JOURNAL OF VIROLOGY, Feb. 2000, p. 2029–2037 Vol. 74, No. 4
0022-538X/00/$04.00+0

Characterization of Gammaherpesvirus 68 Gene 50 Transcription

SHAOFAN LIU, IGLIKA V. PAVLOVA, HERBERT W. VIRGIN IV,* AND SAMUEL H. SPECK*

Departments of Pathology and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 16 July 1999/Accepted 10 November 1999

Gene 50 is the only immediate-early gene that appears to be conserved among the characterized gammaherpesviruses. It has recently been demonstrated for the human viruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) that ectopic expression of the gene 50-encoded product in some latently infected cell lines can lead to the induction of virus replication, indicating that gene 50 is likely to play a pivotal role in regulating gammaherpesvirus reactivation. Here we demonstrate that the murine gammaherpesvirus 68 (γHV68) gene 50 is an immediate-early gene and that transcription of γHV68 gene 50 leads to the production of both spliced and unspliced forms of the gene 50 transcript. Splicing of the transcript near the 5′ end serves to extend the gene 50 open reading frame, as has been observed for the gene 50 transcripts encoded by KSHV and herpesvirus saimiri (Whitehouse et al., J. Virol. 71:2550–2554, 1997; Lukac et al., Virology 252:304–312, 1998; Sun et al., Proc. Natl. Acad. Sci. USA 95:10866–10871, 1998). Reverse transcription-PCR analyses, coupled with S1 nuclease protection assays, provided evidence that gene 50 transcripts initiate at several sites within the region from bp 66468 to 66502 in the γHV68 genome. Functional characterization of the region upstream of the putative gene 50 transcription initiation site demonstrated orientation-dependent promoter activity and identified a 110-bp region (bp 66442 to 66552) encoding the putative gene 50 promoter. Finally, we demonstrate that the γHV68 gene 50 can transactivate the γHV68 gene 57 promoter, a known early gene target of the gene 50-encoded transactivator in other gammaherpesviruses. These studies show that the γHV68 gene 50 shares several important molecular similarities with the gene 50 homologs in other gammaherpesviruses and thus provides an impetus for future studies analyzing the role of the γHV68 gene 50-encoded protein in acute virus replication and reactivation from latency in vivo.

Murine gammaherpesvirus 68 (γHV68; also referred to as MHV68) was isolated from a bank vole, and it infects outbred and inbred mice. The genomic sequence of γHV68 is available and confirms its close relationship with other gammaherpesviruses (39), including Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8). γHV68 can acutely infect multiple organs of mice, including the spleen, liver, lung, kidney, adrenal, heart, and thymus (27, 36). Infection has been associated with splenomegaly and pneumonitis and with a fatal arteritis in mice lacking responsiveness to gamma interferon (36, 38, 41, 42). An association with γHV68 and the development of lymphomas has been reported (35). It has been shown that γHV68 can establish a latent infection in the spleen (36, 37, 41), and B cells and macrophages carry latent γHV68 (43). Because of its genomic structure and association with lymphomas and evidence that it establishes a latent infection in B lymphocytes, γHV68 has been suggested as a murine model for EBV and KSHV infection (8, 23, 30, 33, 35, 39, 40).

In the case of reactivation of EBV, entry into the lytic cycle involves the coordinated expression of two immediate-early gene products, Zta and Rta, encoded by the *BZLF1* and *BRLF1* genes, respectively (see reference 22). Only KSHV appears to encode a homolog of Zta (K8 gene product), although there is no evidence supporting a role for the KSHV Zta homolog in reactivation from latency (15). In contrast, there are obvious homologs of EBV Rta (gene 50-encoded product) encoded by the sequenced gammaherpesviruses (herpesvirus saimiri [HVS], KSHV, and γHV68 gene 50). In addition, both the EBV and KSHV gene 50-encoded products have been shown to be capable of triggering the reactivation of latent virus (19, 26, 34, 47), indicating that a role for the Rta homologs in triggering virus replication is likely conserved among all gammaherpesviruses.

Here we analyze transcription of the γHV68 gene 50 and determine the structure of the gene 50-encoded transcripts. In addition, the transcription start site and putative gene 50 promoter are mapped. We and others have demonstrated that γHV68 infection of mice provides a tractable small-animal model for determining the role of specific host and viral genes in regulating gammaherpesvirus pathogenesis. The current studies provide the necessary molecular information for future characterization of the role of γHV68 gene 50 in acute replication and reactivation from latency and demonstrate a common mechanism for gene 50 transcription shared among γHV68, EBV, HVS, and KSHV.

γHV68 gene 50 is an immediate-early gene. To determine the size(s) of the gene 50 transcript, as well as to assess at what stage of viral infection gene 50 is expressed, RNA from γHV68-infected murine NIH 3T12 fibroblasts was prepared in either the presence or the absence of the protein synthesis inhibitors cycloheximide and anisomycin. Since these antibiotics act at different sites, the use of a double block provides a more effective inhibition at concentrations of drugs which are not generally toxic to cells. In the absence of protein synthesis inhibitors, several transcript species were apparent on Northern blots (Fig. 1). At exposures which readily demonstrated

* Corresponding author. Mailing address: Departments of Pathology and Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., Box 8118, St. Louis, MO 63110. Phone: (314) 362-0367. Fax: (314) 362-4096. E-mail for Herbert W. Virgin: virgin@immunology.wustl.edu. E-mail for Samuel H. Speck: speck@pathology.wustl.edu.

