Herpes Simplex Virus 1 Open Reading Frames O and P Are Not Necessary for Establishment of Latent Infection in Mice

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Herpes simplex virus 1 (HSV-1) and HSV-2 cause two types of infections in humans and experimental animals, productive and latent. Productive infection at the portal of entry involves the coordinated expression of >80 open reading frames (ORFs), replication, assembly of infectious progeny, and destruction of the cell (reviewed in references 49 and 50) (20, 21). More than half of the genes are dispensable for growth in cell culture and appear to have auxiliary functions that optimize viral replication and spread within its host. From the portal of entry, HSV infects innervating sensory neurons and is transported retrograde to the nucleus. The precise sequence of events that follows is unclear; it seems that in some neurons the virus replicates and destroys the neurons whereas in others the virus establishes a latent infection.

It is convenient to differentiate three stages of infection of neurons: the establishment phase, the maintenance phase, and the reactivation phase. Little is known of the establishment phase since the neurons which replicate the virus during the first several days after infection obscure the events taking place in the neurons committed to maintaining the virus in a latent state. In the maintenance state, signaled by the disappearance of all traces of replicating virus, viral DNA is maintained in an episomal form and only a small region of the genome is transcribed, the latency-associated transcripts (LATs) (55). The last phase, reactivation, is induced spontaneously in some experimental animal systems (reviewed in reference 15). It can be induced artificially by explanation and cocultivation of sensory ganglia harboring latent virus. In essence, our knowledge of the requirement for the establishment of latency has been based on whether latent virus can be detected during the maintenance stage or as a consequence of induced reactivation. Over the past decade several genes have been “identified” as playing a role in the establishment of latency (23, 32, 37, 38, 53, 56, 59). The list includes a large number of ORFs and also the LATs. In most instances where thorough investigations have been carried out, it has become apparent that these genes play a key role in viral replication. Consequently, recombinant viruses mutated in or lacking these genes replicate poorly at the portal of entry and during reactivation from latent phase.

The major focus of investigations into genes controlling the establishment or maintenance of latency has been the LATs, a family of transcripts arising from the inverted repeats flanking the unique long (UL) sequence. The full-length 8.3-kb transcript accumulates at low levels in latently infected neurons, while 2.0- and 1.5-kb introns processed from the full-length transcript are abundant (10, 25, 36, 47, 52, 55, 57, 58, 63). These introns are highly stable and appear to be lariat structures (14, 48, 60, 62). Viruses with LATs deleted have been reported to establish latency at levels within a threefold range of the wild type (3, 56). Deletion of LATs reduces the capacity of the virus to cause productive infections in the mouse and reduces the capacity of the virus to replicate following explanation of the neurons (2, 18, 22, 31, 50, 51, 54). The region of LAT associated with decreased reactivation has been mapped to a 348-bp sequence in the 5′ end (3, 19). LAT has not been

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Open reading frame (ORF) O and ORF P partially overlap and are located antisense to the γ134.5 gene within the domain transcribed during latency. In wild-type virus-infected cells, ORF O and ORF P are completely repressed during productive infection by ICP4, the major viral transcriptional activator/repressor. In cells infected with a mutant in which ORF P was derepressed there was a significant delay in the appearance of the viral α-regulatory proteins ICP0 and ICP22. The ORF O protein binds to and inhibits ICP4 binding to its cognate DNA site in vitro. These characteristics suggested a role for ORF O and ORF P in the establishment of latency. To test this hypothesis, two recombinant viruses were constructed. In the first, R7538(P/O−), the ORF P initiator methionine codon, which also serves as the initiator methionine codon for ORF O, was replaced and a diagnostic restriction endonuclease was introduced upstream. In the second, R7543(P/O−) yields were similar to that of the wild type following infection of cell culture or following infection of mice by intracerebral or ocular routes. (iii) R7538(P/O−) virus as assayed by quantitative PCR is eightfold less than that of the repair virus. The repaired virus could not be differentiated from the wild-type parent in any of the assays done in this study. We conclude that ORF O and ORF P are not essential for the establishment of latency in mice but may play a role in determining the quantity of latent virus maintained in sensory neurons.

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shown to express ORFs. A recent report indicated that sequences containing the LAT introns can protect neurons from apoptosis and that a virus with LAT deleted induces apoptosis in rabbit trigeminal ganglia at higher levels than the wild-type virus (39). Thus, at least one function of LAT may be to promote neuronal survival during the maintenance of latent infection. Other studies have suggested that viral functions that repress lytic gene expression in vivo reside within the LAT domain (6, 16). The effectors of these functions are not identified. Irrespective of the final determination of the functions of LATs, the necessary conclusion is that LATs play a role in the maintenance of the latent state rather than in its establishment.

