Murine Gammaherpesvirus 68 LANA Is Essential for Virus Reactivation from Splenocytes but Not Long-Term Carriage of Viral Genome

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ORF73, which encodes the latency-associated nuclear antigen (LANA), is a conserved gamma-2-herpesvirus gene. The murine gammaherpesvirus 68 (MHV68) LANA (mLANA) is critical for efficient virus replication and the establishment of latent infection following intranasal inoculation. To test whether the initial host immune response limits the capacity of mLANA-null virus to traffic to and establish latency in the spleen, we infected type I interferon receptor knockout (IFN-α/βR−/−) mice via intranasal inoculation and observed the presence of viral genome-positive splenocytes at day 18 postinfection at approximately 10-fold-lower levels than in the genetically repaired marker rescue-infected mice. However, no mLANA-null virus reactivation from infected IFN-α/βR−/− splenocytes was observed. To more thoroughly define a role of mLANA in MHV68 infection, we evaluated the capacity of an mLANA-null virus to establish and maintain infection apart from restriction in the lungs of immunocompetent mice. At day 18 following intraperitoneal infection of C57BL/6 mice, the mLANA-null virus was able to establish a chronic infection in the spleen albeit at a 5-fold-reduced level. However, as in IFN-α/βR−/− mice, little or no virus reactivation could be detected from mLANA-null virus-infected splenocytes upon explant. An examination of peritoneal exudate cells (PECs) following intraperitoneal inoculation revealed nearly equivalent frequencies of PECs harboring the mLANA-null virus relative to the marker rescue virus. Furthermore, although significantly compromised, mLANA-null virus reactivation from PECs was detected upon explant. Notably, at later times postinfection, the frequency of mLANA-null genome-positive splenocytes was indistinguishable from that of marker rescue virus-infected animals. Analyses of viral genome-positive splenocytes revealed the absence of viral episomes in mLANA-null infected mice, suggesting that the viral genome is integrated or maintained in a linear state. Thus, these data provide the first evidence that a LANA homolog is directly involved in the formation and/or maintenance of an extrachromosomal viral episome in vivo, which is likely required for the reactivation of MHV68.

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licating cells under selection. The absence of LANA led to a loss of detectable episomal minigenomes (15). Similar data have been generated for KSHV as well (4, 5). Furthermore, it was proposed that the mechanism for this maintenance is through physically tethering the viral episome to host histones so that episomes are distributed evenly to daughter cells (6, 16). Given the variety of contexts in which LANA is transcribed during the virus life cycle, it is clear that LANA homologs also have other important functions during the rhadinovirus life cycle. These other functions include manipulating the DNA damage response and other tumor suppressor pathways (27, 45, 75), transcriptional regulation (32, 58), and loading viruses into cells so that episomes are distributed evenly to daughter cells (6, 16).

Previously, we (52) and others (30) reported the inability of mLANA-null MHV68 mutants to establish latency following the intranasal inoculation of wild-type mice. Here we report studies demonstrating that either altering the route of inoculation in immunocompetent C57BL/6 mice or intranasal inoculation of type I interferon receptor knockout (IFN-α/βR−/−) mice with an mLANA-null MHV68 mutant (73.Stop) results in animals becoming persistently infected and harboring viral genome-positive splenocytes for at least 6 months postinfection. These studies also revealed an essential role for mLANA in virus reactivation from splenocytes and a critical role in peritoneal exudate cells (PECs).

MATERIALS AND METHODS

Cell lines and viruses. MHV68 bacterial artificial chromosome (BAC)-derived strains 73.Stop and 73.MR were described previously (52). Briefly, 73.Stop contains two stop codons and a frameshift early in the open reading frame to disrupt mLANA expression; 73.MR rescues 73.Stop to the wild-type sequence. Viral stocks were grown to passage two in Vero-Cre cells, and titers were determined by plaque assay.