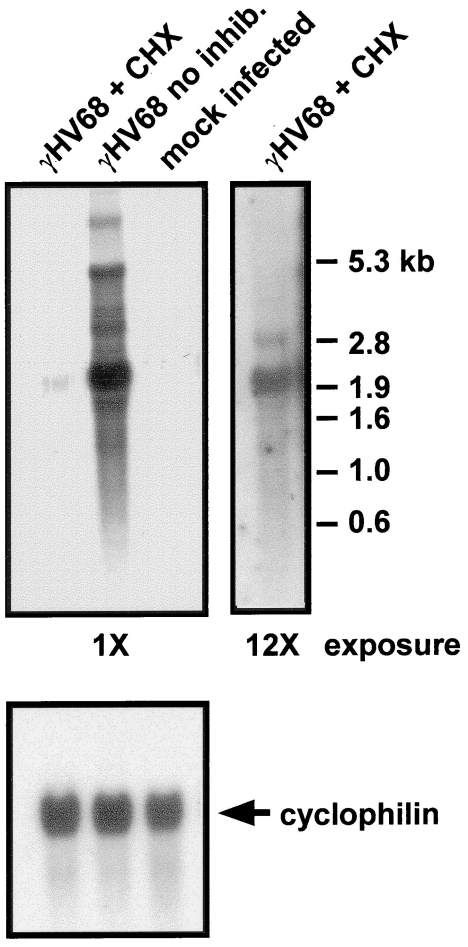


FIG. 1. Northern blot analysis of gene 50-encoded transcripts. Ten micrograms of polyadenylated RNA prepared from NIH 3T12 fibroblasts infected with γHV68 at a multiplicity of infection of 7 for 8 h was loaded in each lane. Cells were either (i) mock infected, (ii) infected in the absence of any inhibitors, or (iii) infected in the presence of the protein synthesis inhibitors cycloheximide (final concentration, 40 μM) and anisomycin (final concentration, 10 μM) (γHV68 + CHX) in a volume of 2 ml of Dulbecco's modified Eagle medium containing 10% fetal calf serum for 1 h. After a 1-h incubation, an additional 15 ml of medium was added (with or without the indicated inhibitors), and the flasks were incubated at 37°C under a 5% CO_2 atmosphere until harvesting. Infected cells were harvested 8 h postinfection, and total RNA was prepared as previously described (3). RNA blotting was carried out by fractionating RNA on 1.2% agarose gels containing 6.6% formaldehyde, 40 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA, followed by capillary blotting in 20× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) onto Hybond nylon membranes (Amersham Corp.). The RNA was covalently cross-linked to the nylon membrane by exposure to shortwave UV light, followed by hybridization with a ^{32}P-labeled gene 50 probe overnight and washed under standard conditions (29). The upper left panel shows a 1-day exposure of the blot, while the upper right panel shows a 12-day exposure of the lane containing RNA isolated from cells infected in the presence of cycloheximide and anisomycin. The migration of molecular weight standards is shown to the right of the blots. The blot was stripped and rehybridized with a ^{32}P-labeled rat cyclophilin probe (6) to assess RNA loading (lower panel). All probes were radiolabeled by the Megaprime DNA labeling system (Amersham, Arlington Heights, Ill.) in accordance with the manufacturer's protocol. The gene 50 fragment was generated by PCR with a sense primer which extended from bp 68660 to 66682 in the viral genome and an antisense primer which extended from bp 69176 to 69154 in the viral genome (see Fig. 2B for the location of probe relative to gene 50).

gene 50-hybridizing transcripts in RNA prepared from cells infected in the absence of protein synthesis inhibitors, few or no detectable gene 50-hybridizing transcripts could be detected in RNA prepared from cells infected in the presence of cycloheximide and anisomycin (Fig. 1, left panel). However, longer exposures revealed the presence of a predominant ca. 2.0-kb gene 50 transcript and a lower abundance of a ca. 2.9-kb transcript, while the larger ca. 5.0- and 10-kb transcripts, detected in RNA prepared from cells infected in the absence of cylcohexmide and anisomycin, were not detectable in RNA from cells infected in the presence of these inhibitors (Fig. 1, right panel). This is consistent with low-level production of the 2.0- and 2.9-kb transcripts in the presence of protein synthesis inhibitors, while generation of the larger transcripts is completely inhibited in the absence of ongoing protein synthesis. Stripping and hybridizing the Northern blot with a probe for the cellular cyclophilin transcript demonstrated that equivalent amounts of polyadenylated cellular RNA were loaded in each lane (Fig. 1, bottom).

This analysis demonstrates that the transcription of gene 50 is enhanced by ongoing viral infection, suggesting that either a newly synthesized viral protein and/or an induced cellular factor augments the transcription of gene 50. In the case of EBV infection, transcription of the immediate-early *BZLF1* and *BRLF1* genes is very weak in the absence of ongoing protein synthesis (11). This has been hypothesized to largely reflect positive feedback regulation by the *BZLF1*-encoded protein Zta (9, 32).

Presence of both unspliced and spliced gene 50 transcripts in γHV68-infected cells. We screened a cDNA library generated with RNA isolated from γHV68-infected NIH 3T12 fibroblasts. RNA was prepared from infected cells harvested at (i) 8 h postinfection in the presence of the protein synthesis inhibitors cycloheximide and anisomycin, (ii) 12 h postinfection in the presence of the herpesvirus DNA polymerase inhibitor phosphonoacetic acid, and (iii) 24 h postinfection in the absence of any inhibitors. Equal amounts of RNA from each preparation were pooled together, followed by purification of polyadenylated RNA and generation of cDNA (custom cDNA library synthesis by Stratagene Inc., La Jolla, Calif.). The initial identification of gene 50 cDNAs was accomplished by PCR screening phage DNA prepared from multiple sublibraries, each containing ca. 50,000 recombinants. A total of ca. 1,000,000 independent recombinants were screened with PCR primers specific for the γHV68 gene 50 sequences. Eight candidate gene 50 cDNA clones were initially identified, and one was successfully purified (clone 50-1). Sequence analysis of cDNA clone 50-1 revealed that this clone extended from bp 66642 to 69462 in the viral genome and was unspliced (Fig. 2A and B). This region contains the entire open reading frames 49 and 50. Clone 50-1 also contained a poly(A) tract downstream of the gene 50 open reading frame, indicating that the polyadenylation signal at bp 69434 was utilized (Fig. 2). Since both gene 49 and gene 50, which are on opposite strands of the viral genome, are present in clone 50-1, the presence of the poly(A) tract downstream of gene 50 confirms that this cDNA clone corresponds to a gene 50 transcript. Five short ATG-initiated open reading frames, ranging from 28 to 40 codons in size, lie upstream of the gene 50 open reading frame in the 50-1 cDNA clone. Based on the size of clone 50-1 [2.8 kb without the poly(A) tract], it is likely that this cDNA corresponds to the less abundant 2.9-kb gene 50 transcript detected by Northern analysis (Fig. 1).

Because clone 50-1 did not account for the structure of the predominant ca. 2.0-kb gene 50 transcript, coupled with the fact that in EBV, HVS, and KSHV it has been shown that the predominant gene 50 transcript contains a splice near the 5′ end of the transcript (19, 21, 34, 44), we designed PCR primers to assess the splicing of the γHV68 gene 50 transcript. Using an upstream PCR primer positioned near the 5′ end of the gene 50 transcript (as defined by the cDNA clone 50-1), a