In earlier studies, we reported that the domain of the inverted repeats represented in LATs contains 16 ORFs of greater than 50 codons and that at least two of these, ORF O and ORF P, are expressed (27, 43). ORF O and ORF P are located in the 3' domain of the LAT domain, almost entirely antisense to the γ34.5 gene. They are expressed from a promoter and associated RNA internal to and 3' coterminal with LAT (4, 61). This transcript is completely repressed during productive infection by the binding of ICP4, the major viral transactivator/repressor, to a consensus ICP4 binding site that straddles the ORF P transcriptional initiation site (13, 26, 28, 29, 33–35, 42, 45, 46, 61). ORF O and ORF P are expressed only under conditions in which ICP4 repression is nonfunctional, i.e., in a virus containing a mutated ICP4 binding site or during infection and maintenance at 39.5°C, the nonpermissive temperature for ICP4, in HSV-1(F) and other limited-passage clinical isolates (11, 12). The repression of ORF O by ICP4 binding site was surprising since ORF O was predicted to begin upstream of the ORF P transcript. Analyses of the products encoded by the ORFs showed that the translation of ORF O initiates methionine codon and then shifts reading frame before the amino acid 35 codon of ORF P (43). Thus, ORF O and ORF P are expressed under identical conditions and have not been detected during productive infection.

Investigations into the functions of ORF O and ORF P have revealed the following. (i) ORF P transcription is sufficient to repress expression of the antisense γ34.5 gene and attenuates virulence (29, 42). (ii) ORF P protein inhibits the expression of ICP0 and ICP22. This correlates with an interference in the splicing of mRNAs inasmuch as (a) ORF P interferes and colocalizes with splicing factors (5); (b) a virus with ORF O and ORF P expression derepressed accumulates significantly less ICP0 and ICP22, which are translated from spliced mRNAs, while the levels of two proteins synthesized from intronless mRNAs, ICP4 and ICP27, are unchanged (5, 42); (c) ORF P protein expression is required for the inhibition of ICP0 and ICP22 expression (42); and (d) ORF P derepression alters the accumulation of the spliced LAT (30). (iii) ORF O protein specifically binds to and inhibits in vitro binding of ICP4 to its cognate site (43). ICP0 is a promiscuous transactivator capable of interacting with and activating transcription from a variety of promoters (6). The effectors of these functions are not identified. Irrespective of the final determination of the functions of LATs, the necessary conclusion is that LATs play a role in the maintenance of the latent state rather than in its establishment.

The goal of this study was to address the significance of ORF O and ORF P in the virus life cycle without interfering with the expression of the antisense γ34.5 gene. ORF P encodes only two methionines, the initiator and one located eight amino acids from the C terminus. Previous studies have shown that mutation of the initiator methionine in the context of the ORF P-depressed virus is sufficient to prevent ORF P protein translation (42). We have previously shown that ORF O may begin at the ORF P initiator methionine codon, such that a substitution of this codon would also prevent ORF O translation (43). Two recombinant viruses were constructed, (i) R7538 (P−/O−), which contains the initiator methionine codon mutation, and (ii) R7543 (P−/O−)R, in which the mutation was repaired to wild type. R7538 (P−/O−) does not express ORF O and ORF P proteins but does express γ34.5 at wild-type levels. We report that mutants lacking the capacity to synthesize ORF O and ORF P proteins were not affected in their ability to replicate in cell culture, mouse central nervous system, mouse eye, or mouse trigeminal ganglia. In the mouse, they established latency but at a reduced copy number per cell.

**Materials and Methods**

**Cells and viruses.** Rabbit skin and 143K− cells were originally obtained from J. McClaren and Carlo Croce, respectively. Vero cells were from the American Type Culture Collection. HSV-1(F) is the prototype HSV-1 strain used in this laboratory; as is the case with fresh HSV-1 isolates with limited history of replication outside the human host, the a+ gene of HSV-1(F) is temperature sensitive and does not repress itself or ORF P at 39.5°C (12, 27). The recombinant virus R3659 has been previously described (28). It lacks the SeI-BulI sequence of the BamHI Q fragment encoding the thymidine kinase (TK) and U24 genes. A sequence consisting of the coding domain of the TK gene under the control of the α27 promoter (40) replaced the BamHI-SphI fragment containing the γ34.5 and ORF P genes (Fig. 1, lane 4).

**Antibodies.** Rabbit polyclonal antisera specific for γ34.5, BR4 (1), and rabbit polyclonal antisera specific for ORF O (43) have been described previously.

