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Herpes Simplex Virus Immediate-Early Proteins ICP0 and ICP4 Activate the Endogenous Human α-Globin Gene in Nonerythroid Cells

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Globin genes are normally expressed only in erythroid cell lineages. However, we found that the endogenous α-globin gene is activated following infection of human fibroblasts and HeLa cells with herpes simplex virus (HSV), leading to accumulation of correctly initiated transcripts driven by the α-globin promoter. The α1- and α2-globin genes were both induced, but expression of β- or ζ-globin genes could not be detected. Experiments using HSV mutants showed that null mutations in the genes encoding the viral immediate-early proteins ICP4 and ICP22 reduced induction approximately 10-fold, while loss of ICP0 function had a smaller inhibitory effect. Transient transfection experiments showed that ICP0 and ICP4 are each sufficient to trigger detectable expression of the endogenous gene, while ICP22 had no detectable effect in this assay. ICP4 also strongly enhanced expression of transfected copies of the α2-globin gene. In contrast, the adenovirus E1a protein did not activate the endogenous gene and inhibited expression of the plasmid-borne α2-globin gene. Previous studies have led to the hypothesis that chromosomal α-globin genes are subject to chromatin-dependent repression mechanism that prevents expression in nonerythroid cells. Our data suggest that HSV ICP0 and ICP4 either break or bypass this cellular gene silencing mechanism.

Herpes simplex virus (HSV) is a large enveloped DNA virus that replicates in the nuclei of mammalian cells. HSV executes a complex genetic program encompassing a variety of controls at the transcriptional and posttranscriptional levels during lytic infection: expression of most cellular genes is strongly suppressed, and three temporally distinct classes of viral genes are sequentially activated to high levels in a cascade fashion (reviewed in references 29, 83, and 99). Five viral immediate-early (IE) genes are expressed first; four of these (ICP0, ICP4, ICP22, and ICP27) encode regulatory proteins that stimulate expression of the viral early (E) and late (L) genes. The E genes are activated next, giving rise to proteins required for replication of the viral genome. Viral DNA replication then ensues, augmenting IE-dependent expression of the L genes. This lytic cascade provides a favorable system for dissecting the mechanisms by which a limited number of regulatory proteins rapidly reprogram mammalian cells to express a new set of polymerase II-transcribed genes.

All systems of differential gene activation must distinguish genes destined for activation from the large excess of other genes present in the same nucleus. In the case of the HSV lytic cycle, the initial source of selectivity is a specific DNA sequence element, TAATGARATTC, which targets the VP16 molecules delivered by the infecting virion to the upstream control regions of the viral IE genes, resulting in selective activation of IE transcription (5, 14, 34, 58, 59, 62–64, 70, 77, 96). IE proteins then stimulate expression of the viral E and L genes. However, in contrast to VP16-induced activation of IE gene transcription, the mechanisms that target E and L genes for induction have yet to be clearly defined. E and L control regions are not marked by obviously conserved class-specific sequences analogous to TAATGARATTC, and exhaustive mutational and biochemical analyses have failed to uncover specific DNA sequence elements required for activation by IE polypeptides (17, 26, 67, 89). Indeed, minimal promoters consisting of an isolated TATA box are induced (48, 54, 56), and HSV IE proteins activate a variety of viral and cellular TATA-bearing promoters in transient cotransfection assays (25, 26, 28). Perhaps the only clear conclusion to emerge from these studies is that the precise sequence of the TATA element plays a major role in dictating the degree of induction (17, 28, 51). This observation fits in well with recent data showing that the IE protein ICP4 interacts with components of the basal transcriptional apparatus, including TATA-binding protein, TFIIB, and TAF250 (15, 91). Despite the relaxed sequence specificity displayed with newly introduced target genes, IE proteins do not appear to globally activate expression of endogenous cellular genes. Taken in combination, these observations raise the likelihood that features other than primary nucleotide sequence play a major role in targeting viral E and L genes for selective activation. In principle, any property that distinguishes HSV E and L genes from the majority of endogenous cellular genes could contribute to this selectivity; examples include subnuclear localization, extrachromosomal versus chromosomal state, prior transcriptional status, or differences in higher-order packaging into chromatin.

Studies of the effects of HSV IE proteins on cellular globin genes have cast some light on the foregoing issues. Early studies by Everett and coworkers revealed that transiently transfected copies of the rabbit β-globin gene are activated by HSV IE proteins in nonerythroid cells, whereas the endogenous chromosomal β-globin gene is not induced (26, 27). Although the biological relevance of the transfection assay could be questioned, entirely analogous results were obtained when the rabbit β-globin gene was inserted into the intact HSV genome and analyzed during lytic infection (90). Further analysis re-

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vealed that β-globin genes integrated into cellular chromosomes through stable transfection are also activated (25), demonstrating that extrachromosomal location is not the critical feature that distinguishes the response of transfected templates from that of the endogenous gene. These data indicate that the β-globin promoter is inherently susceptible to activation by IE proteins and imply that some aspect of the higher-order structure of the endogenous β-globin locus precludes induction.

Recent advances in understanding of globin gene regulation (reviewed in reference 40) provide a plausible explanation for these differential responses. The 150-kb β-globin gene cluster (comprising the ε-, ^{G}γ-, ^{A}γ-, δ-, and β-globin genes) is packaged into inactive heterochromatin in nonerythroid cells; the entire locus displays a DNase I-resistant chromatin structure and replicates late during S phase (32, 41). Tissue-specific activation during erythroid cell differentiation involves binding of erythroid cell-specific factors to the upstream locus control region (β-LCR) located 5 to 11 kb upstream of the cluster, generating a set of five DNase I-hypersensitive sites (33, 39, 92). The activated LCR then mediates global decondensation of the locus into an early-replicating open chromatin conformation (32). It seems quite likely that the tightly packed heterochromatic nature of the β-globin locus in nonerythroid cells shields the β-globin promoter from the effects of HSV IE polypeptides.

