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Differential Regulation of Endogenous and Transduced β-Globin Genes during Infection of Erythroid Cells with a Herpes Simplex Virus Type 1 Recombinant

CRAIG A. SMIBERT AND JAMES R. SMILEY*

Molecular Virology and Immunology Program, Pathology Department, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

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We infected murine erythroleukemia cells with a nondefective herpes simplex virus (HSV) type 1 recombinant bearing the rabbit β-globin gene under the control of its own promoter, in order to compare the regulation of a cellular gene residing in the viral genome to that of its active endogenous counterpart. We found that the viral globin gene was activated by HSV immediate-early polypeptides, whereas expression of the endogenous β-globin gene was strongly suppressed: transcription was greatly inhibited, and β-globin mRNA was rapidly degraded. Degradation of globin mRNA was induced by a component of the infecting virion and required a functional UL41 gene product. These results demonstrate that HSV products can have opposing effects on the expression of homologous genes located in the cellular and viral genomes and suggest that the preferential expression of HSV genes that occurs during infection is not achieved solely through sequence-specific differentiation between viral and cellular promoters or mRNAs.

Herpes simplex virus type 1 (HSV-1) infection strongly inhibits the expression of most endogenous cellular genes (18), while three classes of viral genes are sequentially activated to high levels in a regulatory cascade driven by viral products (34, 82). This differential regulation encompasses a variety of transcriptional and posttranscriptional controls, and many of the HSV-1 gene products that contribute to these events have been identified. However, in most cases little is known about the precise mechanisms involved. In addition, the structural features that render viral genes preferred targets for activation and most cellular genes targets for shutoff remain largely obscure.

HSV genes are transcribed in the nucleus by host RNA polymerase II. Transcription of the five viral immediate-early (IE) genes is stimulated by a structural component of the virion, Vmw65, that is delivered into cells upon infection (2, 7, 66). Vmw65 forms a complex with the cellular transcription factor OCT1, which binds to the TAATGARATTC (where R is a purine) motifs that are present in all of the IE regulatory regions (3, 6, 22, 23, 42, 43, 47, 59, 67, 68). Thus, activation of IE transcription involves recognition of class-specific *cis*-acting regulatory sequences that are distinct from basal promoter elements. Four of the IE proteins (ICP4, ICP27, ICP0, and ICP22) in turn contribute to the activation of the viral early (E) and late (L) genes (for a review, see reference 16). Three lines of evidence suggest that activation of E and L transcription by HSV IE polypeptides does not require recognition of specialized, induction-specific sequences in the responding promoters. First, E and L regulatory regions do not display obviously conserved class-specific sequences analogous to the TAATGARATTC motif. Second, mutational analyses of HSV E and L promoters have shown that activation by IE polypeptides requires only basal promoter elements in *cis* (9, 12, 13, 25, 32, 33, 35, 36, 52–54, 72); indeed, certain L promoters that consist of only a TATA box-cap site region are efficiently induced (32, 35). Third, HSV IE polypeptides can activate some, but not all, heterologous genes that have been newly introduced into cells by transfection or as part of an infecting HSV-1 genome (13–15, 17, 55, 60, 63, 75, 79, 80). Taken in combination, these observations have led to the hypothesis that HSV IE polypeptides activate transcription by interacting with one or more components of the core transcription machinery, such as the TATA box factor TF IID (9, 13, 16, 17, 52).

The relatively relaxed target promoter specificity of IE polypeptides displayed on newly introduced templates contrasts with the overall inhibition of cellular gene expression that occurs during infection. The various responses of cellular globin genes to HSV infection illustrate this point: HSV IE proteins activate expression of globin genes that have been newly introduced into fibroblasts (13–15, 63, 75), although the endogenous β-globin gene is not induced (15). Indeed, cellular globin gene expression is massively suppressed and globin mRNA is degraded during infection of erythroid cells (57, 58). One interpretation of these observations is that at least some of the mechanisms that contribute to the differential expression of viral and cellular genes in infected cells do not involve sequence-specific differentiation between viral and cellular promoters or RNAs. Possibly relevant features that might serve to distinguish the responses of endogenous and newly introduced genes include differences in higher-order packaging and/or subnuclear localization. Alternatively, some of these seemingly disparate responses could be due to variations in regulatory mechanisms, depending on the host cell types or virus strains used; for example, some evidence suggests that the shutoff mechanisms in erythroid cells might differ from those that operate in other cell types (reviewed below). In order to distinguish between these possibilities, it is necessary to examine the regulation of a newly introduced globin gene during infection of a cell type in which endogenous globin gene expression is strongly suppressed by HSV-1 gene products.

HSV-1-induced inhibition of endogenous cellular gene expression involves repression of host transcription and suppression of ongoing translation (for a review, see reference 18). Early studies indicate that HSV-1 infection

* Corresponding author.

strongly inhibits transcription of integrated polyomavirus and adenovirus genomes and suggest that one or more IE polypeptides other than ICP4 are required for this negative regulation (65, 77). More recent studies show that transcription of many (but not all) endogenous cellular genes is suppressed during infection (37, 50, 64); in several cases, the IE polypeptide ICP22 appears to be required for this inhibition (37). The molecular mechanisms underlying these transcriptional effects remain to be clarified. To date, the effects of HSV products on transcription of active endogenous globin genes have not been reported.

HSV-1-induced suppression of host protein synthesis is accompanied by degradation of cellular mRNAs (57). Nishioka and Silverstein found that shutoff of globin synthesis during infection of murine erythroleukemia (MEL) cells with HSV-1 strain F could be resolved into two phases: a virion component first disrupts polysomes in the absence of de novo viral gene expression and then globin mRNA is degraded in response to newly synthesized viral polypeptides (58). In contrast, HSV-1 strain KOS inhibits host translation and degrades cellular mRNAs in Vero cells in the absence of de novo viral gene expression (44, 45, 70, 71, 78). Read and Frenkel (70) isolated a viable KOS mutant designated *vhs*1 that is defective in these processes, and more recent studies have mapped the *vhs*1 mutation to the HSV-1 UL41 open reading frame (45). Apparently, the UL41 gene product is a virion component that directly or indirectly triggers rapid turnover of cellular and viral mRNAs. Thus, distinct mechanisms appear to be involved in the RNA turnover induced by HSV-1 KOS in Vero cells and HSV-1 F in MEL cells. It is possible that these disparities reflect the use of different virus strains; alternatively, Vero and MEL cells may respond in different ways to HSV-1 host shutoff functions. Data consistent with either of these possibilities have been reported. Various HSV-1 and HSV-2 strains appear to differ in their ability to effect virion-induced shutoff when assayed in a single cell type (19–21, 29, 30), and at least some isolates of HSV-1 KOS have been reported to be defective for virion-induced shutoff of globin synthesis in MEL cells (29, 30).

We previously described the isolation and characterization of a nondefective HSV-1 KOS recombinant (L7/14), which bears the rabbit β-globin gene and 1,200 nucleotides (nt) of 5′-flanking globin sequences inserted into the viral thymidine kinase gene (*tk*) in the *tk* antisense orientation (75). These studies demonstrated that the β-globin gene was expressed to high levels from its own promoter as an HSV E gene during lytic infection of Vero cells. This result was intriguing, as it appeared to contrast with those of earlier studies, in which globin gene expression was suppressed following infection of MEL cells with HSV-1 strain F (57, 58). We therefore examined the regulation of endogenous and transduced globin genes during infection of MEL cells with KOS L7/14. Our results show that the viral globin gene was regulated as an HSV E gene in this cell type, while expression of the endogenous β-globin gene was strongly suppressed at the transcriptional and posttranscriptional levels. Preexisting mouse β-globin RNA was rapidly degraded, as originally reported by Nishioka and Silverstein (57, 58). However, in our experimental system, RNA turnover was induced by a virion component and required the product of the UL41 gene (*vhs*). These results demonstrate that HSV regulatory products can have opposing effects on the expression of homologous genes located in the cellular and viral genomes.