**Plasmids.** pRB4929 contains the 830-bp NorI fragment of BamHI S cloned into the NorI site of pUC19. Plasmid pRB4929 contains a mutant ORF P initiator methionine codon and was made by insertion of a 160-bp PCR product into the SphI-DraIHI sites of pRB4930. The PCR primers were 5′-ACCAGGGGCTGCGGC CCTAGGGCAGCGGCGGATACGCCGCTGCGGCTC and 5′-GAGCCGGGAG GCCTGATCGGGCCGTCGCTGAGGCAGCCGGCGCT. Underlined bases represent mutations that replace the ORF P initiator methionine codon (ATG) with an isolate codon (ATA) and create a diagnostic AavII restriction site 15 bp upstream. Plasmid pRB4929 was used to construct the recombinant virus R7537. Plasmid pRB8103, containing the BamHI Q fragment, was used to repair the deletion in the TK gene of R7537, resulting in the recombinant virus R7538. pRB4794 has been described elsewhere (28). It contains the 1,800-bp NcoI fragment of BamHI S, spanning the region between the start codons of the a0 and γ34.5 genes. It was used as a probe for analyses of recombinant viral DNA and was used to repair the mutations in the ORF P domain of R7538 resulting in the recombinant virus R7543.

**Construction of recombinant viruses.** Viral stocks and titrations of viruses were done in Vero cells (American Type Culture Collection). R7537 was constructed by cotransfection of rabbit skin cells (originally obtained from J. McClaren) with pRB4929, which contains the ORF P initiator methionine codon.
FIG. 1. Schematic representations of sequence arrangements in the recombinant viruses used in these studies. (Line 1) Representation of the HSV-1(F) genome. Shown are the U3 and unique short (U3S) sequences, which are flanked by inverted repeats a, b, and b′, a′, and c′. (Line 2) Domains of the ORF O, ORF P, and γ34.5 genes in the inverted repeat sequence b′a′ flanking the U3 sequences. The coding sequences (boxes) and transcripts (lines with arrows denoting transcription direction) of the ORF O, ORF P, and γ34.5 genes are shown. Solid circle, wild-type ICP4 binding site. (Line 4) Sequence arrangement of the relevant domains of R3659. The Stul-BstEII sequences containing ORF P and γ34.5 were replaced in both repeats by the chimeric a27tk gene. (Line 6) Sequence arrangement of the relevant domains of recombinant R7538(P−/O−). The a27-tk gene of R3659 was replaced with sequences containing a mutated ORF P initiation methionine codon introducing a diagnostic AvrII endonuclease site. (Line 8) Sequence arrangement of the relevant region of recombinant R7534(P−/O−)/R. The mutations in the ORF O/P domain of R7538(P−/O−) were repaired by transfection with the NcoI fragment from the HSV-1(F) BamHI S fragment. (Lines 3, 5, 7, and 9) Expected sizes of fragments detected by hybridization of the 1,800-bp NcoI fragment with electrophoretically separated digests of viral DNAs with NcoI-AvrII. Diagnostic of the replacement of the initiator methionine. Arrows, restriction cleavage sites present in the respective viruses and therefore fragment boundaries. HSV-1(F) DNA would be expected to yield band A, R3659 DNA would be expected to yield band B, R7538(P−/O−) DNA would be expected to yield bands C and D, and R7543(P−/O−)/R DNA would be expected to yield band E. Abbreviations: St; Stul; Nc; NcoI; Bs; BstEII.

mutation, with R3659 viral DNA (Fig. 1, lane 4) which contains a deletion in the TK gene, and with an a27 promoter-driven TK gene replacement of the 1,000-bp Stul-NcoI fragment encoding the ORF P and γ34.54 genes. TK− viruses were selected by plating the progeny of the cotransfection on 143Tk− cells overlaid with Dubcovo modified Eagle medium containing 5% newborn calf serum and 40 μg of bromodeoxyuridine per ml of medium. Plaque-purified stocks were prepared as described elsewhere (41). Viral DNA was isolated from infected cells and purified on a 5 to 20% potassium acetate gradient as described elsewhere (27). Viral DNAs from single plaques were analyzed for the presence of novel AvrII endonuclease restriction sites diagnostic of the initiator methionine codon mutation. The TK gene of R7537 was repaired by plating the progeny of the cotransfection of rabbit skin cells with R7537 viral DNA and pBR4794, which contains the 1,800-bp NcoI fragment isolated from BamHI S of HSV-1(F). Plaque-purified stocks were analyzed for a wild-type restriction endonuclease pattern indicating the absence of the introduced EcoRl and AvrII sites within BamHI S. R7543 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-long, 0.85% agarose gel and transferred to a Zeta probe (Bio-Rad, Richmond, Calif.) by capillary action in 0.5 M NaOH. The membrane was rinsed in 2 x SSC (0.3 M NaCl plus 0.015 M Na2HPO4) and prehybridized in 30% formamide-6x SSC-1% milk-1% sodium dodecyl sulfate (SDS)-100 μg of single-stranded calf thymus DNA per ml for 30 min at 68°C. Denatured, 32P-labeled pBR4794 (106 cpm) was then added overnight, and the blot was rinsed as recommended by the manufacturer. Autoradiographic images were obtained on Kodak XAR5 film.