Many other tissue-specific genes are also packaged into condensed, late-replicating chromatin in nonexpressing cells (9, 47). However, this is not universally the case. For example, the human α-globin gene cluster (comprising the ζ2-, α2-, α1-, and θ-globin genes) is embedded in a region of open, early-replicating chromatin in all cell types (18, 98). Moreover, although erythroid cell-specific expression of the α-globin gene depends on an LCR-like DNase I hypersensitive site (HS −40) located 40 kb upstream of the cluster (45, 46), this element is not required to maintain the locus in an open conformation (12). The open conformation of the α-globin cluster raises interesting questions about the mechanisms that maintain the silence of the α-globin gene in nonerythroid cells. Indeed, the set of transcription factors present in nonerythroid cells is sufficient for robust α-globin promoter activity, as evidenced by the finding that transfected copies of the α-globin gene are strongly expressed in a variety of cell types (11, 49, 68, 95). Taken in combination, these observations argue that the endogenous α-globin gene is subject to a form of negative control that does not act on newly introduced copies of the gene (11, 16). Possible explanations include (i) local chromatin-dependent repression of the α-globin promoter and (ii) packaging into a form of inactive chromatin distinct from the DNase I-resistant late-replicating variety observed at the β-globin and many other tissue-specific loci.

We have previously shown that the human α-globin gene is activated by HSV IE polypeptides when it is delivered into nonerythroid cells by infection with an HSV recombinant (72, 88). We report here that the endogenous human α-globin gene is also induced during HSV infection and that the IE proteins ICP0 and ICP4 are each sufficient to trigger this response. These data demonstrate that ICP0 and ICP4 bypass or overcome the mechanisms that maintain the silence of the endogenous α-globin gene in nonerythroid cells.

MATERIALS AND METHODS

Cells and viruses. MRC5, HeLa, and Vero cells were grown in alpha minimal essential medium containing 10, 10, and 5% fetal bovine serum, respectively. The following HSV type 1 (HSV-1) strains were propagated on Vero cells: wild-type KOS and F; KOS Paa^{r}5 (43); *hr*R3 (35), an ICP6 mutant; R325tk^{+} (76), an ICP22 mutant; and N38 (97), a mutant lacking ICP47. The ICP4 null mutant, *d*120 (20), was grown on complementing E5 cells, and the ICP27 deletion mutant, 5*dl*1.2 (66), was propagated on complementing 3-3 cells. The ICP0 nonsense mutant, *n*212 (12), was grown and titrated on U20S cells (102).

HSV infection and RNA extraction. Unless otherwise specified, infections were at a multiplicity of infection of 10 PFU/cell. Total cellular RNA was extracted by using a guanidinium isothiocyanate-based method with the RNazol B reagent (TM Cinnax, Inc.) according to the manufacturer's protocol. In some experiments, aphidicolin (10 μg/ml) or phosphonoacetic acid (300 μg/ml) was added to block viral DNA replication. Where indicated, cycloheximide was added to the growth medium 1 h prior to infection and maintained continuously.

Primer extension. Ten micrograms of RNA was annealed to the 5′-^{32}P-labeled primer (50,000 Cerenkov cpm) in 10 μl of 10 mM Tris (pH 8.0)–1 mM EDTA–250 mM KCl at 62°C for 1 h; 25 μl of 20 mM Tris (pH 8.7)–10 mM $MgCl_2$–5 mM dithiothreitol containing 0.33 mM each dATP, dCTP, dGTP, and dTTP, 10 μg of actinomycin D per ml, and 0.5 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) was then added, and the reaction mixture was incubated at 42°C for an additional hour. Extension products were precipitated with ethanol and resolved on an 8% polyacrylamide sequencing gel. Gels were exposed to X-ray films for autoradiography or to PhosphorImager screens (Molecular Dynamics, Sunnyvale, Calif.) for quantitative analysis. The following synthetic primers were purchased from the central facility of the Institute for Molecular Biology and Biotechnology, McMaster University: α-globin, 5′ AGGCGGCCTTGACGTTGGTCTTGTC 3′; β-globin, 5′ ACAGGGCAGT AACGGCAGACTTCTC 3′; and ζ-globin, 5′ CCACATGGACACAATGATG GTCCTC 3′.

S1 nuclease protection. An aliquot consisting of 20,000 Cerenkov cpm of a single-stranded 3′-labeled 300-nucleotide (nt) *Bst*EII-*Bam*HI fragment spanning the 3′ end of the human α2-globin gene (labeled at the BstEII site) was hybridized with 10 μg of RNA at 42°C for 3 h in 10 μl of 0.6 M NaCl–20 mM Tris (pH 7.5)–1 mM EDTA. The samples were cooled on ice, then 140 μl of 0.28 M NaCl–30 mM sodium acetate (pH 4.4)–4.5 mM zinc acetate containing 66 U of S1 nuclease (Boehringer Mannheim) was added, and the reaction mixture was incubated at 37°C for 1 h. Samples were precipitated with ethanol and resolved on an 8% polyacrylamide sequencing gel.

Plasmids. ICP0 and ICP4 expression vectors (driven from the human cytomegalovirus [HCMV] IE promoter; pDR27 and pBB37) were obtained from Peter O'Hare. An HCMV promoter-driven ICP22 expression vector (pICP22) was constructed as follows. A 3.5-kb *Kpn*I-*Bcl*I fragment of HSV-1 strain 17 DNA containing the ICP22 gene was cloned between the *Kpn*I and *Bam*HI sites of pUC18. An *Eco*NI-to-*Eco*RI fragment spanning the ICP22 open reading frame was then subcloned into the *Hin*dIII site of pRC/CMV (Invitrogen) after filling in of all ends with the Klenow fragment of *Escherichia coli* DNA polymerase I. An adenovirus E1a expression clone (pE1a) was derived from pKH548 (6), which contains genomic adenovirus type 5 E1a and E1b DNA sequences with a *Bam*HI site inserted at nucleotide position 548. A 1-kb *Bam*HI-*Hpa*I fragment encompassing the E1a open reading frame was inserted into the *Not*I site of the pRC/CMV vector (after filling in of the *Bam*HI end with the Klenow fragment of DNA polymerase I) to generate pE1a. pUCα2 was constructed by inserting a 4.2-kb *Sst*I fragment spanning the human α2 gene (−2775 to +1477 relative to the transcription initiation site) into pUC18. This fragment bears the globin promoter and transcription unit but lacks the LCR-like HS −40 sequence. To facilitate detection of transcripts arising from the cloned α-globin gene without interference from transcripts of the endogenous gene, a modified version of pUCα2 (pUCα2-NsiI) bearing a 12-nt insert at the *Nco*I site was generated. To this end, two partially complementary single-stranded oligonucleotides (5′ CAT GGTGCTATG 3′ and 5′ CATGCATAGCAC 3′) were annealed and then inserted into the *Nco*I site on pUCα2.

