The Herpes Simplex Virus Type 1 Regulatory Protein ICP0 Enhances Virus Replication during Acute Infection and Reactivation from Latency

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ICP0 is a potent activator of herpes simplex virus type 1 gene expression in transient assays and in productive infection. A role for ICP0 in reactivation from latency in vivo has also been suggested on the basis of the observation that viruses with mutations in both copies of the diploid gene for ICP0 reactivate less efficiently than wild-type virus. Because the ICP0 gene is contained entirely within the coding sequences for the latency-associated transcripts (LATs), ICP0 mutants also contain mutations in LAT coding sequences. This overlap raises the question of whether mutations in ICP0 or the LATs, which have also been implicated in reactivation, are responsible for the reduced reactivation frequencies characteristic of ICP0 mutants. Two approaches were taken to examine more definitively the role of ICP0 in the establishment and reactivation of latency. First, a series of ICP0 nonsense, insertion, and deletion mutant viruses that exhibit graded levels of ICP0-specific transactivating activity were tested for parameters of the establishment and reactivation of latency in a mouse ocular model. Although these mutants are ICP0 LAT double mutants, all nonsense mutants induced the synthesis of near-wild-type levels of the 2-kb LAT, demonstrating that the nonsense linker did not disrupt the synthesis of this LAT species. All mutants replicated less efficiently than the wild-type virus in mouse eyes and ganglia during the acute phase of infection. The replication efficiencies of the mutants at these sites corresponded well with the ICP0 transactivating activities of individual mutant peptides in transient expression assays. All mutants exhibited reduced reactivation frequencies relative to those of wild-type virus, and reactivation efficiencies, like replication efficiencies in eyes and ganglia, correlated well with the level of ICP0 transactivating activity exhibited by individual mutant peptides. The amount of DNA of the different mutants varied in latently infected ganglia, as demonstrated by polymerase chain reaction analysis. No correlation was evident between reactivation frequencies and the levels of viral DNA in latently infected ganglia. Thus, replication and reactivation efficiencies of ICP0 mutant viruses correlated well with the transactivating efficiency of the corresponding mutant peptides. In a second approach to examining the role of ICP0 in latency, a single copy of the wild-type gene for ICP0 was inserted into the genome of an ICP0−LAT− double mutant, 7134, which exhibits a marked impairment in its ability to replicate in the mouse eye and reactivate from latency. Insertion of one copy of the ICP0 gene into 7134 restored the ability of the mutant to reactivate from latency to approximately one-half of wild-type level. Together, these results demonstrate a role for ICP0 distinct from the role of the LATs in the establishment and reactivation of latency.

Expression of the herpes simplex virus type 1 (HSV-1) genome is regulated through two distinct and mutually exclusive programs that result in productive infection and latency. Productive infection involves the expression of all viral genes required to produce new progeny virus, whereas latency is characterized by the repression of nearly all viral gene expression such that only a series of overlapping transcripts, the latency-associated transcripts (LATs), is detected (12, 53, 55–57). Identification of the factors that determine which program of viral gene expression is operative and those that mediate the switch from productive infection to latency and vice versa is central to understanding the pathogenesis of HSV.

The studies described in this report are concerned with the role of ICP0, an immediate-early (IE) regulatory protein, in the establishment and reactivation of latency in vivo. Because the genes expressed during productive infection are repressed during the establishment of latency and derepressed during reactivation, a brief consideration of the functions of the viral regulatory proteins that orchestrate productive infection is necessary.

