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Expression of a Cellular Gene Cloned in Herpes Simplex Virus: Rabbit Beta-Globin Is Regulated as an Early Viral Gene in Infected Fibroblasts

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We constructed nondefective herpes simplex virus type 1 recombinants bearing the intact rabbit beta-globin gene inserted into the viral gene for thymidine kinase to study the expression of a cellular gene when it is present in the viral genome during lytic viral infections. The globin promoter was activated to high levels during productive infection of Vero cells, giving rise to properly spliced and processed cytoplasmic globin transcripts. Expression of globin RNA occurred with early kinetics, was not affected by blocking viral DNA replication, and was strongly inhibited by preventing viral immediate-early protein synthesis with cycloheximide. These results support the hypothesis that temporal control of herpes simplex virus early gene expression is accomplished by mechanisms that are not restricted to viral promoters. In addition, these data show that a cellular transcript can be correctly processed and can accumulate to high levels during viral infection; this indicates that the mechanisms of virally induced shutoff of host RNA accumulation and degradation of host mRNAs do not depend on sequence-specific differentiation between host and viral RNAs. These findings also suggest that herpesviruses have considerable potential as high-capacity gene transfer vectors for a variety of applications.

Transcription of herpes simplex virus (HSV) genes occurs in the nuclei of infected cells and is accomplished by the host cell RNA polymerase II. Viral genes belonging to at least three differentially regulated classes are sequentially activated to high levels by viral proteins in a cascade fashion (34, 80), while expression of most host cellular genes is suppressed. Promoter transplant experiments have shown that HSV promoter regions determine the kinetics of expression of linked coding sequences during infection (63, 73), suggesting that temporal control of HSV gene expression operates largely at the transcriptional level. This hypothesis is supported by recent nuclear run-on experiments (26). These results imply that several distinct classes of HSV promoters are modulated by viral regulators during infection.

Five viral immediate-early (IE) genes are transcribed in the absence of de novo viral protein synthesis (1, 6, 43, 67, 83). Efficient IE gene transcription is thought to involve stimulation of far upstream enhancerlike elements by a structural component of the virion Vmw65 (2, 5, 42, 44, 45, 60, 65). The IE proteins in turn play crucial roles in transcriptional activation of viral early (E) and late (L) genes. Studies of viral mutants and transfection experiments have demonstrated that the IE protein Vmw175 (or ICP4) is required for efficient E and L gene expression and that Vmw110 (or ICP0) acts synergistically with Vmw175 to further stimulate expression of these genes (10, 11, 18, 25, 58, 62, 64, 66, 69, 78, 81). In addition, Vmw63 (or ICP27) activates the expression of some L genes beyond the levels achieved by the combination of Vmw175 and Vmw110 (20, 68), while Vmw67 (or ICP22) is implicated in the expression of L genes in certain cell types but not in others (72). Although the precise mechanisms of action of the IE proteins are unknown, partially purified preparations of Vmw175 have been reported to interact with particular DNA sequences present in viral and other DNAs (3, 21, 40, 41, 53) (possibly in combination with cellular factors [22]). In addition, Vmw110 displays structures homologous to the metal-binding "fingers" of some eucaryotic DNA-binding proteins (61), and Vmw110 present in crude cell extracts binds to calf thymus DNA in vitro (32).

HSV E genes are maximally expressed before the onset of viral DNA replication, and accumulation of E mRNAs declines late during infection (31). The initial activation of E gene transcription during infection requires the activity of viral IE proteins and the integrity of DNA sequences located within E gene promoters (8, 14–17, 76). Little is known concerning the factors that limit E gene expression late during infection.

Following the onset of viral DNA replication, two subclasses of L promoters are activated to high levels. The so-called E/L or leaky L genes differ from true L genes in the degree to which their expression is augmented by viral DNA replication (80). However, both classes of L promoters are detectably activated by IE proteins in the absence of DNA replication in transfection experiments (9, 10, 12, 33, 36, 73).

Several lines of evidence suggest that at least some of the *cis*-acting E and L gene control elements involved in the response to IE proteins are not virus specific. First, there are no obviously conserved sequences present in HSV E or L promoters other than elements also present in other cellular and viral promoters. The E promoters analyzed to date consist of a TATA box linked to an upstream element composed of binding sites for cellular transcription factors (16, 17, 27, 38, 49–52, 70), and true L promoters appear to consist only of a TATA box-cap site region (33, 36). Second, mutational analyses of E and true L promoters have thus far failed to uncover sequences that are solely and specifically required for transactivation by IE proteins (for a review, see reference 49). In the case of the well-characterized HSV E thymidine kinase (*tk*) promoter, the *cis*-acting elements required for activation by IE proteins (8, 14, 15) coincide with the binding sites of cellular transcription factors (27,

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38). Similarly, studies of the HSV E glycoprotein D (*gD*) promoter have shown that transactivation does not require elements other than those involved in promoter function in the absence of IE proteins (16, 17). An interesting feature of these latter data is that sequences in the upstream *gD* region reported to bind the Vmw175 protein in vitro (3, 21) can be deleted without affecting the response of the *gD* promoter to IE proteins in in vivo assays dependent on Vmw175 function (16, 17). In addition, available evidence indicates that activation of two HSV true L promoters by IE proteins requires only the TATA box-cap site region (33, 36).

A third line of evidence suggesting that viral promoters lack virus-specific elements required for transactivation by IE proteins emerged from the findings that HSV and adenovirus IE proteins activate transcription of some cellular genes, including rabbit beta-globin, when these genes are reintroduced into cells by transfection (17–19, 24, 28, 35). The ability of HSV IE proteins to stimulate transcription of a transfected beta-globin gene (17–19) contrasts strongly with the effects of HSV infection on cellular globin gene expression; i.e., the globin gene located at its normal chromosomal site is not activated following infection of fibroblasts (19), and globin expression is suppressed following infection of erythroid cells in a process involving degradation of globin mRNA (55, 56). These data have been interpreted to suggest that some features of the higher-order packaging of transfected genes renders them more accessible to activation by IE proteins than their endogenous counterparts are (19).

