Transcription of the Derepressed Open Reading Frame P of Herpes Simplex Virus 1 Precludes the Expression of the Antisense $\gamma_134.5$ Gene and May Account for the Attenuation of the Mutant Virus

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Open reading frame P (ORF P), located at the 3' terminus of the 8.5-kb DNA sequence transcribed during latency and almost completely antisense to the $\gamma_134.5$ gene, is naturally repressed by infected cell protein 4 (ICP4), the major herpes simplex virus 1 regulatory protein. Earlier studies on cells infected with a mutant in which the expression of ORF P is derepressed have shown that (i) the accumulation of the α infected cell proteins 0 (ICP0) and 22 (ICP22), the products of spliced mRNAs, is reduced congruent with the binding of ORF P protein to p32, a component of the ASF/SF2 splicing factors, (ii) ORF P protein colocalizes with spliceosomes, (iii) both $\gamma_134.5$ mRNA and protein are virtually undetectable, and (iv) the virus is attenuated on intracerebral inoculation in mice. We report the construction and characterization of two recombinant viruses: R7546, in which ORF P transcription was derepressed and the initiator methionine codon was replaced; and R7547, in which both mutations were repaired to the wild-type genotype. The mutations in R7546 do not alter the amino acid sequence of the $\gamma_134.5$ gene. We report that (i) the reduction in the accumulation of $\gamma_134.5$ mRNA and protein in cells infected with mutant viruses expressing derepressed ORF P genes reflects the effects of antisense transcription of ORF P rather than a function of ORF P protein, (ii) the attenuated phenotype of the viruses carrying derepressed ORF P genes is due largely to the absence of the $\gamma_134.5$ protein, and (iii) the reduction in accumulation of ICP0 and ICP22 requires the expression of ORF P protein.

This report concerns two genes of herpes simplex virus 1 (HSV-1) arranged totally antisense to each other. The $\gamma_134.5$ gene maps next to the terminal *a* sequence in the inverted repeat sequences *ab* and *b'a'* flanking the unique long (*U_L*) sequence and therefore is present in two copies per viral genome. This gene encodes 263 codons in strain HSV-1(F) and requires the onset of viral DNA synthesis for maximal expression (2, 11). Current data indicate that this gene has at least two functions. The least understood is the function which enables the virus to replicate in the central nervous system (CNS) of animals used in experimental model systems. In the absence of both copies of the gene, high-titer virus is unable to destroy the CNS of mice following intracerebral inoculation (9). The virus expresses genes in only a small number of neurons, astrocytes, and oligodendrocytes arranged along the needle track and whose projections impinge on this region of the brain, but viral replication is virtually undetectable (23). The second function of the $\gamma_134.5$ gene is to prevent premature shutoff of protein synthesis in cells of human derivation as a consequence of phosphorylation of the α subunit of translation initiation factor eIF-2 by double-stranded RNA-activated kinase (PKR) (8, 12, 13). In this instance, $\gamma_134.5$ binds to the protein phosphatase 1 α and directs it to dephosphorylate eIF-2 α , thereby allowing unabated protein synthesis in infected cells (17).

Open reading frame P (ORF P) maps antisense to the $\gamma_134.5$ gene at the 3' end of the 8.5-kb latency-associated transcript sequence reported to be transcribed during latent infections (20, 28, 29, 32). Of the 248 codons, only 8 codons of ORF P are

not antisense to $\gamma_134.5$ and only 23 codons of $\gamma_134.5$ are not antisense to ORF P (21). The history of the discovery of ORF P is of interest inasmuch as it relates to its function. Bohenzky et al. (5) identified a promoter within the *ab* and *b'a'* inverted repeat sequences mapping antisense and 3' to the $\gamma_134.5$ gene. Yeh and Schaffer (31) demonstrated that this promoter drives the expression of an RNA species, but only if a major regulatory protein, infected cell protein 4 (ICP4), is dysfunctional or absent. The repressor function of ICP4 depends on the binding of ICP4 to a high-affinity cognate site at or near the transcription initiation site of the target gene (15, 22, 24), and indeed, a high-affinity binding site straddles the transcription initiation site (−9 to +3) (31). The objectives of this laboratory in initiating these studies were to identify expressed open reading frames mapping within the domain of the viral genome transcribed during latency. Of the 16 open reading frames designated A to P and mapped in that domain, the one which yielded the strongest signal was ORF P, but only under conditions in which the ICP4 binding site located at the transcription initiation site of ORF P was destroyed by mutagenesis or following infection and maintenance at 39.5°C, the nonpermissive temperature of limited-passage isolates carrying a temperature-sensitive ICP4 (19–21). The expression of ORF P cannot be detected during productive infection.

Preliminary experiments using the mutant carrying a derepressed ORF P indicated that it is attenuated in experimental animals (19), but the question arose as to whether the attenuated phenotype reflected the failure of $\gamma_134.5$ gene expression as a consequence of antisense transcription of the derepressed ORF P. One possible function of ORF P emerged from yeast two-hybrid system analyses. These studies indicated that ORF P interacts with p32, a component of the ASF/SF2 alternative splicing factors (6). The significance of the interaction was

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verified by demonstration of in vitro interaction between ORF P and another splicing factor, the B/B' components of the SM antigens; by colocalization of ORF P with spliceosomes; and more significantly, by the evidence that in cells infected with the virus carrying derepressed ORF P, the levels of viral protein products of spliced mRNAs, ICP0 and ICP22, were reduced relative to the protein products of intronless mRNAs, ICP4 and ICP27 (6).