During productive infection, the 72 or more HSV-1 genes are expressed coordinately in three major kinetic classes: IE, early (E), and late (L) (7, 25). The protein products of these three classes of genes function primarily in the regulation of viral gene expression, viral DNA replication, and virion assembly, respectively. HSV-1 encodes at least five regulatory proteins: an L protein, VP16, and four IE proteins, ICP4, ICP0, ICP22, and ICP27. The virion-associated protein VP16 stimulates the expression of IE genes upon entry of virus into host cells (4, 29). ICP4 activates E and L gene expression and represses IE gene expression at the transcriptional level (9–11, 13, 15, 21, 22, 40, 41, 43). ICP0 enhances expression of all three classes of viral genes and is apparently able to enhance
expression of any gene that exhibits a basal level of transcription (1–3, 5, 15–19, 21, 30, 38–41, 43, 47, 52, 54). The phenotypic properties of ICP0 mutant viruses during productive infection indicate that this protein plays a fundamental role in enhancing the expression of E and L genes (3, 5) but appears to play a more limited role in enhancing IE gene expression (3). The limited role of ICP0 in IE gene expression during productive infection likely reflects the overriding stimulatory activity of VP16 for IE genes. When infection occurs in the absence of VP16, such as in transfection of cells with infectious viral DNA, ICP0 is essential for the efficient expression of ICP4 (3). The expression of E and L genes activated by ICP4 and ICP0 is modulated at the posttranscriptional level by ICP27 (36, 37, 44, 45, 50). Little is known about the regulatory effects of ICP22 except that it is able to repress the upregulatory activities of ICP4 and ICP0 in transient expression assays (unpublished observation).

By virtue of its potent upregulatory activity, ICP0 has been postulated to play a role in (i) the establishment of latency by increasing the efficiency of virus replication at the site of primary infection and in ganglionic neurons, the site of latent infection, and (ii) reactivation from latency, by boosting viral gene expression in neurons at the onset of reactivation. This hypothesis is supported by evidence from studies of ICP0 deletion mutant viruses in animal models which indicates that functional ICP0 is required for the efficient establishment and reactivation of latency (6, 28). Because LAT coding sequences completely overlap the gene for ICP0, however, the mutants used in these studies also contain large deletions in the LAT gene. Therefore, the role of ICP0 in latency and reactivation has not been firmly established. In this paper, we present confirmatory evidence supporting a role for ICP0 distinct from the role of the LATs in the establishment and reactivation of latency. First, we show that the ability of mutant viruses to express graded levels of ICP0 transactivating activity correlates well with the ability of these viruses to replicate in mouse eyes and ganglia during the establishment of latency. Second, we show that insertion of a single copy of the ICP0 gene into the genome of an ICP0 LAT double mutant restores the ability of this virus to replicate in eyes and ganglia and to reactivate from latency.

MATERIALS AND METHODS

Cells and viruses. Vero cells and 0–28 cells (Vero cells stably transformed with the wild-type ICP0 gene [47]) were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum as described previously (46). Mouse neuroblastoma (NB41A3) cells (ATCC CCL 147) were propagated in F10 (HAM) medium containing 2.5% fetal bovine serum and 15% horse serum. Wild-type HSV-1, strain KOS, and the following mutants derived from KOS were propagated and assayed as described previously (51). 7134 (1) and dIX3.1 (47) are ICP0 null mutants (Fig. 1). The former contains the lacZ coding region in place of the ICP0 coding region, and the latter contains a large deletion in the ICP0 gene. Viruses n212, n428, n525, n680, n720, and n770 contain nonsense mutations in the ICP0 gene (1). 7134R was generated by replacing the lacZ gene in 7134 with wild-type ICP0 DNA sequences (1). The isolation of mutant 171 will be described below. dlLAT1.8 is a LAT null mutant isolated by Leib et al. (27).

Plasmids. Plasmid pW3 contains a 6.2-kb SalI-PstI fragment (nucleotides 125,066 to 118,866 [42]) derived from the b sequence in the long terminal repeat of the viral genome cloned into pUC13 (see Fig. 6) (47); the shaded region between the StuI site at nucleotide 124,816 and the HpaI site at nucleotide 120,300 in pW3 and pW3F in Fig. 6 contains the ICP0 coding region (including both introns) and the 5' and 3' flanking sequences necessary and sufficient for ICP0 expression. Plasmid pSK was constructed by inserting the 2.6-kb SalI fragment (nucleotides 52,196 to 54,830) derived from pSG18 (23), into pUC8 (see Fig. 6). This SalI fragment contains the poly(A) site at nucleotide 52,766 for the rightward transcribed, coterminal mRNAs of the UL24, UL25, and UL26 genes and the poly(A) site at nucleotide 53,063 for the leftward transcribed, coterminal mRNAs of the UL27, UL28, and UL29 genes (32). The small EcoRI-StuI fragment that comprises the left-hand terminus of pW3 was replaced with the EcoRI-FspI fragment (nucleotides 52,196 to 53,150) from pSK. The HpaI-PstI fragment that comprises the right-hand terminus of pW3 was replaced with the Dral-FspI fragment in the center of pSK to yield pW3F. In pW3F the Dral-FspI region from pSK was duplicated to ensure that the sequences derived from pSK contained sufficient information for cleavage and polyadenylation of both the rightward (UL24, UL25, and UL26) and the leftward (UL27, UL28, and UL29) groups of genes. The net result of these manipulations was the insertion of a single copy of the ICP0 gene into the genomic region between these two groups of genes without disrupting their expression.

