Disruption of Gammaherpesvirus 68 Gene 50 Demonstrates that Rta Is Essential for Virus Replication

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Gammaherpesviruses are lymphotropic viruses that establish a lifelong infection of the host and are associated with cellular transformation and tumor formation in immunosuppressed hosts. Murine gammaherpesvirus 68 (γHV68; also referred to as murine gammaherpesvirus 68), infects and forms plaques on monolayers of fibroblast cells, and lymph nodes (5, 32, 43), with B cells, macrophages, and splenic dendritic cells serving as latency sites (11, 33, 44). Additionally, HV68 can persist in lung epithelial cells (30). Significantly, γHV68 has been shown to infect mice is of great importance for the gammaherpesvirus field, because it provides a genetically tractable system for studying viral pathogenesis. γHV68 infects multiple organs of inbred mice acutely, including the spleen, liver, lung, kidney, adrenal gland, heart, and thymus (4, 24). γHV68 can cause inflammation of the great elastic arteries (42). Additionally, γHV68 can establish a latent infection in the spleen, peritoneal cells, and lymph nodes (5, 32, 43), with B cells, macrophages, and splenic dendritic cells serving as latency sites (11, 33, 44). Additionally, γHV68 can persist in lung epithelial cells (30). Significantly, γHV68 has been associated with lymphoproliferative disease and lymphoma in mice (37). γHV68 readily infects and forms plaques on monolayers of fibroblast cells, and several virus mutants have been generated successfully, demonstrating the utility of this virus for genetic studies (for example, see references 7 and 22).

Latency and reactivation from latency are central aspects of gammaherpesvirus pathogenesis. Latency is thought to be the primary mechanism by which herpesviruses chronically infect their host. In the case of gammaherpesviruses, there is a tight correlation between latency infection and gammaherpesvirus-associated tumors. Reactivation and lytic virus replication are associated with reactivation disease and spread of the virus between hosts. However, the critical question of whether reactivation and subsequent virus replication are required for the maintenance of chronic gammaherpesvirus infection remains unanswered. Additionally, the molecular mechanisms involved in virus reactivation in vivo are poorly understood. Gammaherpesvirus immediate-early genes serve as initiators of the viral lytic cascade, and in the case of Epstein-Barr virus (EBV) reactivation, immediate-early viral gene expression has been shown in vitro to be regulated by signal transduction pathways that trigger reactivation from latency.

Extensive studies have shown that the EBV immediate-early BRLF1 (also known as gene 50 or rta [replication and transcription activator]) and BZLF1 gene products (Rta and Zta, respectively) act as critical regulators of the viral lytic cascade (9; reviewed in reference 29). The BZLF1 gene does not appear to be well conserved among the gammaherpesviruses. In Kaposi’s sarcoma-associated virus and rhesus rhadinovirus the K8 and R8 gene products, respectively, are located in the same position in the viral genome and encode bZIP proteins that appear to be distantly related to Zta (15, 17, 28, 47). Herpesvirus saimiri and γHV68 do not appear to encode homologs of Zta. In contrast, gene 50 is well conserved among all known gammaherpesviruses (31, 39, 40, 45). Both an unspliced form and a spliced form of gene 50 transcript have been characterized (see Fig. 1). Open reading frame (ORF) 49 and gene 50 are encoded on opposite strands and are organized “head-to-head” in the viral genome (Fig. 1). Transcription of both the
unspliced and spliced gene 50 transcripts initiates downstream of ORF 49, with transcription extending through ORFs 49 and 50 (Fig. 1). In the case of the spliced transcript, splicing removes the region antisense to ORF 49 and extends the gene 50 ORF. Notably, this organization and transcription of gene 50 are well conserved among the known gammaherpesviruses (12, 18, 20, 31, 38, 45).