MATERIALS AND METHODS

Cells and virus. The Friend virus-transformed MEL cell line 745aJG6 was obtained from A. Bernstein, Mount Sinai Research Institute, Toronto, Ontario, Canada. Vero cells were from the American Type Culture Collection. Vero and MEL cells were maintained in α minimal essential medium (GIBCO) supplemented with 5 and 10% fetal calf serum, respectively. The viral strains HSV-1 KOS PAA^{r}5 (28), PAA^{r}5 L7/14 (75), and KOS *vhs*1 (70) were propagated, and the titers of the virus were determined in Vero cells.

Infection of MEL cells. MEL cells were diluted to a density of 2×10^4 cells per ml and maintained until a density of 1.5×10^5 cells per ml was reached. Terminal erythroid differentiation was induced by adding hexamethylene bisacetamide to a final concentration of 5 mM. After 3.5 days in the inducer solution, cells were pelleted, washed in serum-free medium, and suspended to a final density of approximately 2×10^7 cells per ml. Virus was adsorbed for 1.5 h, and medium containing 10% serum was added to obtain a cell density of 1×10^6 cells per ml.

RNA extraction. Cytoplasmic RNA was prepared by the method of Berk and Sharp (4).

S1 nuclease mapping and primer extension. S1 nuclease mapping and primer extension were performed as previously described (75). The following synthetic primers were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University: (i) glycoprotein D (gD), 5′-CCCCATACCGGAACGCACCA CACAA-3′, predicted extension product of ca. 80 to 90 nt (83); (ii) rabbit β-globin, 5′-CCGCAGACTTCTCCTCAC TGGACAG-3′, predicted extension product of ca. 87 nt (11); and (iii) mouse β-globin, 5′-AAGAGACAGCAGCCTTCT CAGCATC-3′, predicted extension product of ca. 92 nt for the transcript of the β-globin major gene (41).

Nuclear run-on transcription assays. Isolation of nuclei, nuclear run-on transcription, and hybridization of transcripts to single-stranded DNA were performed essentially as described previously (49) with some modifications. Run-on transcripts were extracted from nuclei as described by Greenberg an Bender (26). Cells (5×10^7) were pelleted by low-speed centrifugation, suspended in 1 ml of buffer I (0.32 M sucrose, 3.0 mM $CaCl_2$, 2.0 mM magnesium acetate, 0.1 mM EDTA, 0.1% Triton X-100, 1.0 mM dithiothreitol [DTT], 10 mM Tris hydrochloride [pH 8.0]) containing 40 U of RNasin (Promega Biotec Corp.) per ml, and then disrupted in a Dounce homogenizer. The extract was diluted with 2 volumes of buffer II (2.0 M sucrose, 5.0 mM magnesium acetate, 0.1 mM EDTA, 1.0 mM DTT, 10 mM Tris hydrochloride [pH 8.0]) containing 40 U of RNasin per ml and then layered onto 1.8 ml of buffer II and centrifuged at 130,000 × *g* for 45 min in an SW50.1 rotor. The nuclear pellet was suspended in 100 μl of 5.0 mM magnesium acetate–0.1 mM EDTA–5.0 mM DTT–50.0 mM Tris hydrochloride (pH 8.0) containing 200 U of RNasin per ml and 25% glycerol and was stored in liquid nitrogen.

For run-on transcription assays, 10^7 nuclei in 100 μl were combined with 30 μl of a 3.33 mM solution of ATP, CTP, and GTP, 40 μl of 0.6 M KCl–12.5 mM magnesium acetate, and 25 μl (250 μCi) of [α-^{32}P]UTP, and the solution was incubated for 35 min at 37°C. Labeled transcripts were isolated as follows: *Escherichia coli* tRNA was added to 100 μg/ml, and nuclei were treated with 20 μg of pancreatic DNase I (Boehringer Mannheim Biochemicals) per ml for 10 min at 37°C. Sodium dodecyl sulfate (SDS) was added to 0.5%, followed by digestion with proteinase K (300 μg/ml) for 15

min at 37°C. A portion was removed and assayed for incorporation into acid-insoluble material. The remainder of the reaction mixture was extracted with phenol-chloroform and precipitated with 5% trichloroacetic acid containing 30 mM sodium pyrophosphate. The precipitate was collected on a nitrocellulose filter, washed extensively with 5% trichloroacetic acid–30 mM sodium pyrophosphate, and then eluted by incubation in 1% SDS–10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA at 65°C for 10 min. The elution was repeated, and the pooled eluate was extracted with phenol-chloroform and precipitated with ethanol. Typically, ca. 4×10^7 dpm were incorporated per 10^7 nuclei. Infected and uninfected nuclei routinely displayed similar levels of overall transcriptional activity.

Specific run-on transcripts were detected by hybridization to single-stranded recombinant M13 DNAs immobilized on nitrocellulose. Mouse β-globin RNAs were detected by using a 3.5-kilobase (kb) *Eco*RI-*Xba*I fragment containing the entire globin gene and 1 kb of 5′-flanking sequences, cloned between the *Eco*RI and *Xba*I sites of M13mp19. HSV-1 *tk* RNAs were detected by using a 1.3-kb *Eco*RI-*Sma*I fragment of HSV-1 KOS DNA, inserted between the *Eco*RI-*Sma*I sites of M13mp19. Filters bearing 10 μg of DNA per slot were pretreated and then hybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS–1 mM EDTA–10 mM Tris hydrochloride (pH 7.5)–2× Denhardt solution–250 mg each of salmon sperm DNA and *E. coli* tRNA per ml in 50% formamide for 36 h at 52°C. Following hybridization, filters were washed extensively in 2× SSC–0.1% SDS at 68°C and treated with 10 μg of RNase A per ml for 20 min at 37°C.

Construction of HSV-1 mutants bearing disruptions at the UL41 locus. A 3.6-kb *Hin*dIII-*Hpa*I fragment of HSV-1 KOS PAA^{r}5 DNA, bearing the UL41 open reading frame and ca. 1 kb of 5′- and 3′-flanking sequences, were cloned between the *Hin*dIII and *Sma*I sites of pUC19, generating pUL41.

