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Expression of a Herpes Simplex Virus 1 Open Reading Frame Antisense to the $\gamma_1$34.5 Gene and Transcribed by an RNA 3′ Coterminal with the Unspliced Latency-Associated Transcript

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Sensory neurons harboring latent herpes simplex virus 1 express viral RNAs derived from one or more transcriptional units contained in the inverted repeats which flank the long unique sequence but which terminate in the inverted repeats flanking the small unique sequences of viral DNA. These transcripts are also made in productively infected cells. We have identified 16 potential open reading frames (ORFs) predicted to encode 50 or more codons within the domain of the largest reported unspliced transcript and examined 5 ORFs by in-frame insertion of a sequence encoding an epitope reacting with a monoclonal antibody against a human cytomegalovirus protein. One ORF (ORF P), coincident with but antisense to the $\gamma_1$34.5 gene, was expressed but only under conditions in which ICP4 was not functional. To ensure the authenticity of the expression, a second degenerate sequence encoding the same epitope was inserted into a distant site of the same ORF. The protein expressed by the ORF P with two insertions migrated more slowly than the one carrying one insertion only, indicating that ORF P is expressed.

A remarkable feature of herpes simplex virus 1 and 2 (HSV-1 and HSV-2) is their capacity to cause both productive infection resulting in cell death and latent infection in which the virus is maintained without apparent injury to the cell for the life of the host (32). Specifically, upon entry into its host, the virus infects, multiplies in, and destroys the cells at the portal of entry into the body. Either the infecting virus or the progeny of the productive infection may infect nerve endings and be transported to the nuclei of sensory neurons (reviewed in references 33 and 34). In experimental animal model systems, some of the neurons undergo productive infection, whereas in other neurons, viral replicative functions are restricted and a latent infection is established (33, 34). The neurons harboring the latent virus are not intrinsically nonpermissive inasmuch as explantation of neurons harboring latent virus results in termination of the latent state, productive infection, and cell death (40). Virus also reactivates in neurons in situ following stress, damage to the cells innervated by the neurons, or hormonal imbalance (33).

A key question is whether HSV-1 encodes functions which enable the establishment of the latent state. The literature published to date is ambiguous. Thorough analyses of sensory neurons have shown that the only detectable viral expression in these cells consists of RNAs now known as the latency-associated transcripts (LATs), but their function is far from clear (12, 41, 42).

The original LAT was described as a 2-kb, nuclear, nonpolyadenylated and uncapped RNA (11, 41, 42). The RNA was found by in situ hybridization of latently infected mouse, rabbit, and human trigeminal ganglia (21, 31, 37, 39, 41). A transcript of this size and sequence content is also present in productively infected cells (42). A 1.5-kb LAT species is also present in sensory neurons of infected animals harboring latent virus, but this transcript has not been detected in productively infected cells (43). In subsequent studies, (i) a TATA box which punctuates the initiation of transcription of the 2-kb LAT was mapped to a TATA box approximately 700 bases upstream from its 5′ end inasmuch as the deletion of 203 bases around the TATA abolished the appearance of the 2-kb transcript (12), (ii) the region between the 2-kb LAT and the predicted cap site was also transcribed (12, 28, 49) and (iii) it was concluded that the 2-kb transcript is a stable intron spliced from a larger, approximately 8.3-kb polyadenylated transcript from the observation that the first polyadenylation signal 3′ to the promoter is approximately 8.3 kb away and the 2-kb LAT is flanked by canonical, functional splice donor and acceptor sites (15, 49). The approximately 8.3-kb transcript is henceforth designated the putative unspliced LAT. The 1.5-kb RNA therefore either is a further spliced species from the 2-kb LAT or arises from alternate splicing of the 8.3-kb RNA in latently infected neurons. The sequences 5′ and 3′ of the 2-kb RNA were detected by in situ hybridization of trigeminal ganglia harboring latent virus, but the quantities detected were considerably smaller than those of the 2-kb LAT (28, 42, 49). The 8.3-kb transcript was detected by hybridization of electrophoretically separated RNA extracted from productively infected cells and from rabbit trigeminal ganglia harboring latent virus (49). The spliced 6-kb transcript has never been detected or cloned as cDNA.

The observation that the 2-kb LAT was antisense to the 3′ domain of the α0 gene led to the speculation that the LAT transcript enabled the establishment of latency by suppressing the expression of the α0 gene (15, 41). However, mutants from which the sequences encoding the LAT or its promoter have been deleted establish latency and are maintained in sensory neurons in a latent state (19, 22, 36, 38). Another hypothesis, that the function of LATs is to enable reactivation from the latent state, is supported by reports from several laboratories that the incidence of reactivation of virus from explanted sensory neurons of mice and rabbits and the kinetics of reactivation of these deletion mutants decreased (4, 17, 22). In our hands, a mutant from which the promoter and approximately 1.4 kb of the transcribed 5′ domain of the LAT sequences had been deleted reactivated from explanted gan-