A straightforward interpretation of the data summarized above is that transactivation of HSV genes by IE proteins does not rely on recognition of virus-specific promoter elements but instead occurs through mechanisms that stimulate a relatively nonspecific pathway for gene expression (8, 14, 17, 49) that also acts on some suitably presented cellular genes (19). Similar conclusions have been reported for adenovirus E1a-mediated activation of adenovirus early genes (for a review, see reference 39). A strong prediction of this hypothesis is that a cellular gene known to be stimulated by viral IE polypeptides following transfection would also be activated as a viral gene when delivered into cells as part of an infecting HSV genome. We tested this prediction by constructing a nondefective HSV type 1 (HSV-1) recombinant bearing the intact beta-globin gene. Our results show that correctly initiated and processed globin transcripts accumulated to high levels early during lytic viral infection and that globin was controlled as a viral E gene by several criteria.

These results confirm that transcriptional activation during HSV infection occurs through mechanisms that are not restricted to viral promoters and suggest the possibility that late inhibition of E gene expression does not involve virus-specific *cis*-acting control signals. In addition, our results raise interesting questions about the mechanisms involved in HSV-induced shutoff of host cell gene expression.

(A preliminary report of this work was presented at the 11th Herpesvirus Workshop, Leeds, United Kingdom, July 1986.)

MATERIALS AND METHODS

Virus and cells. HSV-1 strain KOS PAA^{r}5 (7, 30) was used throughout this study. Viral stocks were propagated and the titers of the viruses were determined on monolayers of Vero cells. The DNA polymerase mutation present in strain Paa^{r}5 renders the viral polymerase resistant to phosphonoacetic acid and hypersensitive to aphidocolin (7).

Plasmids. All plasmid constructions and recombinant DNA manipulations were done by using the general methods described by Maniatis et al. (47).

Construction of viral recombinants. *tk*-deficient viral recombinants bearing inserts of the rabbit beta-globin gene were constructed by using a two-step procedure that has been described previously (74, 75). Briefly, beta-globin-bearing *Sst*I fragments (Fig. 1) were inserted into the unique *Sst*I cleavage site within the *tk* gene present on pTK1, then the resulting *tk*-deficient insertion mutations were transferred into the intact HSV genome by DNA-mediated marker transfer. The antimutator DNA polymerase mutant HSV-1 KOS PAA^{r}5 (30) was chosen as the recipient viral strain, following the suggestion of D. Coen, to reduce the frequency of spontaneous *tk*-deficient mutants. Strain PAA^{r}5 is partially resistant to thymine arabinoside as a result of the polymerase mutation, necessitating the use of 5-bromodeoxycytidine as the *tk*-counterselective agent. After selection of *tk*-deficient progeny by plaque purification on Vero cells in the presence of 100 μg of 5-bromo-deoxycytidine per ml, viral isolates bearing the desired insert were identified by Southern blot hybridization (77).

RNA extraction. Vero cells were infected at the indicated multiplicity, and cytoplasmic RNA was prepared by the method of Berk and Sharp (4). Where indicated below, drugs were added at the time of infection and maintained continuously.

S1 nuclease mapping. DNA probes for S1 nuclease mapping were prepared by 5′ end labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP or 3′ end labeling with the Klenow fragment of *Escherichia coli* DNA polymerase I and the appropriate α-^{32}P-labeled deoxynucleotide as described by Maniatis et al. (47). In some cases, single-stranded probes were prepared by electrophoretic strand separation as described by Maxam and Gilbert (48), while in other cases, duplex probes prepared by secondary cleavage were used under R-looping conditions.

For transcript mapping, 10 to 30 μg of cytoplasmic RNA and 1×10^4 to 3×10^4 Cerenkov cpm of uniquely end-labeled probe DNA were ethanol precipitated out of 0.3 M sodium acetate (pH 5.6) and then dissolved in 30 μl of hydridization buffer consisting of 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), and 1 mM EDTA and containing either 50% (single-stranded probes) or 80% (duplex probes) recrystallized, deionized formamide. Following hybridization at 42°C (single-stranded probes) or 53°C (duplex probes), hybrids were digested with 2×10^3 U of S1 nuclease (Boehringer Mannheim) for 1 h at 37°C in 0.3 ml of 150 mM NaCl–50 mM sodium acetate (pH 4.6)–5 mM $ZnSO_4$. The reaction was stopped by adding 50 μl of 4 M ammonium acetate and 30 μl of 0.2 M EDTA. The mixture was extracted with phenol-chloroform and then ethanol precipitated. The pellets were washed with 70 and 95% ethanol, dried, and suspended in sequencing-gel loading dye. The protected portions of the probe fragment were then sized by electrophoresis through 8% polyacrylamide sequencing gels.