Transfection. HeLa cells were transfected with 30 μg of ICP0, ICP4, ICP22, or E1a expression vector by using a modification of the calcium phosphate coprecipitation method (37). Where indicated, 5 μg of pUCα2-NsiI was included. In all cases, pUC18 carrier was added to bring the total amount of DNA to 60 μg. Briefly, CsCl-purified plasmid DNA was resuspended in 500 μl of 250 mM $CaCl_2$ and then added to an equal volume of 2× HeBs buffer (350 mM NaCl, 62 mM HEPES, 1.8 mM Na_2HPO_4). The DNA precipitate was added to HeLa cells grown to a density of 2×10^6 cells in 100-mm-diameter tissue culture plates. After 16 h, the cells were washed three times with fresh medium. The cells were then incubated for a further 24 h. Total RNA was harvested as described above and used in primer extension assays.

RESULTS

Activation of endogenous human α-globin genes during HSV infection of nonerythroid cells. We examined the effects of HSV infection on expression of the endogenous α-globin gene in nonerythroid cells. Parallel dishes of HeLa and MRC5 cells (immortal cervical carcinoma cells and normal human diploid fibroblasts, respectively) were infected with increasing amounts of HSV-1 KOS strain Paa^{r}5, and total cellular RNA harvested at 6 h postinfection was scored for α-globin tran-

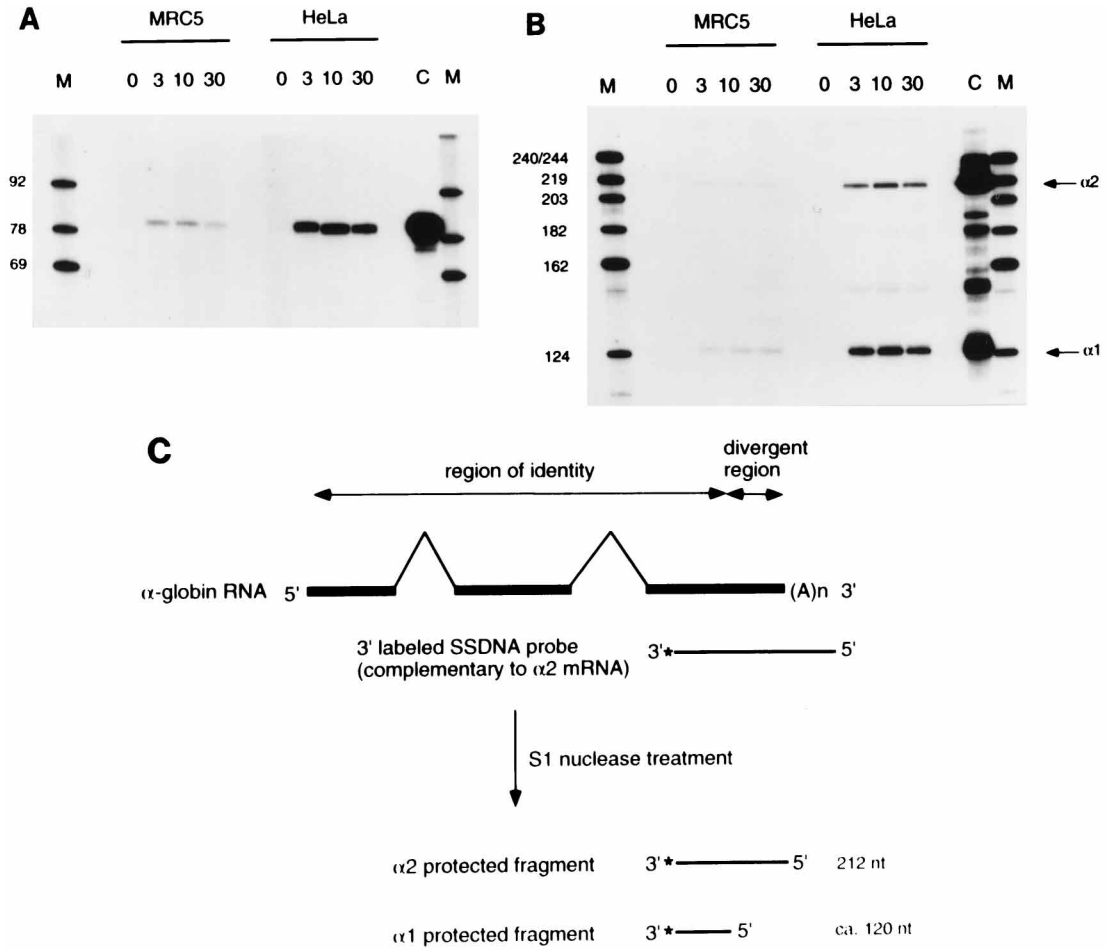

FIG. 1. Induction of α-globin transcripts in HSV-infected HeLa and MRC5 cells. HeLa and MRC5 cells were infected with HSV-1 strain KOS strain PAA^{r}5 at the indicated multiplicities (PFU/cell). Total cellular RNA extracted 6 h postinfection was then analyzed for α-globin transcripts by primer extension (A) and S1 nuclease protection (B). (A) Primer extension. Ten-microgram samples of RNA were analyzed with a 5′-^{32}P-labeled oligonucleotide complementary to positions +65 to +80 of α-globin mRNA, and extension products were resolved on an 8% polyacrylamide sequencing gel. Sizes of markers (M), *Hpa*II fragments of pBR322 DNA, are indicated in nucleotides on the left. C, control RNA extracted from human blood. (B) Detection of α1 and α2 transcripts by S1 nuclease protection analysis. RNA samples (10 μg) were hybridized to a 3′-labeled single-stranded DNA complementary to the 3′ end of α2 mRNA (diagrammed in panel C), and then hybrids were digested with S1 nuclease. The protected portions of the probe were analyzed on an 8% sequencing gel. Signals at 212 and ca. 120 nt arise from α2 and α1 transcripts, respectively. (C) Schematic diagram of the 3′-labeled probe used in panel B. SSDNA, single-stranded DNA.