The gene 50 product, Rta, has been shown previously to act as a transcriptional activator of downstream viral genes (14, 18, 19, 31; reviewed in reference 25). Additionally, it has roles in DNA replication and cell cycle regulation (10, 16, 27, 35, 50). Most importantly, overexpression of EBV, Kaposi’s sarcoma-associated virus, herpesvirus saimiri, and \( \text{H}9253 \text{HV68} \) gene 50 in cell lines latently infected with the corresponding virus has been shown to lead to disruption of latency (13, 14, 19, 23, 31, 49, 51). Recently, Feederle and colleagues generated EBV null mutants with mutations in the BZLF1 and BRLF1 genes (gene 50) and showed that both genes are essential for EBV replication, thus providing the first genetic evidence for the requirement of these gammaherpesvirus immediate-early genes for virus replication (9). Consistent with an essential role of the \( \text{H}68 \) gene 50 in virus replication, two dominant-negative forms of \( \text{H}68 \) gene 50 were shown to significantly inhibit viral protein expression and virion production in lytically infected cell lines (48). Taken together, these data strongly indicate that gene 50 is required for the initiation of the viral lytic cycle in vitro and that this requirement is conserved among the characterized gammaherpesviruses.

Here we describe the generation and initial characterization of a gene 50-null mutant \( \text{H}68 \). We show that the gene 50-null mutant is incapable of virion production or viral DNA replication and is attenuated for viral protein synthesis. The defect in gene 50 can be rescued in trans either by a genomic fragment containing gene 50 or by a gene 50 cDNA clone corresponding to the spliced gene 50 transcript. The latter result demonstrates that Rta expressed from the spliced transcript is sufficient to rescue replication of a gene 50-null mutant.

MATERIALS AND METHODS

Viruses and tissue culture. The \( \text{H}68 \) bacterial artificial chromosome [\( \text{H}68 \text{(BAC)} \)] (2) was obtained from Heiko Adler and Ulrich Koszinowski (Max von Pettenkofer Institute, University of Munich, Munich, Germany). \( \text{H}68 \text{(BAC)} \) virus stocks were prepared as follows. NIH 3T12 cells were infected at a multiplicity of infection (MOI) of 0.05 and harvested at 4 days postinfection (p.i.), and samples were homogenized, clarified, and aliquoted for storage at \(-80^\circ\text{C}.\)Titers of viral stocks were determined by at least two independent plaque assays, as described below. Working gene 50 mutant virus stocks were grown by infecting NIH 3T12-derived gene 50-expressing stable cell lines at an MOI of 0.01, harvested at 7 to 14 days p.i., and otherwise treated the same as described above. NIH 3T12 and the gene 50 stable cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 2 mM l-glutamine. Gene 50-expressing cell lines were additionally supplemented with 0.5 mg of active G418/ml. Cells were maintained in a 5% CO_2 tissue culture incubator at 37°C.

Plaque assays. Plaque assays were performed on monolayers of NIH 3T12 or gene 50-expressing stable cell lines under Noble agar overlay. The cells were plated into six-well plates at 3 \( \times \) 10^5 cells per well on the day prior to the infection. Infections were performed in an 0.2-ml volume, and plates were rocked every 15 min for 1 h at 37°C. Samples were overlaid with 3 ml of a 1:1 mixture