Immunoblots. Immunoblots were done as previously described (27). Briefly, infected cells were scraped into phosphate-buffered saline (PBS), pelleted under low-speed centrifugation, resuspended in disruption buffer containing 0.7 M β-mercaptoethanol, 2% SDS, 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 electrophoretically transferred to a nitrocellulose sheet, blocked, reacted with the appropriate antiserum, rinsed, and 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. Proteins were then rinsed again and developed as recommended by the manufacturer (Bio-Rad).

Viral replication in cell culture. Titrate 25-cm2 plaque dishes containing Vero or SK-N-SH cells were infected with 5 PFU of HSV-1(F), R7538(P−/O−), or R7543(P−/O−)/R. Cells were harvested at 6, 12, or 24 h, washed in PBS, resuspended in 1 ml of sterile milk, taken through three freeze-thaw cycles, sonicated, and used to infect Vero cells in 25-cm2 plaque dishes at 10-fold dilutions. Cells were maintained in 199" medium, and plaques were counted 2 days after infection.

Intracranial inoculation of mice. CBA/J mice (3.5 weeks old) from Jackson Laboratory were anesthetized with pentobarbital sodium (Nembutal) and injected intracerebrally with 10-fold serial dilutions of virus, seven mice per dilution. Mortality was monitored daily from 2 to 21 days postinfection. CPE was attributed to the inoculated virus. The 50% lethal dose (LD50) ratios were calculated by the method of Reed and Muench (44).

Assays of viral replication in murine eye and trigeminal ganglia. CBA/J mice from Jackson Laboratory, 4.5 weeks of age, were anesthetized and inoculated with 10 μl of virus on scarified corneas as previously described (29). Mice were sacrificed 1, 3, 5, and 7 days after infection; the eyes or trigeminal ganglia were removed, placed in 1 ml of 199V containing nystatin, homogenized in a homogenizer, and plated on Vero cells (100 μl for 24-h or 12-h CPE). Vero cells were overlaid with 0.2 ml of 199V containing 10 μl (5 x 105 PFU) of virus on scarified corneas as described above. Mice were sacrificed at 30 days after infection, and the trigeminal ganglia were removed, placed in 1 ml of 199V containing nystatin, and incubated for 5 days. The ganglia were then homogenized in a homogenizer, and plated on Vero cells (100 μl for 24-h CPE). Virus was isolated on scarified corneas as described above. CPE was monitored daily for 8 days after cocultivation.

Quantitation of latent virus. CBA/J mice (4.5 weeks old) were ocularily infected as described above. At 30 days after infection, 26 trigeminal ganglia per virus were removed, flash frozen, and stored at –80°C. Quantitative PCR was performed as previously described (23, 24). Briefly, ganglia were homogenized in 1 ml of solubilization solution (5 M guanidine thiocyanate, 50 mM Tris pH 7.5), 10 mM EDTA, 5% β-mercaptoethanol) using a Dounce homogenizer mechanical tissue grinder. DNA was precipitated, resuspended in PCR buffer and digested with 0.2 μg of proteinase K/ml at 55°C for 2 h, 80°C for 20 min, and 95°C for 5 min. One hundred nanograms of DNA was used for PCR with the following primers specific for the viral TK gene or the cellular β-actin gene: TK-1, 5′-CC TAAACGCGTCAAAGCGTGCCG; TK-2, 5′-CACAAGAGCTCGCCGAGGAT; Act-1, 5′-AACCTTAAGGCAAGCTGGGAAGATGAC; Act-2, 5′-CCAG GGAGGAAAGAGATGGCCG; PCR was performed under previously described conditions (24). TK products were amplified for 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension of 5 min at 72°C. Actin PCR conditions were as above except that the annealing temperature was 60°C. Aliquots of the PCR mixture were separated by nondenaturing polyacrylamide gel electrophoresis, electroblotted to a Zeta probe nylon membrane, and subjected to the PCR conditions as described above. The levels of amplified TK product were quantitated with a Storm phosphorimager and compared with a linear standard curve generated by PCR of 100 ng of

*BamHI Q fragment and the ORF P initiator methionine codon mutation. The mutations in the ORF P gene of R7538 were repaired by cotransfection of rabbit skin cells with R7538 viral DNA and pBR4794, which contains the 1,800-bp NcoI fragment isolated from BamHI S of HSV-1(F). Plaque-purified stocks were analyzed for a wild-type restriction endonuclease pattern indicating the absence of the introduced EcoRI and AvrRI sites within BamHI S. R7543 has a wild-type restriction endonuclease pattern and therefore has a wild-type genotype.
uninfected murine trigeminal ganglion DNA spiked with 10-fold dilutions of purified HSV-1(F) DNA. The values were normalized for DNA content by comparison of the amplified β-actin product.