scripts by primer extension using a 5′-labeled 25-mer complementary to residues 65 to 80 of α-globin mRNA (Fig. 1A). As expected, uninfected cells lacked detectable α-globin RNAs. However, RNA samples extracted following infection generated a strong 80-nt primer extension signal that comigrated with that obtained in assays using control human blood RNA. These data demonstrate that HSV infection activates expression of the previously silent α-globin gene in normal human fibroblasts and HeLa cells, giving rise to correctly initiated α-globin transcripts driven from the α-globin promoter. We consistently observed higher levels of globin transcripts in HeLa cells compared to MRC5 cells. In one experiment where this difference was quantified, about eightfold more globin RNA was obtained in HeLa cells (not shown). We do not yet understand the basis for this difference, although the possibility that MRC5 cells are less infectable appears to be excluded by the observation that the levels of viral ICP4 mRNA are equivalent in the two cell types (not shown).

The human genome contains two tightly linked α-globin genes, α1 and α2, which differ only in their 3′ untranslated regions. To determine whether both genes were activated, we used S1 nuclease protection analysis to differentiate between α1 and α2 transcripts. The 3′-labeled single-stranded probe used was derived from α2 genomic sequences; as diagrammed in Fig. 1C, α2 transcripts are predicted to protect 212 nt of the probe from S1 nuclease digestion, whereas α1 transcripts should protect only ca. 120 nt. Infected MRC5 and HeLa cell RNAs gave rise to 212- and ca. 120-nt products which comigrated with those obtained with control blood RNA (Fig. 1B), indicating that both of the endogenous α-globin genes are activated by HSV infection.

The β- and ζ-globin genes are not activated. Previous studies have indicated that HSV IE proteins do not induce the endogenous β-globin gene in rabbit fibroblasts (25). To determine whether infection of human cells leads to a more global induction of globin genes, HeLa cells were infected with 10 PFU of HSV-1 KOS strain Paa^{r}5 per cell in the presence or absence of aphidicolin to block viral DNA replication, and RNA extracted at 6 and 12 h postinfection was scored for α-, β-, and ζ-globin transcripts by primer extension (Fig. 2). As shown in Fig. 2 and further documented below, aphidicolin prevents the decline in α-globin RNA levels that otherwise occurs at late times postin-

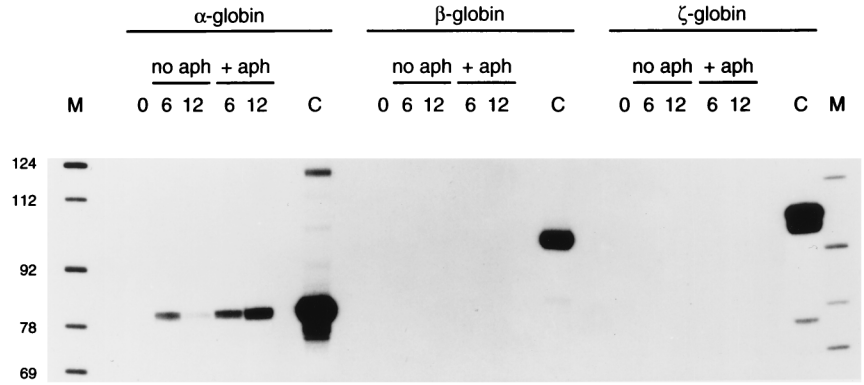

FIG. 2. Primer extension analysis of α-, β-, and ζ-globin transcripts. HeLa cells were infected with 10 PFU of HSV-1 KOS strain PAA^{r}5 per cell in the presence and absence of 10 μg of aphidicolin (aph) per ml. Total RNA extracted at 0, 6, or 12 h postinfection was analyzed by primer extension using 5′-labeled oligonucleotides complementary to α-, β-, and ζ-globin mRNAs, and the extension products were resolved on an 8% polyacrylamide sequencing gel. RNA extracted from human blood was used as the positive control (C) for α- and β-globin transcripts, while RNA from K562 cells was used as the positive control (C) for detection of ζ-globin RNAs. M, size markers (see the legend to Fig. 1).

fection, resulting in enhanced signals at the 12-h time point. Although α-globin transcripts were induced, neither β- nor ζ-globin transcripts could be detected in the infected cell RNA samples. Inasmuch as the β- and ζ-globin primers gave rise to strong signals in primer extension reactions with control RNA from human reticulocytes and K562 erythroleukemia cells, respectively (Fig. 2), we conclude that HSV infection does not detectably activate the endogenous β- or ζ-globin gene. These data confirm the previous observations made with the rabbit β-globin gene and further demonstrate that HSV infection does not induce all members of the α-globin gene family (as evidenced by the finding that the ζ-globin gene is not expressed).

Time course of α-globin RNA accumulation. We examined the time course and general characteristics of α-globin transcript accumulation to gain information about the temporal class of the HSV gene product(s) responsible for induction. Globin RNA was detected by 3 h postinfection, increased in abundance by 6 h, and then declined at later times (Fig. 3). Induction was not blocked by inhibiting viral DNA replication with aphidicolin (Fig. 3) or phosphonoacetic acid (see Fig. 5C); instead, these drugs prevented the decline of accumulated α-globin mRNA at later times. In contrast, induction was blocked when viral protein synthesis was prevented with cycloheximide (Fig. 4), a response that distinguishes the α-globin gene from viral IE genes. In all of these respects, the expression pattern of the endogenous α-globin gene resembled that of an HSV E gene. This, in turn, suggests that one or more IE polypeptides serve to activate α-globin expression.