FIG. 1. Construction of gene 50 recombinant viruses. ORF 50 and the adjacent ORFs are indicated, as are the structures of the spliced and unspliced gene 50 transcripts. The solid black arrow indicates the ORF for each of the transcripts. A schematic representation of the structure of the gene 50 recombinant viruses, G50.Stop.[Frt-Kan-Frt] and G50.Stop.Frt (also designated as G50 KO in the text), is shown below the genome. The kanamycin cassette is indicated, the TAG stop codon mutation (with an associated 2-bp deletion) is represented as a stop sign, and the gray arrowheads represent the FRT sites for Flp-mediated recombination.
of 1% Noble agar and 2% DMEM supplemented with 10% fetal calf serum and a 2% concentration of antibiotic and l-glutamine. NIH 3T12 monolayers were stained on day 6 or 7 p.i., gene 50-expressing stable cells were stained on day 14 p.i. by the addition of a 2-mL overlay consisting of 0.02% neutral red in 1:1 mixtures of Eagle’s medium and 0.3% Noble agar. To generate the G50.Stop.Frt strain, stable NIH 3T12 cells carrying one of the correct G50.Stop.[Frt-Kan-Frt] γHV68(BAC) clones were electroporated with an Fp recombinase expression plasmid (pCP20) (6) and grown overnight at 30°C on plates containing chloramphenicol and ampicillin, and colonies were restreaked onto chloramphenicol plates and grown overnight at 42°C to cure the cells of the Flp recombinase expression plasmid (pCP20), which contains a temperature-sensitive origin of replication. Colonies were again restreaked onto plates containing either chloramphenolic or kanamycin, and DNA from clones that were Cam− Kan+ was subjected to Southern blot analyses. The gene 50 region from one of the correct clones was sequenced to confirm the correct structure of the mutation (see Fig. 2B) and was transfected into a gene 50-expressing cell line to generate virus stocks of G50.Stop.[Frt] γHV68 (BAC) (which will subsequently be referred to as G50 KO(BAC)).

Immunoblot analysis. NIH 3T12 or 14.29 cells (4 × 10^5 to 5 × 10^6) were either mock infected or infected with wild-type (wt) γHV68 virus or γHV68 KO virus at 1 PFU per cell in the presence or absence of phosphonoacetic acid (PAA). Total cell lysates were harvested at 24 h p.i. and 1:20 of the sample was run under reducing conditions on a sodium dodecyl sulfate–12.5% polyacry- lamide gel. Immunoblotting was performed with a 1:500 dilution of rabbit polyclonal anti-γHV68 antiserum (42), followed by detection employing a 1:1,000 dilution of donkey anti-rabbit secondary antibody conjugated to hors eradish peroxidase (Jackson Immunoresearch Laboratories), and blots were developed with ECL chemiluminescent reagent (Amersham) according to the manufactur- er’s protocol.

Viral DNA analysis with quantitative real-time PCR. NIH 3T12 cells were infected with wt γHV68 (BAC) or G50 KO (BAC) at 1 PFU per cell and harvested at various times p.i. (see Fig. 6). Data were collected from two indepen- dent experiments. DNA from cell pellets was isolated with the DNAeasy tissue kit (Qiagen) and gene 50 primers (sense primer, 5′-GGATGCAGGGATGTTC) in a 1:104 and 5.76 × 10^5 copies per ng of DNA, respectively. GAPDH copy numbers per reaction did not vary significantly, ranging from 5.5 × 10^5 to 1.15 × 10^6 copies per ng of DNA.

RESULTS

Targeted disruption of gene 50 with the γHV68 into a BAC. Recently, Adler et al. (2) reported the cloning of the entire γHV68 genome as a BAC [wt γHV68(BAC)], which they generously provided to us. The wt γHV68 (BAC) is maintained in recombinant-deficient bacteria and to date appears to be stable under these conditions. The presence of the BAC se- quences does not affect viral growth in vitro (2) but does influence lytic replication and reactivation in vivo (1). We first aimed to disrupt the γHV68 gene 50 by inserting a translation stop codon (bp 67795), along with a kanamycin resistance cassette (flanked by FRT sites), to allow selection for the desired mutant in E. coli {G50.Stop.[Frt-Kan-Frt] γHV68 (BAC)} (Fig. 1). The incorporation of FRT sites allowed for removal of the kanamycin cassette by FLP recombinase-mediated excision. In addition to incorporation of a translation stop codon, a 2-bp deletion was introduced that generated a frame- shift to ensure that any protein resulting from stop codon read-through or reversion would not encode a functional gene 50 product.