Immunoblot analyses. Cells were lysed with alternative radioimmunoimmunoprecipitation assay buffer (150 mM NaCl, 20 mM Tris, 2 mM EDTA, 1% NP-40, 0.25% deoxycholate, 1 mM NaF, and 1 mM Na3VO4 supplemented with complete mini-EDEA-free protease inhibitors [Roche]) and quantitated by using the Bio-Rad DC protein assay prior to the resuspension of 45 μg of protein in Laemmli sample buffer. Samples were heated to 100°C for 10 min and were resolved by SDS-PAGE. Resolved proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Membranes were incubated overnight at 4°C in hybridization buffer (0.1% SDS, 0.2% sodium dodecyl sulfate, 0.1 M sodium phosphate, pH 7.2, 10% dextran sulfate, 0.01% sodium deoxycholate, 1 mM NaF, and 1 mM Na3VO4), washed with TBS-T, and hybridized with biotin-labeled oligonucleotide probes in hybridization buffer. Membranes were washed in 2x SSC–0.2% SDS, then washed 3x with 2x SSC, and finally washed 2x with 0.5x SSC. The signal was revealed by using AEC and K3-NBT and developed with a BioMax light screen (Kodak). For in-gel Western blot analysis, resolved proteins were transferred onto nitrocellulose and were blocked overnight in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Membranes were washed in 50 mM Tris, 150 mM NaCl, 0.1% Tween-20, 0.2% sodium dodecyl sulfate, 20 mM β-mercaptoethanol, and 0.1% bovine serum albumin. Proteins were visualized using enhanced chemiluminescence (ECL) reagents (Amersham/GE Healthcare) and exposed to film.

Plaque assay. Plaque assays were performed as previously described (78). NIH 3T12 cells were plated in six-well plates 1 day prior to infection at 2 × 106 cells per well. Organs were subjected to 4 rounds of mechanical disruption of 1 min each using a 10-mm zirconia-silica beads (Biospec Products, Bartsville, OK) in a Mini-Beadbeater-8 instrument (Biospec Products). Serial 10-fold dilutions of organ homogenate were plated onto NIH 3T12 monolayers in a 200-μl volume. Infections were performed for 1 h at 37°C with rocking every 15 min. Immediately after infection, plates were overlaid with 1.5% methylcellulose in complete DMEM. After 6 to 7 days, cells were stained with 0.12% (final concentration) Neutral Red. The next day, methylcellulose was aspirated, and plaques were counted. The sensitivity of the assay is 50 PFU/organ.

DC-PCR. Genomic DNA (gDNA) was prepared from splenocytes using standard overnight proteinase K digestion followed by phenol-chloroform extraction. Six micrograms of gDNA was digested overnight with EcoRI or BamHI in a 100-μl reaction mixture. Enzymes were inactivated and DNA was purified by using GeneCleanII (Bio 101). A total of 10%, 1%, or 0.1% of the digested DNA was ligated into a 100-μl reaction mixture overnight at 16°C using T4 DNA ligase. Wells were resuspended in complete DMEM and plated in serial 2-fold dilutions on 3T12 cells were plated in six-well plates 1 day prior to infection at 2×106 cells per well. Organ homogenate were plated onto NIH 3T12 monolayers in a 200-μl volume. Infections were performed for 1 h at 37°C with rocking every 15 min. Immediately after infection, plates were overlaid with 1.5% methylcellulose in complete DMEM. After 6 to 7 days, cells were stained with 0.12% (final concentration) Neutral Red. The next day, methylcellulose was aspirated, and plaques were counted. The sensitivity of the assay is 50 PFU/organ.

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LD analyses. Limiting-dilution (LD) assays for the frequency of latent infection were performed as previously described (78, 80). To determine the frequency of cells harboring latent viral genomes, single-copy-sensitive nested PCR was performed. Splenocytes or PECs were plated in 3-fold serial dilutions in a 0.5 ml of complete DMEM or intranasally with 100 PFU of virus diluted into 0.5 ml of complete DMEM.

Isolation of splenocytes and peritoneal cells and purification of B cells. Mice were sacrificed by asphyxiation at the specified days. Peritoneal cells were recovered by injecting 10 ml DMEM (without serum) into the peritoneal cavity, agitating the mouse, and recovering DMEM by using a 16-gauge needle. Spleens were Dounce homogenized into a single-cell suspension, and erythrocytes were lysed with ammonium chloride. B cells were enriched from total splenocytes by using magnetic beads (Miltenyi B-cell isolation antibody cocktail) and an AutoMACS instrument according to the manufacturer’s instructions (Miltenyi).

RNA isolation and RT-PCR. RNA was harvested from splenocytes by using TRIzol (Invitrogen) according to the manufacturer’s instructions. Three micrograms of RNA was treated with DNase I (Invitrogen), and 1.5 μg of DNase-treated RNA was reverse transcribed by use of random-hexamer-primed RNA and SuperScript III reverse transcriptase (RT) (Invitrogen) according to the manufacturer’s instructions. Minus-RT controls were performed in parallel. The resulting cDNA was serially diluted to 1/5 and 1/25. For PCR amplification of transcripts, 1 μl of each cDNA dilution was subjected to nested PCR in a 25-μl reaction mixture using primer sets previously described (1, 22, 52, 77). The PCR program was as follows: 25 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. Two microliters of the product from round 1 was put into a 50-μl reaction mixture from round 2 under the same conditions for 45 cycles.

