium containing G418 (400 μg/ml) (Gemini) and G418-resistant cells expanded.

MEF cells were transfected with 2 μg of mLANA, m8TR, mLANA-m8TRrev, mLANA-m2TR, mLANA-m4TR, or mLANAF-m4TR in 6-cm dishes at about 75% confluence, using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Two days after transfection, cells were trypsinized, counted, and seeded at 10,000 or 50,000 cells in a 15-cm dish. The next day, cells were rinsed with phosphate-buffered saline, and fresh medium containing G418 (1,000 μg/ml) (Gemini) was added. G418-resistant clones were picked and transferred to 12-well plates for expansion.

Gardella analysis (29) was performed on G418-resistant clones. Cells were loaded into gel wells composed of agarose containing DNase-free protease (Sigma) and sodium dodecyl sulfate. *In situ* lysis of cells occurs as electrophoresis begins in Tris-borate-EDTA buffer. DNA was then transferred to a nylon membrane and detected with a ^{32}P-labeled probe. Probes were DpnI digested prior to radiolabeling, except for the mLANA probe, which was a PCR product amplified using the mLANAfwd and mLANArev1 oligonucleotide primers and was not digested prior to radiolabeling.

Western blots. A20 cells were transfected as described above. Extract from 0.25×10^6 cells was loaded into each lane. Proteins were resolved by SDS-PAGE in 8% polyacrylamide gels, transferred to nitrocellulose, and detected with anti-FLAG antibody conjugated to horseradish peroxidase (HRP) (Sigma), used at a 1:750 dilution, or mouse anti-tubulin monoclonal antibody B-5-1-2 (Sigma), used at a 1:1,000 dilution. Incubation with secondary anti-mouse HRP-conjugated antibody followed by chemiluminescence was performed to detect anti-tubulin antibody.

Fluorescence microscopy. For metaphase spreads, MEF cells were grown in six-well dishes to 80% confluence and then incubated overnight in 1 μg/ml of colcemid (Calbiochem) to induce metaphase arrest. Colcemid-treated cells were swollen in hypotonic buffer for 20 min (1% sodium citrate, 10 mM $CaCl_2$, 10 mM $MgCl_2$), spread onto slides by the cytospin method (Thermoshandon), fixed for 10 min in 4% paraformaldehyde (Polysciences) in phosphate-buffered saline, and permeabilized for 5 min in 0.5% Triton X-100 in phosphate-buffered saline. To detect FLAG epitope-tagged mLANA, cell spreads were incubated with M2 anti-FLAG monoclonal antibody (Sigma) at a 1:750 dilution. Secondary anti-mouse–Alexa Fluor 488 (Molecular Probes) was used to detect M2. Cells were counterstained with propidium iodide (Molecular Probes) (1 μg/ml) to detect DNA, and coverslips were applied with Aqua-Poly mounting reagent (Polysciences). Microscopy was performed with a Zeiss Axioskop microscope, PCM2000 hardware, and C-imaging software (Compix, Inc.).

RESULTS

The MHV68 TR elements act in *cis* to mediate episome persistence. Since the TR elements are the *cis*-acting sequence for KSHV episome maintenance, we assessed whether the mTR elements are the *cis*-acting element for MHV68. KSHV LANA acts on the KSHV TR elements to mediate episome persistence. mLANA is homologous to LANA (Fig. 1A) and is critical for establishment of viral latency in mice. We hypothesized that the mTR elements might have a role in episome maintenance similar to that of the KSHV TRs.

We assayed whether MHV68 TR DNA can mediate extrachromosomal persistence of DNA in murine B lymphoma S11 cells. Most S11 cells are latently infected with MHV68, although some S11 cells contain virus undergoing lytic replication. In latently infected cells, MHV68 persists as a multicopy episome (61). Therefore, the necessary factors responsible for MHV68 episome persistence are expressed in these cells. If the mTRs are the *cis*-acting sequence for episome persistence, then DNA containing the mTR sequence is expected to be capable of persisting as an episome in S11 cells. m8TR (contains eight copies of the MHV68 TR element), m4TR (contains four copies of mTR), or vector alone was transfected into S11 cells. Cells were then seeded into microtiter plates at 10,000 or 1,000 cells per well and placed under G418 selection, for which resistance is encoded on the plasmid. In at least three experiments, outgrowth of G418-resistant cells transfected with m8TR was robust and occurred in all 96 wells in the plates seeded with 10,000 cells per well and in an average of 20 wells in the plates seeded with 1,000 cells per well. Outgrowth was also robust in cells transfected with m4TR, and an average of 86 wells were positive for outgrowth at 10,000 cells per well and 7 wells were positive at 1,000 cells per well. In contrast, after transfection with the vector control, which lacks mTR elements, outgrowth occurred in only an average of 9 wells and 1 well after seeding at 10,000 and 1,000 cells per well, respectively. These outgrowth data indicate that mTR DNA persists more efficiently than the vector in S11 cells. The observed enhanced persistence could be due to maintenance of mTR DNA as episomes or to increased efficiency of integration of mTR DNA.

To assess the presence of m8TR episomal DNA, Gardella gel analyses were performed on G418-resistant cell lines. In Gardella gels (29), live cells are loaded into gel wells and lysed immediately at the start of the gel run. Episomes as large as several hundred kilobases can migrate into the gel, while chromosomal DNA remains at the gel origin. Episomes are then detected by Southern blotting. S11 cells (Fig. 2A, lanes 1, 11, and 20) contain episomes but were not detected because the probe consisted of vector only, which does not share sequence with MHV68. After 90 days of G418 selection, m8TR episomes were detected in 11 (Fig. 2A, lanes 4, 6 to 10, 14, 15, and 17 to 19) of 16 lanes. In a total of two experiments, 26 of 35 G418-resistant cell lines contained episomes. In addition, three of six m4TR-transfected, G418-resistant cell lines had episomes (data not shown). The episomes generally migrated much slower than covalently closed circular plasmid DNA (Fig. 2A, lane 2, faster-migrating band), consistent with recombination events. Selection of enlarged KSHV episomes is fre-

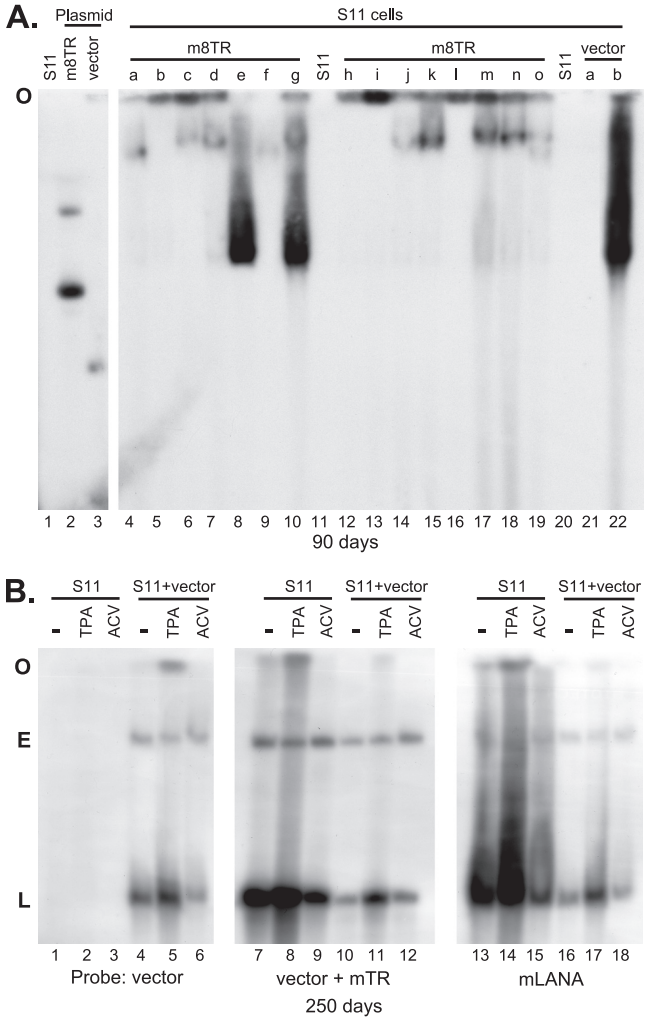

FIG 2 mTR DNA persists as an episome in S11 cells. m8TR or pRepCK vector was transfected into MHV68-infected S11 cells. Seventy-two hours later, cells were seeded into microtiter plates at 10,000 cells/well or 1,000 cells/well and placed under G418 selection. G418-resistant cells were expanded and assessed for episomes by use of Gardella gels. This figure is representative of two experiments. (A) Gardella gel containing S11 cells (lanes 1, 11, and 20), naked m8TR (lane 2) or pRepCK (lane 3) plasmid DNA, m8TR-transfected, G418-resistant S11 cells (lanes 4 to 10 and 12 to 19), and vector-transfected, G418-resistant S11 cells (lanes 21 and 22). A total of ~1.5×10^6 cells was loaded in each lane. G418-resistant cell lines were taken from plates seeded with 10,000 cells per well (lanes 17 to 19, 21, and 22) or 1,000 cells per well (lanes 4 to 10 and 12 to 16). The Gardella gel analysis was performed after 90 days of G418 selection. The faster-migrating signal for m8TR (lane 2) or vector (lane 3) is circular covalently closed DNA. The blot was probed with ^{32}P-radiolabeled pRepCK vector DNA. (B) S11 cells (lanes 1 to 3, 7 to 9, and 13 to 15) and a vector-transfected, G418-resistant S11 cell line (lanes 4 to 6, 10 to 12, and 16 to 18) that contained episomal DNA were assessed after treatment with TPA or acyclovir (ACV). The experiment was performed after 250 days of G418 selection. Probes consisted of vector (lanes 1 to 6), m8TR plasmid (which includes both mTR and vector sequences) (lanes 7 to 12), and the mLANA ORF (lanes 13 to 18). For lanes 1 to 6, 1.5×10^6 cells were loaded per lane; for lanes 7 to 12, 0.15×10^6 cells were loaded per lane; and for lanes 13 to 18, 1.5×10^6 cells were loaded per lane. Fewer cells were used with the mTR probe due to the greater sensitivity of detection resulting from the number of repeated mTR elements in MHV68. The gel origin (O) and S11 episomal (E) and linear (L) forms (due to MHV68 lytic replication) are indicated.

quently observed in KSHV episome maintenance assays and is due to recombination events resulting in increased numbers of TR elements in episomes. Therefore, MHV68 TR DNA can persist as episomes in cells latently infected with MHV68.