RESULTS

Construction and characterization of the recombinant virus R7538(P−/O−), containing a mutated ORF P initiator methionine codon, and the repaired recombinant virus, R7543(P−/O−)R. The procedures for the construction of recombinant viruses R7538(P−/O−) and R7543(P−/O−)R are described in Materials and Methods. R7538(P−/O−) contains two nucleotide substitutions, one at the initiator methionine codon (ATG→ATA) and one 15 bp upstream creating a unique AvrII restriction endonuclease site (CCCCAGG→CCTAGG). The mutation creating the novel AvrII restriction endonuclease site also introduced a TAG stop codon into the predicted ORF O gene, upstream of the ORF P initiator methionine codon. Both mutations are in wobble codons of γ34.5, and as such they do not alter the amino acid sequence of the γ34.5 protein. The genotype of R7538(P−/O−) was verified by the presence of the unique AvrII restriction endonuclease site diagnostic of the ORF P initiator methionine codon mutation, the location of which is shown in Fig. 1, line 6. R7538(P−/O−) DNA was purified and incubated with restriction endonucleases NcoI and AvrII, electrophoretically separated on 0.85% agarose gels, transferred to Zeta probe membranes, and hybridized with 32P-labeled pRB4794, which contains the 1,800-bp NcoI fragment of BamHI S (Fig. 2, lane 3). Restriction endonuclease cleavage of R7538(P−/O−) with NcoI and AvrII resulted in the predicted 1,070- and 730-bp DNA fragments (Fig. 1, line 7, and Fig. 2, bands C and D), verifying the presence of the ORF P initiator methionine codon mutation. This pattern is distinct from those of the 1,800-bp fragment of HSV-1(F) (Fig. 1, line 3, and Fig. 2, band A) and the 700-bp DNA fragment of the parental virus R3659 (Fig. 1, line 5, and Fig. 2, band B).

As is necessary in all cases in which the phenotypes of recombinant viruses are tested, the mutations were repaired by cotransfection of rabbit skin cells with R7538(P−/O−) and pRB4794 (described above). Viral DNAs from plaque isolates of progeny virus were screened for the wild-type restriction pattern. Incubation of R7543(P−/O−)R with NcoI and AvrII resulted in the wild-type 1,800-bp band (Fig. 1, line 9, and Fig. 2, band E), indicating that the introduced mutations were replaced with wild-type sequences.

Expression of ORF O and γ34.5 proteins in cells infected with HSV-1(F), R7538(P−/O−), and R7543(P−/O−)R recombinant viruses. The ORF P initiator methionine codon mutation previously was shown to prevent ORF P translation from a derepressed ORF P transcript (42). The effect of the mutations on ORF O expression was unknown. Therefore, cells in replicate 25-cm2 flasks were mock infected or infected (10 PFU/cell) with HSV-1(F), R7538(P−/O−), or R7543(P−/O−)R at 4°C and maintained at 39.5°C, the nonpermissive temperature for ICP4. After 20 h the cells were harvested, lysed by sonication in disruption buffer, boiled for 5 min, subjected to electrophoresis in denaturing polyacrylamide gels, transferred to a nitrocellulose sheet, and reacted with polyclonal antisera specific for ORF O. As shown in Fig. 3A, ORF O accumulated in cells infected with either HSV-1(F) or the repair virus R7543(P−/O−)R and maintained at the nonpermissive temperature for ICP4. ORF O was not detected in R7538(P−/O−), indicating that the introduced mutations are sufficient to prevent ORF O expression.

Since the mutations introduced into ORF O and ORF P are also in the γ34.5 gene, we verified that γ34.5 expression in the recombinant viruses was comparable to that in the wild-type virus. Cells in replicate 25-cm2 flasks were mock infected or infected with 10 PFU of HSV-1(F), R7538(P−/O−), and R7543(P−/O−)R and maintained at 37°C. Infected-cell lysates were processed as described above and reacted with polyclonal antisera BR4, specific for γ34.5. As shown in Fig. 3B, the γ34.5 protein was present in all infected lysates at comparable levels. The results of Fig. 3 show that, in addition to precluding ORF P protein translation, the mutations introduced into R7538(P−/O−)R prevent ORF O synthesis without affecting the expression of the antisense γ34.5 gene.

Replication of wild-type and mutant viruses in cell culture and in vivo. The replication competence of recombinant viruses was tested in four series of experiments involving (i) the production of infectious progeny in cell culture, (ii) determination of the neurovirulence of the recombinant viruses compared to that of the wild type, (iii) the isolation of infectious virus from murine eyes, and (iv) the isolation of infectious virus from murine trigeminal ganglia. In the first series of experiments, replicate 25-cm2 cultures of Vero or SK-N-SH cells were infected with 5 PFU of HSV-1(F) or R7538(P−/O−) per
cell. Infected cells were harvested at 6, 12, and 24 h after infection. As shown in Table 1, the yields of HSV-1(F) and R7538(P−/−/O−) viruses were comparable in Vero cells and in SK-N-SH cells. Thus, the absence of ORF O and ORF P protein synthesis did not affect viral growth in cells of neuronal and nonneuronal origin.