Construction of mutant viruses. To generate mutant 171, the ICP0 gene was inserted by homologous recombination between the UL26 and UL27 genes in the genomic background of 7134 following cotransfection of Vero cells with pW3F and infectious 7134 DNA (see Fig. 7). The resulting mutant virus also contains the original ICP0 and LAT null mutations in the terminal and internal b repeats of the viral genome surrounding UL (1). A second mutant virus, n212, was constructed by replacing the lacZ substitution mutation in 7134 with the viral DNA fragment from pn212 which contains a nonsense mutation in the ICP0 gene at codon 212 (1).

LAT expression by ICP0 mutant viruses. NB41A3 cells were infected at a multiplicity of 10 PFU per cell with KOS or mutant viruses. Titers of mutant and wild-type viruses used to calculate multiplicities were predetermined on 0–28 cells. Total RNA was isolated 24 h postinfection by lysis in guanidine thiocyanate followed by CsCl gradient centrifugation (48). LATs were detected by Northern (RNA) blot analysis (49) using a riboprobe derived from plasmid pBbsLAT containing the 1886-bp SalI fragment (nucleotides 119,286 to 120,172 [42]) specific for LATs cloned into pGEM3Zf(+)(Fig. 1E).

Animal procedures. CD-1 mice were infected by the ocular route with wild-type or mutant viruses as described previously (28). Virus titers in eye swabs and acutely infected ganglia were determined on day 3 postinfection. Reactivation frequencies were determined by explant cocultivation of latently infected ganglia with 0–28 cells on day 30 postinfection (28), and the significance of differences between the reactivation frequencies of mutant and wild-type viruses were assessed by standard χ² tests.

Quantitation of ganglionic cells containing biologically active genomes. The number of ganglionic cells containing biologically active viral genomes was determined by a modification of the dissociation superinfection procedure (28). Trigeminal ganglia from mice infected 30 days previously were removed and placed in 5 ml of serum-free medium containing HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) (SMFH; Dulbecco’s modified Eagle’s medium containing 0.075% sodium bicarbonate and 20 mM HEPES). Contaminating blood in SFMH was removed by centrifugation of ganglia at 2,000 rpm in a Sorvall RC3 centrifuge using the HB4 rotor at 4°C for 10 min. Clean ganglia were digested in 2.5 ml of enzyme.
solution (SFMH containing 0.125% trypsin and 0.02% collagenase) by shaking in a water bath at 37°C. After 20 min, dissociated cells in suspension were decanted into Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Fresh enzyme solution was added to the remaining ganglion pieces for further digestion. After three rounds of digestion, dissociated cells were pelleted and resuspended in 3 ml of SFMH containing 10% fetal bovine serum and 5% horse serum, seeded into Matrigel (Collaborative Research, Inc., Bedford, Mass.)-coated six-well plates, and incubated at 37°C overnight. The replication-incompetent ICP27 deletion mutant virus 5d1.2 (5 × 10⁶ PFU in 0.5 ml) was added to the ganglionic cell monolayer (approximately 1 × 10⁶ cells) and incubated at 37°C for 1 h. The inoculum was removed, and 5 × 10⁵ Vero cells in 3 ml of growth medium were added. Growth medium containing 2% hydroxymethyl cellulose was applied to the cell monolayer 3 h later, and plaques that developed in 4 days were counted and recorded.

Quantitation of viral DNA in latently infected ganglia. Ganglia were removed from mice on day 30 postinfection, total DNA (approximately 2.5 μg per ganglion) was isolated, and viral DNA in total ganglionic DNA was quantified by polymerase chain reaction (PCR) as described previously (8, 26). The signals from the single-copy mouse gene amplified together with viral DNA were similar in all samples.