Integration of transfected DNA into MHV68. Unexpectedly, one of the two G418-resistant cell lines transfected with vector, which lacks TR sequence, had large amounts of extrachromosomal DNA (Fig. 2A, lane 22). In fact, extrachromosomal signals in vector-transfected S11 cells were observed for 3 of 6 G418-resistant cell lines tested in a total of two experiments. Since the vector lacks mTR DNA and any MHV68 sequence, it was expected that integration into host cell chromosomes would be required to allow persistence of DNA and that transfected DNA would not persist as an episome. Notably, two of the G418-resistant cell lines containing m8TR episomes (Fig. 2A, lanes 8 and 10) also had large amounts of extrachromosomal DNA, which ran in a similar pattern on the gel compared with that of the vector-transfected cell line (Fig. 2A, lane 22). Of the 35 G418-resistant cell lines transfected with m8TR, this finding of large amounts of extrachromosomal DNA was observed in 5 cell lines and in 1 of 6 G418-resistant cell lines transfected with m4TR (Fig. 2 and data not shown).

One possible explanation for the vector being maintained as an episome was that the vector had integrated into MHV68 genomes, which are episomal. To test this possibility, the Southern blot was stripped of signal and reprobed for a sequence present in MHV68 but not the transfected mTR DNA. Strikingly, overlay of the two films demonstrated complete signal overlap for the cell line containing extrachromosomal vector (data not shown). Notably, the two cell lines containing m8TR DNA in a pattern similar to that of the extrachromosomal vector noted above also had a confluence of signals when the films were overlaid (data not shown). These results are consistent with integration of vector and m8TR DNAs into MHV68 genomes in these G418-resistant cell lines.

Since the integration of vector lacking any MHV68 DNA into MHV68 episomal genomes was an unexpected event, we further investigated this finding. S11 cells or a G418-resistant S11 cell line transfected with vector (lacking mTR DNA) but containing extrachromosomal vector DNA was assessed after 250 days of G418 selection. Cells were treated with 20 ng/ml tetradecanoyl phorbol acetate (TPA) or 100 μM acyclovir for 48 h. TPA induces lytic infection, while acyclovir inhibits lytic infection. As expected, probing with vector did not detect a signal in S11 cells (Fig. 2B, lanes 1 to 3), since the probe lacks MHV68 sequence. However, the vector probe detected episomal (E) (Fig. 2B, lane 4) and linear (L) (Fig. 2B, lane 4) DNA (from lytic infection) in the G418-resistant cells containing vector episomal DNA. Incubation with TPA increased the vector-containing linear, replicating DNA signal (Fig. 2B, lane 5) but not the episomal signal, which is the expected effect on S11 virus, since TPA induces lytic replication. In contrast, treatment with acyclovir decreased the amount of linear, replicating DNA but not the episomal signal (Fig. 2B, lane 6), which is the expected effect on MHV68 infection. A Gardella gel with the same cell lines was also probed with mLANA, which is present in MHV68 but not in the vector, to detect MHV68 DNA. In both S11 cells and G418-resistant, vector-transfected S11 cells, similar patterns of increasing linear DNA after TPA treatment and decreased linear DNA after acyclovir treatment were observed (Fig. 2B, lanes 13 to 18).

Simultaneous probing of a Gardella gel with sequences specific for both vector and mTRs detected the same episomal and linear

replicating DNA patterns with TPA and acyclovir. No additional bands or doublets were detected in the cells containing episomal vector DNA compared with the S11 cells (Fig. 2B, lanes 7 to 12). This finding strongly indicates that both probes detected the same bands. Overall, these findings are consistent with vector integration into MHV68 episomal genomes in some G418-resistant S11 cell lines. Furthermore, these results suggest that some G418-resistant m8TR cell lines (such as in Fig. 2A, lanes 8 and 10) also have integration of transfected vector into episomal MHV68 genomes. Importantly, however, most G418-resistant cell lines transfected with m8TR did not have an overlap of signal when probed for mLANA compared with the vector probe. This result is therefore consistent with independent episome persistence of the transfected m8TR DNA in these lines.

CMV promoter-driven mLANA acts on mTR DNA in *cis* to mediate low levels of episome persistence. Since m8TR and m4TR persisted as episomes in S11 cells, we wished to investigate whether mLANA acts on mTR DNA to mediate episome maintenance analogous to the KSHV LANA function. To test this possibility, we constructed plasmids containing CMV promoter-driven mLANA or mLANA with a C-terminal FLAG tag (mLANAF), and also plasmids containing two, four, or eight mTR elements (termed CmLANA-m2TR, CmLANAF-m2TR, CmLANA-m4TR, CmLANAF-m4TR, or CmLANAF-m8TRrev). As controls, we generated plasmids containing only mLANA (termed CmLANA or CmLANAF) or used m2TR, m4TR, and m8TR, which contain only TR elements. Uninfected mouse A20 B lymphoma cells were transfected with m2TR, m4TR, m8TR, CmLANA, CmLANAF, CmLANA-m2TR, CmLANAF-m2TR, CmLANA-4TR, CmLANAF-4TR, or CmLANAF-m8TRrev. Cells were seeded into microtiter plates at 3 days posttransfection and placed under G418 selection, against which resistance is encoded by the plasmid vector. G418-resistant cell outgrowth was similar for each of the transfections containing mTR DNA, whether or not mLANA was present in *cis*. After transfection with mTR-containing DNA, nearly all 96 wells were positive for outgrowth in plates seeded at 1,000 cells/well, ~10 to 30 wells were positive after seeding at 100 cells per well, and ~0 to 5 wells were positive after seeding at 10 cells per well. G418-resistant cell outgrowth for the CmLANA or CmLANAF transfections was lower, at ~50 positive wells for plates seeded at 1,000 cells/well, ~10 positive wells after seeding at 100 cells per well, and ~0 to 2 wells after seeding at 10 cells per well. The comparable rates of G418-resistant cell outgrowth with mTR transfections, regardless of the presence of mLANA, were consistent with either an absence of mLANA episome maintenance or episome maintenance occurring at a rate similar to that of integration.

G418-resistant cells were expanded and assessed by Gardella gel analysis for the presence of episomes. m2TR-transfected cells (Fig. 3B, lanes 10 to 13), m4TR-transfected cells (Fig. 3A, lanes 5 and 6), and m8TR-transfected cells (Fig. 3B, lanes 31 to 34), which contain mTR elements but not the mLANA sequence, did not contain episomes. Similarly, cells transfected with CmLANA (Fig. 3A, lanes 21 to 24 and 33) or CmLANAF (Fig. 3B, lanes 19 to 22), which contain the mLANA sequence but do not have mTR elements, also did not contain any episomes. Therefore, no episomes were present when either mLANA was present without mTR elements or mTR elements were present without mLANA.

G418-resistant cells transfected with DNA containing both mLANA and mTRs were also assessed for the presence of episomes. CmLANAF-m2TR, which contains FLAG-tagged mLANA and two mTRs, had no episomes in six G418-resistant cell lines (Fig. 3B, lanes 13 to 18). Similarly, cells transfected with CmLANA-m2TR, which contains mLANA without an epitope tag and two mTRs, had no episomes in four G418-resistant cell lines (data not shown). However, CmLANA-m4TR, which contains mLANA and four mTR elements (Fig. 3A, lanes 7 to 20 and 27 to 32), had episomal DNA in four lanes (Fig. 3A, lanes 9, 27, 28, and 30), and in a total of two experiments, CmLANA-m4TR-transfected cells had episomes in 9 of 33 (27%) G418-resistant cell lines. In addition, CmLANAF-m4TR, which contains FLAG-tagged mLANA and four mTR elements, had episomal DNA in one lane (Fig. 3B, lane 5), and in a total of two experiments, CmLANAF-m4TR had episomes in 2 of 24 (8%) G418-resistant cell lines. CmLANAF-m8TRrev had episomes in 1 (Fig. 3B, lane 29) of 18 (6%) G418-resistant cell lines (Fig. 3B, lanes 25 to 30, and data not shown). Therefore, when mLANA expressed from a CMV promoter was present in *cis* with 4 or 8 mTR elements, episomes were present in a small percentage of G418-resistant cell lines. This finding is consistent with mLANA acting on mTR elements to mediate low-efficiency episome persistence.