The G50.Stop.[Frt-Kan-Frt] γHV68 (BAC) mutant was generated as described previously (2) through homologous recom-
bination in *E. coli* JC8679 cells by using a plasmid containing the mutation and ca. 1.4 kb of homology to *HV68* sequence on each side of the mutation (Fig. 1). Southern blot analysis of the *G50.Stop.[Frt-Kan-Frt]/HV68(BAC)* by digestion with *SpeI* and hybridization with a biotin-labeled gene 50 probe demonstrated, as predicted, the presence of a 2.8-kb restriction fragment in the wt *HV68* and a 4.3-kb fragment in *G50.Stop.Frt* consistent with the insertion of the kanamycin cassette (Fig. 2A, left panel, lanes 3 and 4). Additionally, the region around the mutation in *G50.Stop.Frt* was sequenced to confirm that the desired mutation had been introduced into the viral genome (Fig. 2B). For ease, the *G50.Stop.Frt* will be referred to as *G50 KO(BAC).*

Isolation of a gene 50-null *HV68* in gene 50-expressing stable cells. Viral stocks of *G50 KO* were generated by transfection of the gene 50-expressing cell line 14.18 with *G50 KO(BAC)* DNA purified from *E. coli*. Notably, no infectious virus could be isolated upon multiple cycles of reinfection of NIH 3T12 cells.

One of the correct *G50.Stop.[Frt-Kan-Frt]/HV68(BAC)* clones was then subjected to Flp-mediated recombination (see Materials and Methods) to generate *G50.Stop.Frt* *HV68(BAC)* (Fig. 1). The excision of the kanamycin cassette was demonstrated by Southern blot analysis (Fig. 2A, left panel, lanes 3 and 4). Additionally, the region around the mutation in *G50.Stop.Frt* was sequenced to confirm that the desired mutation had been introduced into the viral genome (Fig. 2B). For ease, the *G50.Stop.Frt* *HV68(BAC)* will be referred to as *G50 KO(BAC).*

**FIG. 2.** Genomic structure of gene 50 recombinant viruses. (A) Southern blot analysis. (Left) wt, *G50.Stop.[Frt-Kan-Frt]*, and *G50.Stop.Frt* BAC viral genomes were purified from *E. coli* DH10B cells, digested with *SpeI*, electrophoresed, blotted, and hybridized with a biotin-labeled gene 50 probe (bp 66642 to 69462). The biotin-labeled gene 50 probe was generated by using the Detector random primer DNA biotinylation kit (Kirkgaard & Perry Laboratories), and the Southern blot was developed by using the DNA Detector genomic Southern blotting kit (Kirkgaard & Perry Laboratories) according to the manufacturer’s instructions. (Right) wt *HV68* or wt BAC and *G50.Stop.Frt* BAC ( *G50 KO* in the text) viral genomes were purified from virions isolated from NIH 3T12 and gene 50-expressing stable cell line 14.29, respectively, digested with *XbaI*, electrophoresed, blotted, and hybridized with a *32*P-labeled gene 44 probe (bp 61444 to 62183). On both panels, the fragment sizes of the molecular size markers are shown to the left of each blot (1-kb DNA ladder for the left panel, lambda DNA- *BstEII* digest for the right panel; New England Biolabs). To the right of each blot are shown the predicted sizes of the viral DNA fragments detected by the respective probes in each blot. (B) Nucleotide sequence of the region containing the mutation in the *G50.Stop.Frt* BAC. The genome coordinates are to the right of the nucleotide sequence. The nucleotide sequence shown in lowercase letters denotes the gene 50 intron of the spliced gene 50 transcript, and the sequence in uppercase letters denotes the second gene 50 exon of the spliced gene 50 transcript. The splice acceptor site is denoted with an arrowhead. The ORF 50 ATG is boxed. The mutation in *G50.Stop.Frt* BAC is depicted in boldface, the introduced TAG stop codon is boxed, the FRT site is underlined, and the *XbaI* site within the FRT site is boxed and italicized.
3T12 cells with G50 KO(BAC), demonstrating a dependence on gene 50 expression for growth of G50 KO(BAC) virus. Subsequent screening of multiple subclones of the gene 50 stable cell line clone 14 for growth of the G50 KO virus identified clone 14.29 as the most efficient in supporting growth of the mutant virus. Homologous recombination between the G50 KO viral DNA and gene 50 inserted into the cellular genome can result in the generation of revertant virus containing the wt gene 50 locus. Nonhomologous recombination is less likely, but also possible. To assess the reversion frequency in the viral stocks, they were analyzed by plaque assay on NIH 3T12 cells, which do not express gene 50. This method accounts for virus resulting from both homologous and nonhomologous recombination, provided that the resultant virus has wt growth characteristics. The reversion frequency of the G50 KO(BAC) virus stocks generated on the gene 50-expressing cell line 14.29 was estimated to be 1 PFU of revertant in $3 \times 10^6$ to $1 \times 10^5$ PFU of G50 KO virus. To ensure that the genetic lesion in the G50 KO remains unaffected by growth in tissue culture, G50 KO DNA was isolated from virions and analyzed by Southern analysis employing the presence of an additional XbaI site in the FRT sequence of the G50 KO genome (Fig. 2A). Southern analyses of XbaI-digested G50 KO virion DNA, with a $^{32}$P-labeled gene 44 probe, demonstrated hybridization of the probe, as expected, to a 12.2-kb fragment in the wt $\gamma$HV68 and in the wt $\gamma$HV68(BAC) and a 6.5-kb fragment in the G50 KO (Fig. 2A, right panel, lanes 1 through 4). Additionally, this analysis also demonstrated that the mutation is contained within the gene 50 genomic locus, because XbaI generates cuts in the $\gamma$HV68 genome outside the region used for homologous recombination.