At least two HSV IE proteins are required for efficient accumulation of α-globin mRNA in infected cells. HSV encodes five IE proteins, and four of these (ICP0, ICP4, ICP22, and ICP27) have been shown to regulate gene expression in HSV-infected cells (reviewed in references 29 and 83). The E protein ICP6 is also induced by VP16 and expressed very early during infection (23). We used viral mutants bearing lesions in each of these six genes to examine their roles in activating α-globin expression during infection of HeLa cells (Fig. 5). Accumulation of ICP0 mRNA was monitored as a control for infection (data not shown), except in the case of the ICP0 mutant *n*212, where ICP4 mRNA was examined. This analysis revealed that mutations that inactivate ICP27 (5*dl*1.2), ICP47

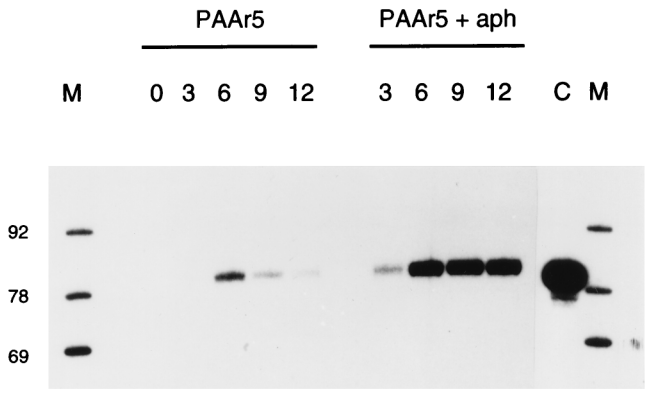

FIG. 3. Time course of accumulation of α-globin transcripts in infected cells. HeLa cells were infected with 10 PFU of HSV1 KOS strain PAA^{r}5 per cell in the presence or absence of 10 μg of aphidicolin (aph) per ml. Total RNA extracted at the indicated times (hours) postinfection was analyzed for α-globin RNA by primer extension. Human blood RNA was used as the positive control (C). M, size markers (see the legend to Fig. 1).

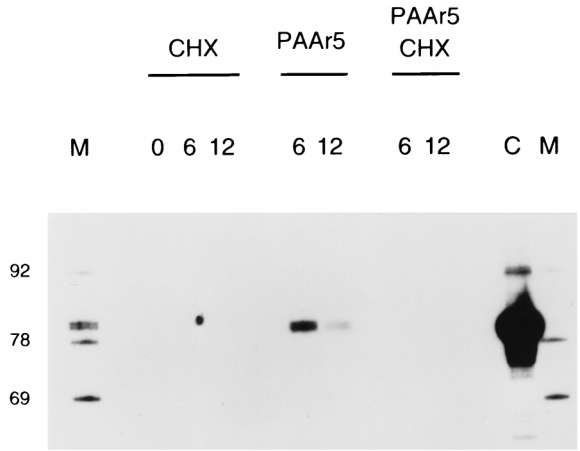

FIG. 4. Effects of cycloheximide on induction of α-globin transcripts. HeLa cells were infected with 10 PFU of HSV1 KOS strain PAA^{r}5 per cell in the presence or absence of 100 μg of cycloheximide (CHX) per ml, and total RNA harvested at the indicated times (hours) postinfection was analyzed for α-globin RNA by primer extension. C, control; M, size markers (see the legend to Fig. 1).

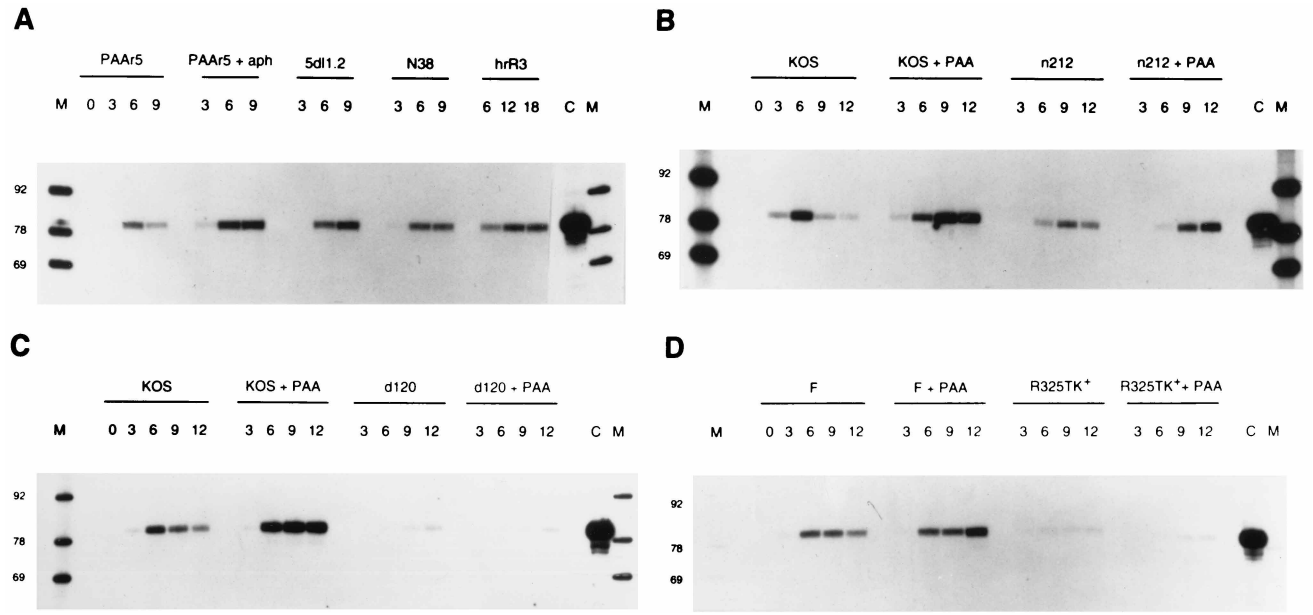

FIG. 5. Effects of mutations in various HSV IE genes on induction of α-globin RNAs. HeLa cells were infected with 10 PFU of the indicated HSV strains per cell, and RNA extracted at the indicated times (hours) postinfection was analyzed for α-globin transcripts by primer extension. Where indicated, aphidicolin (aph; 10 μg/ml) or phosphonoacetic acid (PAA; 100 μg/ml) was added to block viral DNA replication. Panels A to D represent independent experiments. The HSV strains used are described in Materials and Methods. C, control; M, size markers (see the legend to Fig. 1).