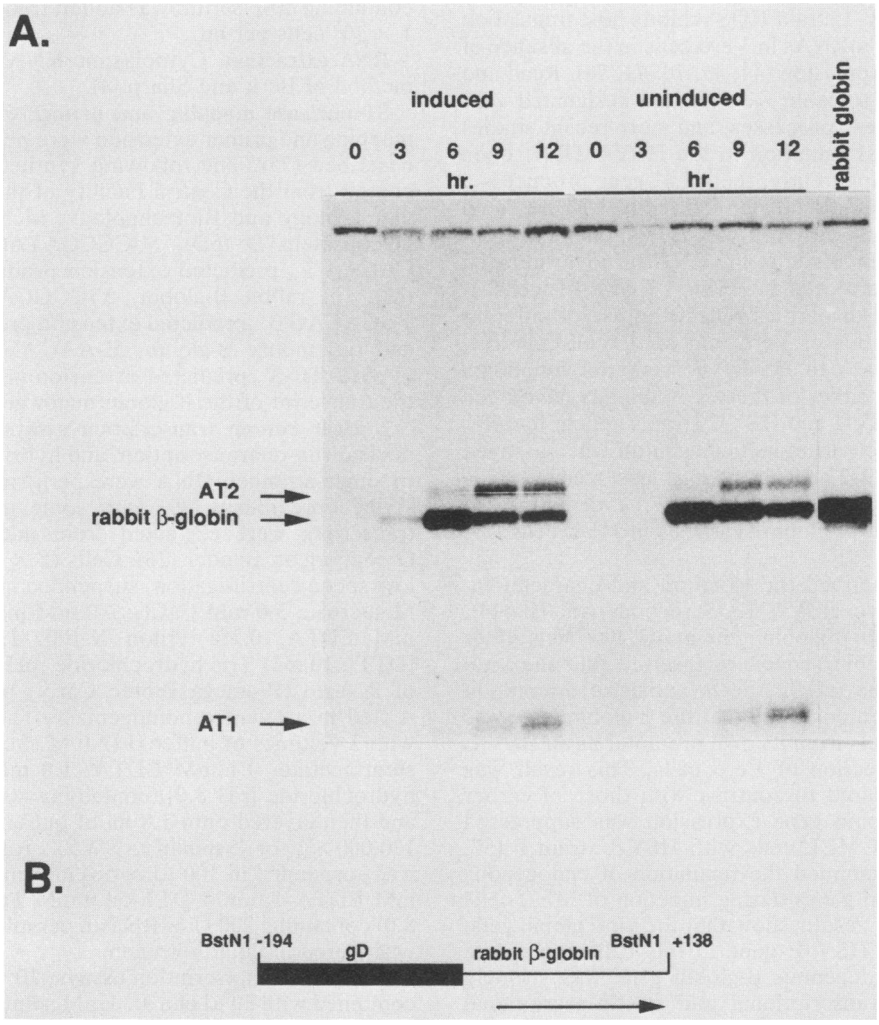

FIG. 1. Expression of the rabbit β-globin gene during infection of MEL cells. (A) Actively proliferating (uninduced) and terminally differentiated (induced) MEL cell cultures were infected with 10 PFU of L7/14 per cell, and cytoplasmic RNAs harvested at the indicated times postinfection were scored for rabbit β-globin transcripts. RNA (10 μg) was hybridized to the 5′-labeled single-stranded globin probe shown in panel B, and the resulting hybrids were digested with S1 nuclease. Arrows mark the positions of the signals corresponding to correctly initiated globin transcripts and the aberrant AT1 and AT2 transcripts described in the text. A portion of the probe fragment was also hybridized to 0.5 ng of purified rabbit globin mRNA (40% β-globin) to provide a standard for correctly initiated transcripts (rabbit globin). The autoradiograph was exposed for 15 h. (B) Structure of the S1 probe. The probe was derived from a previously described gD-globin fusion gene (12) and allows detection of correctly initiated rabbit β-globin RNAs.

Mutant derivatives bearing disruptions of the UL41 open reading frame were generated by inserting a 4,267-base-pair (bp) *Bam*HI fragment bearing the *E. coli lacZ* gene driven from the HSV-1 ICP6 promoter (24; kindly provided by S. Weller) into pUL41 in the UL41 orientation (see Fig. 6). Mutant *vhs*A was generated as follows: pUL41 was cleaved with *Apa*I, treated with T4 DNA polymerase, and ligated to the *lacZ Bam*HI fragment (repaired with T4 DNA polymerase). A plasmid bearing the *lacZ* cassette inserted across the two UL41 *Apa*I sites was identified. This mutation deletes sequences predicted to encode residues 22 to 179 of the 489-residue UL41 gene product and separates sequences downstream from residue 179 from the UL41 promoter. Mutant *vhs*B contains the *lacZ* cassette inserted at the unique *Bam*HI site in UL41; this mutation disrupts the open reading frame downstream of sequences predicted to encode residue 342.

The *vhs*A and *vhs*B mutations were transferred from plasmid DNA into the viral genome by DNA-mediated marker transfer, as previously described (73, 74). Following cotransfection of Vero cells with infectious PAA^{r}5 viral DNA and the appropriate plasmid, recombinants bearing the desired mutation were recognized as blue plaques after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plaques were allowed to develop for 2 days in α minimal essential medium containing 5% fetal bovine serum and 0.05% human immune globulin and then were overlaid with 0.5% agarose in F11 medium containing 5% fetal bovine serum and 300 μg of X-Gal per ml. The structures of the mutant viral genomes were then confirmed by Southern blot hybridization of viral DNA (76).

RESULTS

Expression of a transduced rabbit β-globin gene during lytic infection of mouse erythroid cells with an HSV-1 recombinant. The recombinant HSV-1 strain KOS PAA^{r}5 L7/14 bears the rabbit β-globin gene inserted into the viral *tk* gene in the *tk* antisense orientation. We previously reported that the β-globin gene in this strain is expressed from its own promoter and regulated as an HSV E gene during lytic infection of Vero cells (75). These results appeared to contrast with those reported in earlier studies, which indicated that HSV-1 infection of murine erythroid cells dramatically inhibits the expression of endogenous globin genes (57, 58). We tested the possibility that these disparate outcomes arose as result of the different cell types and/or virus strains used, by monitoring the expression of HSV-associated and endogenous β-globin genes during infection of MEL cells with strain L7/14.

MEL cells can be induced to undergo terminal erythroid differentiation by exposure to a variety of agents, including hexamethylene bisacetamide (for a review, see reference 48). Following induction, globin gene transcription is activated (31) and globin mRNAs are stabilized (81). As a result, globin mRNAs accumulate to extremely high levels over the course of several days. We infected uninduced and differentiated MEL cell cultures with strain L7/14 and then scored for the expression of the viral β-globin gene by S1 nuclease analysis (Fig. 1A). The results indicated that the rabbit β-globin gene was transcribed from its own promoter with a time course similar to that of an HSV E RNA: the levels of correctly initiated β-globin RNA peaked at 6 h postinfection and declined somewhat thereafter. We also observed two additional aberrant globin-related transcripts that accumulated at later times postinfection (AT1 and AT2). The AT1 and AT2 transcripts have been observed in previous studies and were shown to represent aberrantly spliced and readthrough transcripts, respectively (75). The pattern of viral globin gene expression was not greatly influenced by the differentiated state of the host cells: indeed, the levels and temporal course of accumulation of globin RNAs in both cell populations were similar to those previously observed during infection of fibroblasts (additional data not shown). Thus, the factors that lead to increased globin gene expression during erythroid cell differentiation had little effect on the expression of a β-globin gene residing in the HSV-1 genome.

During infection of Vero cells, expression of a β-globin gene residing in the HSV-1 genome requires the prior synthesis of HSV IE polypeptides (63, 75). To determine whether IE polypeptides are also required in erythroid cells, we infected differentiated MEL cells in the continuous presence of cycloheximide. The levels of globin mRNAs arising from the viral genome were then assayed by primer extension by using an oligonucleotide complementary to nucleotides +63 to +87 of rabbit β-globin mRNA (Fig. 2). We found that cycloheximide greatly inhibited the accumulation of rabbit β-globin RNA. Taken in combination with the results shown in Fig. 1, the data suggested that the rabbit β-globin gene was controlled as an HSV E gene when it was delivered into erythroid cells as part of an infecting HSV-1 genome.