**Gene 50 is necessary and sufficient for G50 KO(BAC) virus production in NIH 3T12 cells.** The hypothesis on which these studies are based is that gene 50 is essential for initiation and progression of the $\gamma$HV68 lytic cycle. To test the essentiality of gene 50 in a quantitative manner, we infected cell monolayers of NIH 3T12 and the gene 50-expressing cell line 14.29 with G50 KO(BAC) and wt $\gamma$HV68(BAC) virus at 0.1 PFU per cell, and total virus (cells and supernatants) was collected at different times postinfection. Importantly, the G50 KO(BAC) virus stock used had less than 25 PFU of revertant virus/ml (below the limit of detection). Mock-infected samples did not display any cytopathic effect (CPE) up to 14 days p.i. Both NIH 3T12 and 14.29 cells infected with wt $\gamma$HV68(BAC) virus reached 100% CPE at 4 days p.i. Notably, NIH 3T12 cells infected with G50 KO(BAC) virus did not display any CPE, similar to mock-infected cells, up to 14 days p.i. In contrast, G50 KO(BAC) virus-infected 14.29 cells showed a progression from small plaques without cleared centers starting at ca. 4 days p.i. to plaques with cleared centers (typical for wt $\gamma$HV68 infection), appearing at day 8 p.i., to 100% CPE by 14 days p.i. Viral titers of samples recovered from infection of NIH 3T12 and 14.29 fibroblasts were determined on the gene 50-expressing cell line 14.29 (Fig. 3). Prior to these experiments, we established that $\gamma$HV68 grew with comparable kinetics and had a comparable growth output on both NIH 3T12 and 14.29 cells (data not shown), and therefore wt $\gamma$HV68(BAC) viral titers were also determined on 14.29 cells. wt $\gamma$HV68(BAC) reached a maximum titer of ca. $2 \times 10^7$ to $5 \times 10^7$ PFU/ml at day 4 p.i. on either NIH 3T12 or 14.29 cells. Titers of virus recovered from G50 KO(BAC) virus-infected NIH 3T12 cells slowly declined with time, demonstrating an absence of virus production in cells lacking gene 50 expression. In contrast, G50 KO(BAC) virus growth on the 14.29 complementing cell line was readily detectable, reaching a maximum titer of $3 \times 10^6$ PFU/ml at day 8 p.i. In addition, samples from G50 KO virus-infected 14.29 cells were plated on NIH 3T12 cells to test for the presence of revertant virus, and none was detected (data not shown), indicating that there is <25 PFU of revertant virus/ml present in these samples. Therefore, the high levels of virus production detected upon infection of the 14.29 cell line with G50 KO(BAC) virus can be attributed to G50 KO(BAC) virus growth, and not virus that has restored expression of wt gene 50.