RESULTS

Properties of the ICP0 and LAT mutants used in these studies. The ICP0 and LAT genes are present in both copies of the b inverted repeat sequences surrounding the unique long region of the viral genome and are transcribed from opposite strands (Fig. 1A to C) (12, 14, 20, 53, 55–59). The 3' terminus of the ICP0 mRNA overlaps the 2.0-kb, poly(A)+ LATs by approximately 1 kb (Fig. 1C). Furthermore, the three coding exons that specify the 775-amino-acid ICP0 protein are contained entirely within sequences encoding the large, poly(A)+ LATs, which have been postulated to be the precursors of the shorter poly(A)+ LATs (56, 57). By definition, therefore, ICP0 mutants are also mutated in sequences specifying the LATs.

One approach that can be taken to assess the role of ICP0 in latency and reactivation independent of the role of the LATs in
these processes is to characterize a series of mutant viruses that exhibit graded levels of ICPO activity in vitro and minimally perturb LAT coding sequences. Using such mutants, one can determine whether the ICPO transactivating activity of mutant plasmids can be correlated with the ability of the corresponding mutant viruses to establish and reactivate from latency.

For this purpose, a series of KOS-derived nonsense mutants (n212, n428, n525, n680, n720, and n770) were used in these studies. Five of the six mutants produce truncated ICPO peptides of the expected sizes (427, 524, 679, 719, and 769 amino acid residues, respectively) (1). The 211-amino-acid n212 peptide has not been detected. With the exception of the n212 peptide, which exhibited no detectable ICPO activity for HSV-1 promoters either in transient expression assays (1) or in tests designed to measure enhancement of viral gene expression (3), the ICPO peptides specified by the mutants exhibit graded levels of transactivating activity relative to that of wild-type ICPO (1) (Table 1). In general, the longer the mutant ICPO peptide is, the greater is its transactivating activity in transient expression assays (1). In addition to mutations in ICPO coding sequences, n680, n720, and n770 also contain the nonsense linker in the large potential open reading frame (ORF-1) of the 2-kb poly(A)− LATs (Fig. 1C and D). The mutations in n212, n428, and n525, on the other hand, are located far upstream of the 3′ terminus of the poly(A)− LATs but are present within sequences specifying the large poly(A)+ LATs (Fig. 1C and D). In mutant 7134, both copies of the ICPO coding region have been replaced with the coding region of the lacZ gene, whereas in dlx3.1, a region of 2,965 bp, including the ICPO transcription initiation site (i.e., from nucleotides 1808 to 2722 and from nucleotides 121,602 to 124,566 in the b repeat regions [42]) has been deleted. Like n212, both 7134 and dlx3.1 exhibit no detectable ICPO transactivating activity (Table 1); of these three mutants, only the mutation in 7134 extends into the poly(A)− LAT region (Fig. 1C and D).

The LAT-minus mutant dlLAT1.8 was also included in this study. In this mutant, sequences containing the LAT promoter and transcription start site have been deleted (Fig. 1C and D) such that no LAT synthesis is detectable (27); ICPO transcription and protein synthesis are unaffected, however (unpublished observations).

LAT expression by ICPO nonsense mutants. Because mutations in ICPO are ICPO and LAT double mutations, we first attempted to determine whether the sizes and levels of LAT expression were affected in the ICPO nonsense mutants. RNA isolated from mutant and wild-type virus-infected NB41A3 cells was probed with the LAT-specific sequences in pBbSLAT (Fig. 1E) by Northern blot analysis (Fig. 2). RNA from KOS-infected cells contained only a 2-kb RNA species that hybridized with pBbSLAT sequences. The large 8.5-kb putative precursor of the 2-kb LATs was not detected. This pattern of LAT expression is consistent with previous reports (12, 59). Like wild-type virus, all nonsense mutants and the rescued virus, 7134R, but not the lacZ-containing mutant, 7134, express LAT species in decreasing amounts with longer poly(A) minus LATs (Fig. 2).