A synthetic 35-mer (5′ GACAACCAGCAGC/CTGCCC AGGGCCattaaggatc 3′; purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University) was used to detect correct splicing of globin exon 1 to exon 2. The oligomer consists of 25 nucleotides (nt) complementary to the sequence across the exon 1-exon 2 junction (the splice junction is indicated in the sequence by /), with a 10-nt AT-rich nonhybridizing tail at the 3′ end (indicated by lowercase letters). S1 nuclease

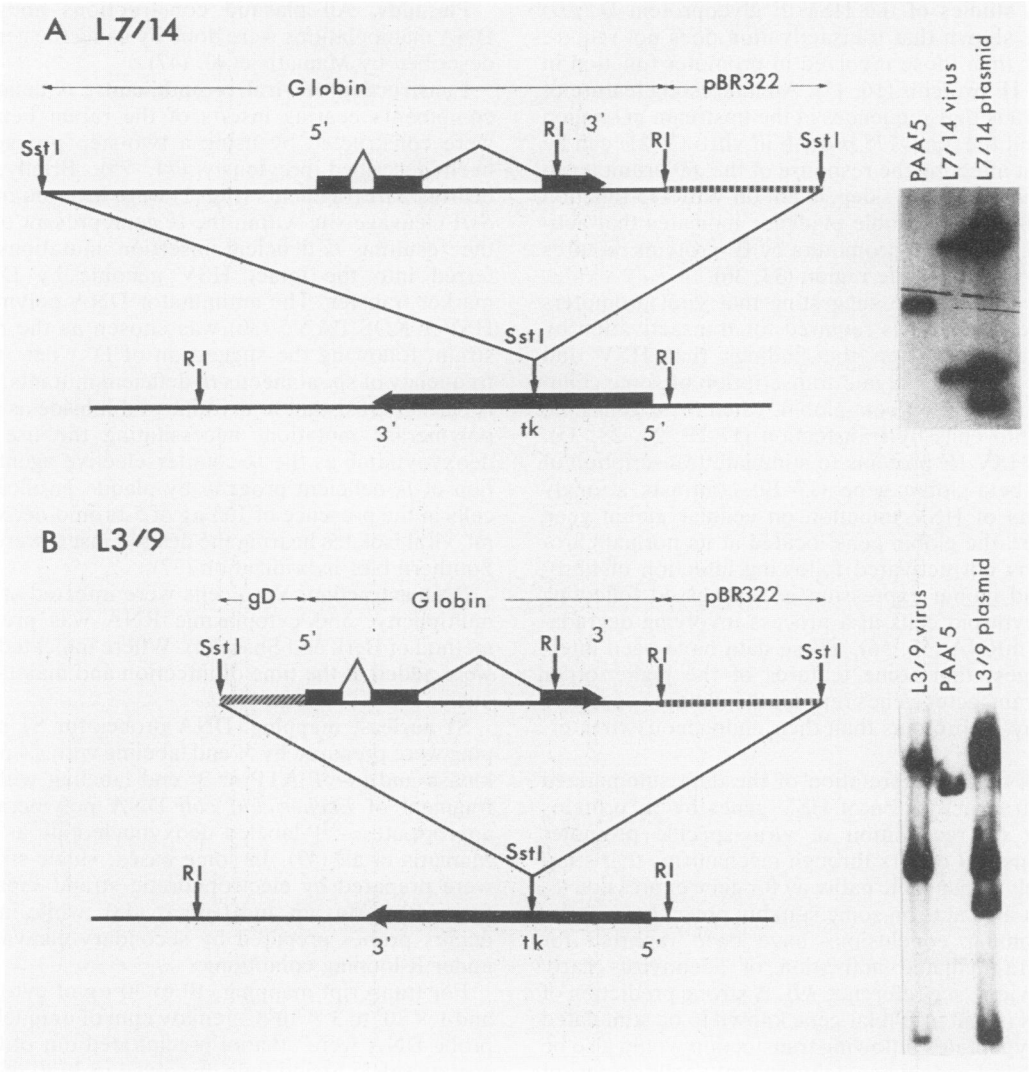

FIG. 1. Construction and identification of viral recombinants. Plasmids L7/14 and L3/9 bear the intact rabbit beta-globin gene and a *gD*-globin gene fusion inserted at an *Sst*I site in the viral *tk* structural gene. These plasmids were used to generate *tk*-deficient recombinant viral strains (see Materials and Methods). The structures of recombinant strains were confirmed by Southern blot hybridization of *Eco*RI (RI)-cleaved viral DNAs, using the corresponding plasmid as a probe. The smallest *Eco*RI fragment of L7/14 DNA (590 base pairs) was run off the gel displayed in panel A.

mapping experiments using this probe were done as described above, except that hybridization was at 45°C in buffer lacking formamide and 4×10^3 U of S1 nuclease was used.

Primer extension. A synthetic 25-mer complementary to residues 63 to 87 of rabbit beta-globin mRNA was purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University (5' CCGCAGACTTCTCCTCACTGGACAG 3'). A 10-μg sample of infected cell cytoplasmic RNA was ethanol precipitated out of 0.3 M sodium acetate (pH 5.2) and washed with 70 then 95% ethanol. The dried RNA pellets were suspended in 8 μl of 10 mM Tris hydrochloride (pH 7.9)–1 mM EDTA containing 2×10^5 Cerenkov cpm of 5'-end-labeled primer, and 2 μl of 10 mM Tris hydrochloride (pH 7.9)–1 mM EDTA–1.25 M KCl was then added. The mixture was hybridized for 1 h at 60°C and cooled to room temperature. After the addition of 25 μl of 20 mM Tris hydrochloride (pH 8.7)–10 mM $MgCl_2$–5 mM dithiothreitol–0.33 mM of each deoxynucleoside triphosphate, 10 μg of actinomycin D per ml containing 10 U of avian myeloblastosis virus reverse transcriptase (Joseph Beard, Life Sciences Inc.), the mixture was incubated for 1 h at 37°C. The extension products were then precipitated by adding 300 μl of 95% ethanol; they were then pelleted, washed with ethanol, dried, and suspended in 10 μl of sequencing-gel loading buffer.