Since the 14.29 cell line was created with a fragment of the $\gamma$HV68 genome containing the entire ORF 49, in addition to gene 50, it is formally possible that the 14.29 cell lines expresses both Rta and the ORF 49 product. ORF 49 is a putative gene with no known function that is conserved among the sequenced gammaherpesviruses (40) and thus is likely to be expressed during virus infection. To exclude the possibility that defects in the expression of other genes in the gene 50 locus, specifically ORF 49, are responsible for the inability of G50 KO virus to grow on NIH 3T12 fibroblasts, we designed a strategy to generate a gene 50-expressing cell line that can express only Rta. To accomplish this, we cloned the spliced gene 50 transcript, which lacks ORF 49 (Fig. 1). This approach was based on the assumption that it is the spliced transcript that encodes the complete Rta protein. This cDNA was cloned into an expression vector and used to generate stable NIH

![Graph](http://jvi.asm.org/)

**FIG. 3.** G50 KO virus is defective for virus replication in NIH 3T12 fibroblasts and can be rescued by expression of gene 50 in COVS. NIH 3T12 fibroblasts or a stable NIH 3T12-derived cell line expressing gene 50 (14.29 cells; see Materials and Methods) was infected with wt BAC or G50 KO at 0.1 PFU per cell. Samples were harvested at the indicated times. All samples were quantified by plaque assay on the 14.29 cell line. Samples from G50 KO-infected 14.29 cells were additionally quantified by plaque assay on NIH 3T12 cells to determine the presence of revertant virus, and none was detected by this assay. Data were compiled from two independent experiments.
3T12 cell lines expressing only the spliced form of the gene 50 transcript (see Materials and Methods).

To study the functionality of the spliced gene 50 cDNA, FLAG epitope-tagged spliced gene 50 (sG50-FLAG) and FLAG epitope-tagged full-length gene 50 (fG50-FLAG) expression vectors were cotransfected with the gene 57 promoter-driven luciferase (57pLuc) reporter construct into NIH 3T12 cells and analyzed for luciferase activity (Fig. 4A). The fG50-FLAG expression vector displayed approximately the same levels of activation of the gene 57 promoter as did the untagged full-length gene 50 (fG50) expression vector, indicating that addition of the FLAG epitope does not affect the function of gene 50 as previously reported (48). Notably, the sG50-FLAG expression vector could also achieve a similar level of activation of the gene 57 promoter as the full-length gene 50 constructs, consistent with the hypothesis that it is the spliced transcript that gives rise to functional Rta (note that the full-length gene 50 constructs encode both the spliced and unspliced gene 50 transcripts). This observation is consistent with previous findings that the spliced form, but not the unspliced form, of the herpesvirus saimiri gene 50 is capable of activating the gene 57 promoter (46). However, this analysis does not rule out a function for a gene 50 product expressed from the unspliced gene 50 transcript.