In the second series of experiments, the neurovirulence of HSV-1(F), R7538(P−/−/O−), and R7543(P−/−/O−)R was tested. Neurovirulence represents the capacity of HSV to replicate in the presence of HSV-1 neutralizing antibody, and plaque titers generally fell within a threefold range. The amounts of infectious R7538(P−/−/O−) did not significantly affect the ability of the virus to replicate in cell culture or the murine central nervous system.

In the third series of experiments, 4.5-week-old CBA/J mice were infected with 5 × 10^5 PFU of HSV-1(F), R7538(P−/−/O−), or R7543(P−/−/O−)R per eye. At days 1, 3, 5, and 7 after infection, three mice per group were anesthetized and sacrificed and the eyes were removed, washed, homogenized in 1 ml of 199V with nystatin using a mechanical tissue grinder, and frozen. Vero cells in replicate 25-cm² flasks were infected with 10-fold dilutions of the respective virus, maintained in the presence of HSV-1 neutralizing antibody, and plaque titers were determined 2 days after infection. As shown in Table 2, the amounts of infectious R7538(P−/−/O−), HSV-1(F), and R7543(P−/−/O−)R isolated from eyes following ocular infection generally fell within a threefold range. The amounts of infectious R7538(P−/−/O−) were not statistically different from those of HSV-1(F) or R7543(P−/−/O−)R at any of the times tested (P > 0.05). Thus, the relative amounts of infectious wild-type and recombinant viruses isolated from the site of inoculation were indistinguishable.

In the fourth series of experiments, 4.5-week-old CBA/J mice were infected with 5 × 10^5 PFU of HSV-1(F), R7538(P−/−/O−), or R7543(P−/−/O−)R per eye. At days 3 and 5 after infection, the trigeminal ganglia were removed and virus was titered as described above. As shown in Table 3, the amounts of infectious R7538(P−/−/O−), HSV-1(F), and R7543(P−/−/O−)R isolated from ganglia 3 days after ocular infection fell within a threefold range (9.0 × 10^6, 2.7 × 10^7, and 2.9 × 10^6 PFU per ganglion, respectively). At day 5, the level of infectious R7538(P−/−/O−) was 2 to 3 log units less than that of

<table>
<thead>
<tr>
<th>Virus</th>
<th>PFU/ganglion (1,000) on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>HSV-1(F)</td>
<td>27 ± 4.2</td>
</tr>
<tr>
<td>R7538(P−/−/O−)</td>
<td>9.0 ± 2.3</td>
</tr>
<tr>
<td>R7543(P−/−/O−)R</td>
<td>29 ± 9.3</td>
</tr>
</tbody>
</table>

a Average counts of plaques ± standard error appearing in flasks containing Vero cells, which were infected with 10-fold dilutions of trigeminal ganglion homogenates (20 ganglia total per virus), harvested at the indicated days after ocular infection.

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TABLE 1. Replication of HSV-1(F) and R7538(P−/−/O−) in Vero and SK-N-SH cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus yield (log_{10} PFU) from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vero cells at: SK-N-SH cells at:</td>
</tr>
<tr>
<td></td>
<td>6 h  12 h  24 h  6 h  12 h  24 h</td>
</tr>
<tr>
<td>HSV-1(F)</td>
<td>4.9  6.8  7.8  4.8  7.4  8.0</td>
</tr>
<tr>
<td>R7538(P−/−/O−)</td>
<td>4.9</td>
</tr>
</tbody>
</table>

a Virus yields from Vero and SK-N-SH cells infected with 5 PFU of HSV-1(F) or R7538(P−/−/O−) per cell and harvested at 6, 12, and 24 h after infection.

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TABLE 2. Isolation of infectious HSV-1(F) or of recombinant viruses R7538(P−/−/O−) and R7543(P−/−/O−)R from murine eye

<table>
<thead>
<tr>
<th>Virus</th>
<th>PFU/eye (100) on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  3  5  7</td>
</tr>
<tr>
<td>HSV-1(F)</td>
<td>880 ± 230 16 ± 6.6 3.3 ± 1.5 2.1 ± 1.2</td>
</tr>
<tr>
<td>R7538(P−/−/O−)</td>
<td>1,400 ± 490 11 ± 3.1 2.6 ± 0.8 0.4 ± 0.2</td>
</tr>
<tr>
<td>R7543(P−/−/O−)R</td>
<td>1,600 ± 770 28 ± 22 2.3 ± 1.8 1.6 ± 1.3</td>
</tr>
</tbody>
</table>

a Average counts of plaques ± standard error appearing in flasks containing Vero cells, which were infected with 10-fold dilutions of eye homogenates (12 samples total per virus), harvested at the indicated days after ocular infection.
and infected with 10⁶ PFU of HSV-1(F), R7538(P/O) versus days after cocultivation. CBA/J mice (4.5 weeks old) were anesthetized, ocularly scarified, and infected with 10⁶ PFU of HSV-1(F), R7538(P/O), or R7543(P/O)-R. After 30 days, mice were sacrificed, and the trigeminal ganglia were removed, incubated in 1 ml of 199V medium plus nystatin for 5 days at 37°C, homogenized, and used to infect 25-cm² plaque dishes containing Vero cells. CPE was monitored daily for 8 days. The graph represents the percentage of ganglia producing infectious virus versus days after cocultivation.