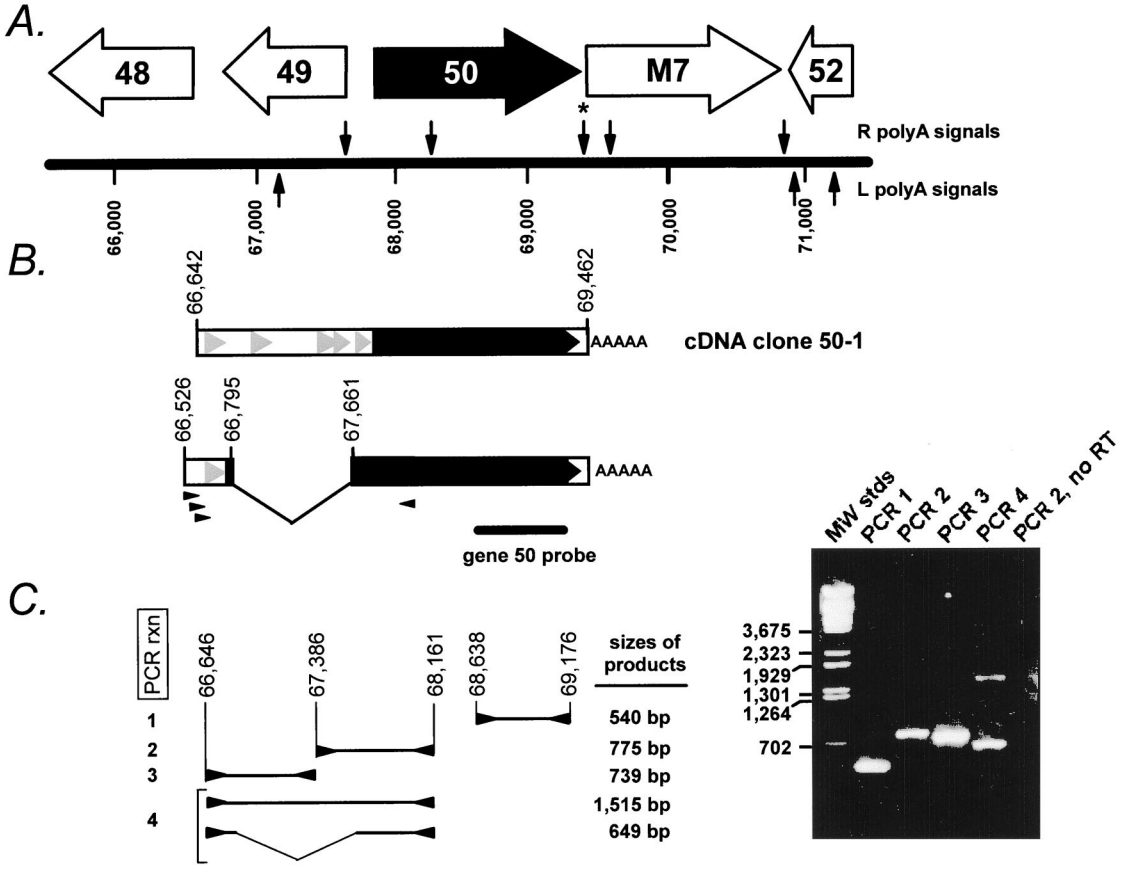
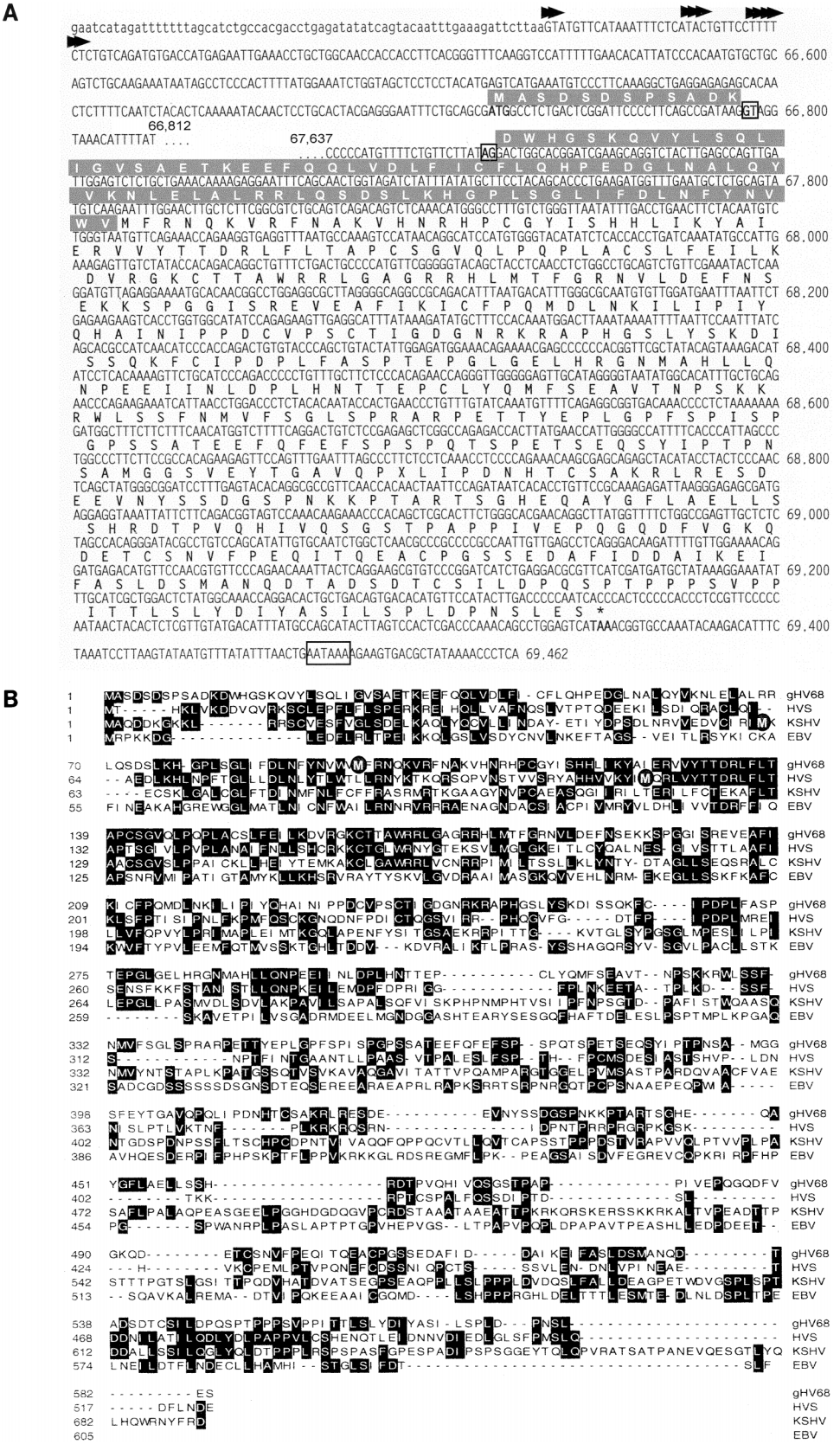


FIG. 2. (A) Schematic illustration of the gene 50 region of the γHV68 genome (39). The locations of the putative viral genes adjacent to gene 50 are indicated, as are the locations of consensus poly(A) signals, which are indicated above (for those associated with the R strand of the viral genome) and below (for those associated with the L strand of the viral genome) the map of the viral genome. The asterisk denotes the poly(A) signal which was utilized in the transcript from which the cDNA clone 50-1 was generated. (B) Schematic structure of the cDNA clone 50-1, which is unspliced and contains viral sequences from bp 66642 to 69462. Below the schematic of the cDNA clone 50-1 is the deduced structure of the ~2.0-kb spliced gene 50 transcript, based on RT-PCR analysis (see discussion in text). The small arrowheads below the spliced transcript denote PCR primers used to amplify the spliced form of the gene 50 cDNA. Also shown is the position of the gene 50 probe used in the Northern blot shown in Fig. 1. The solid black arrow indicates the gene 50 open reading frame, which is extended by the splice to the upstream exon, as illustrated in Fig. 3. The grey arrowheads indicate the presence of small (encoding >25 amino acids) open reading frames upstream of gene 50. (C) RT-PCR analysis of gene 50 transcripts. The structures and sizes of the RT-PCR products obtained employing the indicated PCR primers (arrowheads) are shown to the left of the ethidium bromide-stained agarose gel. The viral genomic coordinates (39) of the PCR primers employed for each reaction were as follows: PCR 1, upstream primer bp 68638 to 68660 and downstream primer bp 69176 to 69154; PCR 2, upstream primer bp 67386 to 67407 and downstream primer bp 68161 to 68141; PCR 3, upstream primer bp 66646 to 66667 and downstream primer bp 67385 to 67364; and PCR 4, upstream primer bp 66646 to 66667 and downstream primer bp 68161 to 68141. Also shown is an ethidium bromide-stained agarose gel of the PCR products generated with the primers indicated. A negative control RT-PCR, to detect the presence of contaminating viral genomic DNA in the RNA preparation, is shown (primers for PCR 2 were used to amplify DNA present in a cDNA synthesis reaction lacking reverse transcriptase [PCR 2, no RT]). All the PCR products visible by ethidium bromide staining were cloned and sequenced.