RESULTS

Identification of recombinants. Nondefective HSV-1 recombinants bearing rabbit beta-globin sequences inserted into the viral *tk* gene were generated by in vivo recombination as described in Materials and Methods (Fig. 1). Recombinant L7/14 carries the intact globin gene and ca. 1,200 base pairs of 5' flanking sequences borne on a 3.7-kilobase (kb) *Sst*I fragment inserted in the *tk* antisense orientation. L3/9 bears a previously described hybrid fragment, in which the globin structural gene is driven by the promoter of the

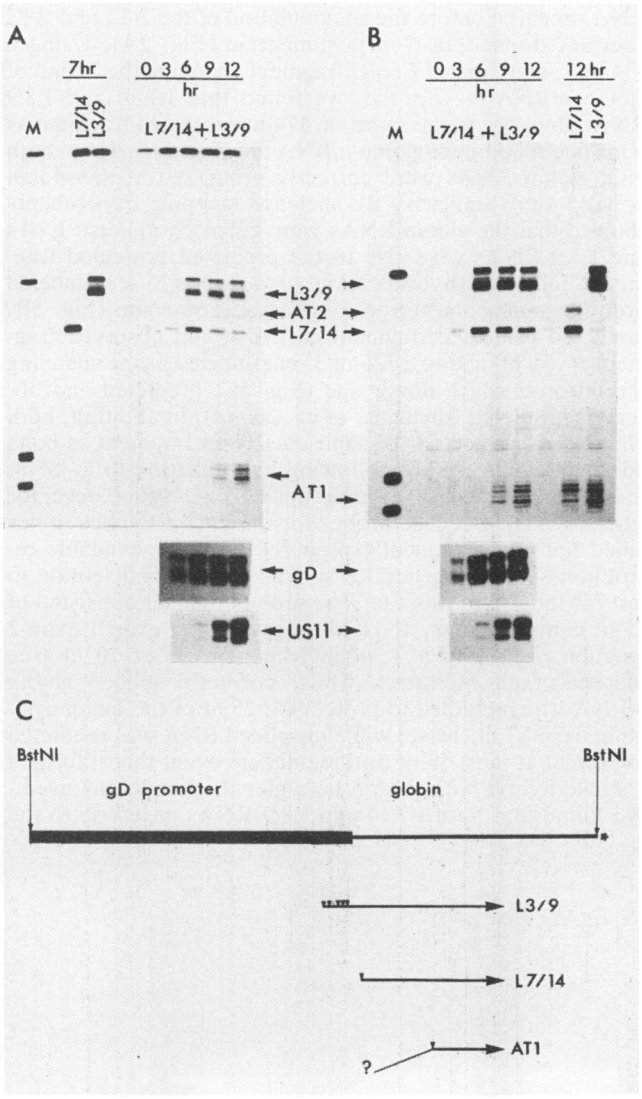

FIG. 2. Kinetics of globin RNA expression. Total cytoplasmic RNA (20 μg) prepared from Vero cells infected with the indicated viruses was hybridized to the 5'-end-labeled probe diagrammed in panel C to detect globin transcripts. Some of the RNA samples were also hybridized to a 5'-end-labeled *Nco*I-*Sst*I fragment spanning the *gD* cap site to detect bona fide *gD* transcripts (82) and to a 5'-end-labeled *Taq*I-*Bam*HI fragment to detect US11 RNAs (37). Following treatment with S1 nuclease, digestion products were displayed on an 8% sequencing gel. Single infections used a multiplicity of infection of 10 PFU per cell, while 5 PFU per cell of each virus was used in mixed infections. (A and B) The results of two independent time course experiments are shown. The labels AT1 and AT2 indicate the aberrant transcripts described in the main text (see also Fig. 3). Markers (M) were 5'-end-labeled *Bst*NI subfragments of a *Pvu*II-*Bam*HI fragment of pRED4 (16). (C) The *Bst*NI probe used was derived from the *gD*-globin fusion gene present in L3/9 and allows separate identification of transcripts specified by each of the viral strains.

HSV-1 early gene encoding gD (16), inserted in the same orientation. Following isolation of *tk*-deficient progeny, viral clones bearing the desired inserts were identified by Southern blot hybridization of *Eco*RI-cleaved viral DNA (Fig. 1). Insertion of the intact globin gene in strain L7/14 resulted in the replacement of the 2.3-kb PAA^{r}5 *Eco*RI *tk* fragment with the predicted *tk*-globin fusion fragments of 3.9 and 1.3 kb and the acquisition of a 590-nt internal globin fragment (Fig. 1A; additional data not shown). Similarly, L3/9 viral DNA displayed the predicted 3.1- and 1.3-kb *tk*-globin fusion fragments and the 590-nt internal globin fragment (Fig. 1B).

Efficient expression of the beta-globin gene from its own promoter during infection. We tested for the expression of the inserted beta-globin genes by S1 nuclease mapping of correctly initiated cytoplasmic globin transcripts produced during lytic infection of Vero cells. The probe used (Fig. 2C) was derived from the *gD*-globin fusion gene present in recombinant L3/9; it allows separate detection of the globin RNAs arising from the intact globin gene carried by L7/14 and the *gD*-globin fusion (16).

Cells separately infected with L3/9 and L7/14 displayed high levels of the predicted globin transcripts 7 h postinfection (Fig. 2). Experiments that included commercial purified rabbit globin mRNA as a standard suggest that globin transcripts constitute approximately 2×10^{-5} of the total cytoplasmic RNA in cells infected with L7/14 (see Fig. 5; additional data not shown), a value similar to that reported for viral *tk* mRNA (0.1% of infected-cell mRNA [50]).