HSV-1(F) or the repair virus (3.2 × 10⁵, 1.4 × 10⁵, and 7.7 × 10⁴ PFU per ganglion, respectively). It is unclear whether the decrease in infectious R7538(P/O) virus at day 5 reflects an alteration in the efficiency of the establishment of latency. We conclude that at day 3, a time at which acute infection and the establishment of latency. We conclude that at day 3, a time at which acute infection and the establishment of latency are occurring in mice, infectious R7538(P/O) was present in trigeminal ganglia at levels comparable to those of HSV-1(F) and the repair virus.

Reactivation from latency in mice infected with HSV-1(F), R7538(P/O), and R7543(P/O)-R. The significance of ORF O and ORF P in the latent life cycle was assessed by measuring the reactivation of wild-type and recombinant viruses. CBA/J mice (4.5 weeks old) were infected by ocular scarification with 5 × 10⁵ PFU of HSV-1(F), R7538(P/O), or R7543(P/O)-R per eye. After 30 days, a time at which replicating HSV-1 or R7538(P/O) could not be isolated in this animal model (reviewed in reference 15) (data not shown), mice were anesthetized; the trigeminal ganglia were removed, incubated for 5 days in 199V plus nystatin, homogenized, andcocultivated in replicate 25-cm² flasks containing Vero cells; and CPE was scored for each ganglion. Figure 4 shows the percentage of ganglia that produced reactivated virus versus the day after cocultivation. Both HSV-1(F)- and R7543(P/O)-R-infected ganglia reactivated virus in 100% of the samples, with 90% showing obvious CPE 1 day after cocultivation. R7538(P/O)-infected ganglia reactivated virus in 95% of samples; however, CPE in 90% of the ganglia was not achieved until 3 days after cocultivation. This likely reflects smaller amounts of reactivated virus. The data indicate that ORF O and ORF P proteins are not required for reactivation from latency, but a virus precluded from synthesizing ORF O and ORF P proteins reactivated with reduced kinetics.

Since the regulation and functions of ORF O and ORF P imply a role for these genes in the establishment of latency, it seemed likely that the decreased levels of reactivated virus correspond to a decrease in the amount of virus which has established latency. To test this hypothesis, a quantitative PCR analysis was employed to calculate the number of latent viral DNA copies, as previously described (23, 24). CBA/J mice (4.5 weeks old) were ocularly infected as described above, and ganglia were harvested after 30 days and flash frozen. Ganglia were homogenized in 1 ml of DNA extraction buffer, precipitated, washed, resuspended, and treated with proteinase K as described in Materials and Methods. DNA (100 ng) from each sample was subjected to PCR using primers specific for the viral TK gene or the murine β-actin gene (24). Aliquots of the PCR mixture were separated by nondenaturing polyacrylamide gel electrophoresis, electroblotted to a nylon membrane, denatured, and probed with 32P-labeled oligonucleotides internal to the PCR primers. The levels of amplified TK gene product were quantified with a Storm phosphorimager and compared with a linear standard curve generated by PCR of 100 ng of uninfected murine trigeminal ganglion DNA spiked with 10-fold dilutions of purified HSV-1(F) DNA. No amplified TK gene product was detected in uninfected murine trigeminal ganglia samples (data not shown). The values were normalized for DNA content by comparison of the amplified β-actin gene product. The results of these studies are shown in Table 4. Ganglia latently infected with R7538(P/O) contained 0.14 +/− 0.03 viral DNA copies per cell equivalent, whereas the HSV-1(F) latent DNA copy number was estimated to be sixfold higher, 0.84 copies per cell (P < 0.005). The amount of latent R7543(P/O)-R was eightfold higher than the amount of R7538(P/O)-R, 1.10 copies per cell (P < 0.0001). These results indicate that ORF O and ORF P proteins may play a role in the ultimate number of viral DNA copies maintained in the latent state.

DISCUSSION

A unique property of HSV is that it carries a large number of accessory genes designed in large part to control both the intracellular and extracellular environment in which it replicates. Latency is a significant mechanism for the perpetuation of HSV in human populations. Other herpesviruses, notably Epstein-Barr virus and other members of the gammaherpesvirus subfamily, have evolved elaborate mechanisms for the establishment of the latent state by virally encoded proteins. The presumption that HSV would also encode functions designed to facilitate the establishment of latency was the basis of the search that led to the identification of ORFs P and O. ORF P and O and their products appear to be ideal candidates for the control of the latent state. Specifically, (i) ORF O and ORF P are located in the domain transcribed during latency, (ii) both ORFs are completely repressed during productive infection by ICP4, the major viral transcriptional transactivator/repressor, (iii) the ORF P protein inhibits the syn-
thesis of the important α-regulatory proteins ICP0 and ICP22 in cells infected with ORF P-derepressed viruses, and (iv) the ORF O protein made under similar circumstances binds to and inhibits ICP4 binding to its cognate DNA site in vitro. The objective of the studies described in this report was to test the role of ORF O and ORF P in the establishment of latency in the mouse model in vivo.