Inhibition of endogenous β-globin gene expression during infection of MEL cells with L7/14. Previous studies have indicated that HSV-1 infection of MEL cells strongly inhibits endogenous globin gene expression, at least in part by inducing degradation of globin mRNA (57, 58). To determine whether endogenous β-globin gene expression was inhibited during infection with L7/14, we assayed the same RNA samples that were analyzed in Fig. 1 for the presence of mouse β-globin mRNAs by S1 nuclease analysis (Fig. 3A). As expected, uninfected induced MEL cells contained extremely high levels of β-globin mRNA. Although a direct quantitative comparison with the levels of rabbit globin RNA arising from the viral genome is difficult because different S1

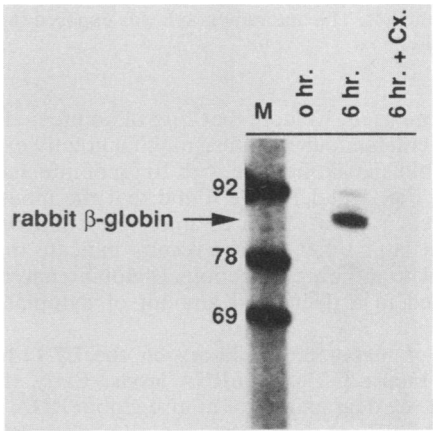

FIG. 2. Effects of cycloheximide on accumulation of rabbit β-globin RNA. Differentiated MEL cells were infected with 10 PFU of L7/14 per cell in the presence or absence of 100 μg of cycloheximide (Cx.) per ml, and RNA samples prepared at 6 h postinfection were scored for rabbit β-globin transcripts by primer extension analysis (see Materials and Methods). Markers (lane M) were 3′-end-labeled *Hpa*II fragments of pBR322 DNA. Marker fragment sizes (in nucleotides) are shown on the left.

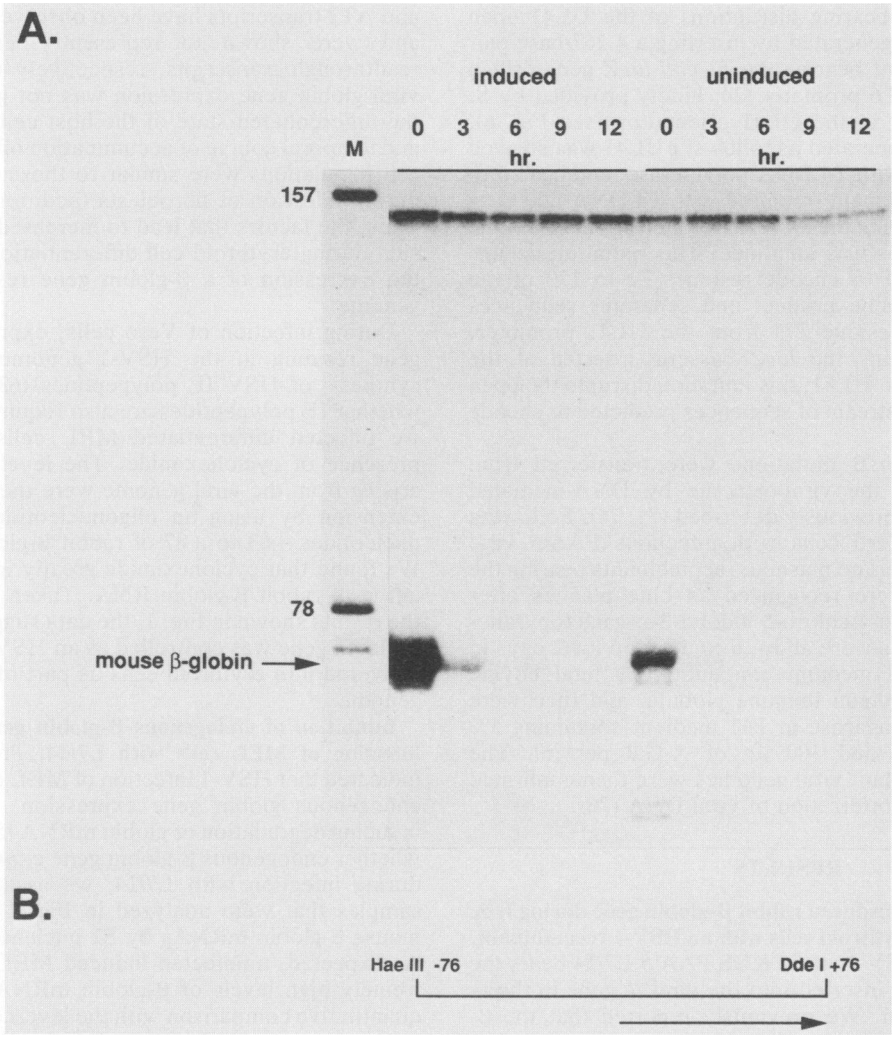

FIG. 3. Mouse β-globin RNA levels during infection of MEL cells with L7/14. (A) One microgram of each of the RNA samples analyzed in Fig. 1 was annealed to the 5′-labeled single-stranded mouse β-globin probe fragment shown in panel B, and the resulting hybrids were digested with S1 nuclease. Markers (lane M) were 3′-end-labeled *Hin*fI fragments of pTK173. Marker fragment sizes (in nucleotides) are shown on the left. The autoradiograph was exposed for 3 h. (B) S1 probe, extending from −76 to +76 relative to the mouse β-globin major gene cap site.

probes were used, we note that 1 μg of uninfected RNA gave rise to an endogenous globin signal that greatly exceeded the rabbit globin signal obtained with 10 μg of infected cell RNA (compare Fig. 1 and 3). We found that the mouse β-globin RNA levels rapidly declined upon infection and did not recover at later times. These results indicate that L7/14 is competent to inhibit endogenous β-globin gene expression, as reflected in a decreased amount of cytoplasmic globin RNA.

Effects of metabolic inhibitors on the L7/14-induced decrease of mouse β-globin mRNA levels. Early studies indicated that the degradation of mouse globin RNA induced by HSV-1 strain F requires de novo viral gene expression (58). These results contrast with more recent data suggesting that degradation of cellular mRNAs induced by HSV-1 strain KOS in Vero cells is mediated by a component of the infecting virion encoded by the *vhs* gene (44, 45, 71, 78). We examined the requirements for the decline of endogenous β-globin RNA observed during infection of MEL cells with L7/14 (a KOS derivative). Differentiated MEL cells were infected with L7/14 in the continuous presence of either dactinomycin, to inhibit transcription, or cycloheximide, to inhibit protein synthesis. RNA samples were then scored for mouse β-globin RNA by primer extension with a primer complementary to +68 to +92 of the transcript (Fig. 4). We found that neither drug prevented the rapid decrease in globin RNA levels in L7/14 infected cells, suggesting that de novo viral gene expression was not required. Control experiments indicated that little if any decline occurred over the course of the experiment in mock-infected cultures treated with cycloheximide or dactinomycin, results that are in keeping with the known stability of β-globin RNA (81). Taken in combination, these data suggested that a component of the infecting L7/14 virion reduced the levels of mouse β-globin mRNA (at least in part) by destabilizing preexisting cytoplasmic RNA.