The sG50-FLAG construct was transfected into NIH 3T12 cells, the cells were grown under G418 selection, and the bulk stable cells were tested for the ability to support G50 KO virus growth. Since G50 KO virus did not cause any CPE on the bulk stable cells were tested for the ability to support G50 KO virus. One stable cell line, s27, was chosen for further studies based on its ability to grow. Since G50 KO virus did not cause any CPE on the bulk stable cells were tested for the ability to support G50 KO virus. The s27 cell line was infected with wt HV68(BAC) virus at 0.1 PFU/ml, similar to wt/H9253 HV68(BAC) levels, by 8 days p.i. This observation is consistent with previous findings that the spliced form, but not the unspliced form, of the herpesvirus saimiri gene 50 is capable of activating the gene 57 promoter (46). However, this analysis does not rule out a function for a gene 50 product expressed from the unspliced gene 50 transcript.

Mock-infected samples did not display any CPE up to 14 days p.i. wt/hHV68(BAC) reached 100% CPE by 2 days p.i. on s27 cells and by 4 days on NIH 3T12 cells, with a maximum titer of ca. $2 \times 10^7$ to $6 \times 10^7$ PFU/ml by 4 days p.i. for both cell lines. G50 KO(BAC)-infected NIH 3T12 cells did not display any CPE, and there was no detectable virus production by day 14 p.i. Importantly, G50 KO(BAC)-infected s27 cells reached 100% CPE and maximal viral titers of ca. $2 \times 10^7$ PFU/ml, similar to wt/hHV68(BAC), levels, by 8 days p.i. This represents a ca. 300-fold increase compared to input G50 KO virus in an assay that measures multiple rounds of infection. Plaque assay of G50 KO(BAC)-infected s27 samples on NIH 3T12 cells failed to detect the presence of any virus that had restored wt gene 50 in the G50 KO(BAC) virus, thus demonstrating that the high levels of virus production in the gene 50-expressing s27 cells were due to trans-complementation of the G50 KO(BAC) defect by gene 50. This experiment provides unambiguous genetic evidence that the growth defect observed with the G50 KO(BAC) can be attributed to disruption of hHV68 gene 50. Thus, the latter analyses demonstrate that the expression of Rta from the spliced gene 50 transcript is both necessary and sufficient for growth of the G50 KO (BAC) virus.

It should be noted that the analyses of G50 KO(BAC) virus
To gain insights into the basis for this discrepancy, we assessed viral DNA replication in NIH 3T12 cells infected with either G50 KO(BAC) or wt γHV68(BAC) virus. Cells were either mock infected or infected at an MOI of 1.0, and DNA was isolated from cell pellets at different times p.i. To assess the amount of viral DNA present in each sample, we developed a quantitative real-time PCR assay with primers specific for the viral genome (gene 50) or a cellular gene (GAPDH) (Fig. 5). GAPDH levels were monitored to ensure the PCR products were of the expected sizes (data not shown). Real-time PCR analyses revealed that, as expected, wt viral genome copy numbers increased as infection progressed, reaching a maximum increase of >200-fold (compared to time zero) at 2 days p.i., which coincided with 100% CPE of the cell monolayer. In contrast, we could not detect replication of the G50 KO(BAC) virus over the 4-day time course of the experiment, and viral DNA copy numbers actually decreased ca. 10-fold by 24 p.i. (Fig. 5, inset). Thus, as expected, the G50 KO(BAC) virus is deficient in viral DNA replication.

We extended the above study by analyzing viral protein expression in NIH 3T12 and 14.29 cells infected with G50 KO (BAC) or wt γHV68(BAC) virus at 1 PFU per cell in the presence or absence of PAA, an inhibitor of herpesvirus DNA polymerase. Expression of viral proteins was analyzed by immunoblotting of cell lysates recovered at 24 h p.i., with a rabbit polyclonal anti-γHV68 antiserum generated by infecting rabbits with γHV68 (Fig. 6). In wt γHV68(BAC)-infected cells, the antiserum detected several lytic antigens (Fig. 6, lanes 3 and 9), whose expression was dependent on viral replication (Fig. 6, lanes 4 and 10), indicating that they are late viral proteins.