The native mLANA promoter drives higher-level mLANA expression than the CMV promoter. We reasoned that a low expression level of mLANA might have been responsible for the low efficiency of episome persistence and therefore assessed mLANA expression levels driven by either the CMV promoter or the native mLANA promoter. CmLANAF, CmLANAF-m2TR, CmLANAF-m4TR, and CmLANAF-m8TRrev, which have mLANA driven by the CMV promoter, were transfected into A20 cells and analyzed for mLANA expression by immunoblotting with anti-FLAG antibody. CmLANAF-m4TR (Fig. 4, lanes 3 and 14) expressed mLANA at a higher level than did CmLANAF (Fig. 4, lane 5) or CmLANAF-m2TR (Fig. 4, lane 4). CmLANAF-m4TR mLANA expression (Fig. 4, lanes 3 and 14) was also higher than that of CmLANAF-m8TRrev (Fig. 4, lane 15). It is possible that the higher expression level from CmLANAF-m4TR accounted for its higher efficiency of episome persistence than that of CmLANAF-m2TR or CmLANAF-m8TRrev (Fig. 3).

mLANAF, mLANAF-m2TR, mLANAF-m4TR, and mLANAF-m8TRrev each have mLANA driven by its native promoter sequence and were also tested for mLANA expression levels. Three native promoters have been described for mLANA, including one immediately upstream of mLANA and two within the mTR elements (2, 12). The orientation of the mTR elements in relation to mLANA in mLANAF-m2TR and mLANAF-m4TR is the same as in genomic MHV68, but it is reversed in mLANAF-m8TRrev. mLANAF (Fig. 4, lane 10) expressed mLANA at a similar level to those of mLANAF-m8TRrev (Fig. 4, lane 7) and CmLANAF (Fig. 4, lane 5). mLANAF-m2TR (Fig. 4, lane 9) and mLANAF-m4TR (Fig. 4, lane 8) expressed mLANA at substantially higher levels than those of mLANAF and mLANAF-m8TRrev. The expression level for mLANAF-m4TR was higher than that for mLANAF-m2TR, as evident in the 10-s exposure (Fig. 4, middle panel, lanes 8 and 9, respectively). Therefore, the presence of upstream mTR elements in the native genomic orientation relative to mLANA substantially enhances mLANA expression, and the level of expression is much greater than that driven by the CMV promoter. Furthermore, four upstream mTR elements were more efficient than two mTR elements in driving mLANA expression.

Native promoter-driven mLANA maintains mTR episomes with enhanced efficiency. Since higher mLANA expression oc-

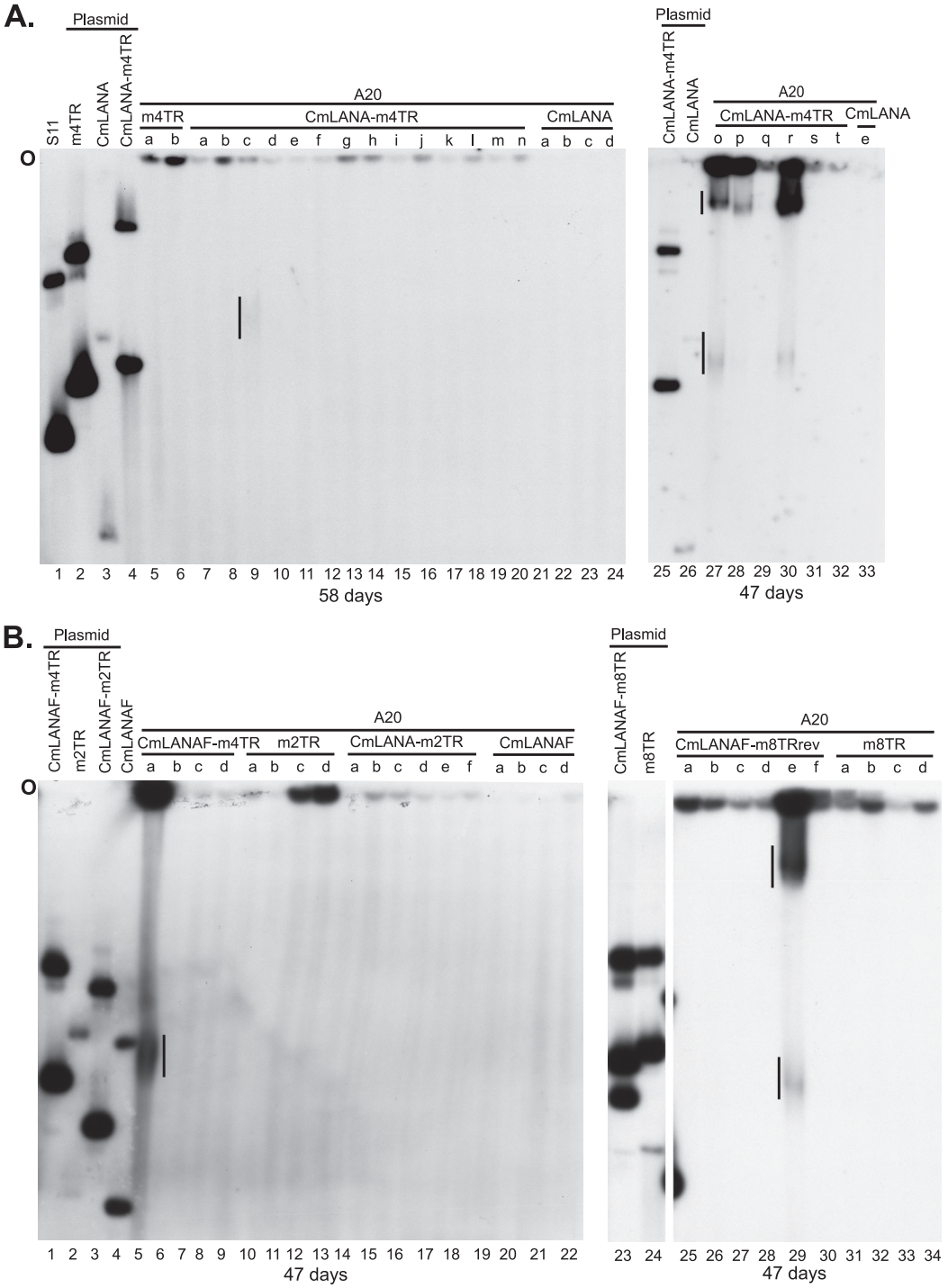

FIG 3 CMV promoter-driven mLANA in *cis* with mTR elements persists in episomal form at low efficiency. A20 cells were transfected with plasmids containing CMV promoter-driven mLANA and mTR elements or with mTR DNA. Seventy-two hours later, cells were seeded in microtiter plates at 1,000, 100, or 10 cells/well and placed under G418 selection. A total of 2×10^6 to 3×10^6 A20 cells was loaded per lane in Gardella gels, and 1.5×10^5 S11 cells were loaded per lane. (A) Gardella gel containing S11 cells (lane 1), naked m4TR DNA (lane 2), naked CmLANA DNA (lanes 3 and 26), naked CmLANA-m4TR plasmid DNA (lanes 4 and 25), m4TR-transfected, G418-resistant A20 cells (lanes 5 and 6), CmLANA-m4TR-transfected, G418-resistant A20 cells (lanes 7 to 20 and 27 to 32), and CmLANA-transfected, G418-resistant A20 cells (lanes 21 to 24 and 33). G418-resistant cell lines were taken from plates seeded with 1,000 cells per well (lanes 22 and 24) or 100 cells per well (lanes 5 to 21, 23, 27 to 33). (B) Gardella gel containing naked CmLANA-m4TR (lane 1), naked m2TR (lane 2), naked CmLANAF-m2TR (lane 3), naked CmLANAF (lane 4), naked CmLANAF-m8TR (lane 23), and naked m8TR (lane 24) plasmid DNA, as well as G418-resistant A20 cells transfected with CmLANAF-m4TR (lanes 5 to 8), m2TR (lanes 9 to 12), CmLANAF-m2TR (lanes 13 to 18), CmLANAF (lanes 19 to 22), CmLANAF-8TRrev (lanes 25 to 30), or m8TR (lanes 31 to 34). G418-resistant cell lines were taken from plates seeded with 100 cells per well (lanes 5 to 8, 10 to 12, 15 to 18, 20 to 22, and 30 to 34) or 10 cells per well (lanes 9, 13, 14, 19, and 25 to 29). The gel origin (O) is indicated. The number of days of G418 selection is shown below each panel. For naked plasmid DNA, the fastest-migrating signal is circular covalently closed DNA. Blots were probed with ^{32}P-radiolabeled m8TR DNA. Vertical lines indicate migration locations of episomal DNA.

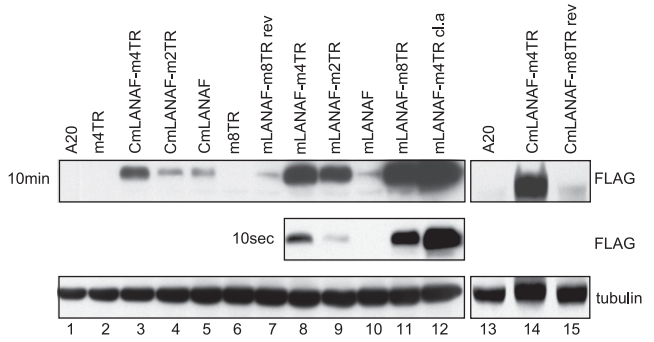

FIG 4 mLANA levels after transfection of different expression vectors. A20 cells were transfected with the indicated plasmids and harvested for immunoblotting 72 h later, except for the sample in lane 12. The gel shows A20 cells (lanes 1 and 13) and A20 cells transfected with m4TR (lane 2), CmLANAF-m4TR (lanes 3 and 14), CmLANAF-m2TR (lane 4), CmLANAF (lane 5), m8TR (lane 6), mLANAF-m8TRrev (lane 7), mLANAF-m4TR (lane 8), mLANAF-m2TR (lane 9), mLANAF (lane 10), mLANAF-m8TR (lane 11), or CmLANAF-m8TRrev (lane 15). Lane 12 contains mLANA-m4TR cl.a, which is the G418-resistant cell line containing episomes shown in Fig. 5A, lane 8. The bottom panel shows a tubulin immunoblot for the same cells. A total of 0.25×10^6 cells was loaded in each lane. The middle panel shows a shorter exposure (10 s) of lanes 8 to 12 than the 10-min exposure for these lanes in the top panel. The top right panel shows a 20-min exposure. This figure is representative of at least two experiments.

curs from the native promoter than from the CMV promoter, we assessed whether episome maintenance is enhanced when native promoter-driven mLANA is oriented in *cis* with mTR elements. mLANAF, m4TR, and mLANAF-m4TR were each transfected into A20 cells, seeded into microtiter plates at a density of 1,000 cells per well, 100 cells per well, or 10 cells per well, and placed under G418 selection. For m4TR and mLANAF-4TR, G418-resistant outgrowth occurred in nearly all wells seeded at 1,000 cells per well, in ~15 wells seeded at 100 cells/well, and in ~2 wells seeded at 10 cells/well. This pattern of similar G418-resistant cell outgrowth after transfection of all mTR-containing DNA constructs in both the presence and absence of mLANA continued in subsequent experiments. In comparison, mLANAF G418-resistant cell outgrowth occurred in only ~30 wells seeded at 1,000 cells/well, ~2 wells seeded at 100 cells/well, and 0 wells seeded at 10 cells/well. The higher rate of G418-resistant cell outgrowth for cells transfected with mTR-containing DNA than that for cells in the absence of mTR DNA provided evidence for a higher rate of integration of mTR-containing plasmids into the host genome. Since there was no increase in the G418 outgrowth rate with mLANA and mTRs expressed in *cis*, results were consistent with either a lack of episome persistence or an efficiency of episome persistence that was no higher than that of integration.