Involvement of the *vhs* function in induced RNA turnover. The observation that L7/14-induced turnover of endogenous β-globin mRNA occurred in the absence of de novo viral gene expression was consistent with the hypothesis that this

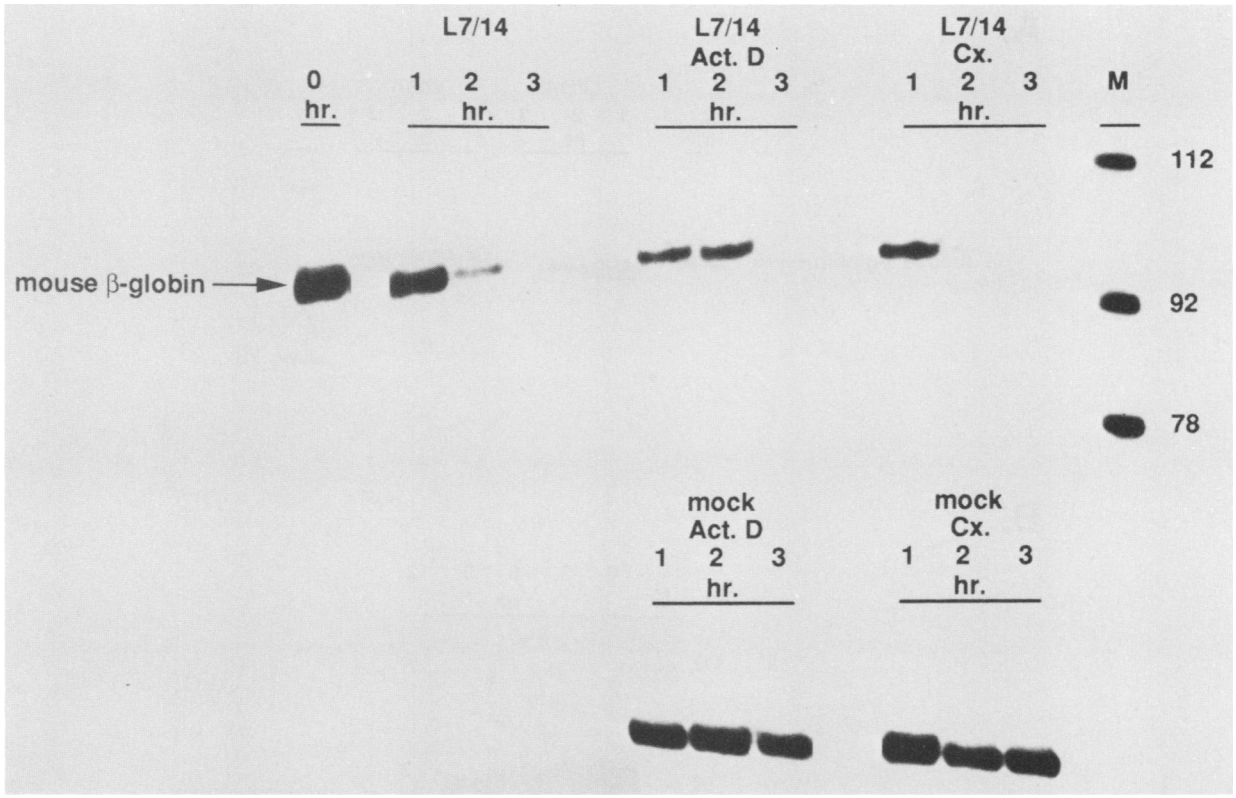

FIG. 4. Effects of inhibitors on the decline of mouse β-globin RNA levels induced by L7/14. Differentiated MEL cells were infected with 10 PFU of L7/14 per cell, and cytoplasmic RNAs were prepared at the indicated times postinfection. The levels of mouse β-globin mRNA were assessed by primer extension (see Materials and Methods). Where indicated, dactinomycin (Act. D; 10 μg/ml) or cycloheximide (Cx.; 100 μg/ml) was added at the time of infection and maintained continuously. Markers (lane M) were 3'-labeled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

process is induced by the virion-associated *vhs* function. The *vhs*1 mutant was isolated on the basis of its inability to induce virion-mediated shutoff of host translation (70). More recently, the *vhs*1 mutant has been shown to be defective in reducing the half-lives of cellular and viral mRNAs when assayed in Vero cells (44, 45, 61, 62, 78). To test the involvement of the *vhs* function in the degradation of mouse β-globin RNA, we infected hexamethylene bisacetamide-induced MEL cells with L7/14 and the *vhs*1 mutant of HSV-1 strain KOS and then monitored cytoplasmic globin RNA levels by primer extension (Fig. 5). L7/14 induced a dramatic decline in globin RNA levels within 3 h, whereas the *vhs*1 mutant had little effect over the same time course. However, globin RNA levels were observed to decline at later times postinfection (Fig. 5B), after the onset of gD RNA accumulation (gD is an HSV E gene). This late decline of globin mRNA levels during *vhs*1 infection most likely represents the secondary host shutoff effect for which the *vhs*1 mutant is known to be competent (70). These data suggest that the rapid degradation of mouse β-globin mRNA induced by L7/14 relies on the *vhs* function.

Construction of HSV-1 KOS PAA^{r}5 mutants bearing disruptions of the UL41 open reading frame. The *vhs*1 mutant was isolated after random mutagenesis (70), and as such this strain may contain other mutations in addition to the characterized defect in UL41. Although previous studies clearly demonstrated that the lesion in UL41 is responsible for the host shutoff defect of the *vhs*1 strain in Vero cells (45), we felt that it was important to confirm that the UL41 gene product is required for RNA turnover in MEL cells; this seemed particularly appropriate in view of reports that certain naturally occurring isolates of strain KOS are unable to effect virion-induced shutoff in this cell type (29, 30). An additional goal was to examine the effects of UL41 mutations in the same genetic background as L7/14. We therefore constructed two derivatives of HSV-1 KOS PAA^{r}5 (the parent of L7/14) bearing disruptions of the UL41 open reading frame. In order to facilitate the identification of mutants, we employed the *E. coli lacZ* gene as an insertional mutagen. A previously described cassette bearing the *lacZ* gene under the control of the HSV-1 ICP6 promoter (24) was inserted into plasmid-borne copies of the UL41 gene, and the resulting mutations were transferred into the viral genome by homologous in vivo recombination (see Materials and Methods).

Mutant *vhs*A contains the *lacZ* cassette inserted between the two *Apa*I sites in the UL41 open reading frame; as a result, sequences predicted to encode residues 22 to 179 of the 489-residue polypeptide (51) were deleted and sequences downstream of residue 179 were dissociated from the UL41 promoter (Fig. 6). Mutant *vhs*B bears the *lacZ* cassette inserted at the unique *Bam*HI site (Fig. 6). This lesion disrupts the open reading frame after residue 342; on the basis of the sequence of the ICP6 promoter region (51), this construct is predicted to produce a ca. 43-kilodalton truncated UL41-related polypeptide. In order to verify the structures of the mutant genomes, viral DNAs were cleaved with a mixture of *Hin*dIII, *Kpn*I, and *Hpa*I and hybridized to