(N38), or ICP6 (*hr*3) did not impair induction of α-globin RNA (Fig. 5A). Rather, these mutants strains displayed an extended period of induction, in that globin RNA levels did not decline during the later stages of infection. By contrast, viral mutants bearing lesions in the genes encoding ICP4 (*d*120) and ICP22 (R325tk$^+$) displayed greatly reduced levels of globin RNA relative to the wild-type parental strains (Fig. 5C and D). The ICP0 mutant *n*212 showed a slightly impaired induction compared to the parental KOS strain (Fig. 5B); however, this may be a result of the decreased amount of ICP4 expressed compared to the parental-strain infected cells (data not shown). These results indicate that although two (and possibly three) IE proteins contribute to induction, none of these gene products is absolutely required.

The interpretation of these results is complicated by the fact that ICP0, ICP4, and ICP22 each modulate the expression of other HSV genes: ICP4 is required for activation of E and L gene expression (20, 100), ICP22 stimulates L gene expression in certain cell types (86), and ICP0 stimulates expression of HSV genes belonging to all three temporal classes (13, 71). Thus, it was possible that some or all of these IE proteins contribute to induction indirectly by stimulating the expression of one or more additional viral proteins which serve as the true inducer. To distinguish between direct and indirect effects, it was necessary to examine activity in the absence of other HSV gene products.

ICP0 and ICP4 are each sufficient to trigger expression of the endogenous α-globin gene in HeLa cells. We used a transient transfection approach to determine the effects of ICP0, ICP4, and ICP22 on expression of the endogenous α-globin gene. HeLa cells were transfected with expression vectors bearing these IE genes under the control of the HCMV IE promoter, and RNA samples harvested 36 h later were scored for α-globin RNA by primer extension (Fig. 6A). Cells transfected with the ICP0 (pDR27) and ICP4 (pBB37) expression vectors displayed easily detectable levels of α-globin RNA, whereas the ICP22 vector (pICP22) had no detectable effect (Fig. 6A). The levels of α-globin expression were consistently higher in cells transfected with pBB37 (ICP4) than with pDR27 (ICP0); inasmuch as both plasmids bear the same vector backbone, this observation may suggest that ICP4 is a more potent activator of α-globin expression than ICP0. These data demonstrate that ICP0 and ICP4 are each able to induce detectable expression of the previously silent endogenous α-globin gene in the absence of other HSV gene products. ICP4 and ICP0 did not display obvious synergism when provided in combination, and ICP22 had no detectable effect on the activity of either of these proteins (Fig. 6A). One possible explanation for the lack of activity of ICP22 in these assays is suggested by data indicating that another HSV protein (UL13) mediates posttranslational modifications of ICP22 that may be required for activity (78, 79). Further studies are required to test this hypothesis.

Adenovirus E1a does not activate the endogenous α-globin gene. Previous studies have shown that adenovirus E1a activates expression of cotransfected copies of the β-globin gene in a fashion similar to HSV IE proteins (38). Moreover, E1a has often been compared to HSV ICP4 because both proteins are required for efficient expression of viral and cellular genes located their respective viral genomes during infection (8, 29, 88). Given these findings, we examined whether E1a can activate the endogenous α-globin genes in nonerythroid cells. α-Globin transcripts could not be detected in 293 cells (36) which express E1a (Fig. 6B), although HSV infection strongly induced expression in this cell type (data not shown). Moreover, HeLa cells transfected with an E1a expression vector (pE1a) did not display α-globin transcripts (Fig. 6B). Inasmuch as pE1a activated cotransfected copies of the adenovirus E1b and E4 promoters in HeLa cells (not shown), these results argue that E1a does not stimulate expression of endogenous α-globin genes.

Effects of ICP0, ICP4, ICP22, and E1a on transfected copies of the human α2-globin gene. Although the endogenous chromosomal α-globin gene is completely silent in nonerythroid

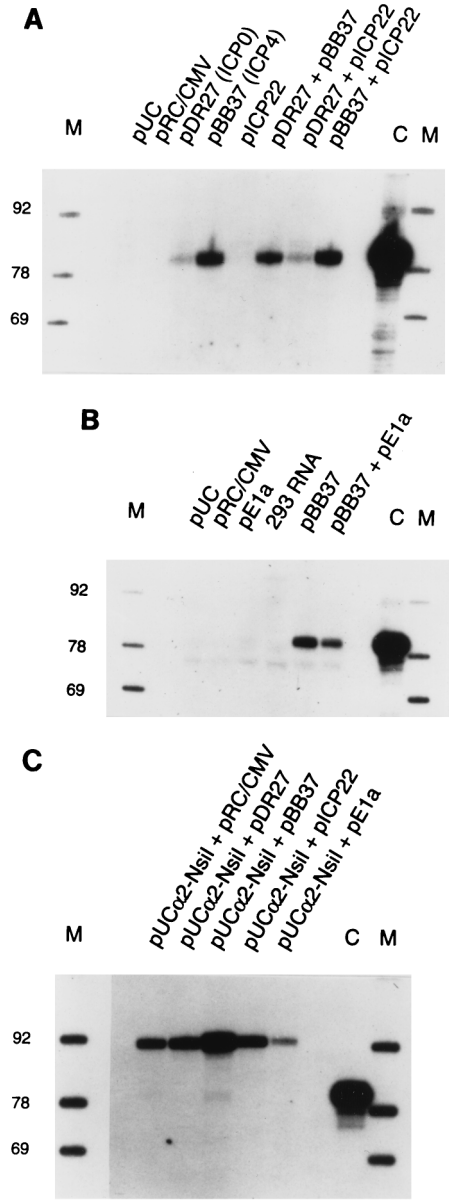

FIG. 6. Induction of α-globin transcripts in cells transiently expressing HSV IE polypeptides. (A) HeLa cells were transfected with control or expression vectors encoding ICP0, ICP4, ICP22 (pDR27, pDR37, and pICP22, respectively), separately or in combination, using the calcium phosphate transfection method. Total RNA extracted 36 h posttransfection was scored for α-globin transcripts by primer extension. C, control; M, size markers (see the legend to Fig. 1). (B) HeLa cells were transfected with control or expression vectors encoding adenovirus type 5 E1a (pE1a) or HSV ICP4 (pBB37), separately or together. Total RNA was extracted and assayed for α-globin RNA as for panel A. (C) HeLa cells were transfected with a plasmid bearing a modified α2-globin gene (pUCα2-NsiI) in combination with vectors expressing ICP0, ICP4, ICP22, and E1a. Total RNA was extracted and assayed for α-globin RNA by primer extension analysis. The plasmid-derived transcripts were 12 nt longer than the blood control RNA, giving rise to products approximately 92 nt long.