The main questions concerning these studies are (i) whether the phenotype of the derepressed ORF P in the context of an infection of cells in vitro or in vivo is due to the suppression of $\gamma_134.5$ gene and (ii) whether the suppression of expression of the $\gamma_134.5$ gene is due to antisense transcription of ORF P or to a specific function of ORF P protein. To answer these questions, we constructed two new recombinant viruses. The first took advantage of the observation that ORF P encodes two methionines, an initiator methionine and a second methionine codon (met₂₄₀) located near the 3' terminus of ORF P. In the recombinant virus designated R7546 (P++⁻), the ICP4 binding site was mutagenized to derepress ORF P transcription and the initiator methionine codon was replaced with an isoleucine codon to prevent the synthesis of ORF P protein. In the second recombinant virus, designated R7547 (P++⁻R), both mutations were repaired to wild-type status. We report that (i) the suppression of $\gamma_134.5$ gene expression is due to antisense transcription and not to the expressed ORF P protein, (ii) the reduced virulence of the virus carrying the derepressed ORF P cannot be attributed solely to ORF P inasmuch as the derepressed mutant incapable of expressing the ORF P protein was also attenuated, and (iii) the selective reduction in the synthesis of ICP0 and ICP22 reported earlier requires the expression of ORF P protein.

MATERIALS AND METHODS

Viruses. HSV-1(F), a limited-passage clinical isolate, is the prototype HSV-1 strain used in this laboratory (14). The recombinant viruses described previously were as follows. The recombinant virus R3659 (21) lacks the *SacI*-*Bgl*II sequence of *Bam*HI-Q encoding the thymidine kinase (*tk*) and *U_L24* genes. A sequence consisting of the coding domain of the *tk* gene under control of the $\alpha 27$ promoter (25) replaced the *Bst*EII-*Stu*I sequence of the *Bam*HI S fragment encoding the $\gamma_134.5$ and ORF P genes (Fig. 1, line 4). R7530 contains a four-base substitution at the ICP4 binding site which straddles the ORF P transcription initiation site (Table 1). This mutation has been shown to be sufficient for derepression of ORF P and creates a diagnostic *Eco*RI restriction site (19). R7531 is the repair of R7530 (19). R3616 contains a 1-kb deletion in the $\gamma_134.5$ and ORF P genes, between the *Bst*EII and *Stu*I sites (9).

Antibodies. Rabbit polyclonal antisera specific for $\gamma_134.5$ (BR4 [2]), ICP22 (R77 [3]), and a C-terminal *Xba*I fragment of ORF P fused to glutathione S-transferase (ORF P_c [19]) and mouse monoclonal antisera specific for ICP0 (H1112 [1]) and ICP27 (H1113 [purchased from Goodwin Institute, Plantation, Fla.] [1]) have been described elsewhere.

Plasmids. Plasmid pRB4794 has been described elsewhere (21). It contains the 1,800-bp *Nco*I fragment of *Bam*HI-S, spanning the region between the start codons of the $\alpha 0$ and $\gamma_134.5$ genes. It was used as a probe for analyses of ORF P recombinant viral DNA and as a source of strand-specific riboprobes for the detection of ORF P and $\gamma_134.5$ mRNAs. Plasmid pRB4855 contains a four-base change in the ICP4 binding site and has been described previously (19). pRB4930 contains the 830-bp *Not*I fragment of *Bam*HI-S cloned into the *Not*I site of pUC19. Plasmid pBR4929 contains a mutant ORF P initiator methionine codon and was made by insertion of a 160-bp PCR product into the *Srf*I-*Dra*III sites of pRB4930. The PCR primers were 5'-ACGGGCTCGGGCCCTAGGCACG GCGGATAACCGCTCGGCCTC and 5'-GAGGCCGAGGCGGTATCG GCGGTGCTAGGGCCCGAGGCCCGT. Underlined bases represent mutations that replace the ORF P initiator methionine codon (ATG) with an isoleucine codon (ATA) and create a diagnostic *Avr*II restriction site 15 bp upstream. Plasmid pRB5159 was made by replacing the *Bsp*EI-*Dra*III fragment from pRB4855 with the corresponding fragment from pRB4929. pRB5159 contains both the ORF P initiator methionine mutation and the ICP4 binding site mutation which derepresses ORF P. It was used to construct the recombinant virus R7545. Plasmid pRB103 containing the *Bam*HI Q fragment was used to repair the deletion in the *tk* gene of R7545, resulting in the recombinant virus R7546. pRB4794 contains the 1,800-bp *Nco*I fragment from *Bam*HI-S and was

FIG. 1. Schematic representations of sequence arrangements in the recombinant viruses used in this study. Line 1, a representation of the HSV-1 (F) genome. Shown are the unique long (*U_L*) and unique short (*U_S*) sequences which are flanked by inverted repeats *cab* and *b'a'c'*. Line 2, domains of the ORF P and $\gamma_134.5$ genes in the inverted repeat sequence *b'a'* flanking the *U_L* sequences. The identical sequences in the inverted orientation map in the *ab* repeat. The coding domains (rectangular boxes) and transcripts (lines with arrows denoting direction) of the ORF P and $\gamma_134.5$ genes are shown. The closed circle denotes a wild-type ICP4 binding site. Line 4, sequence arrangement of the relevant domains of R3659. The *Stu*I-*Bst*EII sequences encoding ORF P and $\gamma_134.5$ were replaced in both repeats by the chimeric $\alpha 27$ -*tk* gene. Line 6, sequence arrangement of the relevant domains of recombinant R7546. The $\alpha 27$ -*tk* gene of R3659 was replaced with sequences containing a mutated ICP4 binding site introducing a diagnostic *Eco*RI endonuclease site and a mutated ORF P initiation methionine introducing a diagnostic *Avr*II endonuclease site. Line 8, sequence arrangement of the relevant region of recombinant R7547. The mutations in the ORF P domain of R7546 were repaired by transfection with the *Nco*I fragment from the HSV-1(F) *Bam*HI S fragment. Lines 3, 5, 7, and 9, expected sizes of fragments detected by hybridization of the 1,800-bp *Nco*I fragment with electrophoretically separated digests of viral DNAs with *Nco*I-*Eco*RI (the first band set per virus), diagnostic of the ICP4 binding site mutation, or *Nco*I-*Avr*II (the second band set per virus), diagnostic of the replacement of the initiator methionine. The arrows in these lines denote restriction cleavage sites present in the respective viruses and therefore fragment boundaries. HSV-1(F) DNA would be expected to yield bands A and B, R3659 DNA would be expected to yield bands C and D, R7546 DNA would be expected to yield bands E, F, and G, and R7547 DNA would be expected to yield bands H and J. Abbreviations: St, *Stu*I; Nc, *Nco*I; Bs, *Bst*EII.

used to repair the mutations in the ORF P domain of R7546, resulting in the recombinant virus R7547.