downstream PCR primer positioned near the 5′ end of the gene 50 coding sequence, and RNA prepared from cells infected in the presence of cycloheximide and anisomycin, we amplified by reverse transcriptase (RT) PCR a 1,515-bp product representing the amplification of the unspliced transcript and a more abundant 649-bp product (Fig. 2C). The presence of both products was dependent on the addition of RT to the reaction mixture (data not shown), demonstrating that this analysis was specific for gene 50 transcripts and could not be accounted for by the presence of contaminating genomic DNA. Sequence analysis of the shorter product revealed the presence of a splice with an intron extending from bp 66796 to 67660 (Fig. 2B and 3A). Notably, introduction of the splice significantly extended the gene 50 open reading frame (Fig. 3A). Based on the genomic sequence, the predicted gene 50 ATG translation initiation codon is located at bp 67907, while the spliced form extends this open reading frame to encode an additional 94 amino acids. The predicted amino terminus of the gene 50 product encoded by the spliced transcript shows significant homology to the gene 50 products encoded by HVS, KSHV, and EBV, indicating that this region is likely to be functionally important (Fig. 3B). Furthermore, splicing of both the HVS and KSHV gene 50 transcripts also serves to extend the coding sequence by juxtaposing to the gene 50 open reading frame, present in the second exon, an upstream translation initiation codon present in the first exon of the spliced form of the gene 50 transcript (12, 19, 34). Notably, alignment of the putative gene 50-encoded proteins reveals extensive homology in the amino-terminal half of these proteins (Fig. 3B). This suggests that the extension of the gene 50 open reading frame by splicing is likely to be functionally important.

The predicted size of the spliced gene 50 transcript [ca. 1.8 kb, without the poly(A) tract], based on the 5′ and 3′ ends of the cDNA clone 50-1, corresponds closely to the size of the predominant ~2.0-kb gene 50 transcript detected by Northern analysis. Utilization of other PCR primer combinations, in


A
B
gHV68
HVS
KSHV
EBV

which either the upstream or downstream PCR primers were located within the intron defined by the RT-PCR discussed above, failed to detect any alternatively spliced products (i.e., only the anticipated products amplified from the unspliced gene 50 transcript were observed) (Fig. 2C). In addition, a control primer pair located near the 3′ end of gene 50 yielded the anticipated size fragment, consistent with the absence of splicing within the gene 50 open reading frame (Fig. 2). Using primers upstream of the 5′ end defined by the cDNA clone 50-1, coupled with a primer downstream of the gene 50 splice acceptor site, we were able to demonstrate the presence of spliced gene 50 transcripts with an upstream primer hybridizing to viral sequences from bp 66526 to bp 66547 (data not shown). The latter result demonstrates that at least some of the spliced gene 50 transcript initiates upstream of the 5′ end defined by the cDNA clone 50-1.

Identification of a candidate gene 50 promoter. Based on the sizes of the gene 50 transcripts observed in the presence of protein synthesis inhibitors (ca. 2.0 and 2.9 kb), it is likely that the site of transcription initiation maps in the region just upstream of the region defined by cDNA cloning and RT-PCR analyses (unless there is an additional upstream splice). We therefore evaluated the genome region from bp 66242 to 66652 for the presence of the gene 50 promoter. Initially, a relatively large region extending from 10 bp downstream of the 5′ end of cDNA clone 50-1 to 400 bp upstream of the 5′ end of clone 50-1 was cloned in both orientations upstream of the luciferase reporter gene (Fig. 4A, reporter constructs 1 and 2). This region exhibited orientation-dependent promoter activity in two different cell lines, consistent with the presence of the gene 50 promoter mapping to this region of the viral genome (Fig. 4A). The sense promoter-driven reporter construct (construct 1) was ca. 100-fold more active than the antisense promoter-driven reporter construct (construct 2) (Fig. 4A). Deletion analysis of this region defined a 110-bp region (from bp 66442 to 66552) that exhibited constitutive activity in both cell lines examined, and again the observed activity was orientation dependent (Fig. 4A, reporter constructs 10 and 11). Further truncation of either upstream or downstream sequences resulted in a significant loss or abrogation of promoter activity (Fig. 4A, reporter constructs 5, 6, 12, and 13). Thus, this analysis demonstrated that there is a candidate gene 50 promoter that maps to the region extending from bp 66442 to 66552 in the γHV68 genome. Furthermore, this appears to be the only promoter present within the 400-bp region immediately upstream of the 5′ end of the cDNA clone 50-1 that is functional in the cell lines tested. As indicated above, this does not rule out the possible presence of a more distal promoter that may be involved in driving gene 50 expression, although the utilization of such a promoter would undoubtedly require additional splicing to generate the appropriately sized gene 50 transcripts.

Notably, the deletion of sequences from bp 66602 to 66652, in the context of sequences extending upstream to bp 66242, resulted in a significant loss of luciferase activity in both cell lines (Fig. 4A, reporter construct 3). Further deletion of sequences from bp 66552 to 66602 restored luciferase activity to levels seen with the full-length fragment (Fig. 4A, reporter constructs 1 and 4). These results indicate the presence of a potent negative regulatory element in the region from bp 66552 to 66602, as well as the presence of a positive regulatory element in the region from bp 66602 to 66552. Alternatively, it is possible that the observed changes in reporter gene activity may reflect alterations in the presence or absence of small upstream open reading frames which may alter the efficiency of translation of the luciferase reporter gene.

Analysis of the sequence of the minimal region exhibiting promoter activity revealed several potential transcription factor binding sites (Fig. 4B) (25). Notably, there is a candidate TATA box located at bp 66439 to 66444. As shown below, S1 nuclease protection analyses defined several potential sites of transcription initiation downstream of this TATA box, suggesting that this TATA box may be functional. In addition to the candidate TATA box, putative MEF2 and IRF binding sites and an E box were identified (Fig. 4B) (25). Elucidation of the functional significance of these sites requires targeting of appropriate mutations to the minimal gene 50 promoter-driven reporter construct. Ultimately, determination of the significance of the identified promoter to gene 50 activity will require the introduction of specific mutations into the viral genome.

