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The Regulation of Synthesis and Properties of the Protein Product of Open Reading Frame P of the Herpes Simplex Virus 1 Genome

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Open reading frame P (ORF P) maps in the inverted repeat sequence *ab* and *b′a′* flanking the long unique (U_L) sequence of the herpes simplex virus 1 genome, within the sequence reported to be transcribed during latent infection of sensory neurons. Both the protein and the RNA were previously reported to be expressed only in cells infected with a deletion mutant or with a mutant carrying a *ts* lesion in the α4 gene encoding the infected cell protein no. 4 (ICP4), a major regulatory protein of the virus. In this report we show that (i) disruption of the ICP4 DNA binding site by replacement mutagenesis resulted in the overexpression of ORF P protein even at permissive temperatures, leading to productive infection; (ii) the expression of ORF P does not require prior viral protein synthesis; (iii) late in infection the ORF protein P is processed into multiple forms characterized by a slower electrophoretic mobility in denaturing gels; (iv) ORF P protein accumulates in nuclei of infected cells; and (v) in some nuclei of infected cells, ORF P protein is organized in the form of rods traversing the nucleus from the basolateral to the apical side. We conclude that ORF P has many of the properties predictive of a viral gene group, which we designate pre-α. Specifically, these could be induced by the α transinducing factor (also known as VP16) carried in the virion; they would be firmly shut off by the onset of expression of α genes required for productive infection; and in the absence of repressive effects of ICP4, their expression could be dependent on the number of viral DNA copies available for transcription. Finally, the productively infected cell would evolve a way of disposing excess pre-α proteins by posttranslational processing.

In this report we describe several unusual characteristics of the expression and properties of the product of the open reading frame P (ORF P) of the herpes simplex virus 1 (HSV-1) genome. Points relevant to this report are as follows.

(i) The HSV-1 genome consists of two covalently linked components, long (L) and short (S), which consist of unique sequences U_L and U_S, respectively, flanked by inverted repeats (33, 40). The inverted repeat sequences flanking U_L have been designated *ab* and *b′a′*, whereas the repeat sequences flanking the U_S have been designated *a′c′* and *ca* (12, 40). Together, the repeated sequences *ab*, *b′a′*, *a′c′*, and *ca* account for 20% of the genome (21, 40) and contain two copies of four genes, α0, $\gamma_1$34.5, and α4 on one strand and ORF P antisense to that strand (2, 6, 18, 24, 27). The most striking feature of ORF P is that it is nearly completely antisense to $\gamma_1$34.5. Only 8 codons of ORF P are not antisense to $\gamma_1$34.5, and only 23 codons of $\gamma_1$34.5 are not antisence to ORF P (18). There are no other examples in the HSV-1 genome of the coding domains of two genes being completely antisense to each other (21).

(ii) The studies in this laboratory stemmed from the observation that the only domain of the HSV-1 genome expressed during latent infection of sensory neurons maps largely to the sequence *ab* and its inverted repeat *b′a′* (36, 39). The expression is that of RNA designated as latency-associated transcripts (LATs). The largest unspliced LAT is 8.5 kb, and its domain encompasses all but approximately 1 kbp each of *ab* and *b′a′* (7, 8, 23, 43). The transcribed strand is antisense to the strand encoding the $\gamma_1$34.5 and α0 genes. Examination of the domain of the transcript revealed at least 16 ORFs (designated by the letters A through P) capable of encoding polypeptides larger than 50 amino acids (18). Since protein products had not been detected in latently infected cells, we tagged five of the most promising ORFs with nucleotide sequences encoding an epitope of human cytomegalovirus (CMV) which reacted with a monoclonal antibody to which we had access (19). Of the five ORFs tested, ORF P was the only one which was expressed in detectable quantities. While this work was in progress, Bohenzky et al. (5) reported the existence of a promoter driving transcription of an HSV-1 domain which included ORF P. Subsequently, Yeh and Schaeffer (42) mapped the 5′ end of the transcript to a position immediately upstream of ORF P.

(iii) The HSV-1 genes have been shown to fall into several major groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (13). The five known α genes are expressed first and do not require prior viral protein synthesis (13, 14). Functional α proteins are required for the expression of β genes, and both prior protein synthesis and viral DNA synthesis are required for optimal expression of γ genes (14). The product of the α4 gene known as the infected cell protein no. 4 (ICP4), a DNA-binding protein, plays a dual role as a transactivator of most viral genes and as a repressor of at least the expression of its own gene and possibly that of others (10, 14, 22, 28). Repression of its own gene is accomplished by its interaction with a strong consensus DNA-binding site at the transcription initiation site since mutagenesis of the site abolishes repression (22). Yeh and Schaffer reported that their transcript was detected only in the absence of ICP4, most likely because its expression was repressed by the presence of an ICP4 binding site at the 5′ terminus of the transcribed sequence (42). They also reported that their transcript was not made in cells infected and maintained in the presence of cy-

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cloheximide, and therefore the synthesis of the transcript required the participation of other α proteins. In the studies reported earlier (18), we showed that ORF P protein accumulated in cells infected with a limited-passage HSV-1 strain which carries a *ts* mutation in the α4 gene and is maintained at the nonpermissive temperature.

We report here that ORF P protein is overexpressed and accumulates in nuclei of cells productively infected with a virus in which the ICP4 binding site at the transcription initiation site of ORF P had been altered by mutagenesis, that ORF P expression does not require prior viral protein synthesis, and that late in infection ORF P protein is processed to forms differing in their electrophoretic mobilities in denaturing gels.