G418-resistant cell lines were expanded and assessed for the presence of episomes by Gardella gel analysis at 47 days postselection. As expected, neither m4TR (Fig. 5A, lanes 5 to 7), which lacks mLANA, nor mLANAF (Fig. 5A, lanes 22 to 24), which lacks mTR elements, had episomes. In contrast, mLANAF-m4TR persisted as episomal DNA in 10 (Fig. 5A, lanes 8 to 10, 12 to 16, 19, and 21) of 14 lanes. Notably, most of the episomal signal migrated much more slowly than the covalently closed circular mLANAF-m4TR plasmid (Fig. 5A, lane 3, bottom band) and even slower than episomal MHV68 from infected S11 cells (Fig. 5A, lane 1, upper band), consistent with selection for recombination into very large episomes. In two experiments, mLANAF-m4TR had episomes in 19 of 45 (42%) G418-resistant cell lines. Furthermore, in two additional experiments, mLANA-m4TR, which is similar to mLANAF-m4TR except that mLANA lacks a C-terminal epitope tag, episomes were present in 18 of 42 (43%) G418-resistant cell lines (data not shown). Since mLANAF-m4TR persisted as episomal DNA with an efficiency similar to that of mLANA-m4TR, the C-terminal FLAG tag did not adversely affect mLANA function.

To assess the long-term stability of the episomes, selected G418-resistant cell lines were again assayed by Gardella gel analysis after 173 days of selection. All five cell lines (Fig. 5B, lanes 8 to 14) continued to stably maintain episomal mLANAF-m4TR DNA. Therefore, episomes were stable for at least ~6 months in continuous culture.

mLANA expression was assessed in these G418-resistant cell lines. As expected, no signal was detected in m4TR (Fig. 5C, lanes 1, 13, and 14)-transfected cell lines, since this plasmid lacks mLANA. mLANAF expression was detected in only one (Fig. 5C, lane 21) of three (Fig. 5C, lanes 11, 12, 20, and 21) mLANAF-transfected, G418-resistant cell lines and was expressed at a relatively low level. In contrast, mLANAF expression was detected in all mLANAF-m4TR cell lines (Fig. 5C, lanes 2 to 10 and 15 to 18) and was robust in all lines except for one that contained very low levels of episomes (Fig. 5A, lane 21). For this cell line, mLANAF could be detected only on longer exposure (Fig. 5C, middle panel, lane 19). It is interesting that robust mLANAF expression (Fig. 5C, lanes 5, 15, 16, and 18) was present even in cell lines that lacked episomes (Fig. 5A, lanes 11, 17, 18, and 20) when the plasmid was integrated.

We observed that A20 cells expressing mLANA proliferated at a lower rate than that of cells that did not express mLANA, suggesting that mLANA may exert growth-inhibitory effects. Therefore, it is possible that when cell outgrowth is robust in nearly all wells, such as after plating 1,000 cells/well after transfection of mTR-containing plasmids, cells with integrated plasmid and lacking mLANA expression may overgrow those cells which contain episomes and express mLANA. However, in these experiments, Gardella gel analyses were generally done from plates seeded with 100 cells/well that had low levels of G418-resistant outgrowth, in which outgrowth is expected to be nearly clonal.

The presence of two or eight mTR elements instead of four mTR elements in *cis* with mLANA was also assessed with regard to episome maintenance. In three experiments, mLANA-m2TR (without an epitope tag) had episomes in 14 of 32 (44%) G418-resistant cell lines, while in two experiments, mLANAF-2mTR (containing a FLAG tag) had episomes in 5 of 18 (28%) cell lines (data not shown). These rates of episome persistence were very similar to those with four mTRs (42% for mLANAF-m4TR and 43% for mLANA-m4TR). mLANA-m8TRrev-transfected, G418-resistant cell lines contained episomes in only 3 (Fig. 5D, lanes 7, 8, and 14) of 12 (Fig. 5D, lanes 3 to 14) cell lines. In two experiments, mLANA-m8TRrev had episomes in 3 of 16 (19%) G418-resistant cell lines, and the signal was very weak in two of the lanes (Fig. 5D, lanes 7 and 14). Two additional experiments assessing mLANAF-m8TRrev (containing an mLANA FLAG epitope tag) had episomes in 2 of 21 (10%) G418-resistant cell lines. Therefore, mLANA-m8TRrev and mLANAF-m8TRrev had lower efficiencies of episome persistence than vectors containing two or four copies of mTR. This lower efficiency was likely due to lower