Mapping of the 5′ ends of gene 50 transcripts. Having mapped the putative gene 50 promoter, we next mapped the sites of transcription initiation from transfected gene 50 promoter-driven reporter constructs. We chose to use the longest promoter construct containing the γHV68 sequences, extending from bp 66242 to bp 66652 (Fig. 4A, reporter construct 1). NIH 3T12 and RAW cells were transfected with either a control reporter plasmid lacking the gene 50 promoter or the gene 50 promoter-driven reporter construct, and total cellular RNA was prepared 48 h posttransfection. Several 60-nucleotide single-stranded oligonucleotide probes antisense to the gene 50 transcript were hybridized to RNA from the transfected cells, and the formation of specific hybrids was assessed after S1 nuclease digestion. The regions to which these probes were designed to hybridize are depicted in Fig. 4A (S1 nuclease probes S10 to S14). Two of these probes, S11 and S12, reproducibly yielded specific protected fragments (Fig. 5). Based on these results, there appear to be several clustered regions of transcription initiation (Fig. 3A, 4B, and 5), which could arise through either (i) multiple sites of transcription initiation from the gene 50 promoter or (ii) degradation of the 5′ end of gene 50 promoter-driven transcripts.

To extend this analysis to virus infection, we subsequently generated polyadenylated RNA from mock-infected and γHV68-infected NIH 3T12 fibroblasts (infected in the presence of cycloheximide and anisomycin). This RNA was analyzed with the S1 nuclease probes described above. As shown in Fig. 5, only the S12 probe reproducibly resulted in specific protection, although the level of protection was relatively modest. Sporadic protection was also detected with the S11 probe

FIG. 3. (A) Nucleotide and deduced protein sequences of gene 50 and its product (39). The deduced protein sequence is above the nucleotide sequence. The genome coordinates are to the right of the nucleotide sequence. The arrowheads above the 5′ untranslated sequence indicate candidate transcription initiation sites, determined by S1 nuclease protection analyses (see Fig. 5 and discussion in text). The nucleotide sequence shown in lowercase letters denotes the genomic sequence, based on nuclease protection analyses, upstream of the site of transcription initiation. The polyadenylation signal utilized in cDNA clone 50-1, located at bp 69434 in the viral genome, is boxed, as are the splice acceptor and donor sites. The spliced form of the gene 50 transcripts extends the open reading frame to encode an additional 94 amino acids (extended amino-terminal sequence is in shaded box above the nucleotide sequence). (B) Alignment of the predicted gene 50-encoded proteins of γHV68, HVS, KSHV, and EBV. The alignment was performed by Clustal analysis using MegAlign (DNASTAR) and is presented without further editing after the initial alignment. The first in-frame methionine encoded within the second exon of the spliced form of the gene 50 transcript is denoted by a shaded circle for the γHV68, HVS, and KSHV gene 50-encoded proteins.