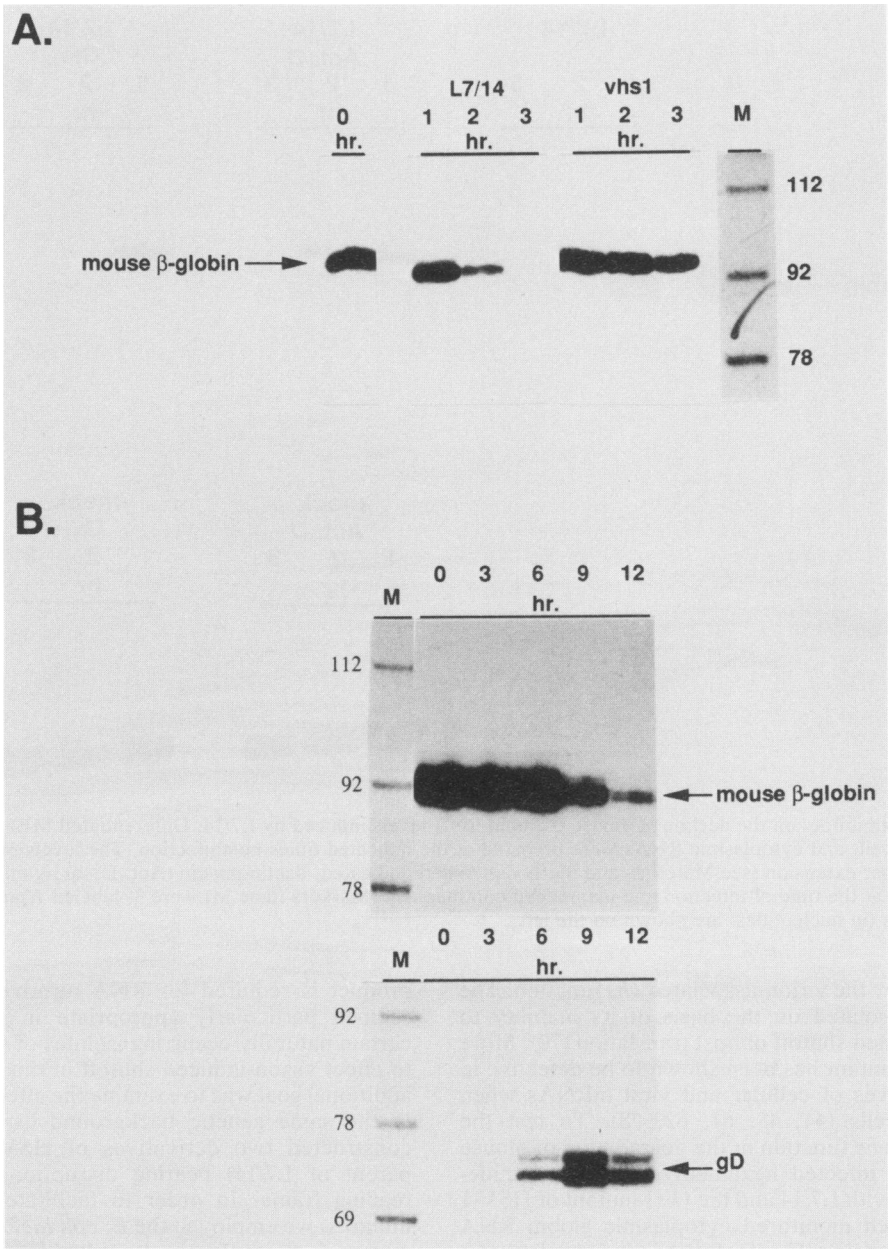

FIG. 5. Effect of the *vhs*1 mutation on induced turnover of mouse β-globin RNA. Differentiated MEL cells were infected with 10 PFU of the indicated virus strain per cell, and cytoplasmic RNAs were scored for mouse β-globin transcripts by primer extension analysis. (A) Comparison of L7/14 and *vhs*1 over a 3-h time course; (B) results of a 12-h time course with *vhs*1. RNA samples were also scored for HSV-1 gD transcripts by primer extension (see Materials and Methods). Molecular weight markers (lanes M) were 3′-labeled *Hpa*II fragments of pBR322 DNA. Marker fragment sizes (in nucleotides) are shown on the left.

a nick-translated plasmid bearing the *vhs*B mutation (Fig. 7). Substitution of the UL41 internal *Apa*I fragment with the *lacZ* cassette in mutant *vhs*A led to the replacement of the 3,354-bp wild-type *Kpn*I-*Hpa*I fragment with UL41-*lacZ* fusion *Kpn*I-*Hpa*I fragments of 3,742 and 1,950 bp and the acquisition of an 834-bp *Kpn*I-*Hpa*I fragment derived from the *lacZ*-ICP6 fusion (an additional 624-bp *lacZ Hpa*I fragment, not visible in Fig. 7, was also observed). Similarly, insertion of the *lacZ* cassette at the *Bam*HI site in mutant *vhs*B disrupted the wild-type 3,354-bp *Kpn*I-*Hpa*I fragment and gave rise to the predicted UL41-*lacZ* fusion fragments of 3,255 and 2,908 bp, along with the 834-bp *lacZ*-ICP6 fragment and the 624-bp *lacZ* fragment (not visible in Fig. 7). These data demonstrate that both mutant viral genomes bear the predicted sequence disruptions at the UL41 locus.

The *vhs*A and *vhs*B strains are capable of replication in Vero cells, although both display a ca. 5- to 10-fold reduced burst size. These data suggest that the UL41 protein does not serve an essential structural or regulatory role in tissue culture. Similar results have been obtained by G. S. Read and K. Knight (personal communication).

Effects of UL41 gene disruptions on induced turnover of globin RNAs. We tested the effects of the *vhs*A and *vhs*B mutations on the ability of KOS PAA^{r}5 to effect virion-