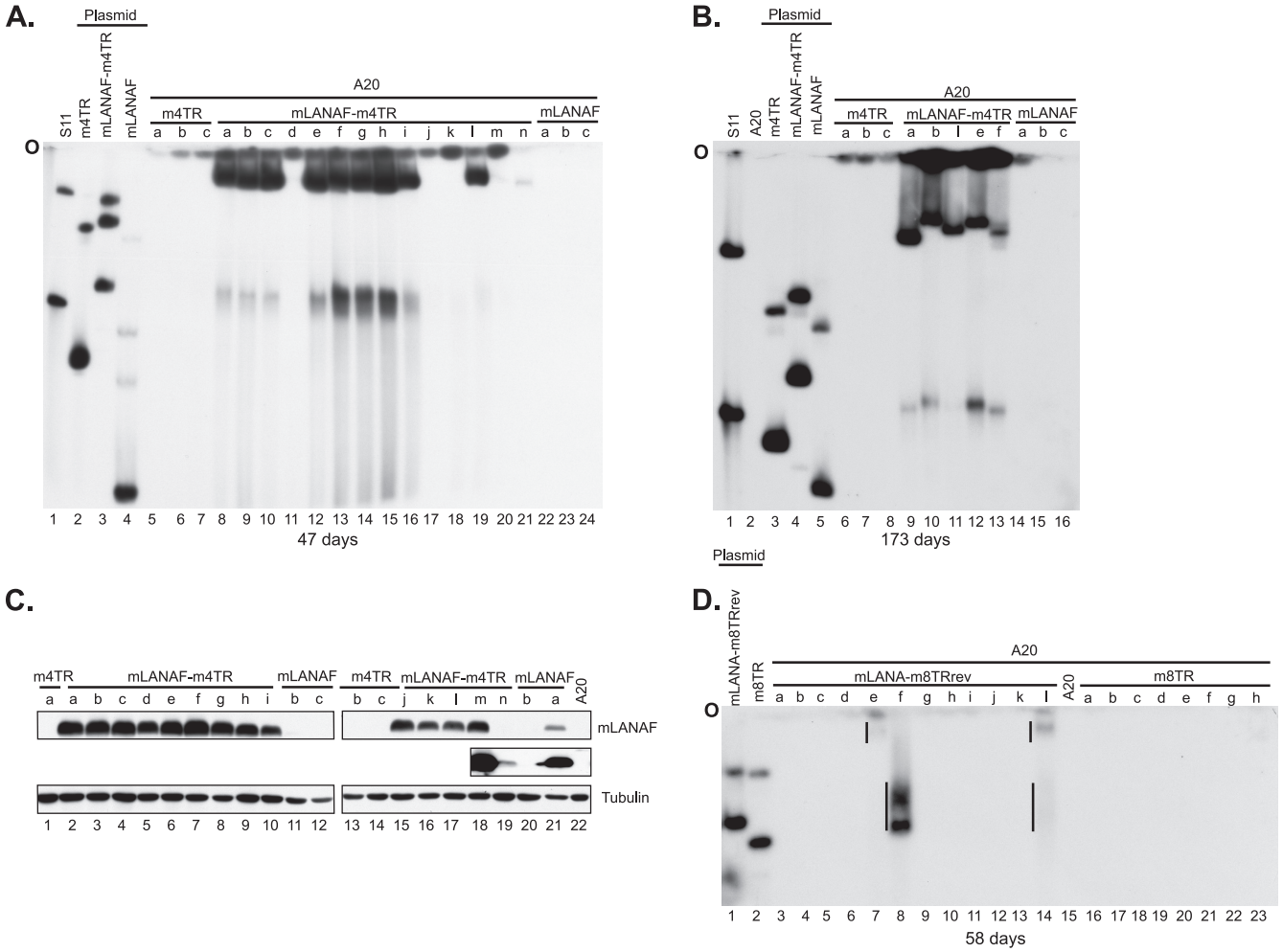

FIG 5 Native promoter-driven mLANA in *cis* with mTR elements persists in episomal form with increased efficiency. A20 cells were transfected with plasmids containing native promoter-driven mLANA with mTR elements or with mTR DNA. Seventy-two hours later, cells were seeded into microtiter plates at 1,000, 100, or 10 cells/well and placed under G418 selection. A total of 2×10^6 to 3×10^6 cells was loaded per lane for Gardella gels. (A) Gardella gel containing S11 cells (lane 1), naked m4TR plasmid DNA (lane 2), naked mLANAF-m4TR plasmid DNA (lane 3), naked mLANAF plasmid DNA (lane 4), G418-resistant, m4TR-transfected A20 cells (lanes 5 to 7), G418-resistant, mLANAF-m4TR-transfected A20 cells (lanes 8 to 21), and G418-resistant, mLANAF-transfected A20 cells (lanes 22 to 24). G418-resistant cell lines were taken from plates seeded with 1,000 cells per well (lanes 22 to 24), 100 cells per well (lanes 5 to 11 and 17 to 20), or 10 cells per well (lanes 12 to 16 and 21). (B) Gardella gel containing S11 cells (lane 1), A20 cells (lane 2), naked m4TR DNA (lane 3), naked mLANAF-m4TR DNA (lane 4), naked mLANAF DNA (lane 5), and A20 cells after 173 days of G418 selection (lanes 6 to 16). Letters above the lanes correspond to the same letters in panel A. (C) Immunoblot of mLANA G418-resistant cell lines from panel A. Lane letters correspond to the same letters as in panel A. Lanes 11 and 20 contain cells from the same G418-resistant "b" cell line. A total of 0.25×10^6 cells was loaded per lane. The middle panel shows a longer exposure for lanes 18 to 22. The bottom panel shows a tubulin immunoblot. (D) Gardella gel containing naked mLANA-m8TRrev DNA (lane 1), naked m8TR DNA (lane 2), G418-resistant, mLANA-m8TRrev-transfected A20 cells (lanes 3 to 14), A20 cells (lane 15), and G418-resistant, m8TR-transfected A20 cells (lanes 16 to 23). G418-resistant cell lines were taken from plates seeded with 1,000 cells per well (lanes 3 to 21) or 100 cells per well (lanes 22 and 23). The figure is representative of at least two experiments. Numbers of days of G418 selection are indicated below the panels. The faster-migrating bands in S11 lanes are linear MHV68 genomic DNA from cells undergoing lytic infection, and the slower-migrating band is episomal MHV68. The fastest-migrating bands in naked DNA lanes are circular covalently closed DNA. Blots in panels A, B, and D were probed with ^{32}P-radiolabeled m8TR DNA. Vertical lines in panel D indicate episomal DNA.

mLANA expression from this construct, which has the mTRs reversed from their native orientation relative to mLANA (Fig. 4, lane 7). In the absence of mLANA (Fig. 5D, lanes 16 to 23), no episomes were observed. Therefore, episome persistence occurred only when mLANA and mTRs were together in *cis*, and episome persistence was more efficient at higher mLANA expression levels.

mLANA maintains mTR episomes in MEF cells. Since mLANA in *cis* with mTRs persisted as episomal DNA in A20 B lymphoma cells, we assessed if mLANA could also maintain episomes in another cell type. Therefore, MEF cells were transfected with mLANA, mLANAF, m4TR, m8TR, mLANA-m2TR, mLANA-m4TR, mLANA-8TRrev, or mLANAF-m4TR and placed under G418 selection. G418-resistant colonies were expanded independently and assessed by Gardella gel analysis for the presence of episomes. As expected, the mLANA (Fig. 6B, lanes 5 to 8), mLANAF (Fig. 6C, lanes 12 and 13), m4TR (Fig. 6C, lanes 6 and 7), and m8TR (Fig. 6A, lanes 18 to 24, and B, lane 13) cell lines did not contain episomes. mLANA-m2TR contained episomes in 4 of 5 lanes (80%) (Fig. 6B, lanes 14, 15, 17, and 18), mLANA-m4TR had episomes in 8 of 12 lanes (67%) (Fig. 6A, lanes 12 to 14,

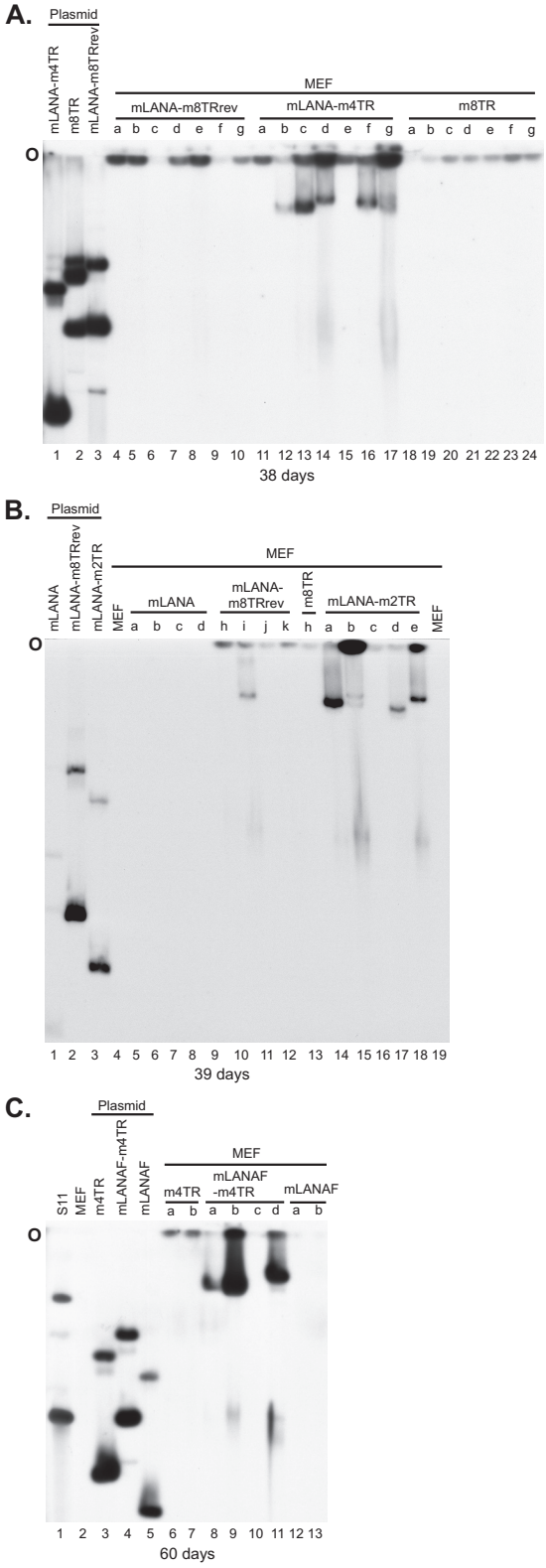

FIG 6 mLANA in *cis* with mTR elements persists in episomal form in MEF cells. MEF cells were transfected with plasmids containing native promoter-driven mLANA in *cis* with mTR elements or mTR elements alone. Forty-eight hours later, cells were trypsinized, reseeded into 15-cm dishes, and placed under G418 selection. (A) Gardella gel containing naked mLANA-m4TR DNA (lane 1), naked m8TR DNA (lane 2), naked mLANA-m8TRrev DNA (lane 3), G418-resistant, mLANA-m8TRrev-transfected MEF cells (lanes 4 to 10), G418-resistant, mLANA-m4TR-transfected MEF cells (lanes 11 to 16), and G418-resistant, m8TR-transfected MEF cells (lanes 19 to 24). (B) Gardella gel containing naked mLANA DNA (lane 1), naked mLANA-m8TRrev DNA (lane 2), naked mLANA-m2TR DNA (lane 3), MEF cells (lanes 4 and 19), G418-resistant, mLANA-transfected MEF cells (lanes 5 to 8), G418-resistant, mLANA-m8TRrev-transfected MEF cells (lanes 9 to 12), G418-resistant, m8TR-transfected MEF cells (lane 13), and mLANA-m2TR-transfected MEF cells (lanes 14 to 18). (C) Gardella gel containing S11 cells (lane 1), MEF cells (lane 2), naked m4TR DNA (lane 3), naked mLANAF-m4TR DNA (lane 6), naked mLANAF DNA (lane 5), G418-resistant, m4TR-transfected MEF cells (lanes 6 and 7), G418-resistant, mLANAF-m4TR-transfected MEF cells (lanes 8 to 11), and G418-resistant, mLANAF-transfected MEF cells (lanes 12 and 13). A total of ~1×10^6 MEF cells was loaded per lane in Gardella gels. Numbers of days of G418 selection are indicated at the bottom of panels. Blots were probed with ^{32}P-radiolabeled m8TR DNA.

16, and 17, and data not shown), mLANA-m8TRrev contained episomes in 1 (Fig. 6B, lane 10) of 11 lanes (9%) (Fig. 6A, lanes 4 to 10, and B, lanes 9 to 12), and mLANAF-m4TR had episomes in 3 of 4 lanes (75%) (Fig. 6C, lanes 8, 9, and 11). Western blot analysis of mLANAF protein expression demonstrated that the mLANAF-m4TR-transfected cell line lacking episomes (Fig. 6C, lane 10) expressed mLANAF at a robust level that was only slightly lower than that of cells containing episomes (Fig. 6C, lanes 8, 9, and 11), while cells transfected with mLANAF without mTRs had significantly reduced or no mLANAF expression (data not shown). These expression patterns were similar to those in A20 cells (Fig. 5C). The lower rate of episome persistence for mLANA-m8TRrev was similar to that in A20 cells and was likely due to lower mLANA protein expression. As observed in A20 cells, MEF cells expressing mLANA proliferated at a lower rate than that of MEF cells not expressing mLANA. Therefore, mLANA acts on mTR DNA to mediate episome persistence in MEF cells.

Native genomic orientation of eight mTR elements in *cis* with mLANA increases episome persistence efficiency. We investigated whether the native genomic orientation of eight mTRs in *cis* with mLANA would affect the efficiency of episome maintenance, since mLANA-m8TRrev, in which the mTR orientation is reversed, was less efficient than mLANA in *cis* with only two or four mTRs in both A20 and MEF cells. Therefore, we generated mLANAF-m8TR, which contains eight mTR elements in native genomic orientation upstream of mLANA (Fig. 1C). Transfection of mLANAF-m8TR (Fig. 4, lane 11) into A20 cells demonstrated substantially more mLANA expression than that of mLANAF-m8TRrev (Fig. 4, lane 7), and the level was somewhat higher than that of mLANAF-m4TR (Fig. 4, middle panel, lane 8). The expression level of mLANAF-m8TR was only slightly lower than the expression level of mLANA in a G418-resistant cell line maintaining mLANAF-m4TR episomes (Fig. 4, middle panel, lane 12).