MATERIALS AND METHODS

Cells and viruses. HSV-1(F), a limited-passage virus, is the prototype HSV-1 strain used in this laboratory (9). The recombinant viruses described previously were as follows. HSV-1(F)Δ305 lacks the 501-bp *Bgl*II-*Sac*I fragment from the domain of the thymidine kinase (*tk*) gene (25). R7519 contains an in-frame CMV gB epitope inserted into the *Bst*EII site of ORF P (18). This epitope reacts with the CH28-2 monoclonal antibody described elsewhere (19). R7522 contains two copies of the CMV epitope tag inserted in frame at the *Bst*EII and *Dra*III sites of ORF P (18). Infected cell lysates of R7519 separated on denaturing polyacrylamide gels and transferred to a nitrocellulose sheet form a single band with an apparent M_r of 30,000 which reacts with the CH28-2 antibody whereas the corresponding band of lysates of cells infected with R7522 has a slower electrophoretic mobility as would be expected of a doubly tagged ORF P (18). Viral stocks were made with and titrations of the recombinant viruses were done in Vero cells (American Type Culture Collection). Transfections of viral DNA were done in rabbit skin cells originally obtained from J. McClaren. As previously described (26), the selection for tk^+ viruses were done in 143TK$^-$ cells overlaid with Dulbecco modified Eagle medium containing 5% fetal bovine serum, hypoxanthine, aminopterin, and thymidine) whereas selection for *tk* mutant viruses were done in 143TK$^-$ cells overlaid with Dulbecco modified Eagle medium containing 5% newborn calf serum and 40 μg of bromodeoxyuridine per ml of medium. Viral DNA was isolated from infected cells and purified on NaI gradients or on a 5 to 20% potassium acetate gradient as described elsewhere (15, 41). All of the viruses used in this study contain wild-type α4 genes; they differ solely in the structure of the genome domain encompassing the ORF P transcriptional unit.

Antibodies. The mouse monoclonal antibodies H1114 to ICP27 and CH28-2 to an epitope of the glycoprotein B of human CMV described elsewhere (1, 19) were obtained from Goodwin Biotechnology, Inc. (Plantation, Fla.).

Plasmids. The HSV-1(F) sequences in plasmid pRB4794 used as a probe to detect ORF P domain sequences have been described previously (18). The oligonucleotide CCGGAGAGAATTCGGCAGGAGCCGCGCATATATACG CTGGGAGCCGGCCCGCCCCCGAGGCGGGCCCGCCCTCGGAG GGCGGGACTGG and its complement, designed to yield cohesive *Bsp*I and *Msc*I ends upon annealing, were synthesized on an Applied Biosystems DNA synthesizer and used to replace the sequences between the *Bsp*EI and *Msc*I sites in *Bam*HI S, yielding pRB4855. These *Bsp*E1 and *Msc*I sites flank the transcription initiation site of ORF P. The *Dra*III-*Dra*I fragment from pRB4795, which contains a CMV tag in ORF P, was used to replace the *Dra*III-*Dra*I fragment of pRB4855. The resulting plasmid was designated pRB4856 and contains the altered sequence between the *Bsp*EI and *Msc*I sites and a CMV epitope tag.

Analyses of viral DNAs. Viral DNAs were digested with *Nco*I and *Eco*RI, subjected to electrophoresis on a 28-cm, 0.8% agarose gel, and transferred to a Zeta probe membrane as recommended by the manufacturer (Bio-Rad, Richmond, Calif.). The membrane was rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate); soaked in a prehybridization solution containing 30% formamide, 6× SSC, 1% milk, 1% sodium dodecyl sulfate (SDS), and 100 μg of single-stranded calf thymus DNA per ml for at least 30 min at 68°C, and then hybridized in the same solution and at the same temperature with 1×10^6 to 5×10^6 cpm of the appropriate denatured ^{32}P-labeled probe as previously described (18). Autoradiographic images were made by overnight exposure to Kodak XAR5 film.

Immunoblots. Immunoblots were done as described previously (18). Briefly, infected cells were rinsed, scraped into phosphate-buffered saline (PBS), and spun down. The cell pellet was resuspended in disruption buffer, sonicated briefly, denatured by boiling, and subjected to electrophoresis in denaturing 12% DATD polyacrylamide gels, electrically transferred to nitrocellulose, blocked, reacted with the indicated antibody and then with the secondary goat anti-mouse antibody conjugated to alkaline phosphatase, and processed for colorimetric detection with alkaline phosphatase as recommended by the manufacturer (Bio-Rad).

Cycloheximide reversal. One hour prior to infection, Vero cells in two 25-cm^2 flasks were treated with cycloheximide (Sigma, St. Louis, Mo.) (100 μg/ml of medium containing 1% newborn calf serum), infected with 50 PFU per cell, and maintained in the same concentration of cycloheximide for 6 h. At that time, the medium was replaced with medium containing cycloheximide and actinomycin D (Sigma) (100 and 10 μg/ml, respectively) and incubated for 1 h. Cells were then extensively washed with medium containing actinomycin D and incubated with medium containing 10 μg of actinomycin D per ml for an additional 8 h, after which the cells were scraped into PBS, harvested, and processed for immunoblotting as described above.

Nuclear localization. Cultures either infected with 20 PFU of R7519, R7522, or HSV-1(F) per cell and incubated at 39.5°C for 22 h or infected with 10 PFU of R7528, R7519, or HSV-1(F) and incubated for 11 h at 37°C were harvested by scraping, rinsed in PBS, collected by centrifugation, and suspended in 200 μl of PBS. Sucrose was added to 5%, and the suspension was placed on wet ice for 10 min. Nonidet P-40 was added to a final concentration of 0.5%, and the test tubes were inverted gently to mix the contents and left on ice for 15 min. The suspensions were centrifuged in a microcentrifuge for 5 min. The supernatant fluid and pellet representing the cytoplasmic and nuclear fractions were separated. The pellet was washed once with PBS, 4× disruption buffer was added to each fraction, and fractions were processed for immunoblotting as described above.

Immunofluorescence. Vero cells were seeded on microscope slides containing four wells. Cells were exposed to 5 or 10 PFU of R7528 or HSV-1(F) per cell and harvested at 12 or 22 h postinfection. Cells were fixed to the slides at −20°C in methanol for 20 min and then blocked in 1% bovine serum albumin (BSA) in PBS for at least 1 h. The coverslip cultures were reacted with the CH28-2 monoclonal antibody for 2 h and with the second antibody conjugated to Texas red (Molecular Probes, Inc., Eugene, Oreg.) for 1 h. Finally, the slides were mounted in 10% PBS in glycerol and inspected with a Zeiss confocal microscope. Digital images of the fluorescent profiles were acquired by using software provided with the Zeiss confocal microscope and printed with a CP210 Codonics digital printer.