cells, transfected copies are robustly expressed. Inasmuch as ICP0, ICP4, and E1a have all been previously shown to enhance expression of newly introduced copies of a variety of viral and cellular genes, it was of interest to determine whether these viral proteins altered expression of a transfected α-globin gene. To discriminate between transcripts of the transfected gene and those arising from the endogenous chromosomal gene, we added a 12-bp insert at the *Nco*I site of a cloned α2 gene (pUCα2-NsiI). As a result, transcripts of this marked gene give rise to a primer extension product 12 nt longer than those of the endogenous gene. As expected, HeLa cells transfected with pUCα2-NsiI expressed high levels of the modified α2-globin mRNA (Fig. 6C), illustrating the constitutive expression of transfected copies of this gene in this cell type. The ICP0 expression vector pDR27 had at best a small stimulatory effect, while the ICP4 vector consistently increased expression ca. 10-fold. A longer exposure of the gel confirmed that in both cases transcripts arising from the endogenous gene were induced (data not shown). The ICP22 vector had no detectable effect, while pE1a consistently reduced expression of the transfected gene.

Effects of an ICP22 mutation on accumulation of ICP4 and ICP0 transcripts during infection of HeLa cells. The finding that ICP0 and ICP4 each induce expression of the endogenous α-globin gene in transfected cells explains why neither protein is essential for detectable activation during HSV infection. However, these data do not explain why inactivating ICP22 function during infection reduces induction ca. 10-fold, while ICP22 has no effect in the transfection assay. Purves et al. (78) have shown that ICP22 is required for the accumulation of ICP0 and several other viral mRNAs during infection of restrictive cell types. Inasmuch as the results presented above demonstrate that ICP0 and ICP4 are directly involved in α-globin induction, we compared the accumulation of ICP0 and ICP4 RNAs during infection of HeLa cells with R325tk$^+$ and the parental F strain by primer extension (Fig. 7). The data indicated that the levels of ICP4 and ICP0 transcripts are reduced relative to wild-type levels in this cell type. With ICP4 RNA, the effect was relatively small (twofold reduction) and was evident only at the early time points. However, ICP0 RNA levels were reduced throughout infection, and the deficit relative to wild-type virus became progressively more pronounced as the infection progressed. These data raise the possibility that ICP22 plays an indirect role in α-globin induction, by stimulating the expression of ICP0 and possibly ICP4.

DISCUSSION

The data presented in this paper demonstrate that correctly initiated transcripts derived from the endogenous human α1- and α2-globin genes accumulate during HSV infection of normal human fibroblasts and HeLa cells. The IE proteins ICP4 and ICP0 are each sufficient to trigger this effect in the absence of other HSV gene products, with ICP4 provoking a more robust response. Inasmuch as Macleod et al. could not detect transcription of the α-globin gene in nonerythroid cells by using a nuclear run-on assay (65), these data imply that HSV IE proteins activate transcription of the previously silent endogenous gene. Previously silent endogenous globin genes are also activated when nonerythroid cells are fused with erythroid cells to form heterokaryons (3, 4). However, the HSV-mediated induction reported here is distinct from the erythroid cell-specific reprogramming of globin gene expression that occurs in heterokaryons, because the α-globin gene is expressed whereas the β-globin gene is not.

The difference in responses of the endogenous α- and β-globin genes to HSV infection is intriguing, as the α- and β-globin promoters are both strongly activated by HSV IE polypeptides when they are newly introduced into cells as part of infecting recombinant HSV genomes (72, 88, 90). As reviewed in the introduction, the α- and β-globin gene clusters adopt very distinct chromatin structures in nonerythroid cells: the α-glo-

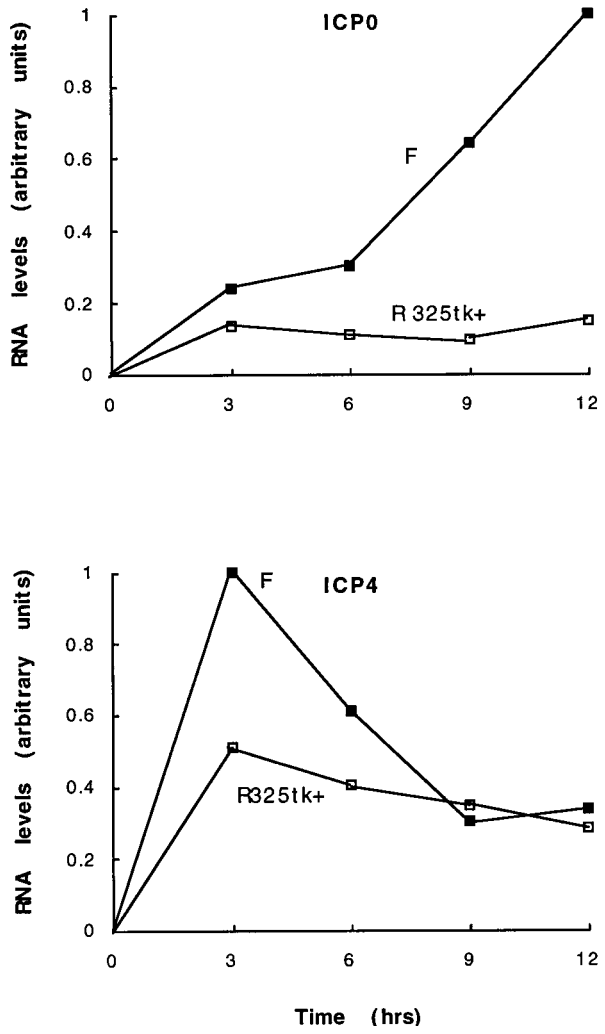

FIG. 7. Accumulation of ICP0 and ICP4 transcripts during infection with R325tk$^+$. HeLa cells were infected with 10 PFU of HSV-1 strain F or the ICP22-deficient mutant R325tk$^+$ per cell, and RNA samples extracted at the indicated times postinfection were analyzed for ICP0 (upper panel) and ICP4 (lower panel) transcripts by primer extension. Primer extension signals were then quantified by PhosphorImager analysis. For each transcript, values were normalized to the most intense signal observed during the time course.

bin locus is packaged in a relatively open early-replicating configuration (18, 98), while the β-globin cluster displays a DNase I-resistant, late-replicating, tightly closed conformation (32, 41). It seems likely that these differences in accessibility account, at least in part, for the differential responses of the endogenous α- and β-globin promoters to HSV IE proteins. If so, then the large number of other tissue-specific genes that are assembled into late-replicating, closed chromatin in nonexpressing cells may also be shielded from activation. However, this explanation probably cannot account for the inactivity of ζ-globin gene, which is located in the α-globin gene cluster and presumably packaged in a relatively open conformation. It will therefore be interesting to determine whether the ζ-globin promoter is inherently susceptible to activation by HSV IE proteins, by assaying the activity of ζ-globin genes delivered into cells as part of an infecting HSV genome.