We assessed the ability of mLANAF-m8TR to persist as an episome. mLANAF-m8TR, m8TR, and mLANAF were each transfected into A20 cells, plated in microtiter plates, and selected for G418 resistance. As expected, m8TR (Fig. 7A, lanes 6 and 7) and mLANAF (Fig. 7A, lanes 22 and 23) did not have episomes. In contrast, mLANAF-m8TR had episomal DNA in 9 (Fig. 7A, lanes 9 to 11, 14 to 16, 18 to 19, and 21) of 15 (Fig. 7A, lanes 8 to 21) cell lines. In two experiments, mLANAF-8TR had episomes in 13 of 18 (72%) cell lines. This episome maintenance efficiency was substantially higher than that of mLANAF-m8TRrev (10%) or mLANA-m8TRrev (19%) and was even higher than that of mLANA in native orientation with two or four mTRs in A20 cells

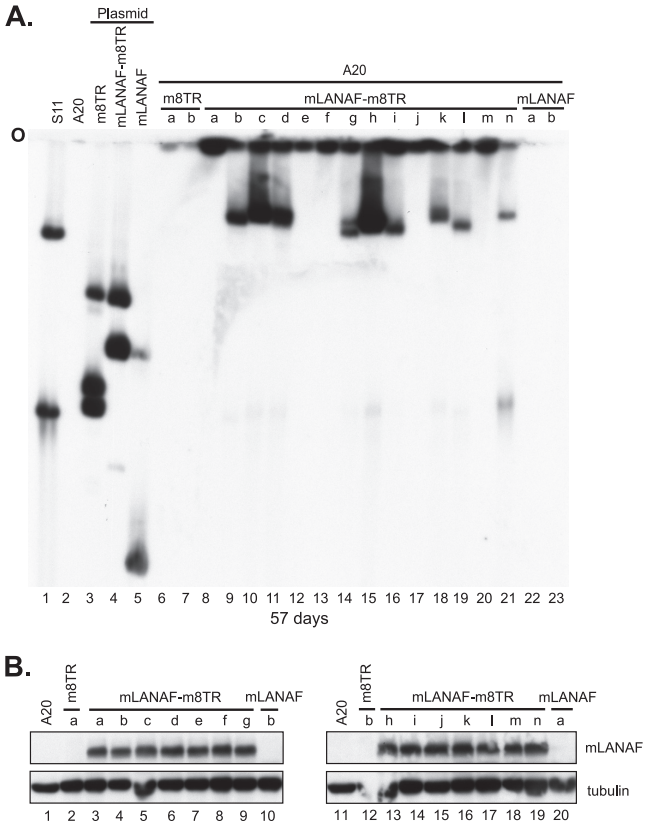

FIG 7 mLANA in *cis* with mTR elements in native orientation enhances episome persistence. (A) Gardella gel containing S11 cells (lane 1), A20 cells (lane 2), naked m8TR DNA (lane 3), naked mLANA F-m8TR DNA (lane 4), mLANAF (lane 5), G418-resistant, m8TR-transfected A20 cells (lanes 6 and 7), G418-resistant, mLANAF-m8TR-transfected A20 cells (lanes 8 to 21), and G418-resistant, mLANAF-transfected A20 cells (lanes 22 and 23). G418-resistant cell lines were taken from plates seeded with 1,000 cells per well (lanes 22 and 23), 100 cells per well (lanes 6 to 18), or 10 cells per well (lanes 19 to 21). A total of ~1.5 × 10^6 cells was loaded per lane. The number of days of G418 selection is shown at the bottom. The blot was probed with ^{32}P-radiolabeled m8TR DNA. (B) Immunoblot of mLANAF and tubulin for cells in panel A. Letters above lanes correspond to the same letters in panel A.

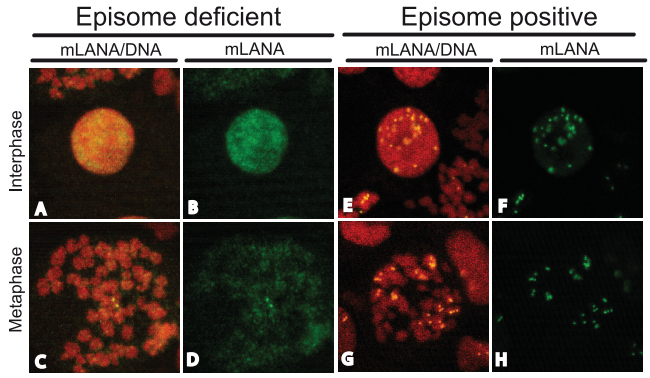

FIG 8 mLANA concentrates to dots along mitotic chromosomes in the presence of episomes but is broadly distributed along chromosomes in episome-deficient cells. mLANAF was detected in MEF cells deficient for episomes (A to D; cells from Fig. 6C, lane 10) or in MEF cells containing mLANAF-m4TR episomes (E to H; cells from Fig. 6C, lane 11). Cells were in interphase (A, B, E, and F) or metaphase (C, D, G, and H). Overlay of green (mLANAF) with red (DNA) generates yellow. Panels C and D contain two mitotic cells. The two paired dots in panels C and D are likely due to the presence of rare episomes that were not detected by the Gardella gel. Brightness and contrast were uniformly adjusted for some panels from the same field by use of Adobe Photoshop. Magnification, ×630.

(Fig. 1C). Therefore, native orientation of the eight mTR elements substantially increased both mLANA expression and episome persistence.

Immunoblot analysis demonstrated that mLANA was expressed at robust levels in all mLANAF-m8TR cell lines (Fig. 7B, lanes 3 to 9 and 13 to 19). Even cell lines lacking episomes (Fig. 7B, lanes 3, 7 to 8, 15, and 18), which therefore contained integrated plasmid, expressed mLANA, similar to the findings with integrated mLANAF-m4TR (Fig. 5C). Notably, cell lines transfected with mLANAF, which contains the native mLANA promoter immediately upstream of mLANA but no mTR elements, did not express mLANA (Fig. 7B, lanes 10 and 20).

mLANA redistributes and concentrates to dots along mitotic chromosomes in the presence of episomes. mLANA was detected in MEF cells (Fig. 8) containing mLANAF-m4TR episomes (cell line from Fig. 6C, lane 11) and in MEF cells deficient in episomes, for which no episomes were detected on Gardella gel analysis (cell line from Fig. 6C, lane 10). In episome-deficient cells (Fig. 8A to D), mLANAF (green) was distributed broadly throughout the nucleus (red) in interphase and over mitotic chromosomes (red) (overlay of green and red generates yellow). In contrast, in the presence of episomes (Fig. 8E to H), mLANAF (green) was concentrated to dots both in interphase and along mitotic chromosomes (red) (overlay of green and red generates yellow). Therefore, in the presence of episomes, mLANA relocalized to dots along mitotic chromosomes.

DISCUSSION

This work demonstrates that mLANA acts on the mTR elements to mediate episome persistence. DNA containing mTR elements persisted as episomes in latently MHV68-infected S11 cells, indicating that the *cis*-acting element for episome persistence is located in the mTRs. Furthermore, plasmids containing mLANA expression cassettes and mTR elements in *cis* were capable of persisting as episomes in both murine A20 B lymphoma cells and MEF cells. In contrast, neither mTR elements nor mLANA alone ever persisted as episomal DNA, consistent with a requirement for both factors for episomal maintenance.

These findings follow earlier work which suggested an episome maintenance role for mLANA. LANA is the episome maintenance protein for KSHV and acts on KSHV TR DNA to maintain episomes (3–5, 14). Similarly, HVS LANA binds to and acts on HVS TR elements to mediate episome persistence (10, 11, 13, 30, 31, 63, 68). mLANA is the MHV68 positional homolog for both KSHV and HVS LANA proteins and also shares sequence homology with these proteins. Furthermore, studies which abolished mLANA expression by introduction of specific mutations demonstrated that mLANA is critical for MHV68's ability to establish latent infection in mice (25, 48). Notably, later work showed that MHV68 lacking mLANA expression could persist at low levels in murine splenocytes, especially after intraperitoneal injection. However, MHV68 was unable to reactivate from splenocytes, and no episomal DNA could be detected in the latently infected cells. This finding was consistent with either integration into host chromosomes or

maintenance of DNA in a linear state, therefore indicating a role for mLANA in the formation or maintenance of episomes (50).

Initial experiments transfecting mTR plasmids into S11 cells were consistent with the mTRs acting as the *cis*-acting element for episome persistence but were complicated by the finding of vector integration into MHV68 episomal genomes in some G418-resistant cell lines. Furthermore, some of the transfected mTR plasmids that persisted as episomes were also integrated into MHV68 genomes. The mTR plasmids contain homologous mTR sequences, which may have facilitated recombination of the transfected plasmid into the mTR elements of MHV68 genomes. However, the integration of nonhomologous pRepCK vector DNA into episomal viral episomes was highly unexpected. S11 cells contain ~40 MHV68 genomes per cell (61). The MHV68 genome is comprised of ~120 kb of unique sequence, plus an unknown number of mTR elements (64). Assuming an mTR copy number of ~40, similar to that in KSHV, the total MHV68 genome consists of ~170 kb. Therefore, there are $\sim 7 \times 10^6$ bp of MHV68 per S11 cell, compared with 2.5×10^9 bp of cell DNA (66), so MHV68 accounts for only ~0.3% of the total DNA present in the cell. The finding that 3 of 6 (50%) G418-resistant cell lines had vector integrated into MHV68 episomes indicates a >150-fold higher rate of integration than expected if integration were completely stochastic. It is possible that integration occurred in cells undergoing lytic replication, which contained increased levels of MHV68 DNA and therefore increased the possibility of integration. Virions released from these dying cells may then have superinfected latently infected cells to form episomes. Although we do not know if the site of integration in MHV68 is the same for each G418-resistant line, it is possible that MHV68 may contain a certain sequence, perhaps the mTR elements, that is highly prone to recombination.