BAC transfections. NIH 3T12 cells were plated and transfected the next day with 0.5 μg BAC DNA with Superfect (Qiagen) according to the manufacturer’s instructions. Transfection efficiency was monitored by the expression of the human cytomegalovirus (HCMV) IE promoter-driven green fluorescent protein (GFP) cassette engineered into the BAC. At 3, 7, and 9 days posttransfection, supernatants were harvested, and titers were determined by plaque assay.

Statistical analyses. All data were analyzed by using GraphPad Prism software. Titer data were statistically analyzed by using the unpaired t test. The frequencies of reactivation and genome-positive cells were statistically analyzed using paired t test.
by using the paired t test. To accurately obtain the frequency for each limiting dilution, data were subjected to nonlinear regression (using a sigmoidal dose curve with a nonvariable slope to fit the data). Frequencies of reactivation and genome-positive cells were obtained by calculating the cell density at which 63.2% of the wells scored positive for reactivating virus based on a Poisson distribution.

RESULTS

Innate immunity prevents dissemination of mLANA-null virus from the lung. Previous studies with mLANA-deficient viruses demonstrated that mLANA is required for the establishment of latency following intranasal inoculation (30, 52). Notably, a 1- to 2-log defect in acute virus replication in the lung was also observed following intranasal inoculation (52). More recently, a replication defect was also observed in vitro at low multiplicities of infection of mouse embryo fibroblasts with an mLANA-null virus (27). Because we do not have a clear understanding of the relationship between the levels of acute virus replication in the lungs and the establishment of latency at distal sites, we initially set out to assess whether innate immunity, specifically the type I interferon response, might be involved in limiting the spread of mLANA-null MHV68 from the lung to the spleen. Type I interferon receptor knockout (IFN-α/βR−/−) and Stat1 knockout mice were previously shown to be hypersusceptible to MHV68 infection (7, 79).

Thus, we inoculated IFN-α/βR−/− mice with 100 PFU of an mLANA-null virus (73.Stop) or a genetically repaired marker rescue virus (73.MR) intranasally and harvested lungs at day 9 postinfection to assess acute virus replication and spleens at day 28 postinfection to assess the establishment of latency (Fig. 1). Notably, the lack of type I interferon-mediated control of replication did not ameliorate the difference in the acute replication of 73.Stop and 73.MR, although significantly more virus production was observed in the lungs of interferon-nonresponsive animals (Fig. 1A). In addition, about half of the IFN-α/βR−/− mice succumbed to 73.Stop or 73.MR infection between days 10 and 20 postinfection, with the greatest drop at day 14 (Fig. 1B). Importantly, no differences in the kinetics of virus-induced death or the percentage of mice that survived were observed between mice infected with 73.Stop and those infected with 73.MR (Fig. 1B).

To assess the establishment of latency in the spleen, we waited for lytic replication to clear, which takes 7 to 10 days longer for IFN-α/βR−/− mice (7). Of the mice that survived, spleens were harvested at day 28 postinfection and subjected to limiting-dilution PCR (LD-PCR) (Fig. 1C) and ex vivo reactivation analyses (Fig. 1D). These analyses revealed that 73.Stop can establish infection in splenocytes at a 7.7-fold-reduced frequency compared to 73.MR. However, no virus reactivation upon the explant of splenocytes from 73.Stop-infected IFN-α/βR−/− mice could be detected (Fig. 1D). This raised the question of whether we were detecting some form of detective/nonproductive 73.Stop virus infection in the spleens of IFN-α/βR−/− mice. Thus, to further address 73.Stop virus infection, we focused on infection of immunocompetent C57BL/6 mice.