Construction of recombinant viruses. Viral stocks and titrations of viruses were done in Vero cells (American Type Culture Collection). R7545 was constructed by cotransfection of rabbit skin cells (originally obtained from J. McClaren) with pRB5159, which contains both the ICP4 binding site mutation and the ORF P initiator methionine mutation, with R3659 viral DNA (Fig. 1, line 4), which contains a deletion in the *tk* gene and an $\alpha 27$ promoter-driven *tk* gene replacement of the 1,100-bp *Stu*I-*Nco*I fragment encoding the ORF P and $\gamma_134.5$ genes. *tk*⁻ viruses were selected by plating the progeny of the cotransfection on 143TK⁻ cells overlaid with Dulbecco modified Eagle medium containing 5% newborn calf serum and 40 μ g of bromodeoxyuridine per ml of medium. Plaque-

TABLE 1. Genotypes and phenotypes of viruses used in this study

Virus	Genotype	Shorthand	Phenotype
HSV-1(F)	Wild type		ORF P repressed; γ_1 34.5 detected
R7530	A ₋₉ TCGTC→GAATTC	(P++)	ORF P derepressed, γ_1 34.5 not detected
R7531	Wild type	(P++)R	ORF P repressed, γ_1 34.5 detected
R7546	A ₋₉ TCGTC→GAATTC, A ₊₁₄₁ TG→ATA, C ₊₁₂₆ CCAGG→CCTAGG	(P++) ⁻	ORF P derepressed; ORF P mRNA made, but ORF P protein and γ_1 34.5 not detected
R7547	Wild type	(P++) ⁻ R	ORF P repressed; γ_1 34.5 detected

^a Nucleotide number relative to transcriptional initiation site of ORF P (+1).

purified stocks were prepared as described elsewhere (26). Viral DNA was isolated from infected cells and purified on a 5 to 20% potassium acetate gradient as described elsewhere (18). Viral DNA from single plaques were analyzed for the presence of novel *Eco*RI and *Avr*II endonuclease restriction sites diagnostic of the ICP4 binding site and the initiator methionine mutations, respectively. The *tk* gene of R7545 was repaired by plating the progeny of the cotransfection of rabbit skin cells with R7545 DNA and pRB103, which contains the *Bam*HI Q fragment, on 143TK⁻ cells overlaid in Dulbecco modified Eagle medium containing 5% fetal bovine serum, hypoxanthine, aminopterin, and thymidine. Virus was isolated, purified, and analyzed as described above. This process resulted in the isolation of R7546, which contains a wild-type *Bam*HI Q fragment, the ICP4 binding site mutation, and the ORF P initiator methionine mutation. The mutations in the ORF P gene of R7546 were repaired by cotransfection of rabbit skin cells with R7546 viral DNA and pRB4794, which contains the 1,800-bp *Nco*I fragment from *Bam*HI-S of HSV-1(F). Plaques were isolated, and viral DNA was analyzed for a wild-type restriction endonuclease pattern indicating the absence of the introduced *Eco*RI and *Avr*II sites within *Bam*HI-S. R7547 has a wild-type restriction endonuclease pattern and therefore has a wild-type genotype.

Analyses of viral DNAs. Viral DNAs were digested with appropriate restriction enzymes as detailed in the legend to Fig. 1. They were then subjected to electrophoresis on a 28-cm 0.85% agarose gel and transferred to a Zeta Probe membrane (Bio-Rad, Richmond, Calif.) by capillary action in 0.5 M NaOH. The membrane was rinsed in 2× SSC (0.3 M NaCl, 0.015 M sodium citrate) and prehybridized in 30% formamide–6× SSC–1% milk–1% sodium dodecyl sulfate–100 µg of single-stranded calf thymus DNA per ml for 30 min at 68°C; 10⁶ cpm of denatured, ³²P-labelled pRB4794 was then added overnight, and the blot was rinsed as recommended by the manufacturer. Autoradiographic images were obtained on Kodak XAR5 film.

Immunoblots. Immunoblot analyses were done as previously described (20). Briefly, infected cells were scraped into phosphate-buffered saline, pelleted under low-speed centrifugation, resuspended in disruption buffer containing 0.7 M β-mercaptoethanol, 2% sodium dodecyl sulfate, 50 mM Tris, and 2.75% sucrose, sonicated briefly, boiled, and electrophoretically separated on a denaturing polyacrylamide gel cross-linked with *N,N'*-diallyltartardiamide (Bio-Rad). The electrophoretically separated, denatured proteins were electrically transferred to a nitrocellulose sheet, blocked, reacted with the appropriate antiserum, rinsed, reacted with either goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase for rabbit polyclonal antisera or goat anti-mouse IgG conjugated to alkaline phosphatase for mouse monoclonal antisera, rinsed again, and developed as recommended by the manufacturer (Bio-Rad).