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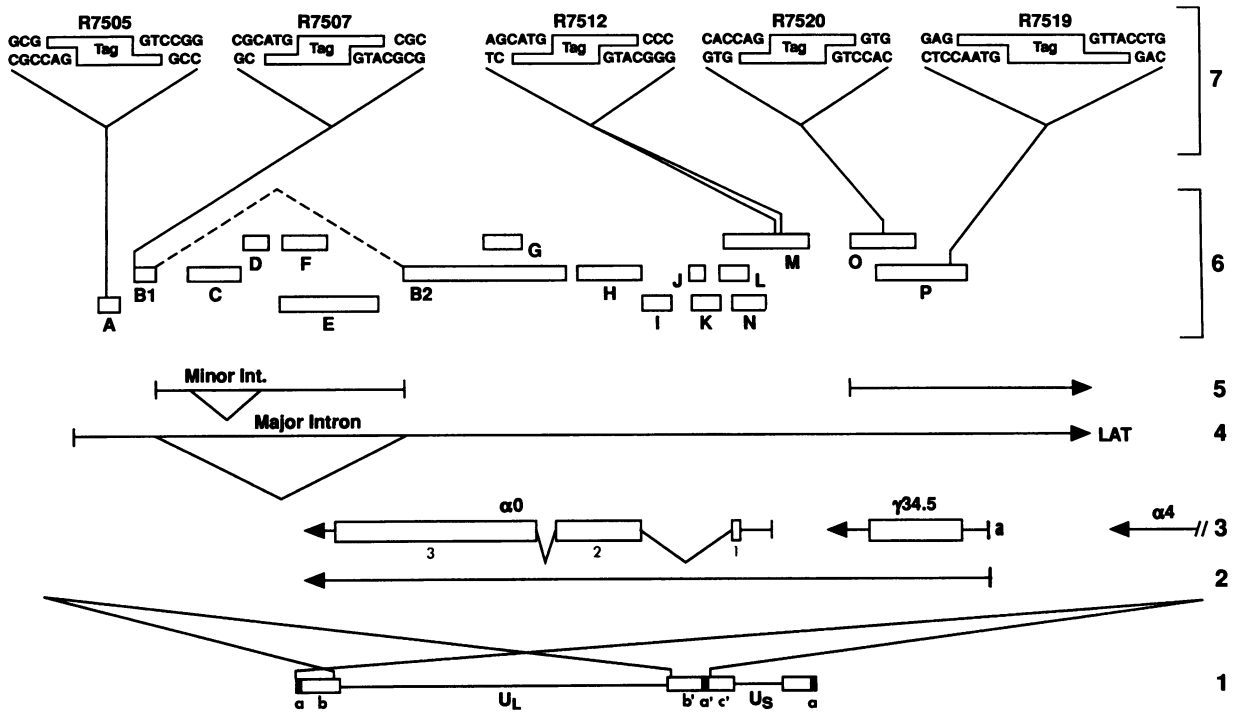

FIG. 1. Schematic representation of the arrangements of transcripts and ORFs in the LAT region. Line 1, orientation 1 of HSV-1; lines 2 to 6, the domain of the LAT unit present in two copies per genome; line 2, a transcript encompassing γ34.5 and α0 (7, 19a); line 3, the 3′ end of α4, the γ34.5 and the α0 transcripts, and coding domains; line 4, the 8.3-kb unspliced LAT, with the position of the major 2-kb intron noted; line 5, the major 2-kb intron (Int.) with the alternative, additionally spliced 1.5-kb species as well as the new transcript that is 3′ coterminal with LAT; line 6, the 16 ORFs in the unspliced LAT described in the text; line 7, the sites of insertion and the linker sequences used for the insertion of the epitope tags into ORFs A, B, M, O, and P and the corresponding recombinant virus numbers.

glia of mice harboring latent HSV-1 with an efficiency approximately 60 to 70% of that of the wild-type virus (21a). The decrease in reactivation frequency, while reproducible, is not impressive, since reductions in the frequency of reactivation of explanted neurons are frequently seen in the case of mutants from which a variety of coding and noncoding domains of the viral genome have been deleted (reviewed in reference 35).

A central issue regarding the domain from which the LAT RNA arises concerns the overall transcription and coding domains of that region of the viral genome. To reiterate, only one transcript, the 2-kb transcript from this region of the viral DNA, is so abundant as to be detected readily and unequivocally in neurons harboring latent virus (37, 41). The unspliced 8.3-kb RNA is present in a much smaller abundance, and the spliced species which gives rise to the 2.0- and 1.5-kb LATS is not present in amounts sufficiently high to be detected (28, 42, 49; reviewed in reference 16). Nevertheless, the domains encoding the 8.3-kb RNA contains many open reading frames (ORFs A to P in Fig. 1). If domains of the 8.3-kb transcriptional unit are expressed, they may be below the level of detection. To test whether the ORFs are expressed, we epitopically tagged specific ORFs to determine whether they are expressed in infected cells in culture.

In this article, we report that of the five ORFs tested, one was expressed under special conditions in infected cells in culture. Relevant to our results are the following.

(i) Inspection of the domain of the unspliced 8.3-kb LAT shows the existence of at least 16 ORFs greater than 50 amino acids in length designated by the letters A through P (Fig. 1, line 6). ORFs A and B are defined by the first two methionine initiation codons downstream of the TATA box of LAT and upstream of the 2-kb splice donor site (Fig. 1, line 4). In HSV-1 strain 17 [HSV-1(17)], there is a third AUG between the TATA box and the splice donor site which is not present in HSV-1(F), and therefore it is not shown here (26, 46). ORF B would be spliced into a large ORF by the removal of the 2-kb stable intron. In the unspliced RNA, ORF B would terminate one codon past the splice donor site. ORFs A and B are not well conserved among HSV-1(F), HSV-1(17), and HSV-1(KOS), though all three strains have both start codons (26, 46).

(ii) The HSV-1(F) ORFs C, D, E, and F are the HSV-1(F) ORFs contained within the 2-kb LAT, but only ORFs E and F are conserved among different strains of HSV-1 (46). ORFs C and D would be spliced out in the 1.5-kb species. Doerig et al. (13) reported that ORF E is expressed in that an antibody to a fusion polypeptide containing part of the ORF E fused to the bacterial TrpE proteins reacted with electrophoretically separated proteins from cells infected with wild-type virus but not from cells infected with a deletion mutant. However, the protein identified by the antibody is over 2.5 times the size of the predicted coding capacity of ORF E. This report has not been confirmed, and no additional data on the products of this ORF have been published.

(iii) The reported sequence of HSV-1(17) shows the presence of seven ORFs (G, H, I, J, K, L, and N; Fig. 1, line 6) antisense to α0. ORF M, which is partially antisense to the 5′ end of α0, contains 222 codons with three in-frame start codons, all of which have good translation initiation context (20, 26).