Gardella gel analyses demonstrated that episomes with mLANA and mTRs migrated much more slowly than the input plasmids (Fig. 2, 3, 5, 6, and 7), consistent with recombination events leading to substantial size increases. In fact, much of the episomal signal migrated slower than the ~170-kb MHV68 episomes in S11 cells (Fig. 5 to 7). We previously observed similar increases in episome size in KSHV LANA episome maintenance, due to TR duplication and generation of input plasmids into multimers (4, 18). It is likely that there is strong selection for recombination events leading to increased numbers of mTR elements and/or an increased copy number of the mLANA expression cassette. If MHV68 contains ~40 TR elements (similar to KSHV) and therefore represents an optimal number for episome maintenance, recombination events leading to larger numbers of mTRs (up to ~40 copies) may occur.

Although MHV68 ORF74 and the C-terminal portion of ORF75C are included in the upstream sequence of the mLANA constructs containing the native promoter sequence (Fig. 1B), it is highly unlikely that either exerts a role in episome persistence. Most importantly, episome persistence occurred in the absence of ORF74 and truncated ORF75C with plasmids containing CMV-driven mLANA and mTRs (Fig. 3). Though episome maintenance efficiency was lower with CMV-driven mLANA, this finding correlated with a lower level of mLANA expression from these constructs. In addition, both mLANAF-m8TRrev (Fig. 5 and 6) and mLANAF-8TR (Fig. 7) contain the same mLANA upstream sequence yet had substantially different episome maintenance efficiencies (Fig. 1C). The difference between these two plasmids is only the orientation of the mTR elements in relation to the mLANA cassette, which resulted in much higher mLANA expression for mLANAF-8TR, with a concomitant increase in episome maintenance efficiency. Furthermore, ORF75C is severely truncated, while ORF74 is a plasma membrane protein homologous to KSHV ORF74, which is a G protein-coupled receptor (65), and its plasma membrane location is not compatible with episome maintenance function.

The presence of the mTR elements in genomic orientation substantially enhanced mLANA expression (Fig. 4, 5, and 7). At least three promoters for mLANA have been described (Fig. 1B). Two are located within the terminal repeat elements, with one either completely (12) or partially (2) contained in the left half of the mTR and one in the right half of the mTR (2, 12). A third promoter is located just proximal to mLANA (12). In addition, the mTR element has enhancer activity for the promoter in the left half of the mTR (2), while the left end of the unique sequence of the MHV68 genome (which was not present in the constructs used here) has enhancer activity for the promoter in the right half of the mTR (12). Since the addition of the mTR elements in native orientation to mLANA substantially enhanced its expression (Fig. 4), it is likely that an mTR promoter was driving the enhanced mLANA expression. It is also possible that the mTR provided enhancer activity to the promoter immediately upstream of mLANA, although the orientation dependence of the mTR elements, in which expression was enhanced only in the native, not reverse, genomic orientation relative to mLANA in the constructs containing eight mTR elements, makes this scenario less likely. When expressed from MHV68, mLANA transcripts contain up to three 5′-untranslated exons, including one at the right end of the unique MHV68 genome and up to two different exons from within the mTR elements (2, 12, 34) (Fig. 1B). Since the unique sequence at the right end of the MHV68 genome is absent in the constructs used here, this exon would be omitted in our experiments when transcription occurs from the mTRs. We observed increased mLANA expression with a higher mTR copy number (Fig. 4), and this could be consistent with multiple active mTR promoters being active and/or increased numbers of mTR enhancer elements acting on the mTR promoter(s). Notably, the splice acceptor site just upstream of the mLANA ORF is present in the constructs with the native upstream mLANA sequence but absent in the CMV promoter constructs, so splicing to this site can occur only in those constructs where the native sequence is present. It is possible that the increased expression of mLANA from the CMV promoter in the presence of four upstream mTR elements (Fig. 4) could be due to mTR enhancer activity or a cryptic splice acceptor site resulting in the mTR promoter driving expression. The generally low expression level from the CMV promoter observed here could possibly be improved with a stronger Kozak consensus sequence.

Transcription of mLANA has several notable differences from that of KSHV LANA. In contrast to the mLANA promoters, the KSHV LANA promoter is located only immediately upstream of the LANA ORF, and there are not additional promoters in the KSHV TR elements. Of note, the promoter for the KSHV K1 gene does extend into the TR, but this is located in the opposite orientation from that of LANA to drive K1 expression at the extreme left end of the KSHV genome (9, 62). In addition, the KSHV LANA transcript is polycistronic and includes two additional ORFs, those of v-cyclin and v-FLIP, while the MHV68 transcript contains only the mLANA ORF (19, 55, 60). Another distinguish-

ing feature between KSHV and MHV68 is that the LANA promoter is activated by LANA (35, 52), while mLANA modestly downregulates the mLANA promoters (12). Also, KSHV ORF50, the lytic switch protein, activates the LANA promoter (43, 47), while mORF50 represses the promoter immediately upstream of mLANA and has little effect on the mTR promoters (12).

In comparison with KSHV LANA, the efficiency of mLANA episome persistence in these experiments appears to be relatively low. The highest efficiency of episome persistence in A20 cells occurred with mLANAF-8TR, for which 72% of G418-resistant cell lines contained episomes, and this efficiency was lower when plasmids contained only four or two mTR elements. Episome maintenance efficiency was somewhat higher in MEF cells than in A20 cells (up to 80% of mLANA-m2TR G418-resistant cell lines had episomes), although this finding was based on assessment of relatively few cell lines. Notably, the absence of episomes was not always due to a loss of mLANA expression, since mLANA continued to be expressed even in the absence of detectable episomes (Fig. 5 and 7). In contrast, in similar experiments with KSHV LANA constructs containing a LANA expression cassette with eight TR elements in *cis*, 100% of G418-resistant cell lines contained episomes (our unpublished data). It is possible that more mTR elements may be present in the MHV68 genome than the ~40 elements in KSHV, and therefore more mTR elements may be necessary for efficient episome persistence. KSHV LANA is a larger protein than MHV68 LANA and may have evolved to be more efficient in its episome persistence.

This work demonstrates that mLANA is nuclear and associates with mitotic chromosomes (Fig. 8). The pattern of distribution is very similar to that of KSHV LANA. In the absence of episomes, LANA distributes broadly throughout interphase nuclei and over mitotic chromosomes. However, when KSHV episomes are present, LANA is concentrated at dots at the sites of KSHV DNA both in interphase and along mitotic chromosomes (3, 5, 6, 14, 36, 38, 42, 51). mLANA was broadly distributed throughout the nucleus and over mitotic chromosomes in episome-deficient cells but was concentrated at dots in interphase nuclei and along mitotic chromosomes in episome-containing cells. It is likely that mLANA concentrates at sites of MHV68 episomes. This finding supports the hypothesis that mLANA directly tethers MHV68 episomes to mitotic chromosomes to allow for efficient segregation to daughter nuclei, similar to the tethering mechanism of KSHV LANA (3, 14). This mechanism of bridging episomes to chromosomes has also been proposed for HVS LANA (11, 13, 63). It is likely that mLANA attaches to episomes through direct binding of mTR DNA, similar to KSHV and HVS LANAs (4, 13, 15, 21, 28, 63, 68).

In summary, this work demonstrates that mLANA acts on MHV68 mTR DNA to mediate episome persistence. These results suggest that mLANA tethers mTR DNA to mitotic chromosomes to efficiently segregate MHV68 DNA into progeny nuclei. Future work is necessary to understand the molecular mechanisms underlying this process and to better understand the similarities and differences between KSHV and MHV68 LANA functions.

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REFERENCES

1. **Albrecht JC.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66**:5047–5058.
2. **Allen RD, 3rd, Dickerson S, Speck SH.** 2006. Identification of spliced gammaherpesvirus 68 LANA and v-cyclin transcripts and analysis of their expression in vivo during latent infection. J. Virol. **80**:2055–2062.
3. **Ballestas ME, Chatis PA, Kaye KM.** 1999. Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. Science **284**:641–644.
4. **Ballestas ME, Kaye KM.** 2001. Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mediates episome persistence through cis-acting terminal repeat (TR) sequence and specifically binds TR DNA. J. Virol. **75**:3250–3258.
5. **Barbera AJ, Ballestas ME, Kaye KM.** 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.
6. **Barbera AJ, et al.** 2006. The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. Science **311**:856–861.
7. **Blasdell K, et al.** 2003. The wood mouse is a natural host for murid herpesvirus 4. J. Gen. Virol. **84**:111–113.
8. **Blaskovic D, Stancekova M, Svobodova J, Mistrikova J.** 1980. Isolation of five strains of herpesviruses from two species of free living small rodents. Acta Virol. **24**:468.
9. **Bowser BS, DeWire SM, Damania B.** 2002. Transcriptional regulation of the K1 gene product of Kaposi's sarcoma-associated herpesvirus. J. Virol. **76**:12574–12583.
10. **Calderwood M, White RE, Griffiths RA, Whitehouse A.** 2005. Open reading frame 73 is required for herpesvirus saimiri A11-S4 episomal persistence. J. Gen. Virol. **86**:2703–2708.
11. **Calderwood MA, Hall KT, Matthews DA, Whitehouse A.** 2004. The herpesvirus saimiri ORF73 gene product interacts with host-cell mitotic chromosomes and self-associates via its C terminus. J. Gen. Virol. **85**:147–153.
12. **Coleman HM, Efstathiou S, Stevenson PG.** 2005. Transcription of the murine gammaherpesvirus 68 ORF73 from promoters in the viral terminal repeats. J. Gen. Virol. **86**:561–574.
13. **Collins CM, Medveczky MM, Lund T, Medveczky PG.** 2002. The terminal repeats and latency-associated nuclear antigen of herpesvirus saimiri are essential for episomal persistence of the viral genome. J. Gen. Virol. **83**:2269–2278.
14. **Cotter MA, 2nd, Robertson ES.** 1999. The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. Virology **264**:254–264.
15. **Cotter MA, 2nd, Subramanian C, Robertson ES.** 2001. The Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen binds to specific sequences at the left end of the viral genome through its carboxy-terminus. Virology **291**:241–259.
16. **Decker LL, et al.** 1996. The Kaposi sarcoma-associated herpesvirus (KSHV) is present as an intact latent genome in KS tissue but replicates in the peripheral blood mononuclear cells of KS patients. J. Exp. Med. **184**:283–288.
17. **Delecluse HJ, Bartnizke S, Hammerschmidt W, Bullerdiek J, Bornkamm GW.** 1993. Episomal and integrated copies of Epstein-Barr virus coexist in Burkitt lymphoma cell lines. J. Virol. **67**:1292–1299.
18. **De Leon Vazquez E, Kaye KM.** 2011. The internal Kaposi's sarcoma-associated herpesvirus LANA regions exert a critical role on episome persistence. J. Virol. **85**:7622–7633.
19. **Dittmer D, et al.** 1998. A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J. Virol. **72**:8309–8315.
20. **Doherty PC, Christensen JP, Belz GT, Stevenson PG, Sangster MY.** 2001. Dissecting the host response to a gamma-herpesvirus. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356**:581–593.
21. **Fejer G, et al.** 2003. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus interacts preferentially with the terminal repeats of the genome in vivo and this complex is sufficient for episomal DNA replication. J. Gen. Virol. **84**:1451–1462.
22. **Fickenscher H, Fleckenstein B.** 2001. Herpesvirus saimiri. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356**:545–567.