The kinetics of globin RNA accumulation were studied in cells coinfected with L7/14 and L3/9 (Fig. 2). As controls, these RNA samples were also hybridized to probes for bona fide *gD* mRNA (an E transcript [82]) and US11 mRNA (an L transcript [37]). Both globin transcripts accumulated with kinetics similar to those of bona fide *gD* mRNA, reaching maximal levels by 6 h postinfection. The levels of globin RNA specified by L7/14 declined somewhat at later times, while the levels of *gD*-globin and *gD* RNAs remained relatively constant. These data show that transcripts arising from the rabbit beta-globin promoter accumulate with kinetics similar to those of viral E mRNAs. In addition, the similar behavior of the *gD* promoter linked to globin-coding sequences in the *tk* locus and the authentic *gD* promoter in

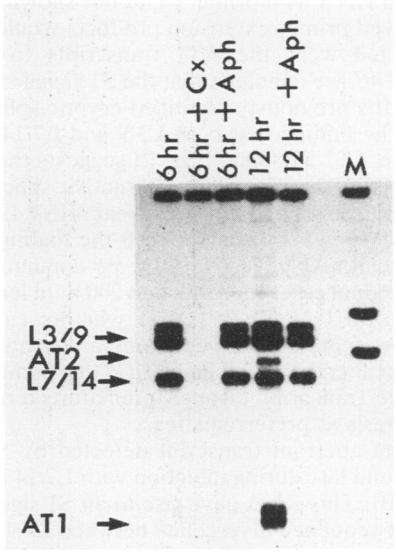

FIG. 3. Effects of inhibitors on globin RNA expression. Cytoplasmic RNA (30 μg) prepared from cells coinfected with L7/14 and L3/9 (5 PFU per cell of each virus) was hybridized to the globin probe described in the legend to Fig. 2, and the hybrids were digested with S1 nuclease. Where indicated, cycloheximide (Cx; 100 μg/ml) or aphidocolin (Aph; 10 μg/ml) was added at the time of infection. Markers (M) were *Hin*dIII cleavage products of the *Bst*NI probe fragment.

the *gD* locus indicates that, in this instance, the behavior of a promoter in the viral genome was not affected by the linked transcribed sequences.

Effects of inhibitors. The accumulation of globin transcripts was strongly inhibited by blocking viral protein synthesis with 100 μg of cycloheximide per ml but was not greatly affected by inhibiting viral DNA replication with 10 μg of aphidocolin per ml (Fig. 3). These results suggest that expression of the globin gene requires viral IE proteins and does not depend on viral DNA replication. Taken with the kinetic data presented above, these results indicate that the globin gene present in L7/14 is controlled as an HSV E gene.

Aberrant transcripts. An unexpected finding was the appearance of two aberrant globin-related transcripts at late times postinfection (Fig. 2, AT1 and AT2). Accumulation of both aberrant transcripts was strongly suppressed by blocking viral DNA replication with aphidocolin (Fig. 3), a finding consistent with the suggestion from kinetic data that these transcripts are under late control. AT1 was specified by both recombinant viral strains, while AT2 arose only from L7/14.

AT1 gave rise to an S1 signal mapping to approximately +46 in globin exon 1. A similar S1 signal has been observed by others in transfection experiments with the intact rabbit beta-globin gene (29) and was attributed to alternative splicing of transcripts initiated upstream of the globin promoter to a cryptic splice acceptor located within exon 1. This interpretation was confirmed by primer extension experiments using a synthetic primer complementary to residues 63 to 87 of globin RNA (Fig. 4). We were unable to detect extension products corresponding to initiations at +46 when RNAs extracted late during infection were used as templates (compare Fig. 4, tracks 5 and 6, with Fig. 3, tracks 4 and 5); instead we observed a heterogeneous set of extension products migrating more slowly than did bona fide globin cDNA in addition to the expected products arising from transcripts initiated at the globin and *gD* promoters. Similar results were obtained by using a 25-mer complementary to residues 115 to 139 of globin RNA as a primer (data not shown). Inasmuch as AT1-derived primer extension products would have been easily detected were the AT1 transcripts to have been initiated at +46, we conclude that the S1 signal at +46 arises from use of the previously identified cryptic splice acceptor in exon 1. The finding that both L3/9 and L7/14 late RNAs displayed the AT1 S1 signal (Fig. 2) suggests the possibility that the AT1 RNAs arise from alternative splicing of transcripts driven from one or more upstream HSV L promoters. This interpretation is consistent with the finding that L7/14 and L3/9 late RNAs gave rise to some comigrating primer extension products (ca. 130, 160, and 200 nt in length; Fig. 4, tracks 7 and 8). It is not yet clear whether each of these extension products is derived from a separate aberrant transcript; an alternative explanation is that some or all arise from a single transcript through premature termination of cDNA synthesis at preferred sites.

The second aberrant transcript detected by S1 analysis, AT2, was found late during infection with L7/14 but not with L3/9 (Fig. 2B). This RNA gave rise to an S1 signal mapping to the site of sequence divergence between L7/14 DNA and the recombinant probe used, suggesting that it arises by readthrough from the upstream globin sequences. The precise initiation site(s) of this transcript remains unknown, but it is possible that it arises from a cryptic L promoter in the 5′ flanking globin sequences.

Correct processing of beta-globin transcripts produced at early times. We tested for correct 3′ processing and splicing of the globin RNAs by S1 nuclease mapping of cytoplasmic RNA prepared before the accumulation of the AT1 and AT2 aberrant transcripts (7 h postinfection; Fig. 2A). Using a 534-nt 3′-end-labeled *Eco*RI fragment spanning the 3′ end of globin mRNA as a probe, we found that L7/14 and L3/9 RNAs gave rise to the same ca. 174-nt protected fragment as bona fide rabbit beta-globin mRNA did (Fig. 5A). Thus, both viral globin RNAs were correctly processed at the globin poly(A) site. Similarly, S1 nuclease mapping experiments showed that the globin RNAs were correctly spliced: L7/14 and L3/9 RNAs gave rise to the predicted protected fragments following hybridization to a 640-nt 5′-end-labeled probe spanning the intron 2 splice acceptor site (Fig. 5B, predicted protected fragment ca. 53 nt and observed fragment ca. 56 nt) and to a 326-nt 3′-end-labeled probe spanning the intron 2 splice donor site (Fig. 5C, predicted and observed protected fragments of ca. 206 nt). In addition, both viral RNAs protected the same ca. 208-nt fragment as bona fide beta-globin mRNA following hybridization to a 487-nt 5′-end-labeled probe spanning the intron 1 splice acceptor site (Fig. 5D). Because probes for the intron 1 splice donor could not be conveniently prepared by using available restriction sites, we generated a synthetic oligonucleotide to test for the use of this site. The probe oligomer consisted of 25 nt complementary to residues across the exon 1-exon 2 junction, linked to a 3′ nonhybridizing tail of 10 nt (see Materials and Methods). Thus, correctly spliced globin mRNA was predicted to protect ca. 25 nt of the oligonucleotide from S1 nuclease, while unspliced RNA was predicted to protect at most 13 nt (in the unlikely event that it formed a stable hybrid with the probe under the conditions used). We found that both L7/14 and L3/9 RNAs gave rise to the