**TABLE 1. Transactivating activities of ICPO peptides specified by mutant plasmids in transient assays and reactivation frequencies of corresponding mutant viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>ICPO (1E)</th>
<th>Thymidine kinase (E)</th>
<th>gB (L)</th>
<th>LA2 (L)</th>
<th>No. of ganglia reactivated/ no. tested (%)</th>
<th>P value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>34/42 (81)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>n212</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>3/36 (8)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>n428</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4/34 (12)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>n525</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>16/36 (44)</td>
<td>&lt;0.0008</td>
</tr>
<tr>
<td>n680</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>16/36 (44)</td>
<td>&lt;0.0008</td>
</tr>
<tr>
<td>n720</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>20/38 (53)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>n770</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>25/30 (83)</td>
<td>0.75</td>
</tr>
<tr>
<td>dlx3.1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>3/34 (9)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>7134</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>8/34 (24)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>7134R</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>24/29 (83)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

<sup>a</sup> The transactivating activities of the peptides specified by ICPO nonsense mutant plasmids used to generate ICPO mutant viruses, on a scale from − (no transactivation) to +++ (strong transactivation), are summarized from the data of Cai and Schaffer (1).

<sup>b</sup> For reactivation studies with mutant viruses, mice were inoculated with 2 × 10<sup>5</sup> PFU of each virus per eye (28). On day 30 postinfection trigeminal ganglia were removed, cut into approximately eight small pieces, and cocultivated with Vero cells for 5 days. Virus production during cocultivation was detected on 0-28 cells. Individual ganglia were considered to have reactivated if cytopathic effect was observed. The reactivation frequency is the ratio of the number of ganglia from which virus reactivated in the 5-day cocultivation test to the total number of ganglia tested.

<sup>c</sup> Results of chi<sup>2</sup> tests to determine the significance of differences in reactivation frequencies of mutants relative to KOS. P values of <0.05 are considered significant.

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pressed the 2-kb LATs. These studies show that the nonsense linkers within the ICP0 genes of these mutants appear to have little or no effect on 2-kb LAT expression. If the LATs encode a protein, however, these linkers may alter the function of this protein.

**Replication of ICP0 nonsense mutants in the eye and in ganglia during acute infection in vivo.** Having previously determined the relative transactivating activities of nonsense mutant peptides in transient expression assays (Table 1) (1) and having demonstrated that the mutants induce the synthesis of the 2-kb LATs (Fig. 2), we next asked to what extent they replicated in an animal model. For this purpose we determined the titers of virus in eye swabs and in ganglia during the acute phase of infection in a mouse ocular model of latency (Fig. 3). The viral titers determined on day 3 postinfection represent progeny rather than input virus, because input virus is barely detectable in eyes by 3 h postinfection (28). In transient expression assays, the wild-type and 7134R forms of ICP0 exhibited equally high levels of transactivating activity (Table 1), and the viruses containing these ICP0 genes replicated with nearly equal efficiency in eyes and in ganglia (Fig. 3). In contrast, plasmids containing a substitution or a deletion in the ICP0 gene (7134 and dlx3.1, respectively) exhibited no ICP0 transactivating activity in transient assays (Table 1), and the viruses containing these mutated ICP0 genes replicated poorly in eyes but somewhat better in ganglia on day 3 postinfection. Like 7134 and dlx3.1, the nonsense mutant n212, the ICP0 gene of which specifies no detectable transactivating activity (Table 1), replicated poorly in both eyes and ganglia. The n428 ICP0 gene specifies only low levels of ICP0-associated transactivating activity (Table 1). Mutant n428 replicated poorly in eyes and ganglia but somewhat more efficiently than n212. Longer ICP0 peptides (i.e., those specified by n525 and n680) exhibited transactivating activities similar to that of n428 (Table 1) and replicated more efficiently in ganglia but less efficiently in eyes than n428. The replication efficiency of n720, whose ICP0-associated transactivating activity is moderately high (Table 1), was nearly identical in eyes and ganglia to the replication efficiencies of n525 and n680 (Fig. 3). Finally, n770 is similar to wild-type virus with regard to the ICP0-associated transactivating activity it specifies (Table 1) and its ability to replicate in eyes and ganglia (Fig. 3). The discordance in the titers of n525, n680, and n720 obtained in eyes and in ganglia is of interest. One might predict that the nonsense mutations in these mutants would affect the efficiency of replication at the two sites equally. The fact that some mutations had a greater effect on the ability of mutant viruses to replicate in eyes than in ganglia suggests that factors regulating viral growth in eyes and in ganglia differ or that our assay for measuring virus replication in eyes on day 3 is less discriminating than the assay that measures virus replication in ganglia.