Intraperitoneal inoculation of immunocompetent mice overcomes the requirement for mLANA to establish a chronic infection but reveals a role for mLANA in virus reactivation. To extend the analyses of IFN-α/βR−/− mice, we investigated alternative routes of inoculation with 73.Stop virus in immu-

FIG. 1. Intranasal infection of IFN-α/βR−/− mice with 73.Stop allows greater lytic replication and mLANA-independent seeding of latency in the spleen but not the reactivation of the virus. (A) Either C57BL/6 mice (black symbols) or IFN-α/βR−/− mice (gray symbols) were infected intranasally with 100 PFU of 73.Stop or 73.MR virus. Lungs were harvested 9 days later, and infectious virus titers were determined by plaque assay. (B) Kaplan-Meier curve of IFN-α/βR−/− mice infected intranasally with 73.Stop or 73.MR virus. Chi-squared analysis revealed no significant difference in survival between these experimental groups. (C and D) Surviving IFN-α/βR−/− mice infected with either 73.Stop or 73.MR virus were sacrificed at day 28 postinfection, and the spleens were harvested for analysis. Splenocytes were subjected to limiting-dilution analyses to determine the frequency of cells harboring the viral genome (C) or spontaneously reactivating virus upon explant onto monolayers of mouse embryo fibroblasts (D). CPE, cytopathic effect.
nocompetent mice in an effort to overcome any restriction that virus replication in the lungs may have had on the establishment of latency. We inoculated C57BL/6 mice intraperitoneally with 1,000 PFU of either 73.Stop or 73.MR virus. At day 18 postinfection, PECs (A and C) and splenocytes (B and D) were harvested and assayed for latency and reactivation by limiting-dilution analyses. Results are the means of data from four independent experimental groups, and error bars represent standard deviations between separate groups.

B cells are the major cell type harboring mLANA-null virus in the spleen, where it establishes an infection that persists for more than 6 months. Because there are many aspects of the early establishment of latency that we do not yet understand, we asked whether there were any gross differences in the cell types that have the capacity to be latently infected in the absence of mLANA. To do this, we enriched for B cells from the spleens of mice infected with 73.Stop and 73.MR at day 18. We determined the frequency of infected B cells, using purified B cells (average, 90% purity), by LD-PCR and in parallel with analyses of the bulk unsorted populations. As expected, these analyses revealed that 73.Stop does indeed establish a chronic infection in B cells (Fig. 3) at a frequency essentially equivalent (4.5-fold decrease versus 5.4-fold decrease) to that determined for the respective unsorted splenocytes. Thus, these results demonstrate that the majority of virus infection for both 73.Stop and 73.MR viruses is in B cells, arguing against the persistence of the mLANA-null virus in a cell population that is physiologically irrelevant for the maintenance of chronic wt MHV68 infection.

One of the many functions proposed for KSHV LANA is that of the episomal maintenance protein, which is responsible for the faithful partitioning of KSHV latent episomes to daughter cells during cell division. Fitting with this idea, we hypothesized that in the absence of mLANA, long-term MHV68 latency would not be maintained. To test this, we again infected C57BL/6 mice intraperitoneally with 73.MR or 73.Stop and sacrificed groups of mice at 6 weeks and 6 months postinfection. Unexpectedly, we observed that the frequencies of 73.Stop and 73.MR rescue viruses were equivalent at both time points (Fig. 4), demonstrating that the absence of mLANA did not lead to a loss of virus-infected splenocytes. Instead, the frequency of 73.MR-infected cells decreased to an often observed set point (71), around 1/5,000 at 6 weeks (Fig. 4A) and 1/19,000 at 6 months (Fig. 4B), where frequencies of 73.Stop-infected splenocytes also settled. This was very surprising given our previous observation that the majority of MHV68-infected B
cells in the spleen are actively proliferating at 6 weeks postinfection (53) and suggests that an mLANA-independent mechanism must be involved in maintaining the viral genome.

**Detection of viral transcripts in splenocytes infected with mLANA-null virus.** Characteristics of latent gammaherpesvirus infection include a limited expression of viral antigens, the absence of infectious virus, the capacity to reactivate, and the distinct expression of a very limited repertoire of viral transcripts (50, 66, 77). We assessed whether the 73.Stop virus-infected splenocytes express other known latency-associated viral genes in vivo. Three genes transcribed during early latency, at least in some splenocyte populations, are the unique M2 and M9 genes and ORF73 (encoding mLANA) (1, 22, 28, 60). To demonstrate an active form of latency in the absence of mLANA expression, we isolated RNA from spleens of individual mice between 25 and 28 days postinfection with 73.Stop or 73.MR and reverse transcribed it to make cDNA. To understand how levels of transcripts vary between 73.Stop and 73.MR, we assayed 5-fold serial dilutions of cDNA. PCR analysis of cDNA detected both spliced M2 and orf73 transcripts as well as M9, M1, and viral DNA polymerase transcripts in 73.MR- and 73.Stop-infected splenocytes (Fig. 5A). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control for integrity for RNA, while RNA prepared from a naïve mouse was used as a control for the specificity of RT-PCR amplification. These results demonstrate that the mLANA-null virus, although crippled for reactivation, is transcriptionally active, expressing both viral genes that are implicated in latency as well as transcripts that are upregulated.