RESULTS

The proximal 5′ nontranscribed (promoter) domain of ORF P contains sequences similar to those of response elements present in the promoter of the α4 gene. Figure 1A depicts a region of the inverted repeats flanking U_L and U_S and containing the domains of the unspliced LAT, ORF P, $\gamma_1$34.5, α4, and α0 genes. Panel B shows the positions of the α transinducing factor (αTIF) response elements in the α4 gene, similar sequences in the ORF P, and the sequences surrounding the transcription initiation sites of ORF P and α4. The nucleotide sequences of the αTIF response elements are shown in panel C. The salient features of the data are as follows.

(i) Upstream of the transcription initiation site of ORF P there is a canonical TATA box and a homolog of the sequence 5′-NC GyATGnTAATGArATTCyTTGnGGG-3′ reported previously to be the consensus sequence for the αTIF response element (4, 17, 20, 25, 34, 35). This sequence is in the same orientation vis-a-vis the TATA box as its homologs in the α4 gene, and therefore it could be expected that ORF P would be expressed as an α gene.

(ii) ORF P is expressed poorly, if at all, in cells expressing fully functional ICP4 (18). As noted earlier, ORF P shares with the α4 gene a canonical ICP4 binding site at its transcription initiation site (5, 11, 42) and its transcripts were detected only in cells infected and maintained at the nonpermissive temperature with *ts* mutants in the α4 gene which no longer regulate the expression of that gene at that temperature and in cells infected with mutants containing deletions or missense mutations in α4 (42). Previous studies have shown that the binding site at the transcription initiation site of the α4 genes represses transcription inasmuch as mutagenesis leading to the destruction of the binding site increased the number of the α4 transcripts 10-fold at 4 h after infection (22).

In the sections described below we show that ORF P is expressed in cells infected and maintained in the presence of cycloheximide and that substitution of the nucleotides forming the ICP4 binding site with a sequence shown previously not to bind ICP4 (22) results in a significant increase in the expression of ORF P protein.

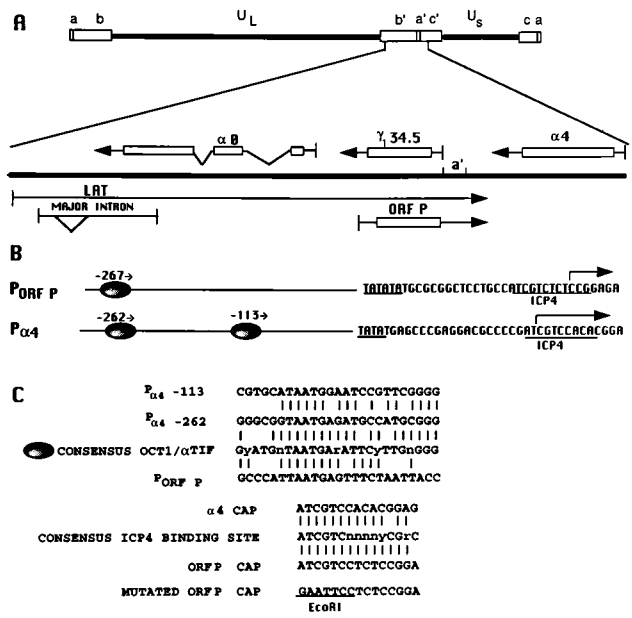

FIG. 1. Schematic representation of arrangements of genes and of select promoter elements of α4 and ORF P genes in repeats flanking U_L and U_S sequences of HSV-1 genome. (A) arrangement of α0, $\gamma_1$34.5, and α4 genes on one strand and of DNA domains of LATs and ORF P on the complementary strand. The sequences encoding the 2.0-kb (major intron) and 1.5-kb nuclear RNAs found in neurons harboring latent virus are also shown. (B) Position and orientation of αTIF response elements (ovals) in domains of α4 and ORF P genes proximal to the respective TATA boxes. The nucleotide sequences at the transcription initiation sites of these genes are indicated by the arrows, and the nucleotide sequences forming the ICP4 binding site at the transcription initiation sites of these genes are underlined. (C) Comparison of response elements of αTIF and binding sites of ICP4 with their respective consensus sequences (11, 20, 34). The last line shows the nucleotide sequence of the substitution to destroy the ICP4 binding site at the transcription initiation site of ORF P (underlined) in the recombinant virus R7528. This sequence is similar to that used to destroy the binding site of ICP4 at the transcription initiation site of the α4 gene (22).

Disruption of the ICP4 binding site by mutagenesis relieves repression of ORF P. Removal of the sequences homologous to the ICP4 binding site at the transcription initiation site of both copies of ORF P was done in two steps, as follows. As illustrated in Fig. 2, a deletion mutant (R3659) in which two kbp of the $\gamma_1$34.5 gene was replaced with the α27-*tk* sequences was the gift of J. Chou. It was constructed by homologous recombination following cotransfection in rabbit skin cells of intact HSV-1(F)Δ305 DNA and the HSV-1(F) *Bam*HI S fragment in which the chimeric α27-*tk* gene replaced the 2-kbp *Bst*EII-*Stu*I fragment in the domain of the $\gamma_1$34.5 and ORF P genes. The recombinant was selected by passage of the progeny of transfection in 143TK$^-$ cells overlaid with HAT medium as previously described (26). One plaque-purified virus designated R3659 was found to contain the α27-*tk* gene inserted into both copies of the domains of $\gamma_1$34.5 and ORF P genes.

