Herpesviruses establish acute lytic infection followed by lifelong, chronic latent infection. The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) are associated with tumorigenesis in immunocompromised patients. EBV and KSHV exhibit a strict species specificity, and murine gammaherpesvirus 68 (γHV68) provides a small-animal model to study gammaherpesvirus infection. γHV68 shares sequence homology and genomic organization with EBV and KSHV (60), and γHV68 infection is associated with lymphomas and lymphoproliferative disease in immunocompromised mice (25, 55, 58).

Similar to other herpesviruses, the lytic replication of γHV68 is mediated by sequential immediate-early, early, and late viral gene expression (1, 34). The lytic gene expression program is restricted during latency; only a small subset of genes are expressed (34). The switch between lytic and latent gene expression programs is controlled by the essential immediate-early lytic switch gene 50. In KSHV and γHV68, gene 50 is the only gene necessary and sufficient to initiate lytic replication during reactivation and de novo infection (14, 31, 32, 38, 42, 49, 54, 65–67). Gene 50 encodes the replication and transcription activator (RTA) protein, which transactivates a number of viral and cellular genes by directly binding to RTA-responsive elements in target promoters or via indirect measures involving interactions with cellular transcription factors (10, 50). Chemical agents and cellular signals that induce the lytic cycle have been well studied; however, the regulation of the gene 50 promoter itself is incompletely characterized, and few negative regulators have been identified. Negative regulators for the EBV lytic switch gene promoters include the YY1 transcription factor and the zinc finger E-box binding factor (ZEB) (17, 37, 69, 71), whereas the virally encoded latency-associated nuclear antigen (LANA) and the transcription factors RBPJ-κ and NF-κB repress the KSHV gene 50 promoter (4, 20, 21).

The importance of repressing lytic switch gene expression is highlighted by studies of γHV68 mutants that constitutively express gene 50. The lytic replication of these viruses is increased, and the ability to establish latency in vivo is abolished (35, 45).

Gamma interferon (IFN-γ) is an important negative regulator of chronic γHV68 infection. Peritoneal cells explanted from infected IFN-γ- or IFN-γ receptor-deficient mice exhibit increased reactivation and higher levels of persistent replication than cells from control mice (51, 59). Mice lacking IFN-γ responses develop large-vessel vasculitis, lymphoproliferative disease, and pulmonary fibrosis (11, 25, 64), which are dependent on productive γHV68 replication (8, 39). The depletion of IFN-γ from latently infected wild-type mice increases γHV68 reactivation from explanted peritoneal cells (51). Furthermore, γHV68 reactivation from wild-type latently infected peritoneal explants is blocked by IFN-γ treatment (51). Importantly, IFN-γ inhibits γHV68 reactivation in a cell type-specific manner whereby latently infected macrophages must be able to respond to IFN-γ for the cytokine to repress reactivation (52). Previous studies of C57BL/6 mice showed that IFN-γ is not required to control acute γHV68 infection (46, 64); however, a more recent report showed that acute infection is inadequately controlled in IFN-γR<sup>−/−</sup> mice on the BALB/c background, and these animals developed lethal pneumonia (24).

While the importance of IFN-γ signaling during γHV68 infection is well characterized, the molecular mechanisms by which IFN-γ controls γHV68 are unknown. Notably, levels of select viral transcripts, including gene 50, are increased in peritoneal cells isolated from latently infected IFN-γR<sup>−/−</sup> mice compared with those infected with wild-type virus (51). Here we report that IFN-γ-mediated control of γHV68 reactivation is cell-extrinsic control over the interchange between the lytic and latent cycles.
mice compared to wild-type mice (51). Given this finding and the fact that IFN-γ blocks reactivation, a process requiring lytic switch gene expression to trigger lytic replication, we tested the hypothesis that IFN-γ inhibits lytic replication and gene 50 expression in HV68-infected macrophages, the cell type that is responsive to the IFN-γ-mediated control of HV68 reactivation.