![FIG. 4. Viral genomes are maintained long-term in mice in the absence of mLANA. C57BL/6 mice were inoculated intraperitoneally with 1,000 PFU of the indicated virus. At 42 days (A) and 6 months (B) postinfection, mice were sacrificed, and splenocytes were assayed by limiting-dilution PCR for the frequency of cells harboring the viral genome.](image)

![FIG. 5. Latency-associated MHV68 transcripts can be detected in mLANA-null virus-infected splenocytes. (A) Semiquantitative RT-PCR analysis of MHV68 latency-associated gene expression in 73.Stop- and 73.MR-infected splenocytes. RNA from individual spleens was reverse transcribed, and dilutions (undiluted or 1:5 or 1:25 dilutions) of the cDNA from each spleen were analyzed by PCR for the indicated genes. Shown are amplifications of products arising from spliced M2 or orf73 transcripts (these RT-PCR products cross the known splice junctions in these viral transcripts), M9, viral DNA polymerase (pol), M1, or the cellular GAPDH transcript using RNA harvested from infected mice at days 25 to 28 postinfection. (B) Compiled data from the RT-PCR analyses shown above (A) indicating the number of PCR-positive reactions and the total number of reactions analyzed.](image)
during virus reactivation/replication. However, although *orf73* transcripts are present, the mLANA protein is not made in the 73.Stop-infected cells.

Finally, we assessed whether the initial virus-induced B-cell responses were similar between 73.Stop and 73.MR infections, since this might impact the frequency of latently infected splenocytes. MHV68 elicits a potent germinal center response, which is dependent on M2 expression (A. M. Siegel and S. H. Speck, unpublished observation). We obtained splenocytes from infected mice at day 18 postinfection and determined the frequency of CD19<sup>+</sup> B cells that stained positive for the germinal center markers GL7 and CD95. As expected, we observed that 73.Stop and 73.MR generated similar germinal center responses: ca. 4.5% of splenic B cells harvested from either 73.Stop- or 73.MR-infected mice exhibited a germinal center phenotype, compared with ca. 0.25% of B cells harvested from naïve mice (Fig. 6). These data argue against the defect in the establishment of 73.Stop latency in the spleen being linked to a diminished germinal center response.

**Failure of mLANA-null virus to reactivate from splenocytes correlates with the absence of viral episomes.** We considered two possibilities that might account for the failure of mLANA-null virus-infected splenocytes to reactivate upon explant. First, we addressed whether the absence of mLANA might impact the initiation of the viral replication cycle in the absence of virion entry and the release of tegument proteins, which would be anticipated to mimic aspects of virus reactivation. Thus, to assess the capacity of mLANA-null virus to initiate *de novo* virion production from naked viral DNA, we transfected either 73.Stop-BAC or 73.MR-BAC DNA into permissive Vero cells and harvested supernatants at 3, 7, and 9 days posttransfection. To control for transfection efficiency, several replicate experiments were performed, and GFP expression in the transfected Vero cells, arising from the cytomegalovirus (CMV) IE promoter-driven GFP expression cassette present in the BAC, was monitored and shown to be equivalent (data not shown). Titers of tissue culture supernatants harvested from each transfection were determined by a plaque assay on NIH 3T12 fibroblasts. Notably, the 73.Stop-BAC-transfected cells displayed a severe, 3-log replication defect at day 3 posttransfection (Fig. 7A). Importantly, a replication defect of this magnitude was not observed for fibroblasts infected with mLANA-null virus (30, 52). The observed replication defect was slightly ameliorated by day 9, most likely due to subsequent rounds of infection with progeny virus produced from the transfected cells. To show that this effect happens very early in the transition from DNA to virus production and is not due to a failure to efficiently plaque 73.Stop virus, we transfected Vero cells with 73.Stop-BAC or 73.MR-BAC in duplicate and harvested lysates at 24, 72, and 96 h posttransfection (Fig. 7B). We observed that the LANA-null BAC displayed a significant lag in the expression of lytic antigens, consistent with the observed decrease in viral titers. This finding is in contrast to the overexpression of lytic antigens previously seen under some experimental conditions upon infection of permissive fibroblasts with the 73.Stop virus (27).