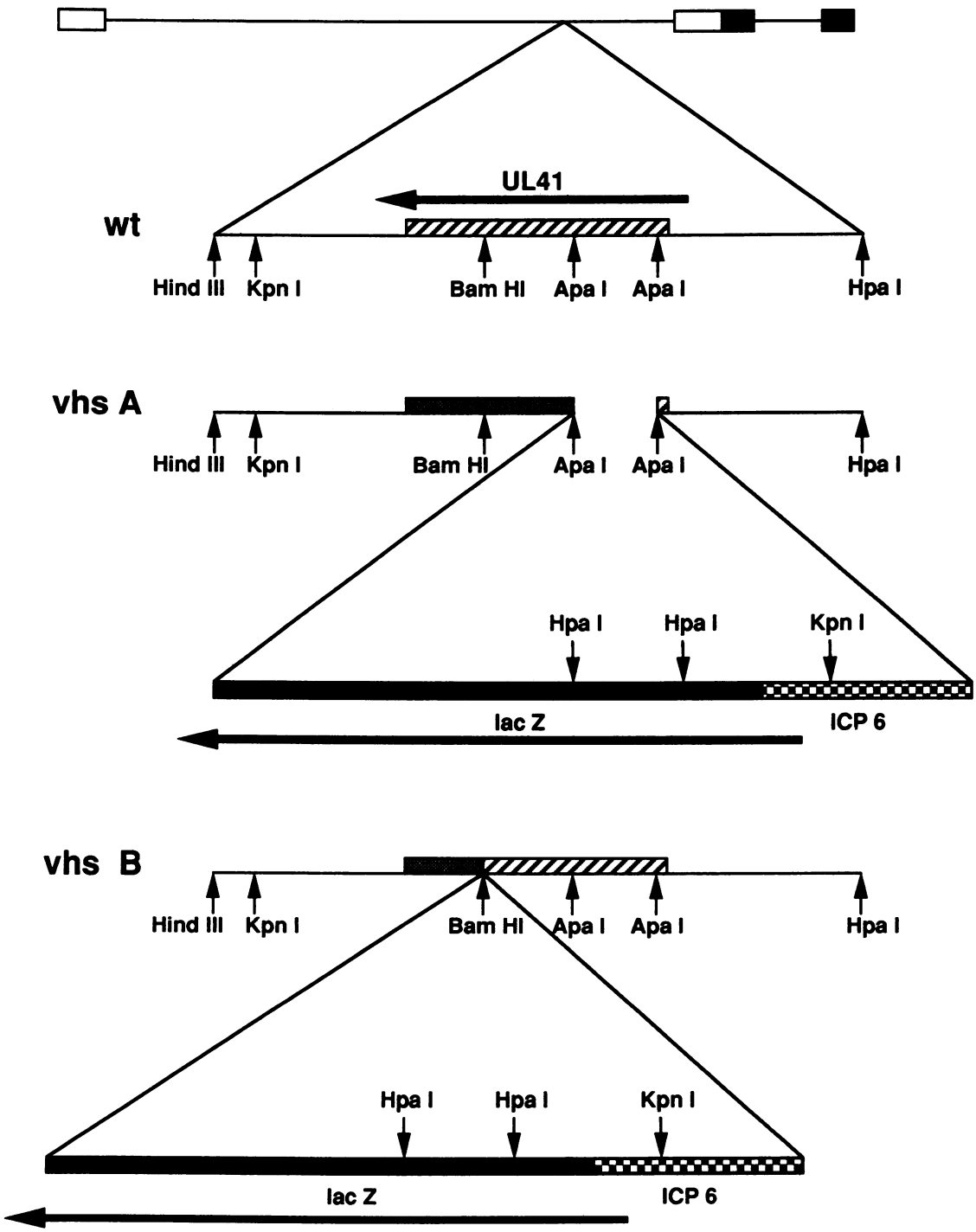

FIG. 6. Structures of mutations disrupting the HSV-1 UL41 gene. Mutations were generated by inserting a *lacZ*-ICP6 cassette into the UL41 gene. The upper portion of the figure depicts the organization of the UL41 region of wild-type (wt) HSV-1 DNA. The UL41 open reading frame (▨) and the direction of transcription (◀) are indicated. Mutant *vhs*A bears the *lacZ* fragment (■) inserted between the *Apa*I sites in UL41; as a result, sequences encoding residues 22 to 179 are deleted and sequences downstream of 179 (▩) are separated from the UL41 promoter. Mutant *vhs*B contains the *lacZ* cassette at the UL41 *Bam*HI site; this lesion disrupts the reading frame downstream of residue 379.

induced degradation of mouse β-globin mRNAs in MEL cells. Induced cell cultures were infected with PAA^{r}5, *vhs*1, *vhs*A, and *vhs*B, and globin RNA levels were assessed at 6 and 12 h postinfection by primer extension (Fig. 8). KOS PAA^{r}5 induced a large decline in globin mRNA in both the presence and absence of a transcription blockade. In contrast, cells infected with the *vhs*A and *vhs*B isolates displayed globin RNA levels similar to those in cells infected

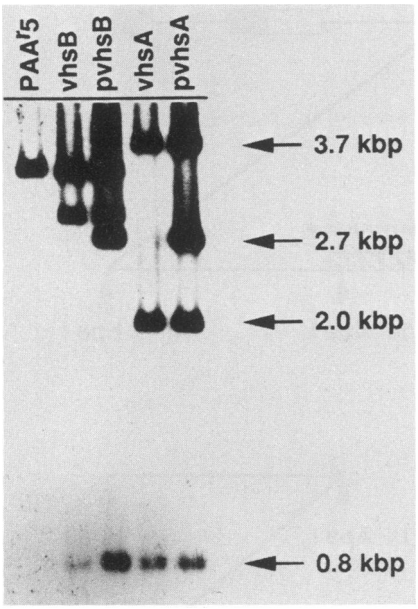

FIG. 7. Southern blot analysis of viral DNAs with disruptions in the UL41 gene. The indicated DNAs were cleaved with a mixture of *Hin*dIII, *Hpa*I, and *Kpn*I, and the resulting fragments were separated by electrophoresis through a 1.0% agarose gel. Following transfer to nitrocellulose, DNA fragments from the UL41 region were visualized by Southern blot hybridization with nick-translated pvhsA. Lanes: PAA'5, parental HSV-1 DNA; vhsB, viral DNA from mutant *vhs*B; pvhsB, plasmid used to construct the *vhs*B strain; vhsA, viral DNA from mutant *vhs*A; pvhsA, plasmid used to construct the *vhs*A strain. Sizes of fragments derived from pvhsA are indicated (kilobase pairs). The 2.7-kb fragment represents the pUC19 vector.

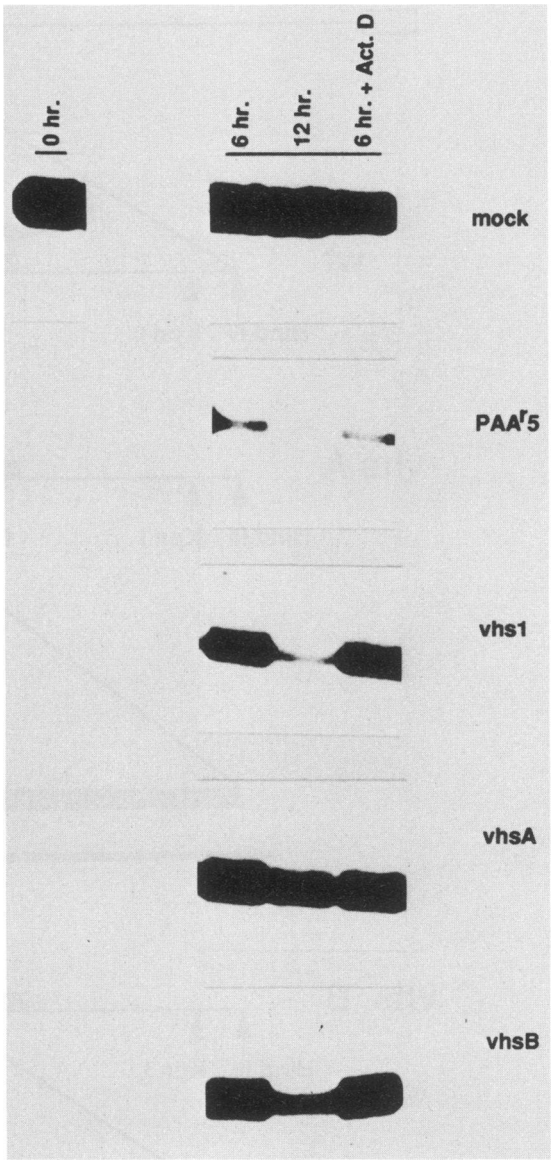

FIG. 8. Effects of UL41 gene disruptions on turnover of endogenous β-globin mRNAs. Induced MEL cells were either mock infected (mock) or infected with 10 PFU of the indicated virus strain per cell, and cytoplasmic RNAs harvested at the indicated times postinfection were analyzed for mouse β-globin mRNA by primer extension (see Materials and Methods). Act. D, Cells infected in the continuous presence of 10 μg of dactinomycin per ml.

with the *vhs*1 mutant at 6 h postinfection. In particular, little if any reduction was observed relative to mock-infected cultures when the experiment was carried out in the presence of a dactinomycin blockade. Thus, both UL41 disruption strains were unable to effect virion-induced turnover of globin RNA. Differences were consistently observed between the various *vhs* mutants when unblocked infections were allowed to proceed for 12 h. The *vhs*1 mutant induced a marked decline in globin RNA levels at later times postinfection, whereas both disruption strains displayed considerably higher globin RNA levels. One interpretation of these data is that the UL41 polypeptide encoded by the *vhs*1 mutant retains partial activity. According to this hypothesis, high levels of newly synthesized *vhs*1 protein directly contribute to the late shutoff induced by the *vhs*1 mutant. Alternatively, the UL41 gene disruptions in the *vhs*A and *vhs*B strains may significantly retard the progress of the lytic cycle, indirectly delaying the onset of late shutoff. Further experiments are needed to distinguish between these possibilities.