To test the role of ORF O and ORF P proteins, we constructed the recombinant virus R7538(P+/O−), in which the initiator methionine codon of both coding sequences was mutated such that it would not affect the amino acid sequence of the product of the antisense γ34.5 gene. This virus contained two nucleotide substitutions, one mutating the ORF P initiator methionine codon and the other creating a unique restriction endonuclease site 15 bp upstream. These mutations were re-paired in the recombinant virus R7543(P−/O−). The salient features of the results and the key conclusions derived from characterization of the recombinant viruses follow.

(i) The initiator methionine codon mutation was previously shown to preclude ORF P protein expression in the context of a virus with ORF P transcription derepressed (42). Characterization of R7538(P−/O−) showed that this mutation also prevents ORF O protein expression in cells infected and maintained at 39.5°C, the nonpermissive temperature for ICP4. The absence of ORF P and ORF O proteins is not surprising since earlier studies have shown that ORF O and ORF P proteins share the ORF P protein initiator methionine and then diverge within the first 34 amino acids of the ORF P protein (43). To preclude a potential low level of expression of ORF O from the single methionine codon in its own reading frame, the base substitution creating a unique restriction endonuclease site also introduced a TAG stop codon in the predicted ORF O frame upstream of the ORF P initiator methionine codon. γ34.5 protein expression and function in the mutant virus, as assayed by replication in neuronal cell lines and determination of neurovirulence, were the same as those in the wild type.

(ii) Consistent with their absence in productively infected cells, ORF O and ORF P proteins play no discernible role in viral replication in cell culture and in vivo. Specifically, R7538(P+/O−) replicated to wild-type levels in cell lines of viral replication in neuronal and nonneuronal origin (SK-N-SH and Vero cells, respectively). Neurovirulence was similarly unaffected. HSV-1(F), R7538(P−/O−), and R7543(P−/O−)R all had PFU/LD50 ratios within a 2.5-fold range. Replication at a peripheral site was tested by the isolation of infectious virus from eyes following ocular infection. Differences in the amounts of infectious HSV-1(F), R7538(P−/O−), and R7543(P−/O−)R were not statistically significant at 1, 3, 5, and 7 days after infection. A fourth assay of replication competence was the recovery of infectious virus from ganglia at the peak of acute infection. After 3 days, the amounts of infectious wild-type and recombinant viruses fell within a threefold range. Thus at a time in which replication and the establishment of latency are occurring concurrently, similar levels of wild-type and recombinant viruses were present in the trigeminal ganglion. Interestingly, at 5 days after ocular infection, the level of infectious R7538(P−/O−) dropped 2 to 3 log units compared with the level of wild-type or repair virus. The mechanism responsible for this decrease is uncertain, but the observation itself does not support the hypothesis that ORF O and P proteins play a significant role in the establishment of latency.

(iii) R7538(P−/O−) virus appears to establish latent infections at reduced levels compared with those of the wild-type parent and repaired viruses. HSV-1(F) and R7543(P−/O−)R reactivated in 100% of infected ganglia with rapid kinetics. The virus with ORF O and ORF P mutated reactivated in 95% of infected ganglia; however, reactivation occurred with reduced kinetics. The decrease in the amount of virus recovered after explanation of trigeminal ganglia correlated with the six- to eightfold-lower numbers of copies of viral DNA/cell in trigeminal ganglia harboring latent R7538(P−/O−) than in those harboring wild-type or repaired viruses.

The results presented here suggest that ORF O and ORF P proteins may play a role but are not essential for the establishment of latency in the mouse model. One interpretation of our results is that the establishment of latency is a multifactorial event involving several additional factors both inside and outside of the HSV LAT domain and that each of them contributes to the switching off of replicative functions in dorsal root neurons. An alternative hypothesis is that ORF P and ORF O are effective in the maintenance phase rather than the establishment phase of latency. For example, it is conceivable that the reduction in the DNA copy number reflects a loss of neurons harboring virus due to reactivation of the latent virus and consequent destruction of neurons harboring them rather than a reduction in the number of neurons in which latent infections had been established. Sorting out this role of ORF P and O would require construction of viral mutants constitutively expressing ORF P and ORF O proteins. This is not an easy task since the expression of ORF P and O of the γ34.5 gene is mutually exclusive. The γ34.5 gene plays a key role inbreaching host defenses against infection; failure to express IC34.5 would significantly impair productive infection and the ability of the virus to establish latent infections. The solution to this problem remains to be found.

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