Second, we considered whether, in the absence of mLANA expression, the viral genome might integrate into the host chromosome, as it has been shown that HVS LANA and KSHV LANA are important for facilitating episomal maintenance of the viral genome in rapidly dividing cells (15, 36, 40). Furthermore, it was previously suggested that KSHV terminal repeat (TR)-containing plasmids integrate into the cellular genome under antibiotic selection when LANA is not present (36). Notably, many characterized gammaherpesvirus integration events have shown that the viral genomes preferentially integrate into chromosomes through their terminal repeats (37, 38). In contrast, episomal virus genomes have fused ter-

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**FIG. 6.** mLANA-deficient virus is not impaired in the induction of a strong germinal center response. Shown are compiled data from flow cytometry analyses of the germinal center (GC) response (expressed as a percentage of total B cells that exhibit a germinal center phenotype) in the spleen at 18 days postinfection. Splenocytes from individual mice were stained with anti-CD19, GL7, and anti-CD95.

**FIG. 7.** A functional mLANA gene is required for the efficient replication of MHV68 following transfection of viral DNA in permissive fibroblasts. (A) NIH 3T12 fibroblasts were transfected with 0.5 μg of either 73.Stop-BAC or 73.MR-BAC (MR) DNA. At 3, 7, and 9 days posttransfection, supernatants were harvested, and titers of infectious virus produced were determined. The data are representative of at least three replicate experiments. (B) Vero cells were transfected in duplicate as described above, and lysates were harvested at 24, 72, and 96 h. Western blots for lytic MHV68 antigen and β-actin are shown.
minal repeats, and the left and right ends of the viral unique sequences are thus physically connected through the fusion of the terminal repeats. To assess the presence of viral episomes, we employed a digestion-and-circularization-mediated PCR (DC-PCR) strategy (12) to detect the presence of fused terminal repeats. Splenocytes from mice infected intraperitoneally with 73.Stop or 73.MR virus were harvested at day 18 postinfection, and total genomic DNA was prepared. The recovered genomic DNA was digested with either EcoRI or BamHI, neither of which cuts within the terminal repeats, followed by the dilution of the digested DNA and ligation under conditions that favor intramolecular ligation (i.e., circle formation) (Fig. 8A). Nested sets of PCR primers were designed a short distance upstream of the respective enzyme digestion sites, oriented toward the cut site, such that a PCR product could be generated only following the ligation of each end to form a circle (Fig. 8A). One set of primers was designed to detect fused terminal repeats, and another was designed to detect the digested and circularized EcoRI-H or BamHI-A2 fragments, containing ORF64, in the middle of the unique region as a control for the detection of viral DNA following digestion and circularization. We observed in four independent sets of infections with both 73.Stop and 73.MR that we could readily detect the ORF64-containing circles (Fig. 8B and C). However, while we also consistently observed the fused terminal repeat product in splenocytes recovered from 73.MR-infected mice, we never observed the presence of the fused terminal repeat product in 73.Stop-infected splenocytes (Fig. 8B and C). This finding implies that mLANA is needed in vivo at early times of latency to facilitate the maintenance of MHV68 as a circular genome in latently infected splenocytes. However, this does not necessarily demonstrate the integration of the viral genome. Another possibility is that the virus may be present in a linear, extrachromosomal form.

**DISCUSSION**

In this report we identify a role for the MHV68 LANA homolog in virus reactivation from splenocytes and PECs as well as provide initial evidence that mLANA plays a critical role in establishing and/or maintaining the viral genome as an episome in vivo. A striking finding here is the capacity of the mLANA-null virus to establish latency in the spleen following the intraperitoneal inoculation of C57BL/6 mice, in contrast to data from previous studies that demonstrated a complete failure to establish latency following intranasal inoculation (30, 52). As discussed above, we previously observed a significant acute replication defect with the mLANA-null virus in the lungs following intranasal inoculation (52). However, a substantial increase of the inoculating dose of the virus, which greatly increased the peak titers of the virus in the lungs, did not overcome the defect in the establishment of latency (52). This finding suggested a more complex relationship between the route of administration and establishment of latency in the spleen in immunocompetent mice. This complexity is underscored by the previously reported observation that the establishment of latency in the spleen following intranasal inoculation is severely impaired in B-cell-deficient mice (MuMT) but that splenic latency is robustly established in these mice following intraperitoneal inoculation (72, 78). Similarly, M2-null mutants also exhibit a more severe establishment-of-latency phenotype in the spleen following intranasal inoculation than following intraperitoneal inoculation (39, 43), and M2-null mutants also exhibit a profound defect in reactivation from B cells.
(39, 48). Taken together, these data suggest that latently infected B cells that traffic to the spleen and reactivate play a pivotal role in the initial establishment of latency following intranasal inoculation but not following intraperitoneal inoculation (64). Thus, the inability of mLANA-null virus to reactivate from splenocytes may be linked directly to the failure of this mutant virus to establish latency following the intranasal inoculation of immunocompetent mice.