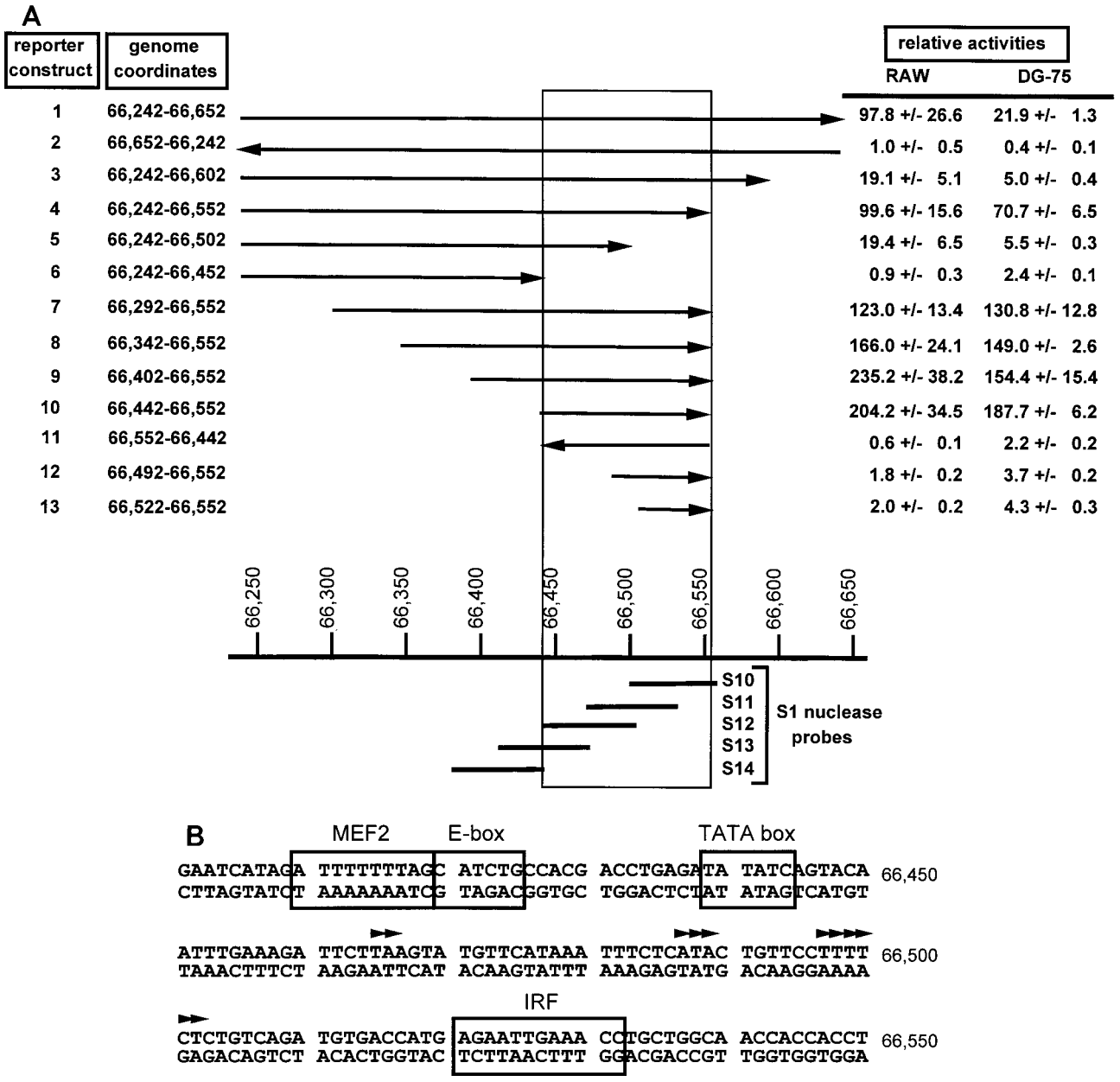


FIG. 4. (A) Identification of the gene 50 promoter. The structures of γHV68 genomic fragments used to map the gene 50 promoter are shown, along with the genomic map coordinates. All viral genomic fragments were cloned upstream of the luciferase reporter gene in the pGL2 Basic vector (Promega, Madison, Wis.). The arrows pointing right indicate the genomic fragments that were cloned in the sense orientation, with the luciferase gene downstream of the putative gene 50 promoter, while the arrows pointing left indicate the genomic fragments that were cloned in the opposite orientation. The indicated reporter constructs (2 μg) were transfected into both the murine macrophage cell line RAW (RAW 264.7) and the human EBV-negative Burkitt's lymphoma B cell line DG-75. RAW cells were transfected with the lipid-based transfection reagent SuperFect according to the manufacturer's protocol (Qiagen, Santa Clara, Calif.). DG-75 cells were transfected with DEAE-dextran, as previously described (28). Cells lysates were prepared 48 h posttransfection and assayed for luciferase activity (7). The data were compiled from three independent experiments for each cell line, and the standard error of the mean is shown. Also shown are the positions of the single-stranded probes used in the S1 nuclease protection analyses (see text for discussion and Fig. 5). The boxed region denotes those sequences which, based on this analysis, are required for gene 50 promoter activity. Relative luciferase activity is the fold increase (mean ± standard error of the mean) over that observed with the parent pGL2 Basic reporter construct (Promega), which was assigned a relative activity of 1.0. (B) Computer analysis of the minimal gene 50 promoter for the presence of transcription factor binding sites. The gene 50 promoter sequenced was analyzed by MatInspector Professional software (23). IRF, interferon response factor; MEF2, myocyte enhancer binding factor 2; E box, binding site for the E family of bHLH transcription factors.

by using RNA isolated from infected cells, but not uninfected cells (data not shown). Taken together, these results are most consistent with the hypothesis that the 5′ end of the gene 50 transcript is unstable, resulting in the production of a heterogeneous population of transcripts containing distinct 5′ ends. Whether the postulated instability of the gene 50 transcript is important for regulating the levels of gene 50 protein produced during infection remains to be assessed. Notably, protection of the full-length S12 probe was also reproducibly observed with RNA isolated from infected cells. This result raises the possi-

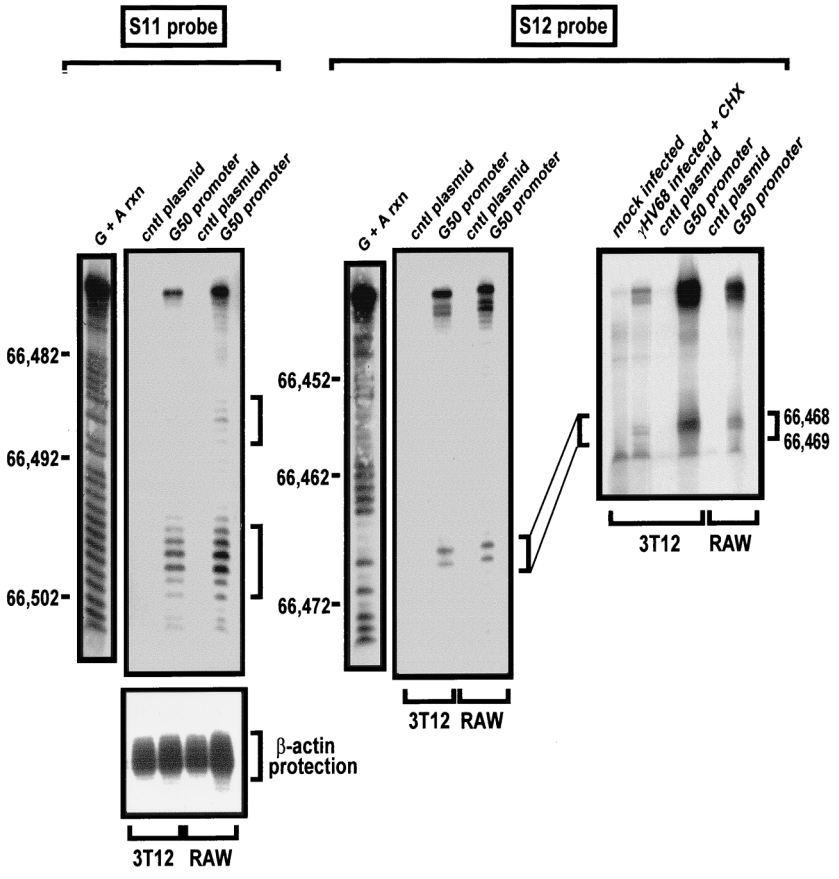


FIG. 5. Mapping of the 5′ ends of the gene 50 transcripts by S1 nuclease protection. Analysis of transcription initiation from the putative gene 50 promoter in transiently transfected NIH 3T12 or RAW cells. The bp 66242 to 66652 gene 50 promoter-driven luciferase reporter construct, or the parent pGL2 Basic reporter construct (Promega), was transfected into NIH 3T12 and RAW cells by the lipid-based transfection reagent SuperFect (Qiagen) as described in the legend to Fig. 4. RNA was prepared (3) from cells harvested 48 h posttransfection, and 40 μg of total RNA was hybridized with 4 ng of the indicated single-stranded oligonucleotide S1 nuclease probes, which had been ^{32}P labeled at the 5′ end with T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, Ind.) (24, 46). The sequences of the probes used were as follows: S11, ^{66531}5′-GTTTCAATTCTCATGGTCACATCTGACAGAGAAAAGGAACAGTATGAGAAATTTATGAAC-3′^{66472}; S12, ^{66501}5′-GAAAAGGAACAGTATGAGAAATTTATGAACATACTTAAGAATCTTTCAAATTGTACTGAT-3′^{66442} (see Fig. 4 for locations of the S11 and S12 probes). Following hybridization overnight at 42°C, the reaction mixture was digested with 300 U of S1 nuclease (Promega) as previously described (24, 46). The protected fragments were recovered and fractionated on a 10% denaturing acrylamide gel. Chemical cleavages of the ^{32}P-labeled oligonucleotide probes (G + A rxn) were used as size markers in the indicated lanes. The right panel shows an analysis of transcription initiation from the gene 50 promoter in γHV68-infected NIH 3T12 cells. NIH 3T12 cells were either mock infected or infected at a multiplicity of infection of 7 in the presence of cycloheximide (final concentration, 40 μM) and anisomycin (final concentration, 10 μM) (+ CHX). Total cellular RNA was prepared (3) from cells harvested 8 h postinfection, followed by isolation of polyadenylated RNA with the PolyA Spin mRNA Isolation kit (New England Biolabs, Beverly, Mass.). Ten micrograms of poly(A) RNA isolated from mock-infected or γHV68-infected cells was hybridized with ^{32}P-labeled S12 probe, followed by digestion with S1 nuclease as described above. In parallel, total RNA isolated from NIH 3T12 and RAW cells transfected with the bp 66242 to 66652 open reading frame 50 luciferase reporter construct was also hybridized with the S12 probe, as described above. Protected fragments were fractionated on a denaturing 10% acrylamide gel.

bility that transcripts that initiate upstream of the identified gene 50 promoter also exist. Further studies are required to address this issue.