23. **Flano E, Husain SM, Sample JT, Woodland DL, Blackman MA.** 2000. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. J. Immunol. **165**:1074–1081.
24. **Flano E, Woodland DL, Blackman MA.** 2002. A mouse model for infectious mononucleosis. Immunol. Res. **25**:201–217.
25. **Fowler P, Marques S, Simas JP, Efstathiou S.** 2003. ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency. J. Gen. Virol. **84**:3405–3416.
26. **Ganem D.** 2007. Kaposi's sarcoma-associated herpesvirus, p 2847–2888. *In* Knipe DM, Howley PM (ed), Fields virology, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
27. **Garber AC, Hu J, Renne R.** 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.
28. **Garber AC, Shu MA, Hu J, Renne R.** 2001. DNA binding and modulation of gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. J. Virol. **75**:7882–7892.
29. **Gardella T, Medveczky P, Sairenji T, Mulder C.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. **50**:248–254.
30. **Griffiths R, Harrison SM, Macnab S, Whitehouse A.** 2008. Mapping the minimal regions within the ORF73 protein required for herpesvirus saimiri episomal persistence. J. Gen. Virol. **89**:2843–2850.
31. **Griffiths R, Whitehouse A.** 2007. Herpesvirus saimiri episomal persistence is maintained via interaction between open reading frame 73 and the cellular chromosome-associated protein MeCP2. J. Virol. **81**:4021–4032.
32. **Grundhoff A, Ganem D.** 2003. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus permits replication of terminal repeat-containing plasmids. J. Virol. **77**:2779–2783.
33. **Hu J, Garber AC, Renne R.** 2002. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells. J. Virol. **76**:11677–11687.
34. **Husain SM, et al.** 1999. Murine gammaherpesvirus M2 gene is latency-associated and its protein a target for CD8(+) T lymphocytes. Proc. Natl. Acad. Sci. U. S. A. **96**:7508–7513.
35. **Jeong JH, et al.** 2004. Regulation and auto-regulation of the promoter for the latency-associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus. J. Biol. Chem. **279**:16822–16831.
36. **Kelley-Clarke B, Ballestas ME, Komatsu T, Kaye KM.** 2007. Kaposi's sarcoma herpesvirus C-terminal LANA concentrates at pericentromeric and peri-telomeric regions of a subset of mitotic chromosomes. Virology **357**:149–157.
37. **Kelley-Clarke B, et al.** 2007. Determination of Kaposi's sarcoma-associated herpesvirus C-terminal latency-associated nuclear antigen residues mediating chromosome association and DNA binding. J. Virol. **81**:4348–4356.
38. **Kelley-Clarke B, De Leon-Vazquez E, Slain K, Barbera AJ, Kaye KM.** 2009. Role of Kaposi's sarcoma-associated herpesvirus C-terminal LANA chromosome binding in episome persistence. J. Virol. **83**:4326–4337.
39. **Kieff ED, Rickinson AB.** 2007. Epstein-Barr virus and its replication, p 2603–2654. *In* Knipe DM, et al (ed), Fields virology, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
40. **Kim KJ, Kanellopoulos-Langevin C, Merwin RM, Sachs DH, Asofsky R.** 1979. Establishment and characterization of BALB/c lymphoma lines with B cell properties. J. Immunol. **122**:549–554.
41. **Komatsu T, Ballestas ME, Barbera AJ, Kelley-Clarke B, Kaye KM.** 2004. KSHV LANA1 binds DNA as an oligomer and residues N-terminal to the oligomerization domain are essential for DNA binding, replication, and episome persistence. Virology **319**:225–236.
42. **Krithivas A, Fujimuro M, Weidner M, Young DB, Hayward SD.** 2002. Protein interactions targeting the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus to cell chromosomes. J. Virol. **76**:11596–11604.
43. **Lan K, et al.** 2005. Induction of Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen by the lytic transactivator RTA: a novel mechanism for establishment of latency. J. Virol. **79**:7453–7465.
44. **Lim C, Seo T, Jung J, Choe J.** 2004. Identification of a virus trans-acting regulatory element on the latent DNA replication of Kaposi's sarcoma-associated herpesvirus. J. Gen. Virol. **85**:843–855.
45. **Lim C, Sohn H, Lee D, Gwack Y, Choe J.** 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.
46. **Marques S, Efstathiou S, Smith KG, Haury M, Simas JP.** 2003. Selective gene expression of latent murine gammaherpesvirus 68 in B lymphocytes. J. Virol. **77**:7308–7318.
47. **Matsumura S, Fujita Y, Gomez E, Tanese N, Wilson AC.** 2005. Activation of the Kaposi's sarcoma-associated herpesvirus major latency locus by the lytic switch protein RTA (ORF50). J. Virol. **79**:8493–8505.
48. **Moorman NJ, Willer DO, Speck SH.** 2003. The gammaherpesvirus 68 latency-associated nuclear antigen homolog is critical for the establishment of splenic latency. J. Virol. **77**:10295–10303.
49. **Nash AA, Dutia BM, Stewart JP, Davison AJ.** 2001. Natural history of murine gamma-herpesvirus infection. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356**:569–579.
50. **Paden CR, Forrest JC, Moorman NJ, Speck SH.** 2010. Murine gammaherpesvirus 68 LANA is essential for virus reactivation from splenocytes but not long-term carriage of viral genome. J. Virol. **84**:7214–7224.
51. **Piolot T, Tramier M, Coppey M, Nicolas JC, Marechal V.** 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.
52. **Renne R, et al.** 2001. Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. J. Virol. **75**:458–468.
53. **Rickinson AB, Kieff ED.** 2007. Epstein-Barr virus, p 2655–2700. *In* Knipe DM, et al (ed), Fields virology, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
54. **Russo JJ, et al.** 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc. Natl. Acad. Sci. U. S. A. **93**:14862–14867.
55. **Sarid R, Flore O, Bohenzky RA, Chang Y, Moore PS.** 1998. Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). J. Virol. **72**:1005–1012.
56. **Simas JP, Efstathiou S.** 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol. **6**:276–282.
57. **Speck SH, Ganem D.** 2010. Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell Host Microbe **8**:100–115.
58. **Speck SH, Virgin HW.** 1999. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. Curr. Opin. Microbiol. **2**:403–409.
59. **Stewart JP, Usherwood EJ, Ross A, Dyson H, Nash T.** 1998. Lung epithelial cells are a major site of murine gammaherpesvirus persistence. J. Exp. Med. **187**:1941–1951.
60. **Talbot SJ, Weiss RA, Kellam P, Boshoff C.** 1999. Transcriptional analysis of human herpesvirus-8 open reading frames 71, 72, 73, K14, and 74 in a primary effusion lymphoma cell line. Virology **257**:84–94.
61. **Usherwood EJ, Stewart JP, Nash AA.** 1996. Characterization of tumor cell lines derived from murine gammaherpesvirus-68-infected mice. J. Virol. **70**:6516–6518.
62. **Verma SC, Lan K, Choudhuri T, Robertson ES.** 2006. Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen modulates K1 expression through its cis-acting elements within the terminal repeats. J. Virol. **80**:3445–3458.
63. **Verma SC, Robertson ES.** 2003. ORF73 of herpesvirus saimiri strain C488 tethers the viral genome to metaphase chromosomes and binds to cis-acting DNA sequences in the terminal repeats. J. Virol. **77**:12494–12506.
64. **Virgin HW, IV, et al.** 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. **71**:5894–5904.
65. **Wakeling MN, Roy DJ, Nash AA, Stewart JP.** 2001. Characterization of the murine gammaherpesvirus 68 ORF74 product: a novel oncogenic G protein-coupled receptor. J. Gen. Virol. **82**:1187–1197.
66. **Waterston RH, et al.** 2002. Initial sequencing and comparative analysis of the mouse genome. Nature **420**:520–562.
67. **Weck KE, Kim SS, Virgin HI, Speck SH.** 1999. Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. J. Virol. **73**:3273–3283.
68. **White RE, Calderwood MA, Whitehouse A.** 2003. Generation and precise modification of a herpesvirus saimiri bacterial artificial chromosome demonstrates that the terminal repeats are required for both virus production and episomal persistence. J. Gen. Virol. **84**:3393–3403.
69. **Wong LY, Matchett GA, Wilson AC.** 2004. Transcriptional activation by the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen is facilitated by an N-terminal chromatin-binding motif. J. Virol. **78**:10074–10085.