IFN-γ inhibits lytic replication of HV68 in primary macrophages. To determine whether IFN-γ inhibits HV68 replication in infected primary macrophages, bone marrow macrophages derived from wild-type C57BL/6 mice were pretreated with 10 units/ml of IFN-γ, and viral growth was assessed (Fig. 1A). IFN-γ treatment significantly inhibited HV68 replication, resulting in a ~265-fold decrease in titers by 72 h postinfection. For cells plated in medium alone, the HV68 titer increased over input virus levels starting at 24 h postinfection. In contrast, HV68 titers of IFN-γ-treated cultures never surpassed input levels. The IFN-γ-mediated inhibition of HV68 growth was not attributable to increased cell death in IFN-γ-
Stat1 mediates IFN-γ-induced inhibition of γHV68 replication. IFN-γ signaling through its receptor induces the transcriptional regulation of relevant effector genes by the transcription factors Stat1, IRF1, and CIITA (3). We next sought to determine whether these transcription factors were involved in the IFN-γ-mediated inhibition of γHV68 replication in macrophages. The IFN-γ suppression of γHV68 growth was abolished in infected macrophages derived from Stat1−/− mice (Fig. 1C). Furthermore, in untreated cultures, the viral titer from Stat1-deficient cells was increased ~7-fold (P = 0.0311) at 48 h postinfection and ~17-fold (P = 0.0344) at 72 h postinfection compared to wild-type cells (compare Fig. 1A and C). Taken together, these results indicate that Stat1 acts as a negative regulator of γHV68 replication and is required for the IFN-γ-induced inhibition of γHV68 replication. In contrast, interferon regulatory factor 1 (IRF1) and CIITA were not required for basal or IFN-γ-mediated suppression of γHV68 growth (Fig. 1D and E).

Cross talk between the IFN-γ and IFN-α/β signaling pathways may occur at multiple levels (3). Furthermore, there is a precedent for interactions between gamma-herpesvirus proteins and the transcriptional regulators of IFN-α/β signaling, IRF3 and IRF7. IRF3 transcriptional activity is manipulated by the BGLF4 kinase from EBV, the corresponding IRF3 homolog ORF36, and viral IRFs (vIRFs) encoded by KSHV (16, 27, 30, 61). IRF7 regulates the expression of EBNA1 and LMP1 in EBV and interferes with RTA-mediated gene activation in KSHV (40, 62, 72). Moreover, IRF7 function is itself targeted by EBV and KSHV proteins (70, 73). Therefore, we examined whether components of the type I interferon signaling pathway contributed to the IFN-γ inhibition of γHV68 growth. Similar to wild-type cells, IFN-γ treatment suppressed viral replication in macrophages derived from IFN-α/βR−/− mice (Fig. 1F). Additionally, IFN-γ effectively inhibited γHV68 replication in macrophages derived from IRF3−/− or IRF7−/− mice (Fig. 1G and H).

IFN-γ reduces transcript levels of the lytic switch gene 50 via Stat1. Previous work from our laboratory suggested that interferons regulate γHV68 gene expression during chronic infection in vivo (2, 51). Therefore, we hypothesized that IFN-γ might inhibit γHV68 replication in infected macrophages by repressing the expression of lytic switch gene 50. To assess whether the expression of gene 50 was IFN-γ responsive, bone marrow-derived macrophages were pretreated with 10 units/ml of IFN-γ before infection with γHV68. RNA from infected cells was harvested 12 h postinfection, and viral transcripts were measured by quantitative reverse transcription-PCR (qRT-PCR). The time point of 12 h postinfection was selected because no difference in viral titers between medium-treated cultures compared to medium alone, as measured by a lactate dehydrogenase (LDH) release assay (data not shown). Importantly, IFN-γ activity required IFN-γ receptor expression since IFN-γ treatment did not inhibit viral replication in macrophages derived from IFN-γR−/− mice (Fig. 1B). This result indicates that signaling events emanating from the IFN-γ receptor are responsible for the IFN-γ-mediated inhibition of γHV68 replication. This finding is consistent with the requirement for receptor expression in the IFN-γ-mediated suppression of γHV68 reactivation and with the phenotype of infected IFN-γR−/− mice (11, 25, 51, 64).