The γHV68 gene 50-encoded protein transactivates the γHV68 gene 57 promoter. To assess whether the gene 50 transcription unit identified here encodes a functional gene 50 transactivator, we cloned the 50-1 cDNA (see Fig. 2B for structure) into a eukaryotic expression vector (pBK-CMV; Stratagene Inc.) under the control of the human cytomegalovirus immediate-early promoter. Introduction of this construct into NIH 3T12 fibroblasts led to readily detectable expression of the spliced form (and presumably the unspliced form) of the gene 50 transcript (data not shown), demonstrating that the ectopically expressed gene 50 transcript is appropriately processed. To assess transcriptional activation activity of the γHV68 gene 50 protein, we determined whether it could transactivate the γHV68 gene 57 promoter. The gene 57 promoter has been shown in other gammaherpesviruses to be a target of the gene 50-encoded transactivator (14, 18, 45). Thus, the luciferase vector with (57pLuc) and without (pGLuc) the gene 57 promoter was cotransfected with either the control expression vector (pBK) or the gene 50 expression vector (pBK50) (Fig. 6). Notably, the gene 57 promoter exhibited very low basal activity but was strongly transactivated (~700-fold induction) by the gene 50 protein (Fig. 6). Weak gene 50 protein transactivation of the luciferase vector was also observed (~10-fold induction) (Fig. 6). Thus, these results demonstrate that a functional gene 50 protein is expressed from the 50-1 cDNA clone and provides further evidence that the spliced form of the transcript identified here encodes the gene 50 transactivator.

Conclusions. We have shown here that the γHV68 gene 50 is transcribed at low levels in the absence of de novo protein synthesis, but it is expressed at much higher levels in the pres-

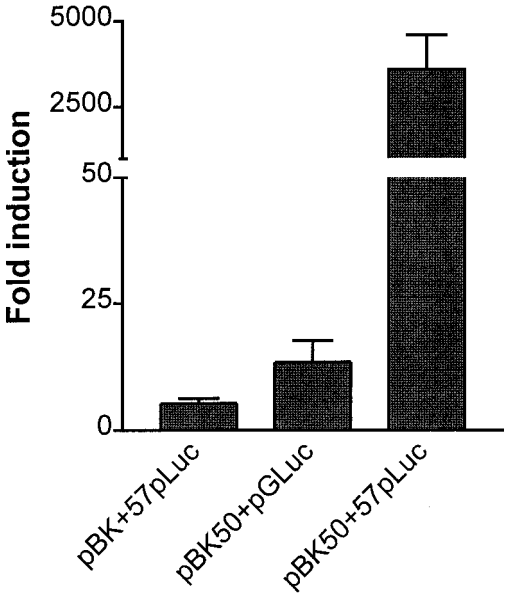


FIG. 6. Transcriptional activation of the γHV68 gene 57 promoter by the γHV68 gene 50-encoded protein. A 565-bp fragment, spanning from bp 75218 to 75782 in the γHV68 genome and containing the gene 57 promoter (based on previous transcript mapping [20]), was cloned into pGL2 Basic (Promega). The 50-1 cDNA clone (see Fig. 2) was cloned into the *Nhe*I and *Kpn*I sites of pBK-CMV (Stratagene). NIH 3T12 cells were cotransfected with 1 μg of either pGL2-Basic (pGLuc) or pGL2 Basic containing the gene 57 promoter (57pLuc) and 1 μg of either pBK-CMV (pBK) or pBK-CMV containing gene 50 (pBK50) with the lipid-based transfection reagent Superfect according to the manufacturer's protocol (Qiagen). Cell lysates were prepared 48 h posttransfection and assayed for luciferase activity (7). The data were compiled from four independent experiments, and the standard error of the mean is indicated.

ence of ongoing viral infection. In addition, our analysis demonstrated that the predominant gene 50 transcript is spliced at the 5′ end of the transcript, resulting in a significant extension of the gene 50 open reading frame. Whether expression of gene 50 from the unspliced transcript(s) also gives rise to a functional protein remains to be assessed. It is notable that for KSHV, HVS, and γHV68, extension of the gene 50 reading frame requires the presence of the spliced transcript, while in EBV both the spliced and unspliced forms of the gene 50 transcript (*BRLF1* gene) are predicted to encode the same gene product (Rta). Thus, it is possible that this reflects a fundamental difference in the regulation of gene 50-encoded protein expression in EBV.

Initial mapping and analysis of the putative gene 50 promoter indicated that it is constitutively active in both a murine macrophage cell line (RAW) and a human B lymphoma cell line (DG-75). Further characterization of this promoter may identify cell lines or cell types in which the gene 50 promoter exhibits lower basal activity. Such cells may be useful for gaining insights into the regulation of the establishment of viral latency and virus reactivation, as has been the case for EBV and regulation of the *BZLF1* immediate-early gene (1, 2, 4, 5, 9–11, 13, 16, 17, 21, 31). In addition, this analysis provides the basis for generating a gene 50 knockout virus. The latter will allow a direct assessment of the role of gene 50 in virus replication, both during infection of permissive cells and during reactivation from latency.

This research was supported by NIH grants CA74730 and HL60090 to H.W.V. and S.H.S., NIH grants CA43143, CA52004, and CA58524 to S.H.S., NIH grant AI39616 to H.W.V., and ACS grant RP6-97-134-01-MBC to H.W.V.

We also acknowledge helpful discussions with members of the Speck and Virgin labs, as well as discussions during joint lab meetings with members of the labs of David Leib and Lynda Morrison.