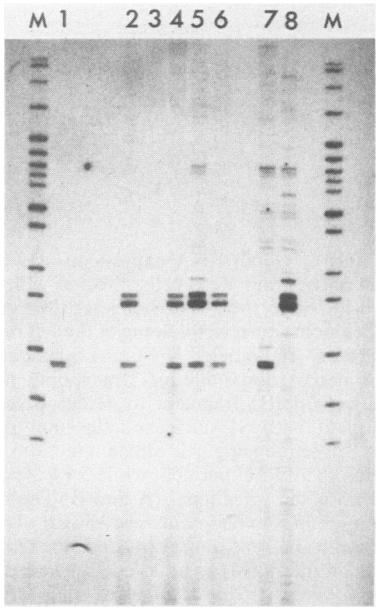

FIG. 4. Primer extension analysis of globin RNAs. Infected cell cytoplasmic RNA (10 μg) was annealed to a 5′-end-labeled 25-mer complementary to residues 63 to 87 of globin mRNA. Following treatment with reverse transcriptase, extension products were displayed on an 8% sequencing gel. Lanes: 1, authentic globin cDNA; 2 to 6, RNA prepared from cells coinfected with L7/14 plus L3/9 6 h postinfection (lane 2), 6 h postinfection plus cycloheximide (100 μg/ml) (lane 3), 6 h postinfection plus aphidocolin (10 μg/ml) (lane 4), 12 h postinfection (lane 5), 12 h postinfection plus aphidocolin (lane 6); 7, L7/14, 12 h postinfection; 8, L3/9, 12 h postinfection. Markers (M) are 3′-end-labeled *Hpa*II fragments of pBR322 DNA.