(iv) ORFs O and P are antisense to the $\gamma_1$34.5 transcript (Fig. 1, lines 2 and 3), and in HSV-1(F) they contain 173 and

248 codons, respectively. Both ORF O and ORF P are extremely well conserved among HSV-1 strains (8). The single major difference is due to the variability in the number of nine-nucleotide repeats encoding the triplet Ala-Thr-Pro in the $\gamma_1$34.5 gene (1, 8). While the studies described here were in progress, Bohenzky et al. (5) reported that the promoter domain of the α0 gene acts as a promoter in an opposite orientation. Yeh and Schaffer (48) reported that an RNA initiates shortly before the 3′ terminus of the $\gamma_1$34.5 gene and is coterminal with the 8.3-kb mRNA. They also reported that the shorter 3′ coterminal transcript was found to be most abundant 12 or more h after infection and could be detected only in cells infected with mutants lacking a functional ICP4 (48). The methionine initiation codon of ORF P is 171 bases downstream from the cap site of this transcript and hence is completely contained within the transcript described by Bohenzky et al. (5). The methionine initiation codon of ORF O has a start codon four nucleotides 3′ of the predicted TATA box, and therefore it is unlikely that ORF O would be translated from this mRNA. Yeh and Schaffer (48) suggested that ICP4 inhibits the expression of the ORF P transcript by binding to an ICP4 binding site 22 bases downstream from the TATA box and 4 bases upstream of the cap site or the ORF P transcript. Inasmuch as the transcript initiates from a site upstream of the α0 gene and extends the length of the $\gamma_1$34.5 gene, none of the published deletions made in the promoter and transcribed domains of the LATs would be expected to affect the expression of this transcript (19, 22, 36, 38).

MATERIALS AND METHODS

Cells and viruses. HSV-1(F), a limited-passage isolate, is the prototype strain used in this laboratory (14). 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 (29). In the R3616 genome, approximately 1,000 bp were deleted from both copies of the $\gamma_1$34.5 gene (6). In the R4002 genome, a *tk* gene driven by the α27 gene promoter (α27-*tk*) was inserted into the 5′ coding sequences of both copies of the $\gamma_1$34.5 gene (6). R7405 containing a U_L20 ORF tagged with a sequence encoding an epitope of the glycoprotein B of human cytomegalovirus (CMV) was described elsewhere (45). This virus served as a positive control for the reactivity of the monoclonal antibody with the epitopically labeled protein. Titrations and preparations of recombinant virus stocks were done in Vero cells (American Type Culture Collection). Transfections of viral DNA were done in rabbit skin cells originally obtained from J. McClaren. Selection for *tk*-plus viruses was done in 143TK$^-$ cells overlaid in HAT medium (Dulbecco modified Eagle medium containing 5% fetal bovine serum, hypoxanthine, aminopterin, and thymidine). Selections for *tk*-minus viruses were done on 143TK$^-$ cells overlaid with Dulbecco modified Eagle medium containing 5% newborn calf serum and 40 μg of bromodeoxyuridine (BUdR) per ml of medium. Viral DNA was isolated from infected cells and purified on an NaI gradient as described previously (44).

Antibodies. The mouse monoclonal antibodies CH28-2 to an epitope of the glycoprotein B of human CMV (23), H640 to ICP4, and H745 to ICP35 were obtained from Goodwin Biotechnology Inc., Plantation, Fla.

Plasmids. The α27-*tk* gene from pRB3621 was inserted by blunt-end ligation into the *Not*I site of pRB4784, which contains the *Sac*I-*Sal*I subfragment of *Bam*HI-B (pRB112) cloned into pGEM 3zf+. In this plasmid, designated as pRB4786, α27-*tk* was placed 335 bp upstream of the LAT TATA box. To insert the 20-amino-acid CMV epitope, an oligonucleotide with the sequence GTCAAGGGTCAGAA GCCTAATCTTCTAGATAGATTAAGGCATCGTAAGAA CGGATATCGACAT and its complement were synthesized, annealed to form an *Rsr*II fragment, and cloned into the *Rsr*II site of pRB4784 to generate pRB4787. ORF B was tagged by insertion of the fragment generated by annealing the oligonucleotide CGGTCCGTGCATGCAAAGGGTCAGAAGCCT AATCTTCTAGATAGATTAAGGCATCGTAAGAACGG ATATCGACATCGGTCCGAGCATGCCATG and its complement into a partial *Sph*I digest of pRB4785 to yield pRB4788. pRB4785 is identical to pRB4784 except that the *Sph*I site in the vector pGEM 3z was destroyed by cleavage with *Sph*I, digestion with T4 DNA polymerase, and ligation. To tag ORF M, the *Bam*HI S fragment cloned into pGEM 3z with the *Sph*I site removed as pRB4789 was digested with *Sph*I, and the fragment generated by cleavage with this enzyme was replaced with the *Sph*I fragment generated by annealing the oligonucleotide AAGGGTCAGAAGCCTAATCTTCTAGA TAGAT TAAGG CACCG TAAGAACGGATATCGACACC GATG and its complement to yield pRB4790. The tagged ORF O was generated by inserting the fragment generated by annealing oligonucleotide AAGGGTCAGAAGCCTAATCT TC TAGATAGAT TAAG GCACCG TAAGAACGGATATC GACACCAG and its complement into the *Dra*III site of pRB143 (*Bam*HI-S) to yield pRB4791. The tagged ORF P (ORF P1) was generated by inserting the fragment generated by annealing the oligonucleotide GTTACCAAGGGTCAG AAGCCTAATCTTCTAGATAGATTAAGGCACCGTAA GAACGGATATCGACAC and its complement into the *Bst*EII site of *Bam*HI-S (pRB143) to yield pRB4792. To insert two tags into ORF P (ORF P2) a *Dra*III fragment generated by annealing the oligonucleotide GTGGAAAGGACAAAAGC CCAACCTTCTAGACCGACTCCGACATAGAAAGAATG GGTACCGACATGGCAG and its complement was inserted into the *Dra*III site of pRB4792 to yield pRB4793. All of the inserts were sequenced to ensure that the tags were in frame with the ORF sequences into which they were inserted.