In the second step the 90-bp *Bsp*EI-to-*Msc*I fragment in pRB143 was replaced with an oligonucleotide of identical size but differing from the original sequence in that the ICP4 binding site was destroyed by mutagenesis to create pRB4855. The sequences replacing the binding site shown in Fig. 1C were similar to those used to destroy the binding site at the transcription initiation site of the α4 gene and also contain a diagnostic *Eco*RI cleavage site (22). The *Dra*III-*Dra*I fragment of pRB4792, which contains ORF P with an epitope tag at the *Bst*EII site, was then used to replace the *Dra*III-*Dra*I fragment of pRB4855, creating a plasmid, pRB4856, containing ORF P with a single tag at the *Bst*EII site and a mutation of the ICP4 binding site at the cap site. Plasmid pRB4856 was cotransfected with intact R3659 DNA on rabbit skin cells, and the progeny of the transfection were plated on 143TK$^-$ cells in the presence of bromodeoxyuridine as previously described (26). A *tk* mutant recombinant (R7528) produced by homologous recombination between the HSV-1(F) sequences in pRB4856 and R3659 was plaque purified and digested with *Nco*I and *Eco*RI. Digestion of the *Bam*HI S fragment with *Nco*I and *Eco*RI should yield an intact *Nco*I fragment with a size of approximately 1,800 bp (Fig. 2, band A, line 3) inasmuch as this fragment does not have an *Eco*RI cleavage site. R3659 DNA should yield two *Nco*I fragments, with sizes of approximately 700 bp (band B) and 80 bp, derived from the original *Nco*I sites in *Bam*HI S to the *Nco*I sites in the *tk* gene (Fig. 2, lines 4 and 5). R7528 DNA would be expected to yield the same *Nco*I fragment as HSV-1(F). However, the inserted *Eco*RI restriction endonuclease site would be cleaved, yielding two *Nco*I-*Eco*RI fragments with sizes of 920 and 930 bp, respectively. These fragments have been labeled in Fig. 2, lines 6 and 7, as band C inasmuch as they are not separated on a 0.8% agarose gel. As shown in Fig. 3, the radiolabeled pRB4794 probe hybridized to the expected fragments contained in electrophoretically separated *Nco*I and *Eco*RI digests of the DNAs. The 80-bp fragment predicted from the digestion of the R3659 DNA with *Nco*I was too small to be detected in this gel.

To determine the effect of the mutagenesis of the ICP4 binding site, replicate Vero cell monolayer cultures were in-

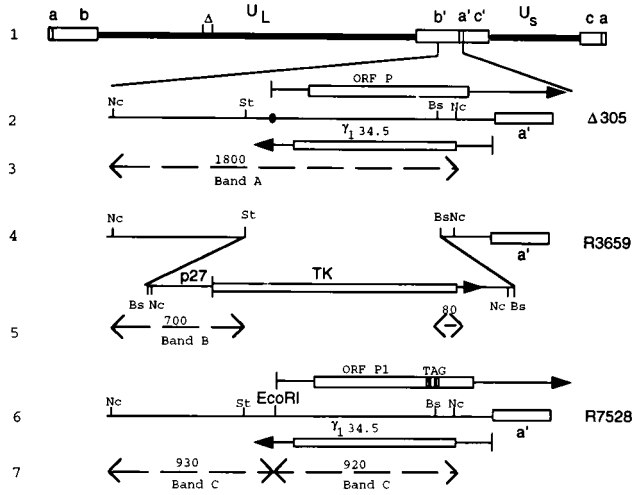

FIG. 2. Schematic representations of sequence arrangements in recombinant viruses used in these studies. Line 1, the HSV-1(F)Δ305 genome lacks the 501-bp *Bgl*II-*Sac*I sequence from the domain of the *tk* gene in the *Bam*HI Q fragment. Line 2, the domains of the ORF P and $\gamma_1$34.5 genes in the inverted repeat sequence *b'a'* flanking U_L sequence. The identical sequences in inverted orientation map in the *ab* repeat (data not shown). The coding domains of the ORF P and $\gamma_1$34.5 as well as the domain of the *a* sequence are shown as open quadrangles. Line 4, sequence arrangement of the relevant domain of the recombinant R3659 in which the *Stu*I (St)-*Bst*EII (Bs) fragment from the domains of ORF P and $\gamma_1$34.5 was replaced by the chimeric α27-*tk* gene. Although not shown diagrammatically, this substitution was made in both the *ab* and *b'a'* domains of the recombinant genome. Line 6, sequence arrangement of the relevant domains of the recombinant R7528. In this recombinant, the α27-*tk* gene was replaced with an ORF P gene carrying a CMV epitope tag in frame at the *Bst*EII site and a mutated transcription initiation site containing a diagnostic *Eco*RI site. Lines 3, 5, and 7, expected sizes of bands generated by restriction enzyme digestion with *Nco*I (Nc) and *Eco*RI and probed with a plasmid containing the HSV-1 *Nco*I-*Nco*I fragment of *Bam*HI S; HSV-1(F) would yield band A, R3659 would yield band B, and R7528 would yield a double band, C.

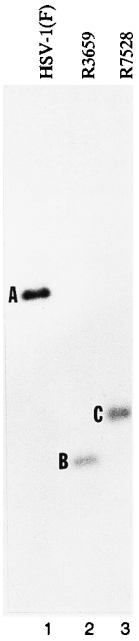

FIG. 3. Autoradiographic image of electrophoretically separated viral DNA fragments containing sequences homologous to a region of ORF P. Viral DNAs were digested with *Nco*I and *Eco*RI, electrophoretically separated on a 0.8% agarose gel, transferred to Zeta probe membranes, hybridized to a radiolabeled DNA of plasmid pRB4794 containing the *Nco*I-*Nco*I subfragment of *Bam*HI S, and exposed to Kodak XAR5 film. The predicted sizes of the fragments generated by the cleavages shown in Fig. 2 are approximately 1,800 bp for band A, 700 bp for band B, and 920- and 930-bp fragments for band C. Note that bands A and B contain *Nco*I-*Nco*I termini whereas those in bands C contain *Nco*I-*Eco*RI termini.

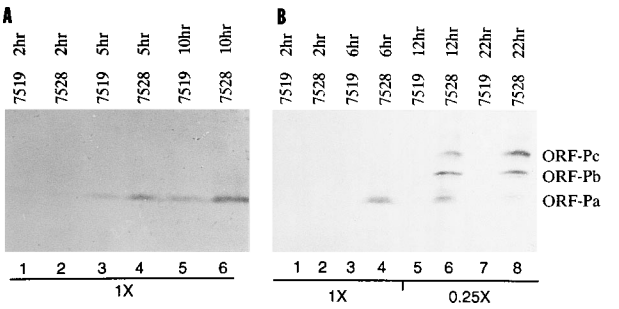

FIG. 4. Photograph of infected cell proteins electrophoretically separated in denaturing gels and reacted with CH28-2 monoclonal antibody to CMV tag inserted into ORF P. Replicate Vero cell 25-cm^2 flask cultures were infected for 2, 5, or 10 h at 39.5°C (A) or 2, 6, 12, or 22 h at 37°C (B) with R7519 or R7528 (20 PFU per cell), harvested, solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted first with the CH28-2 antibody to the epitope tag and then with goat anti-mouse immunoglobulin G coupled with alkaline phosphatase. All viruses carry ORF P tagged with a single epitope. In panel A, the contents of an entire flask were loaded per lane. In panel B, lanes 1 to 4, the contents of an entire 25-cm^2 flask were loaded per lane, and in lanes 5 to 10, 25% of the contents of a 25-cm^2 flask were loaded per lane. The single bands in panel A and panel B (lane 4) with the electrophoretic mobility of a singly tagged ORF P were labeled ORF Pa (designated ORF P1 in a previous paper [18] and Fig. 6). In lanes 6 and 8, the more slowly migrating bands were designated ORF Pb and ORF Pc.