LANA was originally identified in a KSHV latently infected tumor cell line using serum from patients with Kaposi’s sarcoma (31). Subsequent studies demonstrated that LANA expression could be detected in every KS-associated tumor and proliferative disease (11, 31, 56, 59, 67, 75). Those studies and others established a correlation between LANA and KSHV disease, a notion further bolstered by studies that identified numerous functions of LANA, many of which appeared to be consistent with its putative role as a viral oncogene. Functions attributed to LANA proteins include regulating the transcription of cell cycle genes (32), blunting cellular responses to virus infection and DNA damage (27), interacting with p53 and other tumor suppressors (45, 75), and preventing its own presentation by the major histocompatibility complex (MHC) on infected cells (46). LANA expression is associated with these tumor-like phenotypes, and thus, much of what is known about LANA in the context of KSHV infection has been worked out with cancer cells. However, there is no basis for assuming that the constitutive expression of LANA is normal during chronic rhadinovirus infections. Like other herpesvirus latency-associated genes, including EBNA-1, another well-studied gammaherpesvirus protein suggested to promote epimorphic maintenance (63, 83), its expression is likely tightly controlled.

As discussed above, epimorphic maintenance is perhaps the most well-known function attributed to LANA homologs. Similar to what has been shown for EBNA-1 of Epstein-Barr virus (EBV) (82, 83), it has been suggested by studies with both HVS LANA and KSHV LANA that LANA (i) maintains viral genomes or minigenomes as extrachromosomal episomes and (ii) physically associates, or tethers, the viral genome to host DNA to ensure the faithful partitioning of the viral genome into daughter cells (5, 15, 16, 76). We speculated that in the absence of LANA, the virus would integrate into host cells and/or eventually be lost with host cell division. Consistent with episode maintenance assays performed for HVS and KSHV LANA, we were unable to detect episomes with fused terminal repeats in vivo at day 18 in the absence of mLANA. At this point in time, a large percentage of MHV68-positive cells exhibited a germinal center phenotype and were actively proliferating (14, 53). Thus, this may be the point at which in vivo maintenance assay data may most strongly correlate with virus infection of B cells in vivo, and in the absence of mLANA, the integrated viruses are the ones that survive the rapid cell division in the germinal center. Notably, it is the germinal center subset of B cells that have the most robust expression of orf73 transcripts (50). Furthermore, germinal center B cells that survive become either memory cells or plasma cells; memory B cells are the long-lived latency reservoir (26, 81), and plasma cells are a major cell type reactivating virus (48). It is possible that mLANA thus plays a central role in retaining the virus in a state (i.e., viral episome) that can rapidly switch from latency to reactivation.

Our data demonstrate that gammaherpesvirus infection can persist in vivo in the absence of an episomal form, either maintained as a linear piece of DNA or integrated into the host chromosome. There is precedent both for MHV68 being carried as linear DNA upon the infection of primary lymphocytes (24) and for gammaherpesvirus integration (18, 20, 42). Both possibilities may lead to a block in virus reactivation from splenocytes (which largely reflect the infection of B cells), likely due to the absence of a mechanism for the circularization or for the excision of the viral DNA from the host chromosome. Notably, 73Stop virus can reactivate, albeit at a greatly reduced efficiency, from infected peritoneal cells and perhaps from an undetectable population of splenocytes. This minor reactivation may reflect reactivation from a population of infected macrophages that do not require mLANA to maintain the capacity of the virus to reactivate due to either the lack of the active proliferation of those cells or another cell type-specific factor. Similarly, when permissive cells are transfected with MHV68 BAC DNA, 73.STOP-BAC lags behind 73.MRBAC in both lytic antigen production and virion production, indicating a secondary role for mLANA early in the transition from latency to reactivation.