The DNA probes used to confirm the sites of insertion of the tags were cloned as pRB4805 and pRB4794. To clone pRB4805, the *Eco*RV-*Sal*I fragment containing the LAT TATA box and LAT 5′ sequences in pRB112 was inserted into the *Sma*I and *Sal*I sites of pGEM 3zf+. pRB4794 was made by subcloning the *Nco*I fragment from pRB143 into pGEM 3zf+. The recombinant viruses were constructed as described in Results.

Analyses of viral DNAs. Viral DNAs were digested with appropriate restriction enzymes, subjected to electrophoresis on 28-cm 0.8% agarose gels, and transferred to a Zeta probe as recommended by the manufacturer (Bio-Rad, Richmond, Calif.). The blot was rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), soaked in a prehybridization solution containing 30% formamide, 6× SSC, 1% milk, 1% sodium dodecyl sulfate, and 100 μg of single-stranded calf thymus DNA per ml for at least 30 min at 65°C, and then hybridized in the same solution and temperature with 1×10^6 to 5×10^6 cpm of the appropriate denatured ^{32}P-labeled probe as previously described (1). Autoradiographic images were made by overnight exposure to Kodak XAR5 film.

Immunoblots. Infected cells were rinsed, scraped into phosphate-buffered saline, and spun down in microcentrifuge tubes. The cell pellet was resuspended in disruption buffer, denatured by boiling, subjected to electrophoresis in denaturing polyacrylamide gels of 9.3% (R7507 lysates), 12% (R7512, R7519, R7520, and R7522 lysates), and 17% (R7515 lysates), electrically transferred to nitrocellulose and blocked, reacted with the

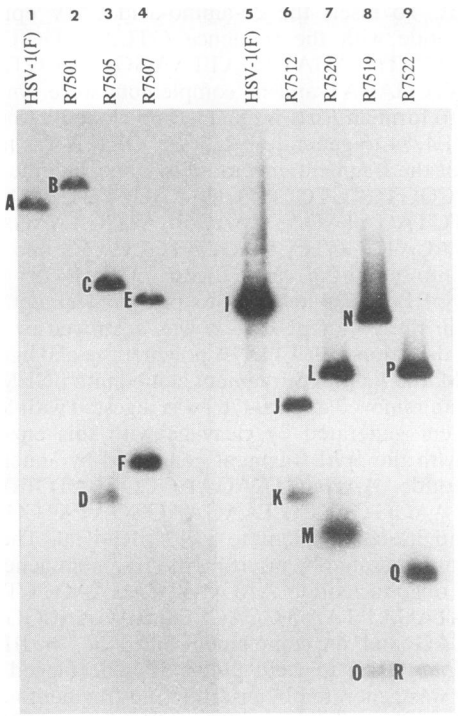

FIG. 2. Autoradiographic image of restriction enzyme digests of the recombinant virus DNAs. Viral DNAs were digested, electrophoretically separated on 0.8% agarose gels, transferred to Zeta probe membranes, hybridized to radiolabeled probes, and exposed to Kodak XAR5 film. Lanes 1 to 4 contain *Dra*I, *Eco*NI, and *Xba*I triple digests of the DNAs of HSV-1(F) or of the recombinant viruses hybridized with an *Eco*RV-*Sal*I probe in *Bam*HI fragment B that contains the LAT promoter and the 5′ region of LAT (Figure 3, left). Lanes 5 to 9 contain *Bam*HI, *Dra*I, and *Xba*I triple digests of the DNAs of HSV-1(F) or of the recombinant viruses hybridized with an *Nco*I subclone of BamHI-S (Fig. 3, right). The letters refer to the expected DNA bands for each virus shown in Fig. 3.

monoclonal antibody, and processed as recommended by manufacturer of either the enhanced chemiluminescence (Amersham International, Amersham, England) or alkaline phosphatase colorimetric (Bio-Rad) detection kit.

RESULTS

Construction of recombinant viruses with tagged ORFs. We chose to examine the expression of five ORFs, i.e., ORFs A, B, M, O, and P. To accomplish this objective, we inserted into each ORF a sequence encoding a 20-amino-acid sequence reactive with a known monoclonal antibody (CH28-2) to an epitope from the glycoprotein B of human CMV as described in Materials and Methods (23). Figure 2 shows the results of analyses of electrophoretically separated digests of wild-type and recombinant virus DNAs. Figure 3 shows the predicted sizes of the restriction endonuclease fragments generated as a result of insertion of the sequences encoding the epitopes. The procedures were as follows.

(i) The recombinant R7501 was constructed by cotransfection of plasmid pRB4786 carrying the α27-*tk* gene in the *Not*I restriction endonuclease cleavage site near the LAT promoter with intact HSV-1(F)Δ305 (*tk*-minus virus) into rabbit skin cells. The progeny of the transfection was passaged in 143TK$^-$ cells in HAT medium for selection of *tk*-plus virus. The *Not*I site is in the inverted repeats flanking the unique long (U_L) sequences. As shown by the *Dra*I-*Eco*NI-*Xba*I digest (Fig. 2), the selected recombinant virus contained an insert containing an *Eco*NI site in both copies of the inverted repeat.