RNA blots. RNA blot analyses were done as previously described (19). Briefly, roller bottles of Vero cells were exposed to 10 PFU of HSV-1(F), R7530, R7531, R7546, or R7547 per cell and incubated at 37°C for 13 h for the detection of γ_1 34.5 mRNAs, since this gene is expressed predominantly after the onset of viral DNA synthesis. Vero cells grown in 150-cm² flasks were exposed to virus as described above and incubated at 37°C for 6 h for the detection of ORF P mRNAs, since this RNA is made early in infection. Cells were collected, washed, treated with 0.1% Nonidet P-40 for plasma membrane solubilization, and then subjected to low-speed centrifugation for the isolation of cytoplasmic fractions. Nucleic acid extraction, precipitation, and DNase treatment followed. The amounts of RNAs which were loaded onto agarose-formaldehyde gels for the detection of different RNA species were as follows: (i) polyadenylated cytoplasmic mRNAs were purified from 800 µg of cytoplasmic RNA with the Promega PolyATtract system IV and loaded for the detection of cytoplasmic γ_1 34.5 mRNAs; and (ii) 20 µg of total cytoplasmic RNAs was loaded onto agarose-formaldehyde gels for the detection of ORF P. Denatured RNAs were separated by electrophoresis in 1.5% agarose-formaldehyde gels and transferred to a Zeta Probe membrane by capillary action in 10× SSC. Strand-specific ³²P-radiolabelled riboprobes were generated by T7 transcription of pRB4794 for ORF P-specific riboprobes and SP6 transcription of pRB4794 for γ_1 34.5 riboprobes. Hybridization and rinsing procedures were as recommended by the Zeta Probe manufacturer. Autoradiographic images were obtained on Kodak XAR5 film.

[³⁵S]methionine labelling. Replicate SK-N-SH neuroblastoma cell cultures (American Type Culture Collection) in 25-cm² flasks were exposed to 10 PFU of HSV-1(F), R3616, R7530, R7531, R7546, or R7547 per cell. At 14 h after infection, the medium was replaced with 199V lacking methionine but supple-

mented with 50 µCi of [³⁵S]methionine (Amersham). After 1 h of incubation at 37°C, cells were harvested, solubilized in disruption buffer, sonicated, boiled for 5 min, subjected to electrophoresis on a 10% denaturing polyacrylamide gel cross-linked with *N,N'*-diallyltartardiamide (Bio-Rad), transferred electrically to a nitrocellulose sheet, and exposed to Kodak XAR5 autoradiography film for 2 days.

Intracranial inoculation of mice. CBA/J mice (3.5 weeks old) from Jackson Laboratory were anesthetized with pentobarbital sodium (Nembutol) and injected intracerebrally with 10-fold serial dilutions of virus, seven mice per dilution. Mice were monitored daily; mortality from 2 to 21 days after infection was attributed to the inoculated virus. The 50% lethal dose (LD₅₀) ratios were calculated by the method of Reed and Muench (27).

RESULTS

Construction and characterization of the recombinant virus R7546 (P++)⁻, expressing a derepressed, untranslated ORF P transcript, and the repaired recombinant virus R7547 (P++)⁻R. The procedures for the construction of recombinant viruses R7546 (P++)⁻ and R7547 (P++)⁻R are described in Materials and Methods, and the viral genotypes are presented in Table 1. The genotype of R7546 (P++)⁻ was verified by the presence of the *Eco*RI restriction endonuclease site diagnostic of the ICP4 binding site mutation, the *Avr*II restriction endonuclease site diagnostic of the mutation of the ORF P initiator methionine, and a wild-type *Bam*HI Q fragment indicative of a repaired *tk* gene. The locations of the *Eco*RI and *Avr*II restriction sites within R7546 (P++)⁻ are shown in Fig. 1, line 6. R7546 (P++)⁻ DNA was incubated with restriction endonucleases *Nco*I-*Eco*RI or *Nco*I-*Avr*II, electrophoretically separated on 0.85% agarose gels, transferred to Zeta Probe membranes, and hybridized with pRB4794, which contains the 1,800-bp *Nco*I fragment of *Bam*HI-S (Fig. 2, lanes 5 and 6). Restriction endonuclease cleavage of R7546 (P++)⁻ DNA with *Nco*I and *Eco*RI resulted in the predicted 880- and 930-bp *Nco*I-*Eco*RI doublet (Fig. 2, band e), confirming the presence of the ICP4 binding site mutation. Digestion of R7546 (P++)⁻ viral DNA with *Nco*I and *Avr*II resulted in the predicted 1,070- and 740-bp *Nco*I-*Avr*II fragments (Fig. 2, bands f and g), confirming that the recombinant virus also contained the initiator methionine mutation. This pattern is distinct from that of the parental virus R3659 (Fig. 1, line 4), in that digestion with *Nco*I-*Eco*RI or *Nco*I-*Avr*II yielded a 700-bp fragment (Fig. 2, bands c and d) detected by hybridization with ³²P-labelled pRB4794 DNA. The size of these fragments is consistent with that predicted for the fragment upstream of the α27-*tk* replacement (Fig. 1, lines 4 and 5).

As is necessary in all instances in which the phenotypes of deletion mutants are tested, the two mutations in the ORF P domain were repaired by cotransfection of rabbit skin cells with R7546 (P++)⁻ viral DNA and pRB4794, which contains the 1,800-bp *Nco*I fragment from the *Bam*HI S fragment of HSV-1(F). Viral DNAs from progeny were then screened for the wild-type pattern by restriction endonuclease cleavage. The repaired virus R7547 (P++)⁻R did not contain the novel *Eco*RI or *Avr*II sites, as indicated by the presence of 1,800-bp

FIG. 2. Autoradiographic image of electrophoretically separated viral DNA fragments containing sequences in the domain of the ORF P and $\gamma_134.5$ genes. Viral DNAs were digested with either *Nco*I and *Eco*RI (lanes 1, 3, 5, and 7) or *Nco*I and *Avr*II (lanes 2, 4, 6, and 8), electrophoretically separated on a 0.85% agarose gel, transferred to Zeta Probe membranes, hybridized to radiolabelled DNA of plasmid pRB4794 containing the 1,800-bp *Nco*I fragment of the *Bam*HI S fragment, and exposed to Kodak XAR5 film. The expected sizes of the fragments generated by cleavages (bands a through j) are shown in Fig. 1.