IFN-γ negatively regulates γHV68 lytic switch gene 50 in a Stat1-dependent manner. (A) IFN-γ treatment decreased gene 50 transcript levels compared to medium alone in wild-type but not Stat1−/− bone marrow-derived macrophages. (B) In contrast, IFN-γ did not alter gene 73 transcript levels. Day 10 bone marrow-derived macrophages were pretreated for 12 h with 10 units/ml of IFN-γ and then infected with γHV68 at an MOI of 10. Total RNA was isolated 12 h postinfection by using TRIzol reagent. RNA was treated with Dnase I (Ambion, Austin, TX) before reverse transcriptase cDNA synthesis was performed using oligo(dT)12-18 and Superscript II (Invitrogen, Carlsbad, CA). qRT-PCR was performed with SYBR green (Invitrogen, Carlsbad, CA) and the following primer sequences: 5′-T GCCCCCATGTGTGTGTATG and 5′-TGTGTCATGCTGAGCGTC TTCG for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-AGAACCCACAGCTTCGACTT and 5′-CAATATGCTGGACAGCCC GTAC for gene 50, and 5′-CAAGAGCTCTGACGGCTCGT and 5′-AGATACCACACGCGTACAAACCGT for gene 73. Transcript levels were normalized to GAPDH within each sample. Data were collected from 4 to 7 independent experiments and calculated using the ∆∆Ct method (29). Data are presented as means ± SEM, and statistical analyses were performed by a Student’s t test.
IFN-γ did not alter transcript levels for immediate-early gene 73, which encodes the LANA protein (Fig. 2B). Importantly, this finding shows that IFN-γ treatment did not significantly interfere with proximal events during infection, such as binding to the cell surface, entry, or uncoating of the viral particle. Furthermore, gene 73 transcript levels were similar in wild-type and Stat1−/− cells (Fig. 2B). Given that gene 73 was not altered by IFN-γ treatment or Stat1 deficiency, IFN-γ and Stat1 control γHV68 by regulating specific viral genes, including gene 50.

Promoters for lytic switch gene 50 are IFN-γ responsive in a Stat1-dependent manner. Previous studies demonstrated that promoters in EBV and KSHV are IFN-α responsive in B cells. The KSHV viral interleukin-6 (vIL-6) promoter is induced in BCP-1 cells, and EBV Qp activity is repressed in Louskes BL cells treated with IFN-α (6, 41). We tested whether IFN-γ suppressed gene 50 expression by acting directly on the promoters for gene 50. Bone marrow-derived macrophages were pretreated with IFN-γ before transfection with a gene 50 promoter-driven luciferase reporter and a β-galactosidase reporter used to normalize for transfection efficiency. Promoter activity was measured 12 h posttransfection. There are two known promoters that drive the expression of distinct gene 50 transcripts (15, 28). A proximal 410-bp promoter is located upstream of exon E1, and a distal 250-bp promoter is found upstream of exon E0 (Fig. 3A). IFN-γ suppressed the 410-bp promoter in a dose-dependent fashion, with a ~32-fold decrease observed at 10 units/ml (Fig. 3C). Similarly, the activity of the 250-bp promoter was repressed by IFN-γ, resulting in a ~15-fold decrease at a dose of 10 units/ml (Fig. 3D). This repression of the activity of gene 50 promoters likely contributes to the decrease in gene 50 transcript levels observed for virally infected cells (Fig. 2A).

IFN-γ repressed both gene 50 promoters in a Stat1-dependent manner, as promoter activity was not significantly changed in Stat1−/− cells treated with 10 units/ml of IFN-γ (Fig. 3E and F). These data show that the promoters for gene 50 are IFN-γ responsive in a Stat1-dependent manner independent of the possible effects of other viral genes on these promoters. Furthermore, basal promoter activity was increased in Stat1−/− cells compared to wild-type cells (compare Fig. 3C and E [P = 0.0009] and D and F [P = 0.0013]). This observation is consistent with Stat1 acting on the gene 50 promoters to negatively regulate basal gene expression.

Stat1 homodimers bind to gamma-activated sequence (GAS) elements, which have been well characterized (9). Sequence analysis using MatInspector software identified two putative consensus GAS elements in the 410-bp promoter and one putative GAS element in the 250-bp promoter (Fig. 3B). To determine whether these sites were required for IFN-γ to repress the activity of the gene 50 promoters, point mutations were introduced to disrupt the predicted GAS sites (Fig. 3B). These mutations were previously shown to disrupt Stat1 binding and transcriptional activation (9, 13, 19). IFN-γ repressed the activity of the 410-bp and 250-bp GAS mutant promoters in a dose-dependent manner (Fig. 3G and H). Furthermore, mutating the predicted GAS elements in the gene 50 promoters did not significantly change basal promoter activity (compare Fig. 3C and G, and compare D and H). Taken together, these data suggest that IFN-γ represses the gene 50 promoters in a Stat1-dependent manner but independent of the predicted gene 50 promoter GAS elements.