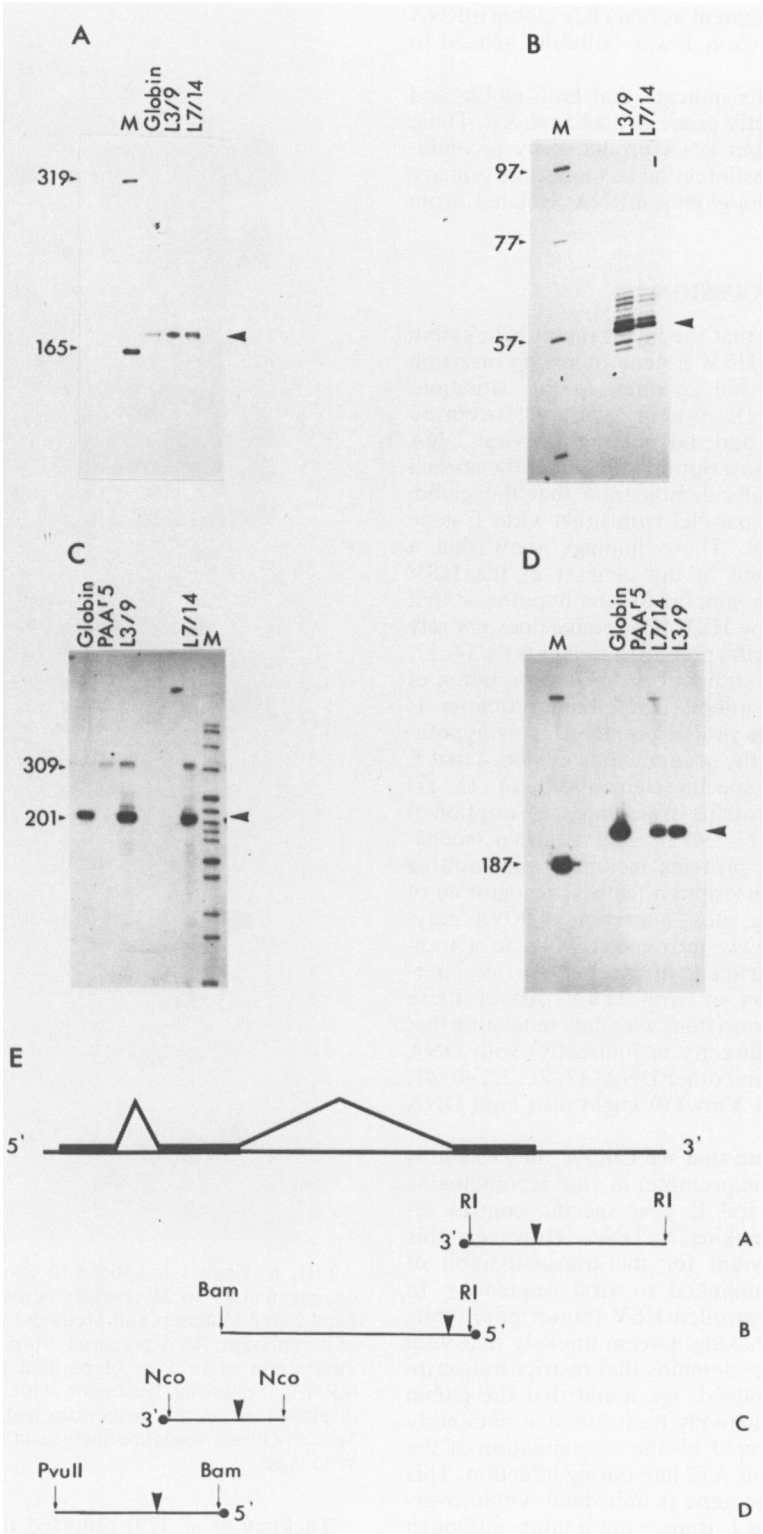

FIG. 5. Processing of the globin RNAs. The indicated end-labeled probes (panel E) were hybridized to 30 μg of cytoplasmic RNA prepared 7 h postinfection and to 5 ng of commercial rabbit globin mRNA (40% beta-globin mRNA). Following digestion with S1 nuclease, products were displayed on 8% sequencing gels. The hybridizations shown in panel D used a duplex probe under R-looping conditions, while all other hybridizations used single-stranded probes. (A) 3′ end. The probe is 534 nt, with an expected product of ca. 174 nt. Markers (M) are 5′-end-labeled *Bst*NI subfragments of a *Pvu*II-*Bam*HI fragment derived from pRED4 (16). (B) Intron 2 splice acceptor. The probe is 640 nt, with an expected product of ca. 53 nt. Markers (M) are a mixture of *Pst*I and *Taq*I digestion products of a 5′-end-labeled *Bgl*II-*Eco*RI fragment spanning the HSV-1 *tk* cap site. (C) Intron 2 splice donor. The probe is 326 nt, with an expected product of ca. 206 nt. Markers (M) are 3′-end-labeled *Hpa*II fragments of pBR322 DNA. (D) Intron 1 splice acceptor. The probe is 487 nt, with an expected product of ca. 208 nt. The marker (M) was derived by cleaving the probe duplex with *Nco*I.

same ca. 25-nt protected fragment as bona fide globin mRNA did (Fig. 6), showing that exon 1 was faithfully spliced to exon 2.

In summary, these results indicate that both globin and *gD*-globin RNAs are correctly processed and spliced. Thus, the major cytoplasmic globin RNA produced by recombinant L7/14 at early times postinfection has the same primary sequence as authentic beta-globin mRNA isolated from reticulocytes.

DISCUSSION

This study demonstrates that the intact rabbit beta-globin gene was controlled as an HSV E gene following insertion into the *tk* locus of the viral genome. In this situation, expression required viral IE protein synthesis, occurred maximally at early times, and did not require viral DNA replication. Since globin transcription initiated at the normal globin cap site, these results demonstrate that the globin promoter was activated in parallel with other viral E gene promoters during infection. These findings show that a cellular promoter functioned in the context of the HSV genome and provide strong support for the hypothesis that activation of transcription by HSV IE proteins does not rely on recognition of virus-specific promoter elements (8, 14, 17, 49). In our view, the most straightforward interpretation of these findings is that the rabbit beta-globin promoter is functionally equivalent to a viral E promoter. This hypothesis is consistent both with the accumulating evidence that E gene promoters lack virus-specific elements (8, 14, 15, 17) and the ability of viral IE proteins to activate transcription of transfected globin genes (17–19, 28, 35). Possible mechanisms of activation by IE proteins include stimulation or mobilization of cellular transcription factors, recognition of transcription factor-binding sites, alteration of RNA polymerase II, transport of newly introduced DNA to a transcriptionally active compartment of the nucleus, or alterations in the conformation of viral DNA. All of these potential mechanisms are consistent with data indicating that Vmw175 interacts (either directly or indirectly) with DNA sequences present in viral and other DNAs (3, 21, 22, 40, 41, 53) and the possibility that Vmw110 might also bind DNA (32, 61).

An alternative explanation that we cannot yet exclude is that the activity of the globin promoter in viral recombinants is regulated entirely by viral E gene-specific control sequences present in the flanking *tk* DNA. However, this explanation does not account for the transactivation of transfected globin genes unlinked to viral sequences. In addition, independently controlled HSV transcription units overlap extensively (80), making it seem unlikely that viral DNA is organized into large domains that restrict transcription to particular times. Indeed, we found that the globin gene present in L7/14 was actively transcribed at both early and late times, as documented by the accumulation of the aberrant transcripts AT1 and AT2 late during infection. This result shows that the globin gene is imbedded within overlapping and opposing E and L transcription units. Although globin is transcribed in the same polarity as the L transcription unit, transcripts initiated from the globin promoter are under E control. The simplest explanation is that temporal control of globin expression depends on features of the globin promoter rather than on flanking viral sequences. We are currently constructing viral recombinants bearing the globin gene inserted through an L gene as a further test of this hypothesis.