*Nco*I fragments (Fig. 2, bands h and j) which were also present in HSV-1(F) (Fig. 2, bands a and b). This result indicates that the mutations in the ORF P genes of the parental virus R7546 ($P++$)⁻ have been replaced with the wild-type sequences.

Expression of ORF P and $\gamma_134.5$ proteins in cells infected with R7530 ($P++$), R7546 ($P++$)⁻, and R7547 ($P++$)⁻R recombinant viruses. Replicate 25-cm² flask cultures of Vero cells were infected with 10 PFU of HSV-1(F), R7530 ($P++$), R7546 ($P++$)⁻, or R7547 ($P++$)⁻R per cell. After 18 h of incubation at 37°C, cells were harvested, lysed by sonication in disruption buffer, boiled for 5 min, subjected to electrophoresis in a denaturing 12.5% polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and probed with rabbit polyclonal antiserum ORF Pc specific for ORF P. As shown in Fig. 3A, the antibody reacted with the typical, previously reported three ORF P polypeptide bands in electrophoretically separated lysates of cells infected with the recombinant R7530 ($P++$) containing an expressible, derepressed ORF P (19). These protein bands were not expressed in cells infected with the recombinant R7546 ($P++$)⁻ carrying a derepressed ORF P but lacking the initiator methionine. Lastly, ORF P protein was also not expressed in cells infected with wild-type virus HSV-1(F) or the recombinant virus R7547 ($P++$)⁻R, in which both the ICP4 binding site and the initiator methionine codon were restored.

An earlier report from this laboratory showed that derepression of ORF P resulted in undetectable amounts of $\gamma_134.5$ protein and mRNA (19). To determine whether this down-regulation was correlated with the synthesis of ORF P protein, a nitrocellulose sheet containing replicate samples from the experiment shown in Fig. 3A was probed with the rabbit poly-

FIG. 3. Photograph of infected cell proteins electrophoretically separated on a denaturing polyacrylamide gel and reacted with polyclonal rabbit antisera specific for ORF P (A) or $\gamma_134.5$ (B). Replicate Vero cell cultures grown in 25-cm² flasks were infected with 10 PFU of HSV-1(F), R7530 ($P++$), R7546 ($P++$)⁻, or R7547 ($P++$)⁻R per cell, maintained at 37°C for 22 h, harvested, solubilized, electrophoretically separated on 12.5% polyacrylamide denaturing gels, transferred to a nitrocellulose sheet, and reacted with rabbit polyclonal antiserum (A) ORF Pc or (B) BR4, then goat anti-rabbit IgG conjugated to alkaline phosphatase, and finally alkaline phosphatase substrate. Both blots were then reacted with mouse monoclonal antiserum specific to ICP0, goat anti-mouse IgG conjugated to alkaline phosphatase, and alkaline phosphatase.

clonal antiserum BR4 to detect $\gamma_134.5$ protein. As shown in Fig. 3B, $\gamma_134.5$ protein was present in lysates of cells infected with HSV-1(F) and the repaired virus R7547 ($P++$)⁻R. The protein was undetectable in cells infected with R7530 ($P++$) or R7546 ($P++$)⁻. ICP0, which is unaffected by ORF P expression in infected Vero cells (unpublished data), was used as a loading control. While the levels of ICP0 fluctuated slightly, the results presented in Fig. 3 indicate that the shutoff of expression of $\gamma_134.5$ protein correlates with derepression of ORF P transcription rather than the expression of ORF P protein.

Expression of ORF P and $\gamma_134.5$ mRNAs in cells infected with R7530 ($P++$), R7546 ($P++$)⁻, and R7547 ($P++$)⁻R recombinant viruses. The synthesis of ORF P mRNA was tested in replicate 150-cm² flask cultures of Vero cells infected with 10 PFU of HSV-1(F), R7530 ($P++$), R7531 ($P++$)⁻R, R7546 ($P++$)⁻, or R7547 ($P++$)⁻R per cell. After 6 h of incubation at 37°C, cells were harvested, and the cytoplasmic RNAs were collected as described in Materials and Methods. Total cytoplasmic RNAs in 20-μg amounts per sample were subjected to electrophoresis in 1.5% agarose-formaldehyde gels, transferred to Zeta Probe membranes by capillary action, and hybridized with radiolabelled riboprobes generated by T7 transcription of pRB4794 specific for the ORF P transcript.

As shown in Fig. 4A, a specific 2.3-kb mRNA species corresponding to the ORF P transcript was present in cytoplasmic RNAs extracted from cells infected with recombinants R7530 ($P++$) and R7546 ($P++$)⁻, in which transcription was derepressed by mutagenesis of the ICP4 binding site. This RNA species was not detected in cytoplasmic RNAs extracted from cells infected with wild-type virus or the recombinants R7531 ($P++$)⁻R and R7547 ($P++$)⁻R, in which the wild-type sequences were restored. Thus, as expected, the ICP4 binding site mutation derepressed the transcription of ORF P (Fig. 4A) but the initiator methionine mutation precluded ORF P protein expression (Fig. 3A) in R7546 ($P++$)⁻-infected cells.