Inhibition of transcription of the endogenous β-globin gene. The results outlined above demonstrate that L7/14 and its parent, KOS PAA'5, strongly suppress endogenous β-globin gene expression, at least in part through UL41-induced RNA turnover. In order to determine whether transcription is also inhibited, we employed the nuclear run-on technique to measure the rate of mouse β-globin gene transcription before and after infection with KOS PAA'5. Nuclei were isolated from infected and uninfected hexamethylene bisacetamide-induced MEL cells, and labeled run-on RNAs were hybridized to single-stranded DNA prepared from M13 clones complementary to HSV *tk* mRNA and mouse β-globin mRNA (Fig. 9). We found that the levels of β-globin run-on transcription were greatly reduced (greater than 95%) by 5 h postinfection. These data indicate that HSV-1 infection strongly suppresses transcription of the endogenous β-globin gene.

DISCUSSION

The results presented in this paper demonstrate that HSV-1 infection of murine erythroid cells strongly inhibits expression of the endogenous β-globin gene, whereas a

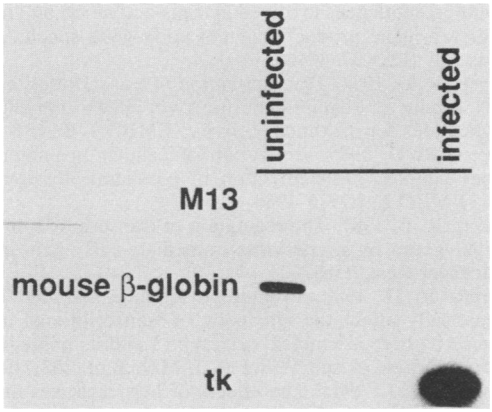

FIG. 9. Effect of HSV-1 infection on transcription of the mouse β-globin gene in MEL cells. Differentiated MEL cells were infected with 10 PFU of HSV-1 KOS PAA^{r}5 per cell, and nuclei were harvested at 5 h postinfection. After in vitro run-on transcription in the presence of [α-^{32}P]UTP, RNAs were isolated and hybridized to a nitrocellulose membrane with 10 μg of single-stranded DNAs prepared from M13 clones bearing DNA fragments complementary to HSV-1 *tk* and mouse β-globin major mRNAs.

β-globin gene residing in the viral genome is activated in parallel with HSV E genes. These data have interesting implications concerning the mechanisms of HSV-induced host shutoff and the basis for the selective activation of viral genes that occurs during infection.

Our results suggest that at least two distinct mechanisms contribute to HSV-1-induced inhibition of endogenous globin gene expression: preexisting cytoplasmic mouse β-globin RNAs were rapidly degraded (as originally reported by Nishioka and Silverstein [57]), and ongoing globin gene transcription was strongly inhibited. We found that degradation of endogenous globin RNA did not require de novo viral gene expression, suggesting that this process is triggered by a component of the infecting HSV-1 virion in our experimental system. Consistent with this hypothesis, the *vhs*1 mutant isolated by Read and Frenkel (70) did not induce rapid turnover of β-globin RNA. We confirmed the requirement for *vhs* function by demonstrating that two mutant strains bearing disruptions of the UL41 open reading frame were also defective in this assay. Several previous studies have indicated that virion-induced host shutoff in Vero cells involves accelerated turnover of cellular mRNAs and that the *vhs* function is required for this process (44, 45, 71, 78). Our results indicate that similar mechanisms underlie the rapid decline in mouse β-globin RNA levels during infection of MEL cells with HSV-1 KOS. This conclusion differs from those reached by Nishioka and Silverstein (58), who found that the degradation of globin mRNA induced by HSV-1 strain F required de novo viral polypeptide synthesis. Previous reports have indicated that various HSV-1 strains differ in their ability to induce virion-mediated shutoff (19). Perhaps HSV-1 strains F and KOS differ in the potency of their *vhs* functions.

The levels of cytoplasmic mouse β-globin RNA were rapidly reduced during infection with L7/14, whereas rabbit β-globin RNAs arising from the viral genome accumulated with a time course characteristic of an HSV E RNA. These results are consistent with the hypothesis that the *vhs*-induced RNA turnover system does not identify cellular RNAs as preferred targets on the basis of their primary nucleotide sequence (44, 62). Taken alone, our observations leave open the possibility that *vhs*-induced RNA turnover occurs in a short-lived burst immediately after infection, resulting in a temporal specificity for preexisting cellular RNAs. Indeed, Fenwick and Owen recently provided evidence that HSV-induced mRNA destabilization occurs only transiently during infection (21). On the basis of these results, these investigators have proposed that newly synthesized viral proteins down regulate the virion-induced turnover system to allow accumulation of viral mRNAs. However, studies by other investigators have demonstrated that the *vhs*-induced RNA turnover system destabilizes viral mRNAs at relatively late times postinfection (44, 45, 62, 78). On the basis of these observations, it has been proposed that the *vhs* function indiscriminately destabilizes mRNAs throughout the infective cycle, facilitating the transition from one phase of infection to the next by tightly coupling cytoplasmic RNA levels to transcription rates (44, 62). If this latter hypothesis is correct, then the implication is that the *vhs*-induced RNA turnover system magnifies the consequences of differential transcriptional regulation of the endogenous and viral globin genes during infection of MEL cells with L7/14. According to this view, mouse β-globin RNA levels are rapidly reduced because transcription is not stimulated whereas rabbit globin RNA later accumulates to the (much lower) levels characteristic of HSV E RNAs as a result of transcriptional activation by IE polypeptides. Further studies are required to distinguish between these two possible explanations for the differential behavior of viral and endogenous globin RNAs in our experimental system.

We found that transcription of the endogenous β-globin gene was strongly inhibited during infection. In contrast, expression of the rabbit β-globin gene borne by L7/14 was activated by viral IE gene products, presumably at the transcriptional level. In principle, these differential responses could result from differences in the sequences of the rabbit and mouse β-globin promoters. However, this explanation seems unlikely, because the mouse β-globin gene is also induced by HSV IE gene products when it is inserted into the HSV genome and delivered into Vero cells (unpublished data).

What is the molecular basis for these opposing transcriptional effects? One possibility is that HSV products disrupt preexisting transcription complexes on actively transcribed cellular genes, releasing transcription factors which are then preferentially reassembled on promoters located in the viral genome. Two previously described features of intracellular HSV DNA might serve to selectively target free transcription factors to promoters located in the viral genome. First, most of the viral DNA is not organized into nucleosomes and is probably present in a relatively open conformation compared with that of host chromatin (10, 46, 56). Second, the viral genome may be localized to a limited number of privileged intranuclear sites (32, 38). In support of this latter hypothesis, viral DNA is concentrated in discrete foci or replication compartments at late times postinfection (69) and ICP4 and ICP8 colocalize to these sites (39, 69). The combination of these two features might also render genes located in the viral genome more accessible to HSV products that act directly on promoters to stimulate transcription. Alternatively, the differential control could reflect the negative influence of HSV regulators on specific *cis*-acting signals flanking the β-globin gene in the cellular genome. Transcription of the endogenous β-globin gene is heavily dependent on several enhancer-like sequences that are located within the globin gene, in the 3′-flanking sequences, and at either end of the entire β-globin-like gene cluster (1, 5, 8, 27, 40). The

latter elements have been postulated to represent anchor points of the globin gene domain to the nuclear matrix (27) and are sufficient to confer erythroid-specific transcription on heterologous promoters (5). Only the intragenic enhancer element is present on the globin fragment that we inserted into the HSV genome, and it is possible that one or more of the other elements represent specific targets of HSV-induced transcriptional repression.

Our results provide strong evidence that features other than promoter sequence and mRNA structure can play a major role in dictating the response of a gene to HSV regulators. We suspect that a complete description of HSV regulatory strategies will require a greater understanding of the roles of higher-order DNA packaging and intranuclear architecture in the control of gene expression in mammalian cells.

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