These data may confound the working definition of latency commonly used in the herpesvirus field. It is generally accepted that a latent virus is one that “may be induced to multiply and that does not exist in an infectious form” (62). Furthermore, episomal herpesvirus genomes are associated with latently infected cells (49, 62). However, a definitive molecular definition of latency is still lacking in the gammaherpesvirus field, and although we know that there is little viral gene expression during latency, there are distinct programs of a limited number of tightly regulated genes observable in EBV-transformed cells and in disease (61, 68). It stands to reason that the transcription of viral genes must be an important aspect of the establishment and maintenance of latency, giving some advantage to virus-infected cells to survive and become a long-term reservoir for the virus. With the mLANA-null virus, we observed the presence of the viral genome in the absence of infectious virus, but the viral genome does not appear to be episomal. We did, however, observe similar patterns of viral gene expression in mutant and wild-type virus-infected cells as well as the long-term carriage of the viral genome in vivo. Thus, the mLANA-null virus is indeed present within the host indefinitely, in the preferred cell type, and at levels equivalent to those of the wild-type virus. In addition, because the mLANA-null virus-infected cells are capable of expressing viral genes, the mutant virus presumably retains the capacity to alter the cell, even though it is incapable of efficiently reentering the lytic cycle to produce progeny virions. Thus, the ability of the mLANA-null virus to ultimately access the same cellular reservoirs that are latently infected with wild-type MHV68 argues in support of a broader definition of latency.

This idea of a more complex definition of latency has been building for several years with both KSHV and EBV tissue culture models. KSHV-infected primary effusion lymphoma (PEL) cells have been treated with lentiviruses that knock down LANA, and while the genome copy number is reduced, latency persists (34). Furthermore, for both EBV (55) and KSHV (74), it was shown that viral replication origins exist and function outside the EBNA-1-dependent OriP and proposed
LANA-dependent terminal repeat (TR) origin, respectively. In the same regard, it is also now appreciated that LANA itself is not sufficient to maintain a TR-containing plasmid, suggesting that specific cellular mechanisms are required for this function (35). Finally, a study using an EBV mutant lacking EBNAL showed that this virus not only can latently infect B cells but also can drive B-cell immortalization albeit with a significantly reduced efficiency (likely due to the need for the virus to integrate into the host genome) (41). The data presented in the present study argue that what we observed in vivo with the 73Stop virus is a form of latency in the absence of both viral episomes and the latency maintenance protein mLANA, because (i) we observed a similar pattern of latency gene expression, (ii) the frequency of viral genome-positive cells persists and is equivalent long-term with the wild-type virus, and (iii) B cells are predominantly infected with 73Stop, similar to the wild type.

With respect to the persistence of herpesviruses in the absence of episome formation, it was previously shown that human herpesvirus 6 (HHV-6), a betaherpesvirus, integrates into host chromosomes as a normal part of its life cycle (17). Furthermore, HHV-6 is apparently able to reactivate from this integrated state, as the virus is frequently detected in the saliva of healthy people and in the blood of bone marrow transplant patients (10, 47). Integrated HHV-6 was also observed in vertical transmission from parent to child, arguing for integration as an efficient mechanism of survival (2). Additionally, the alphaherpesvirus Marek’s disease virus (MDV), which is highly pathogenic in chickens, is found integrated exclusively into host chromosomes but also retains the capacity to be excised and replicate (19, 21). Notably, with respect to gammaherpesvirus biology, the integration of the viral genome at the terminal repeats would prevent the expression of viral genes encoded across the fused terminal repeats (e.g., EBV LMP2a and KSHV K15) (8). The consequences of the loss of such gene products on either the establishment or maintenance of viral latency in vivo are currently unknown. In the case of LMP2a, it was hypothesized previously that it plays a critical role in the survival of EBV-infected germinal center B cells (9) and, thus, likely plays a critical role in the generation of those virus-infected memory B cells that arise from the virus-driven differentiation of infected naïve B cells (3, 9). At present, no such viral gene product encoded across the fused terminal repeats of MHV68 has been identified.

In summary, the results presented here reveal a crucial role for mLANA in reactivation and provide in vivo support for the notion that an episome is a prerequisite for virus reactivation but not the long-term carriage of MHV68. The data also show that mLANA is important early in infection both in mounting differentiation of infected naïve B cells and in vitro. Proc. Natl. Acad. Sci. U. S. A. 107:5563–5568.