The relatively open chromatin structure of the α-globin gene may stem in part from its CpG-rich promoter region (10): CpG islands are in general hypomethylated and less condensed than bulk chromatin (94). (Although Anteqera et al. reported that the entire α-globin gene and promoter are heavily methylated in HeLa cells [11, 2], we find that only the transcribed body of the gene is methylated in our subline [data not shown].) However, the possibility that all tissue-specific genes bearing CpG islands are activated by HSV infection appears to be excluded by our observation that hepatocyte-specific retinol binding protein mRNA does not accumulate during infection of HeLa cells (data not shown).

The complete silence of the endogenous α-globin gene in nonerythroid cells contrasts strikingly with the constitutive activity of the α-globin promoter borne on transfected templates (11, 16, 68). These data have led to the hypothesis that chromatin-dependent repression mechanisms act to prevent expression of the endogenous gene (11, 16). Our data indicate that HSV ICP0 and ICP4 either bypass or disrupt these silencing mechanisms. It is interesting to relate this activity to previous information about the functions of ICP0 and ICP4 in the HSV life cycle.

Little is known of the biochemical mechanism of action of ICP0; however, biological studies have demonstrated that it stimulates expression of all three temporal classes of HSV genes during lytic infection (12, 71, 85, 93). Moreover, it plays a pivotal role in triggering the onset of the viral lytic cycle, as evidenced by the finding that infecting HSV genomes often enter a transcriptionally quiescent state in the absence of ICP0 function (84, 93). A remarkable feature of this quiescent state is that otherwise constitutively active heterologous promoters embedded in the viral genome are silenced along with the HSV lytic promoters (53). Provision of ICP0 in *trans* breaks this quiescent state, leading to expression of the heterologous transgene and activation of the lytic cycle (44, 53). ICP0 also plays an important role in reactivation of the HSV genome from *in vivo* latency in sensory neurons (60). Taken in combination, these data suggest that ICP0 acts to convert HSV genomes from an inactive state to an active conformation. The observation that ICP0 induces expression of the resident α-globin gene without markedly stimulating expression of transfected copies of the same gene raises the possibility that an analogous transition is involved. If so, then further study of this system may provide a valuable model for understanding the mechanisms of HSV latency and reactivation.

ICP4 is an essential transcriptional activator that induces expression of HSV E and L genes and represses IE gene transcription (20, 100). It displays both sequence-specific and generalized DNA binding activity (24, 30, 31, 55, 69, 101), and mutations that inactivate DNA binding abolish the transcriptional regulatory properties of the protein (19, 21, 22, 73, 74, 87). Although appropriately positioned ICP4 binding sites target promoters for repression by ICP4 (57, 61, 81, 82), specific binding sites are not required for transactivation (42, 52, 89), which appears to be mediated through interactions with TATA-binding protein, TFIIB, and TAF250 (15, 91). The transcriptional activation function of ICP4 almost certainly underlies its ability to stimulate α-globin expression. Perhaps ICP4 and components of the basal transcriptional apparatus can access the TATA region of the chromosomal globin promoter despite the restrictive chromatin conformation that excludes other activator proteins present in HeLa cells. Inasmuch as ICP4 provides the functional equivalent of the activation signal delivered by Sp1 (50), this might prove sufficient to induce expression. Alternatively, ICP4 might actively disrupt the restrictive chromatin structure as a prelude to activation. These possibilities could be distinguished by high-resolution *in vivo* footprinting of the gene before and after induction by ICP4. Irrespective of the precise mechanism involved, the ability of

ICP4 to induce expression from a restrictive chromatin environment is likely to be significant during emergence of the HSV genome from latency.

We found that inactivating ICP22 function reduced induction during HSV infection ca. 10-fold. However, the ICP22 expression vector did not stimulate the α-globin gene in transfected cells, nor did it show synergistic effects with ICP0 or ICP4 in cotransfection assays. The possibility that the defect observed during infection stems from other unsuspected mutations in the R325tk$^+$ isolate appears to be excluded by the finding that another independently isolated ICP22 mutant (75) displays the same phenotype (data not shown). One possible explanation is that ICP22 is required for efficient expression of ICP4 and/or ICP0 during infection of HeLa cells. Consistent with this view, we found that ICP4 and ICP0 mRNA levels were reduced relative to wild-type levels during infection with R325tk$^+$. However, these data do not exclude the possibility that ICP22 also directly contributes to activation. If so, then the results of the transfection assays suggest that this effect requires the presence of one or more additional viral proteins. Supporting the latter view, Roizman et al. have provided evidence that ICP22 is phosphorylated by the viral protein kinase encoded by gene UL13 during infection (79). Furthermore, the activity of ICP22 may depend on this posttranslational modification, as evidenced by the finding that ICP22 and UL13 mutants display similar defects in viral gene expression (78). ICP22 is involved in HSV-induced changes to the phosphorylation status of the carboxy-terminal domain of the large subunit of the cellular RNA polymerase II (80). Inasmuch as such changes may alter the elongation properties of polymerase II (7), it may prove informative to explore the effects of ICP22 (with and without UL13) on the processivity of transcription of the endogenous α-globin gene in infected cells.

We suspect that further studies of the mechanisms by which HSV IE proteins stimulate expression of the endogenous α-globin gene will reveal novel activities of these regulators and shed light on mammalian gene silencing mechanisms.

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