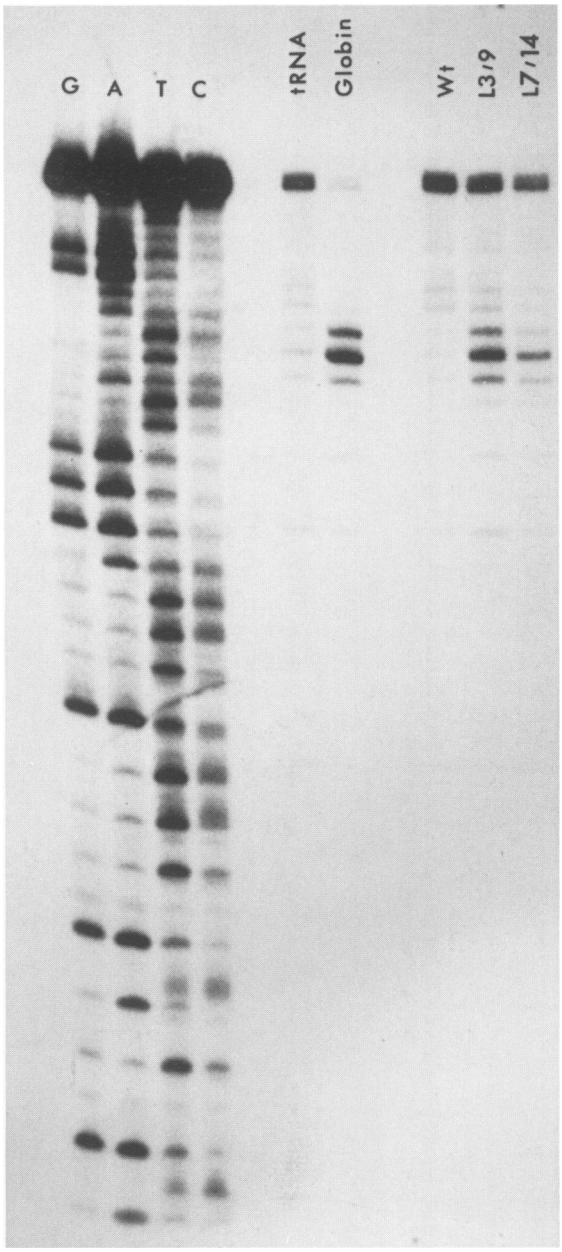

FIG. 6. Exon 1 is spliced to exon 2. A 5'-end-labeled 35-mer complementary to 25 residues across the junction of exon 1 and exon 2 (see Materials and Methods) was hybridized either to 30 μg of cytoplasmic RNA prepared 7 h postinfection with the indicated viral strain or to 5 ng of purified rabbit alpha- plus beta-globin mRNA. Following treatment with S1 nuclease, products were displayed on an 8% sequencing gel. Markers were generated by Maxam-Gilbert sequence analysis of the probe oligonucleotide. Wt, Wild type.

Tackney et al. (79) reported that the intact cellular gene encoding adenine phosphoribosyltransferase (*aprt*) is not detectably transcribed after insertion into the *tk* locus on the intact HSV genome. Based on this finding, they suggested that HSV regulators distinguish between cellular and viral promoters. Our results demonstrate that this distinction does not generally apply to all cellular promoters. Indeed, Patel et al. (59) have shown that a significant subset of cellular transcripts is induced during HSV infection of tissue culture

cells and that the induction of a proportion of these is dependent on viral IE gene expression. Some of these induced gene products are also overproduced in morphologically transformed and tumorigenic cell lines isolated after exposure to HSV DNA or other transforming agents (46). These findings suggest that the regulation of cellular gene transcription by HSV polypeptides has some significance in a variety of aspects of the biology of virus infection.

What features of a gene are required for it to be activated by HSV IE gene products? As outlined in the introduction, many cellular genes located in the cellular chromosomal environment may be in tightly packed chromatin and therefore inaccessible to the activators. This explanation cannot account for the lack of activation of the *aprt* gene described by Tackney et al. (79), since in their experiment, the gene was in the open environment of the viral genome. However, in common with many other cellular housekeeping genes, the promoter of the *aprt* gene does not contain a TATA box (13, 54). It may be that the presence of a normal TATA box is essential for the activation of a promoter by HSV IE gene products. This hypothesis is supported by three observations. First, HSV true late promoters can be activated in the absence of DNA replication by HSV IE gene products in transfection experiments, and the only detectable *cis*-acting sequences that are required for this regulation lie in the TATA box-cap site region (33, 36). Second, the simian virus 40 early promoter responds poorly to HSV-induced activation (57), but this defect can be overcome by making changes in the sequence of the TATA box (R. D. Everett, unpublished data). Third, linker scanning mutations in the TATA box of the HSV *tk* promoter have the most deleterious effects on promoter activity (when present in the viral genome) of all linker scanning mutations of the promoter sequence (8).

The rate of accumulation of globin transcripts driven from the globin promoter declined late during infection in a manner reminiscent of typical viral E RNAs (31), suggesting that similar inhibitory mechanisms are involved. If this suggestion is correct, then it seems unlikely that inhibition of E gene expression late during infection relies on recognition of virus-specific negative control elements. Perhaps reduced E gene expression reflects increasing competition from L promoters activated by viral DNA replication. Alternatively, late viral products may inactivate some of the host transcription factors required for E promoter function.

Splicing of HSV E and L transcripts is rare (80), suggesting the possibility of a mechanism of host shutoff involving alterations of the host splicing system. We found that globin RNAs produced at early times were correctly spliced, making this possibility seem unlikely. In addition, the accumulation of the aberrant AT1 transcript(s) late during infection suggests that the host splicing machinery remains largely intact until at least 12 h postinfection.

The origin of the AT1 transcript is of particular interest. Since AT1 was produced during infection with both L3/9 and L7/14, it seems likely that it arises by alternative splicing of transcripts initiated from one or more upstream viral L promoters. This interpretation is supported by the finding that primer extension of L7/14 and L3/9 late RNAs gave rise to several comigrating extension products larger than bona fide globin cDNA. Alternative splicing of HSV RNAs has been previously described for the glycoprotein C gene (23), and as discussed by these authors, this mechanism may considerably increase the number of viral gene products produced during infection (80).

It is striking that globin RNA accumulated to high levels during infection with L7/14, since one reported mechanism of HSV-induced host translational shutoff involves degradation of host transcripts including beta-globin mRNA (55, 56, 71). The implication is that the virus-induced RNA turnover system does not identify its targets on the basis of their primary nucleotide sequence. Further study of recombinant viral strains that express cellular RNAs may provide insights into the precise molecular mechanisms involved in HSV-induced host shutoff. In this context, it will be interesting to determine whether cellular mRNAs specified by viral recombinants are efficiently translated during infection.

Our finding that an unmodified cellular gene not ordinarily induced by HSV infection is abundantly transcribed after insertion into the HSV genome suggests that herpesviruses have considerable potential as high-capacity gene transfer vectors for a variety of applications.

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