We conclude from these tests that (i) replication efficiency in ganglia correlates well with the length of the ICP0 peptide and the level of ICP0-associated transactivating activity expressed by the mutant peptide in transient assays and (ii) some ICP0-LAT mutants are more affected for replication in the eye than in ganglia (e.g., n520, n680, and n720).

**Reactivation of ICP0 nonsense mutant viruses from latency.** To compare the transactivating activities of ICP0 mutant peptides with the reactivation frequencies of ICP0 nonsense mutant viruses, ganglia latently infected with mutant and wild-type viruses were cocultivated with Vero cells for 5 days and scored for the presence of reactivated virus. In general, the greater the transactivating activity demonstrated by an ICP0 mutant peptide in transient assays was (Table 1, left half), the higher the reactivation frequency was (Table 1, right half). Thus, KOS and 7134R, which specify full-length, fully active ICP0 peptides, reactivated with high and nearly equal frequencies (81 and 83%, respectively). Similarly, mutant n770, which specifies a highly active ICP0 peptide lacking only 5 C-terminal amino acid residues, exhibited a reactivation frequency (83%) similar to those of KOS and 7134R (P = 0.75). The next most efficient reactivation frequency (53%) was observed with n720, which specifies an active ICP0 peptide lacking 55 C-terminal amino acids. On the basis of statistical analysis, this reactivation frequency was significantly different from those of KOS and 7134 (P = 0.005). Among the remaining nonsense mutants, those specifying the shortest ICP0 peptides (n428 and n212) and the least ICP0 activating activity (n428) reactivated with the lowest frequency (n428, 12%; n212, 8%), whereas
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FIG. 4. Quantitation of viral DNA in latently infected ganglia by PCR. Total DNA (approximately 2.5 µg per ganglion) was isolated from trigeminal ganglia of mice inoculated 30 days previously via the ocular route. A 60-bp HSV-1 thymidine kinase gene-specific fragment (arrows) was amplified from 100 ng of DNA by quantitative PCR (8, 26). HSV-1 genomic DNA corresponding to 0, 2, 20, 200, and 2,000 copies per 100 ng of ganglionic DNA was amplified as standards. PCR products were electrophoresed on polyacrylamide gels adjacent to radiolabeled Hinfl-digested φX174 DNA, which served as size markers.

n525 and n680, which specify over half of the ICP0 protein, exhibited only low levels of ICP0-associated activity and reactivated with intermediate efficiency (44%). Like n212, the null mutant dΧ3.1 specifies no ICP0 transactivating activity and reactivated with a frequency of 9%. For reasons that are not clear, null mutant 7134 routinely reactivates from 20 to 25% of latently infected ganglia. Progeny virus isolated from ganglion cultures following reactivation from latency contained mutations indistinguishable from those of the mutants used to infect mice, as demonstrated by Southern blot analysis (data not shown). These results demonstrate that mutations in the ICP0-LAT region reduce the ability of the virus to establish reactivatable latency. Since all mutants, including ICP0 null mutants, can reactivate, although with lower frequency than wild-type virus, ICP0 is not essential for the establishment of or reactivation from latency as previously reported (6, 28).

Levels of viral DNA in mutant-infected ganglia. In an attempt to determine whether the transactivating activities of mutant forms of ICP0 and the reactivation frequencies of ICP0 mutant viruses correlated with the levels of viral DNA in latently infected ganglia, we measured the amount of viral DNA in latently infected ganglia by PCR and the proportion of ganglionic cells containing latent viral genomes. We chose to test two mutants that reactivated inefficiently (n212 and 7134), one that reactivated with intermediate efficiency (n525), and the wild-type virus.