(ii) To recombine the epitopically tagged ORF A and ORF B, intact R7501 DNA was cotransfected with either pRB4787 or pRB4788. The progeny of the transfection were plated on 143TK$^-$ cells in the presence of BUdR. As previously described, only the viral enzyme is available to phosphorylate BUdR, and therefore only spontaneous *tk*-minus mutants or recombinant viruses lacking the *tk* gene replicate under these conditions (30). The *tk* gene of these recombinants was subsequently repaired. The DNAs of R7505 and R7507 recombi-

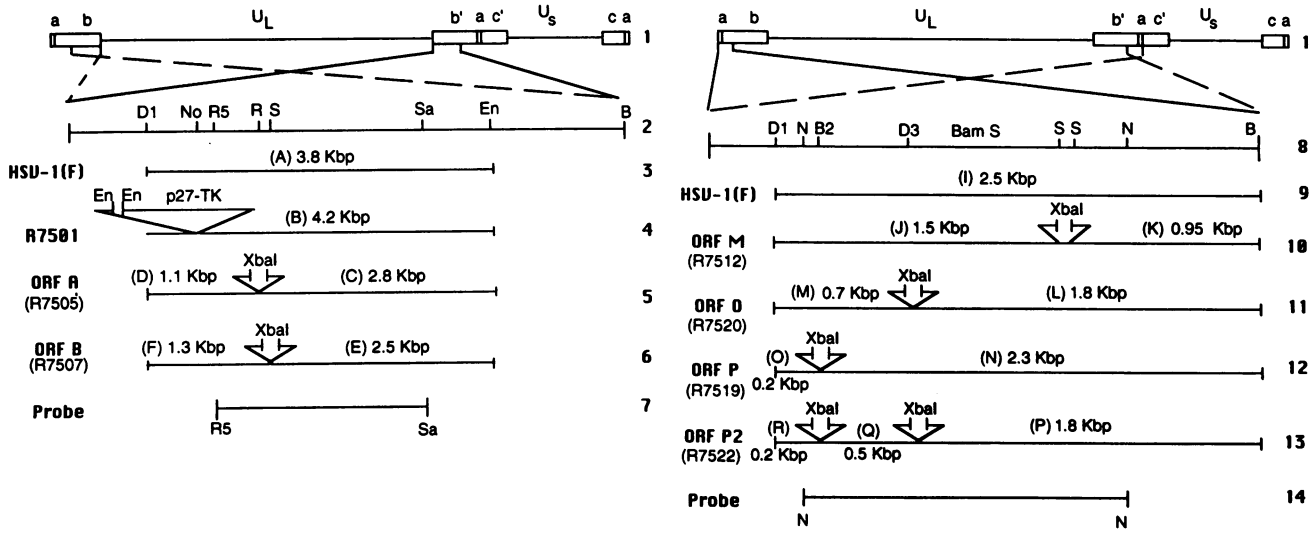

FIG. 3. Schematic representation of sequence arrangements and expected bands of wild-type and relevant genomic domains of recombinant viruses digested with restriction endonucleases. Line 1, the HSV-1(F) genome with the sequence between the U_L region and the *Bam*HI site in the b repeat shown in line 2 and the remainder of the b repeat or BamHI S fragment shown in line 8. Lines 3 to 6, the expected DNA bands generated by restriction endonuclease digestion and shown in Fig. 2, lanes 1 to 4. Lines 9 to 13, the expected DNA bands generated by restriction endonuclease digestion and shown in Fig. 2, lanes 5 to 9. The letters in lines 3 to 6, and 9 to 13 correspond to the labels of the bands in Fig. 2. Lines 7 and 14 show the locations of the probes used in lanes 1 to 4 and 9 to 13, respectively, of Fig. 2.

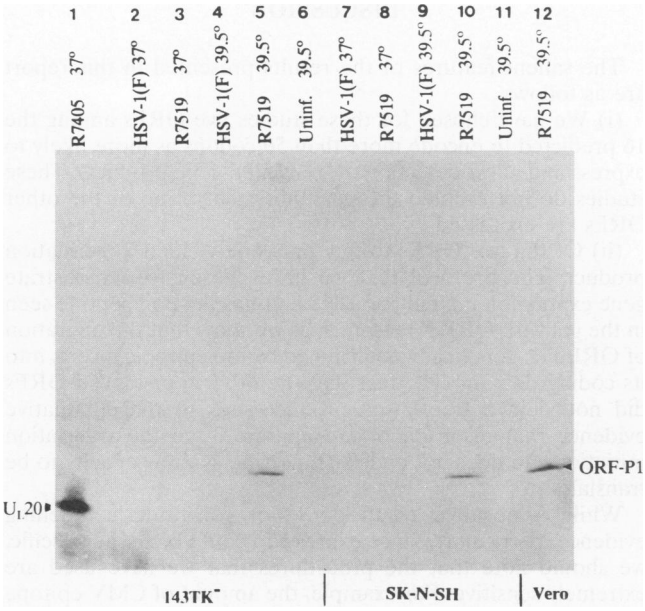

FIG. 4. Photograph of electrophoretically separated polypeptides from cells mock infected or infected with HSV-(F), R7519, or R7405. Cells were infected for 18 to 20 h, harvested, solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, probed with the CH28-2 antibody to the epitope tag, and reacted with anti-mouse immunoglobulin IG coupled with phosphatase secondary antibody, using 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium colorimetric detection. The cell lines were human 143TK$^-$ (lanes 1 to 6), human neuroblastoma SK-N-SH (lanes 7 to 11), and Vero (lane 12). Lanes 4 to 6 and 9 to 12 were infected at 40°C and maintained at 39.5°C. All other lanes show electrophoretically separated lysates of cells maintained at 37°C. The band observed in the lanes containing lysates of cells infected with the recombinant R7519 and maintained at 39.5°C contains a single epitope insertion and is labeled ORF-P1. The positive control in lane 1 contains lysates of cells infected with a recombinant carrying a U$_L$20 gene with an in-frame insertion of the CMV epitope (R7405). The lysate loaded in lane 1 is 1/10 of the amount of cell protein loaded into all other lanes. Note that the apparent molecular weight of the product of the ORF-P protein was determined by extrapolation on the basis of the electrophoretic mobility of ICP4, ICP35, and of the U$_L$20 proteins. The positions of the bands containing these proteins were determined by immunoblotting with corresponding antibodies. Only the position of the band containing the U$_L$20 protein is shown.

nants containing epitope insertions in ORFs A and B, respectively, were shown to contain the diagnostic *Xba*I site at the expected position (Fig. 2).