FIG. 4. Autoradiographic image of electrophoretically separated RNAs from cells infected with HSV-1(F), R7530 (P++), R7531 (P++R), R7546 (P++⁻), or R7547 (P++⁻)R and probed with sequences specific to ORF P (A) or $\gamma_134.5$ (B) transcripts. (A) Twenty micrograms of total cytoplasmic RNA from Vero cells infected for 6 h at 37°C was separated by electrophoresis on 1.5% agarose-formaldehyde gels, transferred to Zeta Probe membranes, and hybridized with radiolabelled riboprobes specific for the ORF P transcript by T7 transcription of pRB4794; (B) 800 μ g of total cytoplasmic RNA from Vero cells infected for 13 h at 37°C was poly(A) purified, separated by electrophoresis on 1.5% agarose-formaldehyde gels, transferred to Zeta Probe membranes, and hybridized with radiolabelled RNA probes specific for the $\gamma_134.5$ transcript by SP6 transcription of pRB4794. The locations of the 28S and 18S ribosomal subunits are shown.

To measure the accumulation of $\gamma_134.5$ mRNA, Vero cells grown in roller bottles were infected with 10 PFU of HSV-1(F), R7530 (P++), R7531 (P++R), R7546 (P++⁻), or R7547 (P++⁻)R per cell. After 13 h of incubation at 37°C, cells were harvested and processed as described above except that polyadenylated mRNA purified from 800 μ g of total cytoplasmic RNA per sample was subjected to electrophoresis. The electrophoretically separated, denatured RNAs were transferred to Zeta Probe membranes and hybridized with radiolabelled riboprobes generated by SP6 transcription of pRB4794 specific for $\gamma_134.5$ mRNAs. In this instance, $\alpha 27$ mRNAs were used as a loading control (data not shown). As shown in Fig. 4B, a specific 1.2-kb mRNA species corresponding to $\gamma_134.5$ mRNAs was detected in cytoplasmic RNA extracted from cells infected with HSV-1(F), R7531 (P++R), and R7547 (P++⁻)R but not in RNA extracted from cells infected with R7530 (P++) or R7546 (P++⁻), which express ORF P transcripts.

The key conclusion to be derived from these results is that accumulations of detectable amounts of the two antisense mRNAs, those of ORF P and $\gamma_134.5$, are mutually exclusive and that transcription of ORF P prevented the accumulation of the antisense $\gamma_134.5$ mRNAs. It should be noted that the DNA fragment used to construct the repair does not extend 5' of the $\gamma_134.5$ coding region, and therefore the $\gamma_134.5$ promoter has not been altered in the repaired virus. The accumulation of $\gamma_134.5$ mRNA in cells infected with R7547 (P++⁻)R indicates that the control of $\gamma_134.5$ mRNA accumulation resides at the site of mutation, i.e., at the ICP4 binding site and not at some undefined site outside the domains of the ORF P and $\gamma_134.5$ genes.

FIG. 5. Autoradiographic image of [³⁵S]methionine-labelled infected cell proteins electrophoretically separated on denaturing gels. Replicate SK-N-SH cells grown in 25-cm² flasks were infected with HSV-1(F), R3616 ($\gamma_134.5^-$ /ORF P⁻), R7530 (P++), R7546 (P++⁻), or R7547 (P++⁻)R (10 PFU per cell) and maintained at 37°C in medium 199V. At 14 h after infection, the medium was replaced with medium 199V lacking methionine but supplemented with 50 μ Ci of [³⁵S]methionine, and the cells were incubated at 37°C for 1 h. The cells were then harvested, solubilized, electrophoretically separated on a denaturing 10% polyacrylamide gel, transferred to a nitrocellulose sheet, and exposed to Kodak XAR5 film.

Functional analyses of the $\gamma_134.5$ gene product in cells infected with wild-type and recombinant viruses. An earlier report from this laboratory showed that protein synthesis was shut off in human neuroblastoma SK-N-SH cells infected with $\gamma_134.5^-$ virus but not in cells infected with wild-type virus or R7530 (P++) (19). These results suggested that small amounts of $\gamma_134.5$ protein expressed in these cells were sufficient to prevent the total shutoff of protein synthesis. To exclude the possibility that ORF P protein expression can compensate for the loss of $\gamma_134.5$ protein synthesis, the following experiment was done.

Replicate cultures of SK-N-SH cells in 25-cm² flasks were infected with 10 PFU of HSV-1(F), R3616 ($\gamma_134.5^-$ /ORF P⁻), R7530 (P++), R7531 (P++R), R7546 (P++⁻), or R7547 (P++⁻)R per cell. After 14 h of incubation at 37°C, the medium was replaced with 1 ml of 199V lacking methionine but supplemented with 50 μ Ci of [³⁵S]methionine, and the cells were reincubated for 1 h. The cells were then harvested, lysed in disruption buffer, sonicated, electrophoresed in 10% denaturing polyacrylamide gels, transferred to nitrocellulose, and exposed to Kodak XAR5 film (Fig. 5). As predicted from earlier studies published elsewhere (12, 13), labelled proteins were essentially absent from lysates of cells infected with R3616 ($\gamma_134.5^-$ /ORF P⁻). The lysates of all other infected cell cultures accumulated approximately equivalent amounts of labelled proteins. Inasmuch as protein synthesis was not shut off in cells infected with R7546 (P++⁻), we may conclude that ORF P protein does not compensate for the decreased accumulation of the $\gamma_134.5$ protein by preventing the shutoff of global protein synthesis.