fected with R7528 or R7519, the virus containing wild-type sequences at the transcription initiation sites of ORF P and the same epitope tag at the *Bst*EII site, and maintained at either 39.5 or 37°C. The infected cells were harvested either at 2, 5, and 10 h or at 2, 6, 12, and 22 h after infection and incubated at 39.5 or 37°C, respectively. The cells were then solubilized in disruption buffer containing SDS, electrophoretically separated on a denaturing polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with the monoclonal antibody to the single CMV tag present in both copies of the gene of both recombinant viruses.

As shown in Fig. 4A, at both 5 and 10 h after infection the amounts of ORF P protein in lystes of cells infected with R7528 detected by the CMV antibody were only marginally larger than those detected in lysate of cells infected with R7519. This would be expected since at 39.5°C ICP4 would not be functional and the distinction between the expression of ORF P of viruses with wild-type and mutated sequences at the transcription initiation site of ORF P would be expected to be minimal. In contrast to these results, much larger amounts of ORF P protein were detected in lysates of R7528 relative to those of R7519 in cultures incubated at 37°C (Fig. 4B). Specifically, ORF P protein was not present in appreciable amounts in lysates of cells infected with R7519 and harvested at any time after infection (Fig. 4B, lanes 1, 3, 5, and 7). Lysates of cells infected with the R7528 virus harvested at 6 h after infection formed a single band (ORF Pa) (Fig. 4B, lane 4) reactive with the antibody to the tag. Lysates harvested at 12 and 22 h after infection each formed three bands (ORF Pa, ORF Pb [itself a doublet], and ORF Pc) (Fig. 4B, lanes 6 and 8) reactive with the antibody to the tag. The ORF Pb and ORF Pc bands migrate more slowly than ORF Pa. Furthermore, in lysates of cells harvested at 22 h the bulk of the tagged ORF P protein appeared in the more slowly migrating bands.

We conclude from these results that the sequences homologous to the ICP4 binding site mutagenized at the cap site of ORF P do repress the expression of ORF P and that in the absence of these sequences ORF P is expressed in productively infected cells maintained at 37°C. The observation that late in infection ORF P protein forms more slowly migrating bands and that the bulk of the protein shifts to the more slowly migrating bands late in infection suggests that ORF P protein is processed in some fashion in the productively infected cells.

ORF P is detected as early as 2 h after infection at 39.5°C. To determine the earliest time at which ORF P driven by its native promoter can be detected in infected cells, replicate 25-cm^2 flask cultures of Vero cells were infected with HSV-1(F) or with R7522 containing ORF P genes each tagged with two copies of the CMV epitope. The cultures were incubated at 34, 37, or 39.5°C and harvested at 2, 5, or 10 h after infection. The results (Fig. 5) were as follows. (i) As in all previous studies, CMV antibody CH28-2 did not react with lysates of cells infected with HSV-1(F) and incubated at any of the temperatures tested (18). (ii) Lysates of cells infected with R7522 and maintained at 39.5°C formed a single band reactive with the CMV antibody at all times tested (Fig. 5, lanes 6, 12, and 18). The amounts of reactive antigen were small in lysates harvested at 2 h after infection (lane 6) and increasingly more abundant in cultures harvested at later times (lanes 6 and 18). In other experiments (data not shown), ORF P protein continued to accumulate as late as 20 h after infection. (iii) ORF P was detected in lysates of cells infected with R7522 at 37°C and harvested at 5 h (Fig. 5, lane 10) and 10 h (Fig. 5 lane 16) after infection. The amounts of ORF P protein were barely detectable in the 5-h sample. At 10 h after infection the amounts of ORF P protein were significantly smaller than those present in the corresponding culture of infected cells incubated at 39.5°C. (iv) ORF P protein was not detected in lysates of cells incubated at 34°C.

We conclude from these results that the amounts of ORF P produced in productively infected cells are small, but that the

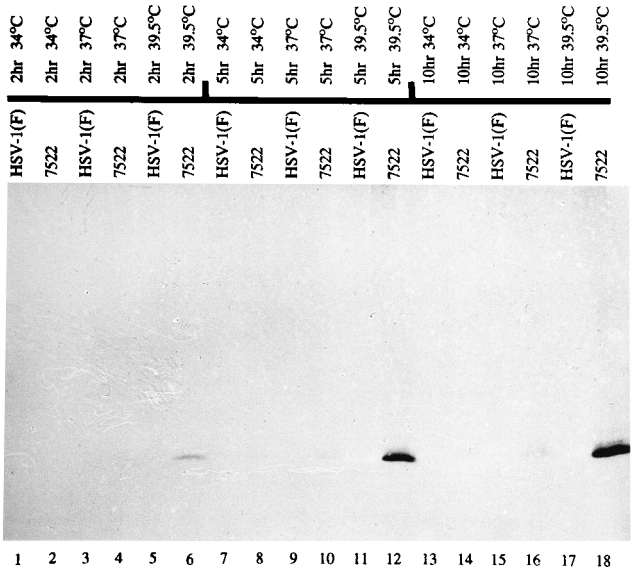

FIG. 5. Photograph of infected cell proteins electrophoretically separated in denaturing gels and reacted with CH28-2 monoclonal antibody to CMV tag inserted into ORF P. Replicate Vero cell 25-cm^2 flask cultures were infected for 2, 5, or 10 h at 34, 37, or 39.5°C with HSV-1(F) or R7522 (10 PFU per cell), harvested, solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted first with the CH28-2 antibody to the epitope tag and then with anti-mouse immunoglobulin G coupled with alkaline phosphatase. Lanes: 1 to 6, lysates of cells infected for 2 hours with HSV-1(F) at 34, 37, and 39.5°C (lanes 1, 3, and 5, respectively) and R7522 at 34, 37, and 39.5°C (lanes 2, 4, and 6, respectively); 7 to 12 and 13 to 18, lysates from cells harvested 5 or 10 h, respectively, after infection. The bands correspond to ORF P tagged with two copies of the CMV epitope.