(iii) To recombine the epitopically tagged ORF M, intact R3616 DNA was cotransfected with pRB4790 plasmid DNA. The R3616 virus lacks 1 kbp from the $\gamma_1$34.5 gene and is unable to replicate following either corneal or intracerebral inoculation of mice, and therefore virus inoculated by the eye route does not appear as infectious virus in the trigeminal ganglia 3 to 4 days postinoculation (47). The progeny of transfection was used to infect mice by the eye route as described in Materials and Methods, and the trigeminal ganglia were removed 3 days postinfection and plated on Vero cells. The expectation was that only recombinant viruses in which $\gamma_1$34.5 was repaired would reach and replicate in the trigeminal ganglia at that time. Approximately 50% of plaques formed on Vero cells contained intact, repaired $\gamma_1$34.5 genes and an insert in the ORF M (data not shown). As shown in Fig. 2, the resulting

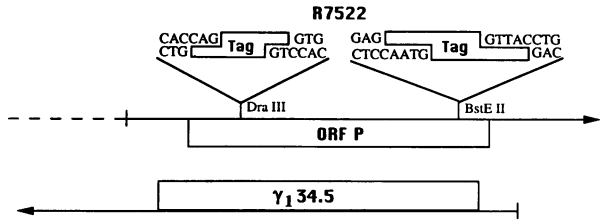

FIG. 5. Schematic representation of the positional relationship of ORF P and $\gamma_1$34.5. Also shown are the positions of the in-frame insertions of the sequences encoding the first (*Bst*EII) and second (*Dra*III) degenerate sequences encoding the CMV epitope.

recombinant R7512 contained the diagnostic *Xba*I site in both copies of the *Bam*HI S fragment. Furthermore, the fragments generated by the digestion were of the predicted sizes.

(iv) To recombine the epitopically tagged ORF O or ORF P, intact R4002 viral DNA was cotransfected with either pRB4791 or pRB4792. The progeny of transfection were plated on 143TK$^-$ cells in the presence of BUdR, and the *tk*-minus progeny was screened for the presence of the epitope insert into both copies of the *Bam*HI S DNA fragment. The *tk* gene of the recombinant viruses selected for further studies was repaired. As shown in Fig. 2, R7020 with an insert in ORF O and R7019 with an insert in ORF P contained the diagnostic *Xba*I cleavage site at the expected position in the *Bam*HI S fragment.

Expression of the ORFs. Two series of experiments were done. In the first, replicate Vero, Sk-N-Sh, and 143Tk$^-$ cells in 25-cm^2 flask cultures were infected with the wild-type parent or recombinant viruses. Cells were harvested at 10 and 18 h after infection at 37°C and at 20 h after infection at 39.5°C. Cycloheximide reversal experiments were also done with one cell line (24). Cells were then solubilized, electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and probed with the anti-CMV epitope monoclonal antibody as described in Materials and Methods. Lysates of cells infected with R7405 carrying a U$_L$20 ORF tagged with the CMV epitope served as a positive control. The tagged U$_L$20 protein migrates with an apparent M_r of 26,000 (3). None of the cell lines infected with either wild-type or recombinant viruses and incubated at 37°C expressed detectable protein bands reactive with the CMV monoclonal antibody (data not shown). We also failed to detect protein bands following cycloheximide reversal done as described in Materials and Methods (data not shown). Of all of the conditions tested, only lysates of cells infected with R7519 and incubated at 39.5°C expressed a protein reactive with the anti-CMV monoclonal antibody (Fig. 4). The polypeptide band reactive with the monoclonal antibody to CMV migrated more slowly than the tagged U$_L$20 protein. The apparent M_r of the ORF-P protein was estimated to be 30,000, as described in the legend to Fig. 4. The apparent M_r of the protein is in good agreement with the predicted M_r of 28,000 calculated from its amino acid sequence.

The purpose of the second series of experiments was to verify that the expression of the tagged protein detected in cells infected with R7519 was in fact genetically determined by ORF P. Specifically, a second oligonucleotide encoding the CMV epitope was inserted into ORF P (ORF P2; Fig. 5) and cloned in pRB4793. The nucleotide sequence but not the predicted amino acid sequence of the second insert was modified to preclude recombinational events between the two inserts. ORF P2 was recombined into the viral genome as was described for

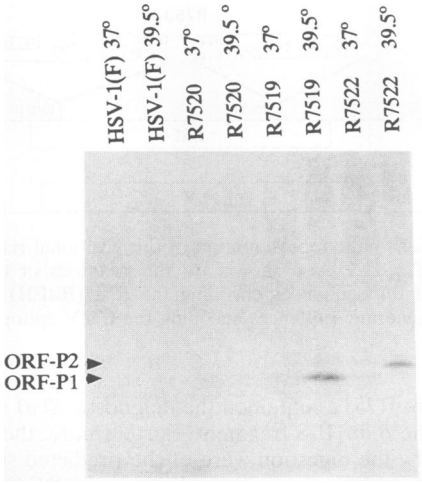

FIG. 6. Photograph of electrophoretically separated polypeptides from Vero cells infected with HSV-1(F), R7519, R7520, or R7522 and incubated at 37 or 39.5°C, as indicated. The procedures for processing the infected cell lysates were done as described in the legend to Fig. 4. The band obtained in cells infected with the virus carrying a single tag in ORF P is labeled ORF-P1, whereas the band obtained with the double tag is labeled ORF-P2.

R7519 to yield the recombinant virus R7522, which is shown to contain the two inserts, as evidenced by the two diagnostic *Xba*I sites in Fig. 2. As shown in Fig. 6, the lysates of Vero cells infected with R7522 express a protein with a mobility slower than that of ORF P1 expressed by R7519 and which reacts with the CMV antibody. This same result was also seen in rabbit skin cells (data not shown). Our results indicate that the protein band tagged by the CMV epitope is genetically determined by the sequence of ORF P and that this ORF is expressed and encodes a protein.