Absence of viral DNA replication and late gene expression in cells infected with gene 50-null γHV68. To gain insights into the basis of the replication defect in the G50 KO(BAC) virus, we assessed viral DNA replication in NIH 3T12 cells infected with either G50 KO(BAC) or wt γHV68(BAC) virus. Cells were either mock infected or infected at an MOI of 1.0, and DNA was isolated from cell pellets at different times p.i. To assess the amount of viral DNA present in each sample, we developed a quantitative real-time PCR assay with primers specific for the viral genome (gene 50) or a cellular gene (GAPDH) (Fig. 5). GAPDH levels were monitored to ensure that the DNA amounts analyzed by real-time PCR were consistent between time points and between the wt γHV68(BAC) and G50 KO(BAC) viruses (data not shown). The levels of viral DNA present at 1 h p.i. (Fig. 5, 0-day time point) were very similar between G50 KO(BAC) and wt γHV68(BAC) viruses (1.34 × 10^4 and 5.76 × 10^3 viral DNA copies per ng of DNA, respectively). Additionally, agarose gel electrophoresis demonstrated that the PCR products were of the expected sizes (data not shown). Real-time PCR analyses revealed that, as expected, wt viral genome copy numbers increased as infection progressed, reaching a maximum increase of >200-fold (compared to time zero) at 2 days p.i., which coincided with 100% CPE of the cell monolayer. In contrast, we could not detect replication of the G50 KO(BAC) virus over the 4-day time course of the experiment, and viral DNA copy numbers actually decreased ca. 10-fold by 24 p.i. (Fig. 5, inset). Thus, as expected, the G50 KO(BAC) virus is deficient in viral DNA replication.

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replication defect and suggests that there is a global defect in late viral gene expression associated with the loss of gene 50 function.

**DISCUSSION**

Previously, Wu et al. reported that γHV68 expression of gene 50 is sufficient to drive γHV68 reactivation in the latently infected S11 B-lymphoma cell line (49). Recently, these researchers have also shown that dominant-negative forms of γHV68 gene 50 can inhibit virus replication (48). The analyses presented here confirm and extend these observations. The genetic proof for the requirement of a gene in a particular process is provided by analysis of the process when the gene is disrupted completely (i.e., a null gene mutant). We show here that a genetic lesion in γHV68 gene 50 renders the virus completely defective for virus production in NIH 3T12 cells and that complementation of this defect in trans by γHV68 Rta restores virus production to wt levels. The mutant virus is incapable of viral DNA replication, and late viral protein expression is severely attenuated in G50 KO(BAC)-infected cells, even at a high MOI. Therefore, we conclude that gene 50 is essential for γHV68 replication in vitro.

The question still remains whether there are other genes in γHV68 that are critical for initiation of the lytic cycle. As mentioned previously, γHV68 does not appear to encode a homolog of the EBV BZLF1 gene, an immediate-early gene with a demonstrated importance for lytic cycle initiation in EBV. Recently, it was shown that, in addition to gene 50, the genes K3, M8, and ORF 73 are expressed with immediate-early kinetics (26), although γHV68 ORF 73 is likely a latency-associated gene (41). Further studies are required to identify other candidate immediate-early genes and determine whether they play a role in initiating the viral lytic cascade. Importantly, analysis of viral gene expression in the absence of functional gene 50 expression should aid in the identification of other immediate-early genes.

Currently γHV68 infection of mice is the only tractable animal model for dissecting host and viral factors involved in regulating in vivo latency and reactivation. To date, there has been no report of the in vivo characterization of a null mutant in a gammaherpesvirus immediate-early gene. The generation of a null mutation in γHV68 gene 50, a conserved immediate-early gene, will allow for the first time an investigation of the role of gammaherpesvirus immediate-early genes for the maintenance of latency in vivo, as well as reactivation from latency. In addition, it will be of interest to determine whether infection of immunocompromised mice with replication-defective γHV68 mutants leads to an enhanced ability to induce lymphomas and other tumors due to the absence of reactivation-associated disease.

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