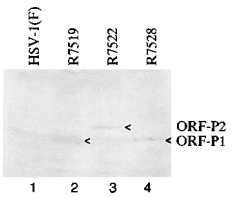

FIG. 6. Photograph of infected cell proteins electrophoretically separated in denaturing gels and reacted with CH28-2 monoclonal antibody to CMV tag inserted into ORF P. Replicate Vero cell cultures were infected with 50 PFU per cell for 6 h at 37°C in the presence of cycloheximide (100 μg/ml), incubated for 1 h in medium containing both cycloheximide and actinomycin D (10 μg/ml), rinsed, and incubated in medium containing actinomycin D only for an additional 8 h before harvesting. The infected cell lysates were processed as described in Materials and Methods. The contents of two 25-cm^2 flasks were loaded in each lane. The bands labeled ORF-P1 in lanes 2 and 4 represent the singly tagged ORF P whereas the band labeled ORF-P2 in lane 3 represents the doubly tagged ORF P.

protein begins to accumulate very early after infection and in the absence of functional ICP4 it can be detected at the earliest time tested (2 h after infection).

ORF P is expressed in the absence of prior viral protein synthesis. To determine whether prior viral protein synthesis is necessary for the expression of ORF P, a cycloheximide reversal experiment was done. Cycloheximide allows viral RNA synthesis but precludes protein synthesis. Vero cell monolayers were pretreated with medium containing 100 μg of cycloheximide per ml and infected with HSV-1(F), R7519, R7522, or R7528 at 37°C in the presence of the same concentration of cycloheximide. At 6 h after infection, the medium was replaced with one containing cycloheximide (100 μg/ml) and actinomycin D (10 μg/ml). At 7 h after infection, the cells were rinsed extensively with warm medium containing actinomycin D and finally overlayed with medium containing actinomycin D, which prevents further RNA synthesis but allows the translation of accumulated viral mRNA. The infected cells were harvested after 8 h in the presence of actinomycin D and processed for immunoblotting as described in Materials and Methods. The proteins were transferred to nitrocellulose and incubated with CH28-2, which recognizes the epitopes inserted in the ORF P contained in R7519, R7522, and R7528 but not in HSV-1(F). As shown in Fig. 6, a protein that migrates with the mobility of a singly tagged ORF P could be detected in the lanes containing R7519 and R7528, and a band that migrates with the mobility of the doubly tagged ORF P could be detected in the lane containing R7522. This demonstrates that prior viral protein expression is not necessary for expression of ORF P.

ORF P is a nuclear protein. To determine the intracellular localization of ORF P protein, two series of experiments were done. In the first, replicate cultures of Vero cells were infected with R7519, R7522, or HSV-1(F) and incubated at 39.5°C. The infected cells were harvested at 22 h after infection and lysed with Nonidet P-40 as described in Materials and Methods. The nuclei were separated from the cytoplasm by centrifugation. Both the cytoplasmic fraction (supernatant fluid) and nuclear fraction (pellet) were solubilized in disruption buffer containing SDS, subjected to electrophoresis, and processed for immunoblotting with the CH28-2 monoclonal antibody as described in Materials and Methods. To control for loading of nuclear protein, the immunoblots were reblotted with monoclonal antibody to ICP27, which localizes in the nucleus (1). ORF P protein was readily detected in the nuclear fraction of cells infected with R7522 or R7519 but, as expected, not in that of cells infected with the wild-type virus HSV-1(F) (Fig. 7). It is noteworthy that the monoclonal antibody to ICP27 reacted with two bands in lysates of these cells, suggesting that ICP27 was subject to partial degradation in these cells.

In the second series of experiments, infected cells were probed with antibody conjugated to a fluorescent probe. In this series of experiments, coverslip cultures of Vero cells infected with HSV-1(F) or R7528 and incubated at 37°C were reacted at various times after infection with monoclonal antibody CH28-2 to the CMV epitope. Occasionally, HSV-1(F)-infected cells showed faint diffuse fluorescence (Fig. 8A) quite distinct from the fluorescence of the intranuclear granules of cells infected with R7528 recombinant (Fig. 8B and C). Cells infected with R7528 virus showed the presence of fluorescent granules at 12 h after infection. These granules coalesced into large masses at 22 h after infection.

The distribution of the ORF P protein in the nucleus was also examined by analyses of digitized images of serial 0.5-μm-thick sections reconstructed with a Zeiss confocal microscope through the *z* axis (basolateral to apical) of the infected cells. The results shown in Fig. 9 are as follows. In some cells exemplified by the cell in the panel A, the distribution of ORF P antigen is asymmetric, and because of the large amount of the protein, it is difficult to reconstruct precisely the configuration of protein mass. In other nuclei, exemplified in the cell shown in panel B, the ORF P antigen takes the form of solid rods which project from the inner basolateral surface to the apical surface of the nucleus. For example, the three small masses of ORF P protein identified by arrows in panel B, section d, are part of rods which can be readily traced through all of the 0.5-μm-thick sections, from sections c to m. We cannot relate the distribution of ORF P protein to that of other infected proteins at this time.

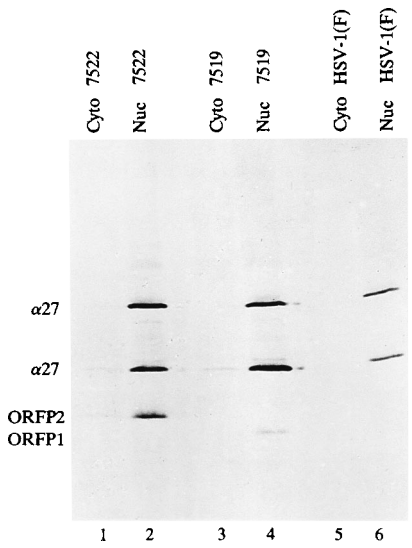

FIG. 7. Photograph of infected cell proteins electrophoretically separated in denaturing gels and reacted with CH28-2 monoclonal antibody to CMV tag inserted into ORF P. Vero cells grown in two 25-cm^2 flasks infected with HSV-1(F), R7519, or R7528 and maintained for 22 h at 39.5°C were harvested into PBS, pelleted by centrifugation, resuspended in PBS with sucrose, lysed in 0.5% Nonidet P-40, and centrifuged to separate nuclear and cytoplasmic fractions. These fractions were solubilized, subjected to electrophoresis on a denaturing gel, transferred to nitrocellulose, and sequentially blotted with the CH28-2 antibody and the H1114 antibody, which reacts with ICP27. Lanes: 1 and 2, cytoplasmic and nuclear fractions, respectively, of cells infected with R7522; 3 and 4, cytoplasmic and nuclear fractions, respectively, of cells infected with R7519; 5 and 6, cytoplasmic and nuclear fractions, respectively, of cells infected with HSV-1(F). ORF P1 and ORF P2 are bands of proteins tagged with one and two copies of the CMV epitope, respectively.