DISCUSSION

The salient features of the results presented in this report are as follows.

(i) We have chosen for these studies five ORFs among the 16 predicted to encode more than 50 codons as more likely to express and allow detection of translated gene products. These studies do not exclude the possibility that some of the other ORFs are expressed.

(ii) Of the five ORFs tested, only one yielded a translation product. The protocol that we have chosen to demonstrate gene expression unambiguously identifies the polypeptide seen in the gel with ORF P inasmuch as we show that the migration of ORF P is genetically determined by the epitopic inserts into its coding domain. The fact that the other four tagged ORFs did not yield a translation product adds to the cumulative evidence that insertion of the epitope 3′ to the translation initiation site does not endow transcripts with a capacity to be translated.

While a negative result does not constitute compelling evidence, particularly since expression could be tissue specific, we should note that the procedures that we have used are extremely sensitive. For example, the amount of CMV epitope attributed to ORF P protein detected in these studies constitutes approximately 1% of the amount of U_L20 produced in infected cells.

(iii) The observation that ORF P is expressed was not expected because it is nearly completely antisense to the $\gamma_1$34.5 ORF. 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 antisense to ORF P. As such, this is the first instance in which two ORFs in the HSV genome are quasi-totally antisense to each other. Because of the overlap, the sequence of ORF P is well conserved among HSV-1 strains (Fig. 7A): the only major variability noted to date is in the number of triplet Ala-Gly-Val repeats corresponding to the Ala-Thr-Pro repeats of the $\gamma_1$34.5 gene (8).

An ORF also exists in HSV-1(HG52) at approximately the

A

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HSV-1(F)      MTASASATRR RNRARSARSR AHEPRRARRA AEAQTTRWRT RTWGEKRTR
HSV-1(17)     MTASASATRR RNRARSARSR AHEPRRARRA AEAQTTRWRT RTWGEKRTR
HSV-1(CVG)    MTASASATRR RNRARSARSR AHEPRRARRA AEAQTTRWRT RTWGEKRTR
HSV-1(MGH10)  MTASASATRR RNRARSARSR AHEPRRARRA AEAQTTRWRT RTWGEKRTR
 Consensus    MTASASATRR RNRARSARSR AHEPRRARRA AEAQTTRWRT RTWGEKRTR

HSV-1(F)      (AGV)10 AGGSGAPS PPARRRRRRA RCSAVTRRRR ARRGGRRKGR EGGWE
HSV-1(17)     (AGV)5 AGGSGAPS PPARRRRRRA RCSAVTRRRR ARRGGRRKGR EGGWE
HSV-1(CVG)    (AGV)6 AGGSGAPS PPARRRRRRA RCSAVTRRRR ARRGGRRKGR EGGWE
HSV-1(MGH10)  (AGV)6 AGGfGAPS PPARRRRRRA RCSAVTRRRR ARRGGRRKGR EGGWE
 Consensus    (AGV)n AGGSGAPS PPARRRRRRA RCSAVTRRRR ARRGGRRKGR EGGWE

HSV-1(F)      GlAPPPGPAPGGGDR GRGAAAVGRA SGAGSGGGLS GQSSSSSSSD ADSG
HSV-1(17)     GsAPPPGPtPGGGgR GRGAAAVGRA SGAdSGGGLS GQSSSSSSSD ADSG
HSV-1(CVG)    GsAPPPGPAPGGGDR GRGAAAVGRA SGAGSGGGLS GQSSSSSSSD ADSG
HSV-1(MGH10)  GlAPPPGPAPGGGDR GRGAAAVGRA SGAGSGGGLS GQSSSSSSSD ADSG
 Consensus    G-APPPGPAPGGGDR GRGAAAVGRA SGAGSGGGLS GQSSSSSSSD ADSG

HSV-1(F)      TWSHWRSSSEQEGGGP lAGGGGGAAA GALLTaGSEL GVEVTWDCAV GTA
HSV-1(17)     TWSHWRSSSEQEGGGP PAGGGGGAAA GALLTaGSEL GVEVTWDCAV GTA
HSV-1(CVG)    TWSHcRSSSEQEGGGP PAGGGGGAAA GALLTtGSEL GVEVTWDCAV GTA
HSV-1(MGH10)  TWSHWRSSSEQEGGGP P.......AA GALLTtGSEL GVEVTWDCAV GTA
 Consensus    TWSHWRSSSEQEGGGP PAGGGGGAAA GALLT-GSEL GVEVTWDCAV GTA