REFERENCES

1. **Borras, A. M., J. L. Strominger, and S. H. Speck.** 1996. Characterization of the ZI domains in the Epstein-Barr virus BZLF1 gene promoter: role in phorbol ester induction. J. Virol. **70:**3894–3901.
2. **Buisson, M., E. Manet, M. C. Trescol-Biemont, H. Gruffat, B. Durand, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) early protein EB2 is a posttranscriptional activator expressed under the control of EBV transcription factors EB1 and R. J. Virol. **63:**5276–5284.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Countryman, J., H. Jenson, R. Seibl, H. Wolf, and G. Miller.** 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J. Virol. **61:**3672–3679.
5. **Daibata, M., S. H. Speck, C. Mulder, and T. Sairenji.** 1994. Regulation of the BZLF1 promoter of Epstein-Barr virus by second messengers in anti-immunoglobulin-treated B cells. Virology **198:**446–454.
6. **Danielson, P. E., S. Forss-Petter, M. A. Brow, L. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe.** 1988. p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA **7:**261–267.
7. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
8. **Doherty, P. C., D. J. Topham, R. A. Tripp, R. D. Cardin, J. W. Brooks, and P. G. Stevenson.** 1997. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunol. Rev. **159:**105–117.
9. **Flemington, E., and S. H. Speck.** 1990. Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1. J. Virol. **64:**1227–1232.
10. **Flemington, E., and S. H. Speck.** 1990. Identification of phorbol ester response elements in the promoter of Epstein-Barr virus putative lytic switch gene BZLF1. J. Virol. **64:**1217–1226.
11. **Flemington, E. K., A. E. Goldfeld, and S. H. Speck.** 1991. Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis. J. Virol. **65:**7073–7077.
12. **Francis, A., T. Ragoczy, L. Gradoville, L. Heston, A. El-Guindy, Y. Endo, and G. Miller.** 1999. Amino acid substitutions reveal distinct functions of serine 186 of the ZEBRA protein in activation of early lytic cycle genes and synergy with the Epstein-Barr virus R transactivator. J. Virol. **73:**4543–4551.
13. **Goldfeld, A. E., P. Liu, S. Liu, E. K. Flemington, J. L. Strominger, and S. H. Speck.** 1995. Cyclosporin A and FK506 block induction of the Epstein-Barr virus lytic cycle by anti-immunoglobulin. Virology **209:**225–229.
14. **Kenney, S., E. Holley-Guthrie, E. C. Mar, and M. Smith.** 1989. The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. J. Virol. **63:**3878–3883.
15. **Lin, S. F., D. R. Robinson, G. Miller, and H. J. Kung.** 1999. Kaposi's sarcoma-associated herpesvirus encodes a bZIP protein with homology to BZLF1 of Epstein-Barr virus. J. Virol. **73:**1909–1917.
16. **Liu, S., A. M. Borras, P. Liu, G. Suske, and S. H. Speck.** 1997. Binding of the ubiquitous cellular transcription factors Sp1 and Sp3 to the ZI domains in the Epstein-Barr virus lytic switch BZLF1 gene promoter. Virology **228:**11–18.
17. **Liu, S., P. Liu, A. Borras, T. Chatila, and S. H. Speck.** 1997. Cyclosporin A-sensitive induction of the Epstein-Barr virus lytic switch is mediated via a novel pathway involving a MEF2 family member. EMBO J. **16:**143–153.
18. **Lukac, D. M., J. R. Kirshner, and D. Ganem.** 1999. Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. J. Virol. **73:**9348–9361.
19. **Lukac, D. M., R. Renne, J. R. Kirshner, and D. Ganem.** 1998. Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology **252:**304–312.
20. **Mackett, M., J. P. Stewart, V. P. de, M. Chee, S. Efstathiou, A. A. Nash, and J. R. Arrand.** 1997. Genetic content and preliminary transcriptional analysis of a representative region of murine gammaherpesvirus 68. J. Gen. Virol. **78:**1425–1433.
21. **Manet, E., H. Gruffat, M. C. Trescol-Biemont, N. Moreno, P. Chambard, J. F. Giot, and A. Sergeant.** 1989. Epstein-Barr virus bicistronic mRNAs generated by facultative splicing code for two transcriptional trans-activators. EMBO J. **8:**1819–1826.
22. **Miller, G.** 1990. The switch between latency and replication of Epstein-Barr virus. J. Infect. Dis. **161:**833–844.
23. **Nash, A. A., and N. P. Sunil-Chandra.** 1994. Interactions of the murine gammaherpesvirus with the immune system. Curr. Opin. Immunol. **6:**560–563.
24. **Puglielli, M. T., M. Woisetschlaeger, and S. H. Speck.** 1996. oriP is essential

for EBNA gene promoter activity in Epstein-Barr virus-immortalized lymphoblastoid cell lines. J. Virol. **70:**5758–5768.
25. **Quandt, K., K. Frech, H. Karas, E. Wingender, and T. Werner.** 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. **23:**4878–4884.
26. **Ragoczy, T., L. Heston, and G. Miller.** 1998. The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. J. Virol. **72:**7978–7984.
27. **Rajcani, J., D. Blaskovic, J. Svobodova, F. Ciampor, D. Huckova, and D. Stanekova.** 1985. Pathogenesis of acute and persistent murine herpesvirus infection in mice. Acta Virol. **29:**51–60.
28. **Ricksten, A., A. Olsson, T. Andersson, and L. Rymo.** 1988. The 5′ flanking region of the gene for the Epstein-Barr virus-encoded nuclear antigen 2 contains a cell type specific cis-acting regulatory element that activates transcription in transfected B-cells. Nucleic Acids Res. **16:**8391–8410.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Simas, J. P., and S. Efstathiou.** 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol. **6:**276–282.
31. **Sinclair, A. J., M. Brimmell, F. Shanahan, and P. J. Farrell.** 1991. Pathways of activation of the Epstein-Barr virus productive cycle. J. Virol. **65:**2237–2244.
32. **Speck, S. H., T. Chatila, and E. Flemington.** 1997. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. Trends Microbiol. **5:**399–405.
33. **Speck, S. H., and H. W. Virgin IV.** 1999. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. Curr. Opin. Microbiol. **2:**403–409.
34. **Sun, R., S. F. Lin, L. Gradoville, Y. Yuan, F. Zhu, and G. Miller.** 1998. A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. USA **95:**10866–10871.
35. **Sunil-Chandra, N. P., J. Arno, J. Fazakerley, and A. A. Nash.** 1994. Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am. J. Pathol. **145:**818–826.
36. **Sunil-Chandra, N. P., S. Efstathiou, J. Arno, and A. A. Nash.** 1992. Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J. Gen. Virol. **73:**2347–2356.
37. **Sunil-Chandra, N. P., S. Efstathiou, and A. A. Nash.** 1992. Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes in vivo. J. Gen. Virol. **73:**3275–3279.
38. **Usherwood, E. J., A. J. Ross, D. J. Allen, and A. A. Nash.** 1996. Murine gammaherpesvirus-induced splenomegaly: a critical role for CD4 T cells. J. Gen. Virol. **77:**627–630.
39. **Virgin, H. W., P. Latreille, P. Wamsley, K. Hallsworth, K. E. Weck, A. J. Dal Canto, and S. H. Speck.** 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. **71:**5894–5904.
40. **Virgin, H. W., IV, and S. H. Speck.** 1999. Unraveling immunity to gammaherpesviruses: a new model for understanding the role of immunity in chronic virus infection. Curr. Opin. Immunol. **11:**371–379.
41. **Weck, K. E., M. L. Barkon, L. I. Yoo, S. H. Speck, and H. W. Virgin IV.** 1996. Mature B cells are required for acute splenic infection, but not for establishment of latency, by murine gammaherpesvirus 68. J. Virol. **70:**6775–6780.
42. **Weck, K. E., A. J. Dal Canto, J. D. Gould, A. K. O'Guin, K. A. Roth, J. E. Saffitz, S. H. Speck, and H. W. Virgin.** 1997. Murine gamma-herpesvirus 68 causes severe large-vessel arteritis in mice lacking interferon-gamma responsiveness: a new model for virus-induced vascular disease. Nat. Med. **3:**1346–1353.
43. **Weck, K. E., S. S. Kim, H. W. Virgin IV, and S. H. Speck.** 1999. Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. J. Virol. **73:**3273–3283.
44. **Whitehouse, A., I. M. Carr, J. C. Griffiths, and D. M. Meredith.** 1997. The herpesvirus saimiri ORF50 gene, encoding a transcriptional activator homologous to the Epstein-Barr virus R protein, is transcribed from two distinct promoters of different temporal phases. J. Virol. **71:**2550–2554.
45. **Whitehouse, A., M. Cooper, K. T. Hall, and D. M. Meredith.** 1998. The open reading frame (ORF) 50a gene product regulates ORF 57 gene expression in herpesvirus saimiri. J. Virol. **72:**1967–1973.
46. **Woisetschlaeger, M., J. L. Strominger, and S. H. Speck.** 1989. Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **86:**6498–6502.
47. **Zalani, S., E. Holley-Guthrie, and S. Kenney.** 1996. Epstein-Barr viral latency is disrupted by the immediate-early BRLF1 protein through a cell-specific mechanism. Proc. Natl. Acad. Sci. USA **93:**9194–9199.