DISCUSSION

The salient features of the ORF P protein are as follows. (i) Its coding sequence is in the domain transcribed during latent infection (7, 8, 23, 43), and to date, no protein product has been reproducibly demonstrated to arise from translation of any of the RNAs from that region of the genome in latently infected neurons. (ii) ORF P is expressed in the absence of prior protein synthesis, consistent with the presence of an αTIF response element closely matching the consensus sequence described by Mackem and Roizman (20) and located upstream of its transcription initiation site. Bohensky et al. (5) also noted a low level of induction of the RNA now known to encode ORF P in cells cotransfected with the sequences encoding this RNA and αTIF. In the absence of de novo viral protein synthesis the level of expression of ORF P was low, reflecting the possibility that ORF P mRNA is unstable or that the distance between the αTIF response element and the TATA box is larger than that observed for the α4 gene. Nevertheless, ORF P appears to share with α genes inducibility by αTIF (20). The failure of Yeh and Schaeffer (42) to detect transcripts in cells infected and maintained in the presence of cycloheximide may reflect both the small amount and the low degree of stability of the mRNA. (iii) The expression of ORF P is strongly repressed by ICP4, the product of the α gene. This conclusion is drawn from the observation that the ORF P protein was detected in significant amounts only in the absence of functional ICP4. (iv) Although not directly relevant to this report, preliminary experiments showed that in the absence of the repressive effect of ICP4, the accumulation of ORF P protein is affected by phosphonoacetate at concentrations sufficient to block viral DNA synthesis (data not shown). While its behavior is that of a γ_1 gene, a simpler interpretation is that the expression of ORF P might be tightly linked to the template copy number. The regulation of ORF P is different from that of α genes, which are not absolutely repressed by ICP4. There are similarities, however, to the regulation of LATs which are

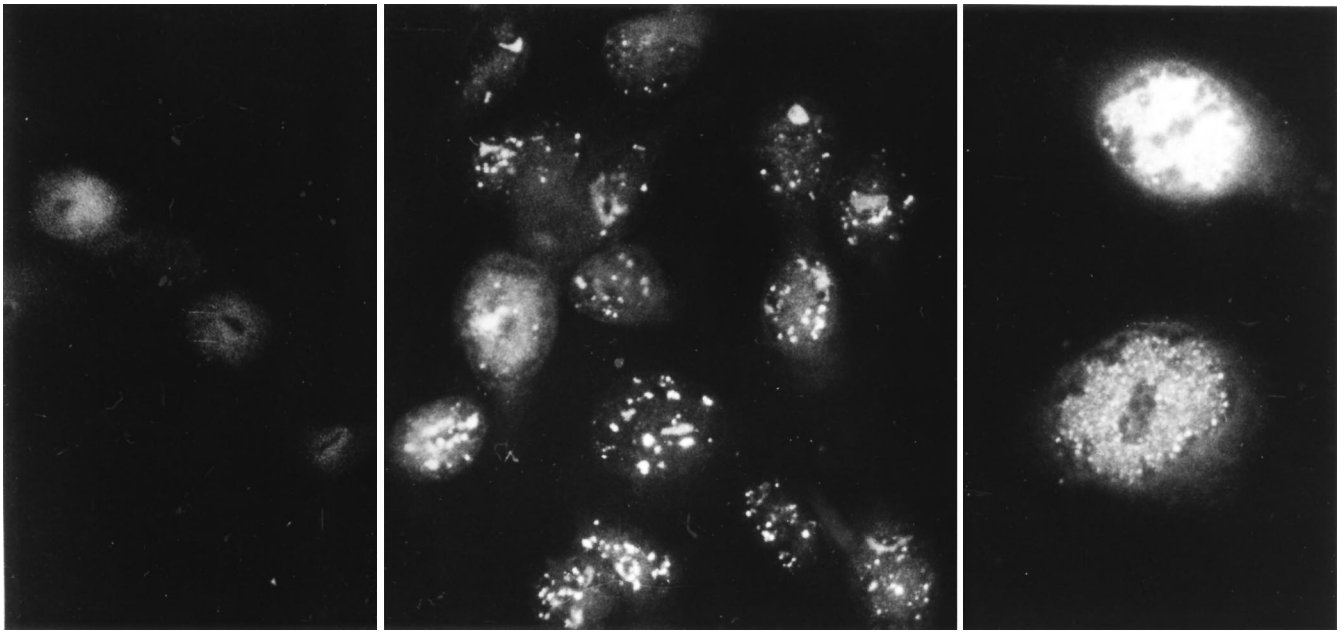

FIG. 8. Immunofluorescence staining of cells infected with HSV-1(F) (left panel) or R7528 for 12 h (middle panel) or 22 h (right panel). Vero cells grown on microscope slides were infected and maintained at 37°C, fixed in cold methanol, blocked with PBS containing BSA, reacted with the CH28-2 monoclonal antibody, rinsed with PBS containing BSA, reacted with anti-immunoglobulin G coupled to Texas red, and rinsed again. Fluorescence was examined with a Zeiss confocal microscope.