HSV-1(F)      PVGPGGRGRRGPRW.RR RRAMETESVP GW*
HSV-1(17)     PVGPGGRGRRGPRW.RR RRAMETESVP GW*
HSV-1(CVG)    PVGPGGRGRRGPRWRRR RRAMETESVP GW*
HSV-1(MGH10)  PVGPGGRGRRGPRWRRR RRAMETESVP GW*
 Consensus    PVGPGGRGRRGPRWRRR RRAMETESVP GW*
```

B

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HSV-1(F)      MTASAsATRR RNRaRSARSR AhEPRRARRA AeaQtTRWRT rTWGEKrT..
HSV-2(HG52)   MTASAaATRR RNRsRSARSR AqDPRRARRA AvsQaTRWRT cTRGEKhTcg
 Consensus    MTASA-ATRR RNR-RSARSR A--PRRARRA A--Q-TRWRT -T-GEK-T--

HSV-1(F)      ..raGVaGva ....GVaGva GvaGVaGva. ...GVaGvaG vaGgsGapsp
HSV-2(HG52)   rgdtGVgGas ggrrGVrGse GrqGVgGasg grrGVrGseG rqGvgGeayl
Consensus     ----GV-G-- ----GV-G-- G--GV-G--- ---GV-G--G --G--G----

HSV-1(F)      PARRrRrRaR csa..... .......... .......... ....vtRRRR
HSV-2(HG52)   PARRvRgRgR ggpppaqaqa rqvlrrgaqp *rqvgrkgal rpallaRRRR
 Consensus    PARR-R-R-R ---------- ---------- ---------- ------RRRR

HSV-1(F)      arrGGRRkgR eggWeglApp pGp..... .......... .......aPgg
HSV-2(HG52)   gagGGRRgvR glrRlppAla rGavrplcvv vagvvrvvav vrlghqqPap
 Consensus    ---GGRR--R -------A-- -G-------- ---------- -------P--

HSV-1(F)      gDRGRGaaAv gRaSgagsgg ..GlsGqsss ssssdadsGt WshWrssseq
HSV-2(HG52)   qERGRGrrAl dRgSrvvrrd hlGvcGwera garhgwsaGa RhgWsagarp
 Consensus    --RGRG--A- -R-S------ --G--G---- --------G- ---W------

HSV-1(F)      egGgPlAGgg ggaaaGaLlt Agselgvevt WDcavGT.Ap Vgpggrgrrg
HSV-2(HG52)   apGtPaAGtp aaghgGrLgs A*arsrsasg REsslGThAr Vtaspclpse
 Consensus    --G-P-AG-- -----G-L-- A--------- -----GT-A- V---------

HSV-1(F)      prWrrRRAme tesvpgw*
HSV-2(HG52)   ltRpgWRAqp gpccgps*
 Consensus    ------RA-- --------
```

FIG. 7. Comparison of the amino acid sequences predicted for HSV-1 (A) and HSV-2 (B) ORF P. (A) Sequence alignment of ORF P in HSV-1(F), HSV-1(17), HSV-1(CVG2), and HSV-1(MGH10); (B) sequence alignment of ORF P in HSV-1(F) and an ORF in the corresponding position in HSV-2(HG52). The comparison was done with the aid of the Gap program of the University of Wisconsin Genetics Computer Group package.

same place in the genome (25). Thus, the HSV-2 sequence contains a TATA box, upstream sequences encoding sequences similar to SP1 response elements, and a binding site for ICP4 at position +22. Moreover, the first 50 predicted amino acids of HSV-1 and HSV-2 ORF P show good homology (Fig. 7B). However, downstream sequences beginning with the triplet repeat AGV in HSV-1 show little or no homology to the published HSV-2 sequence. The extent of the homology of HSV-1 and HSV-2 predicted proteins can be improved by assuming sequencing errors involving frameshifts. This would not be surprising since this region of the HSV-2 genome contains a very high G+C content. The possibility that the reported sequence of HSV-2 ORF P requires reassessment is suggested by the report that whereas the HSV-1 antisense gene $\gamma_1$34.5 yields an unspliced mRNA, the HSV-2 equivalent was reported to yield a spliced mRNA (25). HSV genes which yield spliced mRNAs are few in number and well conserved among HSV-1 and HSV-2 strains (2).

(iv) The regulation of the expression of ORF P is puzzling and remains to be determined. Bohenzky et al. reported that the RNA was present in infected cells at 10 h after infection and found the RNA by nuclear run-on experiments under cycloheximide treatment (5). Yeh and Schaffer failed to detect the RNA in cells infected and maintained in the presence of cycloheximide (48). The finding of an ICP4 binding site close to the transcription initiation of ORF P DNA suggested that ORF P is inhibited by ICP4 but requires other α proteins for its expression. At face value, our results are concordant with but not necessarily supportive of this conclusion. Both α4 and ORF P contain ICP4 binding sites near the transcription initiation sites, and in the case of the α4 gene, it has been demonstrated that ICP4, the product of the α4 gene, inhibits transcription of α4 (27). This has not been shown as yet for ORF P, but the close proximity of the binding site to the transcription initiation sites raises the possibility that in this case ICP4 can also block transcription of ORF P, and the observation that cells infected with recombinants derived from HSV-1(F), a wild-type virus temperature sensitive in ICP4 express ORF P is consistent with this conclusion. The distinction between the expression of the α4 gene and ORF P is based on the observation that α4 is expressed in the presence of cycloheximide whereas ORF P is not (18). Such findings, while potentially significant, are not compelling evidence since the ORF P transcript may be unstable in the presence of cycloheximide.

Perhaps more significant is the report by Yeh and Schaffer (48) that they failed to detect the ORF P mRNA in murine trigeminal ganglia harboring latent virus. At face value, this observation suggests that ORF P is not a member of the family of the 3′ coterminal LATs. However, in the same assays, Yeh and Schaffer (48) failed to detect the 8.3-kb unspliced LAT, raising the possibility that the assays were not sensitive enough to detect small amounts of ORF P mRNA which could be present only in the small fraction of total neurons harboring latent virus (34).

A more satisfying scenario would be if ORF P were to be expressed during latency and if its transcription were to be suppressed by newly synthesized ICP4. Its accumulation late in infection would reflect loss of activity of ICP4 due to posttranslational modification late in infection. The evidence to satisfy this scenario, and to support a role for ORF P in latently infected cells, is not yet available. We should note, however, that none of the deletions previously reported in the domain of the LAT transcript encompassed ORF P (19, 22, 36, 38). The only mutations introduced into the domain of ORF P are those genetically engineered in the $\gamma_1$34.5 gene (6). It is noteworthy that the deletion mutants in the $\gamma_1$34.5 gene exhibit a reduced capacity to establish latency or reactivate from the latent state (47). Studies designed to determine whether the reduced capacity to establish latency reflects the mutations in $\gamma_1$34.5 or the ORF P are in progress. The phenotype ascribed to $\gamma_1$34.5, i.e., blocking the stress response which results in the premature total shutoff of protein synthesis (9, 10), is indeed that of $\gamma_1$34.5 inasmuch as the human neuroblastoma cell line SK-N-SH expressing only the $\gamma_1$34.5 gene does not exhibit the stress response associated with infection of the parental line with the $\gamma_1$34.5$^-$ (5a).

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