Mechanisms of Stat1 negative regulation of γHV68. Stat1 positively regulates hundreds of cellular genes in response to IFN-γ. A multitude of mechanistic studies provide a well-characterized model for Stat1-dependent gene activation that involves chromatin remodeling, the recruitment of transcriptional coactivators, cooperation with general and inducible transcription factors, interactions with core transcriptional machinery, and binding to GAS promoter elements (reviewed in reference 44). In contrast, IFN-γ negatively regulates a small number of genes that are generally involved in the cell cycle, the regulation of the extracellular matrix, or chemokine signaling (43, 44). IFN-γ-mediated gene repression is complex and not well understood. Negative regulation is typically Stat1 dependent, but in the case of cyclin A and the bullous pemphigoid antigen gene BPAG1, repression is mediated by promoter regions that do not contain consensus GAS sites (47, 56).

Our findings show that, in addition to being essential for the actions of IFN-γ in the control of viral replication, gene 50 expression, and the activity of the known gene 50 promoters, Stat1 negatively regulates the basal expression of gene 50 and the gene 50 promoters. The constitutive expression of several cellular genes is positively regulated by Stat1 (7, 18, 22, 23, 53). For instance, the level of expression of some caspase genes is reduced in Stat1−/− cells, and expression can be rescued with a Stat1 mutant that cannot be phosphorylated or form canonical Stat1 homodimers (18, 23). Emerging evidence suggests that the regulation of gene expression by unphosphorylated Stat1 (U-Stat1) is mechanistically distinct from that by ligand-induced phosphorylated Stat1 (reviewed in reference 68). For example, U-Stat1 monomers can bind to half of a GAS palindrome, leaving the N-terminal domain free for protein-protein interactions, as seen for the promoter for the proteosome component LMP2 (7). Defining whether Stat1 phosphorylation sites, interaction domains, or other structural features are required to regulate gene 50 expression may provide valuable insights into the mechanisms of IFN-γ-mediated gene repression and Stat1 ligand-independent functions.

Implications of an IFN-γ-responsive lytic switch gene in γHV68. Given that gene 50 is the only γHV68 gene that is known to be necessary and sufficient to trigger the lytic cycle, it seems likely that the IFN-γ regulation of gene 50 contributes to the control of viral replication. This idea is bolstered by our finding that the gene 50 promoters themselves are IFN-γ responsive in uninfected cells, where the potential for indirect contributions from the virus are eliminated. Host cytokines were previously shown to regulate the interchange between gammaherpesvirus life cycles. Transforming growth factor β (TGF-β) induces EBV reactivation and stimulates the lytic switch gene BZLF1 (12, 26). Notably, de novo protein synthesis is required to induce BZLF1 expression, and the Zp promoter is not TGF-β responsive in uninfected cells, suggesting an indirect mechanism of action (12). IL-6 and IFN-γ induce gene 50 expression and infectious virus production in latently infected KSHV BCBL-1 cells, but their ability to manipulate
Promoter activity is unknown (5, 36, 48). We provide here the first example, to our knowledge, of an inflammatory cytokine directly regulating the promoter of a gammaherpesvirus lytic switch gene.

It is striking that if HV68 maintains an IFN-γ-responsive lytic switch gene given that IFN-γ is a key regulator of chronic infection in vivo. Perhaps the induction of the lytic cycle is restricted by the IFN-γ-mediated repression of gene 50 promoter activity, thus ensuring that if HV68 can establish latency and lifelong infection without untoward damage to the host. Determining whether latency is controlled directly by cell-extrinsic factors in vivo, and not only by viral and host genes within an infected cell, will be of fundamental importance.

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H.W.V. was supported by NIH grant RO1 CA 96511, and M.M.G. was supported by a National Science Foundation graduate research fellowship.
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