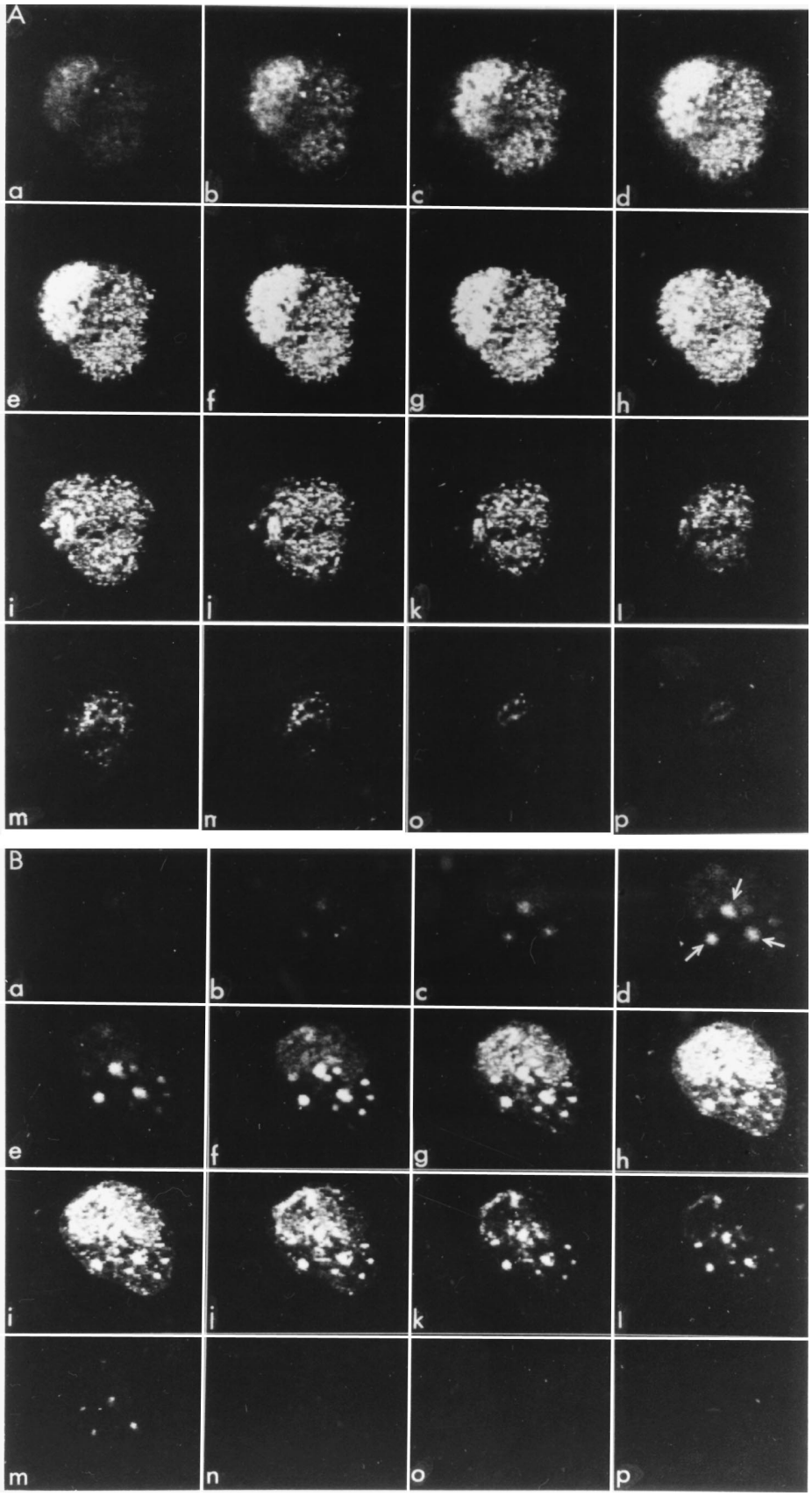

FIG. 9. Digital-image sections of two Vero cells infected with R7528, maintained at 37°C for 22 (A) or 12 (B) h, fixed, and reacted first with the CH28-2 monoclonal antibody and then with a secondary antibody conjugated to Texas red. Sixteen 0.5-μm-thick digital-image sections through the *z* axis were captured and printed with a Codonics CP210 printer. The top left section of each panel is from the basolateral side of the cell. The arrows in panel B, section d, point to the cross section of three clusters of ORF P protein which take the shape of rods inasmuch as the clusters are visible in sections c through m.

down regulated by ICP4 and inhibited by phosphonoacetic acid during lytic infection (3, 29, 37). (v) Late in infection, the accumulated ORF P protein was processed to forms with slower electrophoretic mobility in denaturing gels. (vi) ORF P protein accumulates in nuclei of infected cells. At its face value, the regulation of ORF P expression is unlike that of any other viral protein. One prospective means of ORF P expression may be predicted from an analysis of the overall strategy of the interaction of HSV-1 with its host. Specifically, (i) α genes are induced by a structural protein, αTIF (also known as VP16) (4, 20, 26, 34, 35). In earlier studies in this laboratory, it was assumed that αTIF does not reach the nucleus of the infected cell and that the induced expression of αTIF in sensory neurons could act as a switch to either preclude latency or switch from latent to productive infection (30). However, insertion of a copy of αTIF under the control of a metallothionein promoter failed to affect the establishment of latency (31). There is an alternative interpretation to those results in that αTIF does reach the neuronal nucleus and that it does induce the expression of all or selected genes containing the αTIF response elements. (ii) The predictive function of a viral protein expressed in latently infected cells is maintenance of latency by repression of genes which initiate productive infection, a nuclear function. This is not the function of LATs inasmuch as deletion of promoters and of the sequences encoding the stable introns does not affect the establishment or maintenance of the latent state (16, 32, 38). The expression of such a gene could be determined by response elements of cellular transactivators, but they could include that of the αTIF. The specific role of ORF P protein during latency has not been tested; if it were to have the function described above, it would be expected that its expression would be shut off by α gene products once productive infection ensued and that residual products would be degraded or posttranslationally processed to alter their activity.

The properties of ORF P elucidated to date are consistent with those predicted for the as-yet-unproven, purely hypothetical viral protein which might be involved in the establishment and maintenance of the latent state. First, as operationally defined, HSV-1 proteins expressed in the absence of prior expression of viral genes have been designated α proteins (13, 14), but given the strong repression of its expression by ICP4, the absence of a requirement for prior protein synthesis may be the only property of ORF P shared with the other α proteins. If the expression of ORF P in latently infected neurons is borne out, it would add to the differentiation between α proteins and the proteins exemplified by ORF P and lend credence to the classification of ORF P as a member of a new class of viral proteins with properties expected for pre-α proteins.

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