A second function of $\gamma_134.5$ is related to virulence, that is, the loss of the capacity of null or deletion mutants in the $\gamma_134.5$ genes to destroy the CNS of animals used in experimental model systems (9, 30). This function differs from that which blocks the shutoff of protein synthesis inasmuch as (i) the chimeric gene formed by replacement of the carboxyl terminus of $\gamma_134.5$ protein with the homologous domain from the murine GADD34 (MyD116) protein precluded the shutoff of protein synthesis but did not restore the capacity of the recombi-

TABLE 2. Pathogenicity of wild-type, mutant, and repaired viruses in mice following intracerebral inoculation

Virus	PFU/LD ₅₀ ^a
HSV-1(F).....	150
R7530 (P++).....	1.5 × 10 ⁵
R7546 (P++) ⁻	6.2 × 10 ⁴
R7547 (P++) ⁻ R.....	250

^a Mortality was counted from days 2 to 21 after inoculation. PFU/LD₅₀ was calculated by the Reed and Muench test.

nant virus to replicate in the CNS of susceptible mice (4), and (ii) in-frame deletions at the 5' domains of the γ_1 34.5 gene did not affect protein synthesis but did attenuate virulence (9). The mechanism by which γ_1 34.5 renders the virus virulent is unclear. We have previously shown that derepression of ORF P caused a 2,000-fold increase in PFU/LD₅₀ of the recombinant virus relative to that of its repair construct R7531 (P++)R or HSV-1(F) (19). A key unresolved question is whether the decrease in the capacity of the virus to destroy the murine CNS is due to expression of ORF P protein or to the antisense effect of the transcription of ORF P. To address this question, mice were injected intracerebrally in groups of seven with 10-fold serial dilutions of HSV-1(F), R7530 (P++), R7546 (P++)⁻, or R7547 (P++)⁻R, and mortality was monitored from days 2 to 21. As shown in Table 2, HSV-1(F) and the repaired virus R7547 (P++)⁻R had PFU/LD₅₀ values of 150 and 250, respectively whereas the PFU/LD₅₀ ratios of both R7530 (P++) and R7546 (P++)⁻ were reduced more than 200-fold (1.5 × 10⁵ and 6.2 × 10⁴, respectively). These results suggest that the attenuated virulence of the ORF P derepressed virus was primarily due to antisense transcription. Given this effect, the contribution of the overexpressed ORF P cannot be evaluated properly.

ORF P protein synthesis is required for downregulation of the α proteins ICP0 and ICP22 which are derived from spliced mRNAs. ORF P has been shown to interact with p32, a component of the ASF/SF2 splicing factors. In a test of the significance of this interaction, it was shown that the accumulation of ICP0 and ICP22 but not ICP27 or ICP4 was decreased in HeLa cells infected with R7530 (P++) (6). Whereas ICP0 and ICP22 are synthesized from spliced mRNAs, the mRNAs directing the synthesis of ICP4 and ICP27 are not spliced. To verify the hypothesis that the reduction in synthesis of ICP0 and ICP22 correlates with expression of ORF P protein and not merely to the transcription of the gene, 25-cm² flasks of HeLa cells were infected with 10 PFU of HSV-1(F), R7530 (P++), or R7546 (P++)⁻ per cell. After 8 h of incubation at 37°C, cells were harvested, subjected to electrophoresis in 9% denaturing polyacrylamide gels, transferred to a nitrocellulose sheet, and reacted sequentially with mouse monoclonal antisera for ICP27 (H1113) and ICP0 (H1112) and with rabbit polyclonal antiserum for ICP22 (R77). The data in Fig. 6 show that ICP0 and ICP22 accumulated at lower levels in cells infected with R7530 (P++) than in cells infected with R7546 (P++)⁻. Although the levels of ICP27 fluctuated slightly, the results are consistent with the hypothesis that ORF P protein expression is required for the downregulation of ICP0 and ICP22 and that antisense transcription alone does not contribute significantly to this phenotype.

DISCUSSION

The issues dealt with in this report arose from the observation that ORF P and γ_1 34.5 genes are almost completely an-

FIG. 6. Photograph of infected cell proteins electrophoretically separated on denaturing gels and reacted sequentially with mouse monoclonal antiserum for ICP27, mouse monoclonal antiserum for ICP0, and rabbit polyclonal antiserum for ICP22. Replicate HeLa cell cultures in 25-cm² flasks were infected with 10 PFU of HSV-1(F), R7530 (P++), or R7546 (P++)⁻ per cell. After 8 h of incubation at 37°C, cells were harvested, solubilized, electrophoretically separated on a denaturing 9% polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted sequentially with mouse monoclonal antiserum for ICP27, goat anti-mouse IgG conjugated to alkaline phosphatase, and alkaline phosphatase substrate. The same blot was reacted sequentially with mouse monoclonal antiserum for ICP0 and rabbit polyclonal antiserum for ICP22 and then processed as described above.

tisense to each other. In cells infected with wild-type virus, ORF P is expressed only in the absence of functional ICP4; indeed, to define the function of ORF P, the ICP4 binding site at the transcription initiation site of ORF P had to be destroyed by mutagenesis (6, 19, 21). In contrast, the γ_1 34.5 gene is expressed in productive infection before but especially after the onset of viral DNA synthesis (2). Although the two genes are expressed under entirely different physiological conditions, we were concerned that, because of their location, the phenotype of the ORF P mutants may reflect a novel regulatory pathway—inhibition of gene expression by antisense transcription.

Specifically, the γ_1 34.5 protein appears to have at least two functions. One, mapping in the carboxyl-terminal domain of the protein, prevents the shutoff of protein synthesis by binding to and diverting the protein phosphatase 1 α to dephosphorylate the α subunit of eIF2, the substrate of the double-stranded RNA-activated protein kinase, PKR (8, 12, 13, 16, 17). The second function of γ_1 34.5 appears to map throughout the gene inasmuch as in-frame deletions of any portion of the gene reduced the capacity of mutant viruses to multiply and cause mortality of animals in experimental model systems (9). The studies on ORF P, albeit less advanced, also revealed three phenotypic manifestations. Specifically, (i) ORF P protein associates with a splicing factor, localizes in spliceosomes, and causes a reduction of α proteins expressed from spliced mRNAs but does not affect the accumulation of the products of intronless mRNAs in cells infected with a mutant carrying derepressed ORF P genes (6), (ii) γ_1 34.5 mRNA and protein do not accumulate in detectable cells infected with the derepressed ORF P mutant virus although their presence may be deduced from the phenotype of the virus in infected cells in vitro (19), and (iii) the mutant overexpressing ORF P is highly attenuated in assays involving intracerebral inoculation of mice (19). Although repair of the sequences mutagenized to derepress ORF P rendered the recombinant virus virtually identical to its wild-type parent, the question remained as to whether

any feature of the phenotype of the mutant R7530 (P++) carrying the derepressed ORF P could be due to inhibition of expression of the γ_1 34.5 gene. To resolve the question posed above, we constructed two recombinant viruses. In the first, we doubly mutagenized the ORF P gene such that (i) transcription was derepressed by mutagenesis of the ICP4 binding site and (ii) the initiator methionine was mutagenized to preclude translation of the mRNA. In the second recombinant, we repaired both mutations to wild-type sequence. Although in principle the technology for the introduction of mutations or whole genes into the HSV genome is well advanced, introduction of mutations into the ORF P and γ_1 34.5 genes poses special problems because the viral genome contains two copies of these genes and because the inverted repeats tend to be unstable. Extensive studies were done to verify the context and phenotype of each of the mutants and repaired viruses involved in these studies. The key conclusions of these studies and their implications are as follows.

(i) We have extended the results published earlier (19) that derepression of ORF P results in a decrease in the accumulation of γ_1 34.5 protein (Fig. 3B) and mRNA (Fig. 4B). In the studies presented here, we show that the decrease in the accumulation of γ_1 34.5 protein and mRNA correlates with the transcription of ORF P rather than with the synthesis of ORF P protein. This conclusion is evident from the observation that the mutant carrying a derepressed, nontranslated ORF P is just as effective as the mutant which expresses the ORF P protein in reducing the synthesis of γ_1 34.5 mRNA and protein.

In an earlier report, it was noted that ORF P and γ_1 34.5 are the first antisense genes discovered within the HSV-1 genome and that the regulation by antisense transcription is a novel mechanism of regulation of gene expression (19, 20). Subsequently this laboratory reported another set of antisense genes, U_L43 and $U_L43.5$, whose expression is also mutually exclusive and apparently regulated by a similar pathway except that in this instance both U_L43 and $U_L43.5$ are expressed during productive infection (7).

We have also extended the results published earlier showing that protein synthesis is not shut off in SK-N-SH cells infected with the recombinant virus expressing the derepressed ORF P genes even though we could not detect the accumulation of the γ_1 34.5 protein (19). As reported earlier, at least two lines of evidence suggest that the amount of γ_1 34.5 protein required to block the shutoff of protein synthesis is very small. First, analyses of clonal lines of SK-N-SH cells stably transformed with the γ_1 34.5 gene varied in the amount of γ_1 34.5 protein expressed. Protein synthesis was unaffected in clonal lines expressing undetectable amounts of γ_1 34.5 and infected with the γ_1 34.5⁻ virus (10). Second, whereas γ_1 34.5 deletion mutant R3616 induced shutoff of protein synthesis in both SK-N-SH cells and in human foreskin fibroblasts (HFF), the mutant R4009 carrying a stop codon in the γ_1 34.5 gene induced shutoff of protein synthesis in SK-N-SH cells but not in HFF cells (10). Although γ_1 34.5 protein could not be detected in HFF cells infected with the R4009 mutant, stop codon suppression is not uncommon in eukaryotic cells and the small amount of intact γ_1 34.5 protein made in those cells could have been sufficient to block the shutoff of protein synthesis.

(ii) The experimental animal studies presented earlier (19) and extended in this report showed unambiguously that viruses carrying a derepressed ORF P are attenuated upon intracerebral inoculation of mice. The results in this instance show that the derepressed mutant expressing the ORF P protein is only slightly more attenuated than the mutant expressing ORF P RNA but not the protein. These results suggest that the attenuation is due largely to the suppression of γ_1 34.5 gene expres-

sion and that the contribution of ORF P, if any, cannot be evaluated in these studies. While very small amounts of γ_1 34.5 are sufficient to preclude the shutoff of protein synthesis, they may not be sufficient to promote wild-type virulence.

(iii) We verified and extended an earlier report (6) showing that the levels of accumulation of ICP0 and ICP22, but not those of ICP4 and ICP27, are reduced in cells infected with the virus expressing the derepressed ORF P. The significance of these results stems from the evidence that ICP0 and ICP22 are the products of spliced mRNAs whereas ICP4 and ICP27 are translated from intronless transcripts and that ORF P binds to a splicing factor and colocalizes with spliceosomes. In this study, we show that the reduction in accumulation of ICP0 and ICP22 correlated with synthesis of ORF P protein and is not the consequence of antisense transcription of ORF P. Specifically, ICP22 and ICP0 accumulated at a much lower level in cells infected with R7530 (P++) expressing the ORF P protein than in cells infected with the recombinant virus containing a derepressed ORF P expressing the RNA but not the protein (Fig. 6).

In essence, the studies presented in this report indicate that transcription of ORF P resulted in a decrease in the expression of the γ_1 34.5 gene and that some of the manifestations of the mutant carrying a derepressed ORF P gene are the consequence of antisense inhibition of transcription of the γ_1 34.5 gene. A key finding, reinforced by results reported earlier (6), is that the reduction in the accumulation of ICP0 and ICP22 but not of other α proteins is a phenotype directly related to the function of the ORF P protein, consistent with the affinity of the protein for p32 and its localization in spliceosomes.

ORF P mRNA and protein are not detectable during productive infection, and earlier studies from this laboratory indicate that it is expressed only under conditions of infection in which ICP4 is either not made or is nonfunctional (19–21, 31). As pointed out earlier, the mapping of ORF P in the domain of the genome transcribed during latency and the phenotype of the derepressed gene are consistent with the hypothesis that it is one of several genes which may be involved in the establishment of the latent state.

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