To determine the levels of viral DNA in ganglia, total ganglionic DNA was isolated from individual ganglia and viral DNA sequences were amplified by quantitative PCR (Fig. 4). Viral DNA was present in individual ganglia latently infected with KOS at levels ranging from approximately 200 to greater than 2,000 copies per 100 ng of ganglionic DNA. (Approximately 2.5 µg of DNA was obtained from individual ganglia.) As in the case of ganglia latently infected with KOS, the levels of mutant viral DNA in ganglia varied considerably from ganglion to ganglion. Thus, in n212-infected ganglia, copy numbers ranged from 200 to 2,000, and in 7134- and n525-infected ganglia, copy numbers ranged from no detectable DNA to ~600 copies per 100 ng. On average, there was less viral DNA in 7134- and n525-infected ganglia than in n212-
and KOS-infected ganglia. Comparison of the efficiency of reactivation of n212, 7134, n525, and KOS with the relative numbers of viral genomes in individual ganglia revealed no correlation between the two parameters. (Note: results with mutant 171 will be discussed below.) Thus, with viruses in the order of most to least viral DNA in latently infected ganglia, reactivation frequencies were as follows: KOS, 81%; n212, 8%; 7134, 24%; and n525, 44%. Hence, although a strong correlation exists between ICP0 transactivating activity and reactivation efficiency, no correlation was evident between genome copy number and reactivation frequency, supporting a critical role for ICP0 in reactivation.

In order to measure the number of ganglionic cells harboring viral genomes, cells in latently infected ganglia were dissociated, plated, and superinfected with an ICP27 mutant, 5df1.2. This mutant is replication incompetent but can provide the necessary factors in trans to activate latent genomes (28, 31). Activation of latent genomes by complementation and recombination mechanisms with 5df1.2 has been described previously (28). Although the number of plaques produced following superinfection reflects the relative number of cells in individual ganglia containing retrievable viral genomes, the actual number of genome-containing cells is likely greater than the number of cells that score positive in this test. Because of the complexity of the dissociation assay, in addition to KOS-infected ganglia, we chose to test only n212- and n525-infected ganglia because they contained the most and the fewest viral genomes per ganglion, respectively (Fig. 4).

The number of plaques produced following superinfection of ganglionic cells containing n525 DNA was nearly the same as for ganglionic cells containing KOS DNA (Fig. 5). Furthermore, virus was retrieved from nearly all ganglia from n525-infected mice (the exception being 2 of 12 ganglia). Ganglia latently infected with n212 yielded fewer plaques per ganglion, but all ganglia yielded some plaques. We conclude from these studies that the ICP0 nonsense mutants n212 and n525 establish latent infection in nearly as many ganglia and in nearly as many cells per ganglion as wild-type virus. A comparison of these data with the data concerning genome copy number (Fig. 4), reactivation frequency, and relative ICP0 transactivating activity (Table 1) indicates that reactivation frequency correlates best with ICP0 transactivating activity and not with genome copy number or the number of cells containing reactivatable viral genomes.

**Insertion of one copy of the wild-type ICP0 gene into a distant site in the genome of an ICP0−LAT− mutant.** As mentioned above, reactivation data obtained from mice latently infected with viruses containing mutations in the ICP0-LAT region is difficult to interpret with regard to the role of ICP0 in this process because these mutants are by definition double mutants. Although the ability of the mutants to replicate during acute infection and to reactivate from latency in vivo correlates well with the activity of the ICP0 peptide, more definitive evidence establishing a role for ICP0 in latency and reactivation was obtained by providing ICP0 function, but not LAT function, to an ICP0 LAT mutant, 7134. In these studies, ICP0 function was partially restored to 7134 by insertion of a single copy of the ICP0 gene into the genome at a site distant from the mutations in the ICP0 and LAT genes. As the first step in generating such a virus, plasmid pW3F was constructed (Fig. 6). pW3F contains the entire ICP0 gene flanked by sequences in the UL26 and UL27 genes. Thus, the 4.5-kb Stul-HpaI fragment also contains the ICP0 coding region as well as 0.9-kb 5′- and 0.4-kb 3′-flanking sequences that are sufficient for ICP0 expression in transient expression assays (1; unpublished observations). This fragment contains a subset of LAT coding sequences without the LAT promoter and expresses no detectable LATs (data not shown). The flanking sequences on the left and right of the Stul-HpaI fragment in Fig. 6 should contain sufficient information for polyadenylation of the UL24, UL25, UL26, and UL26.5 and the UL27, UL28, and UL29 transcripts, respectively, and sufficient sequence length for insertion between the UL26 and UL27 genes by homologous recombination.

The HSV-1 sequences in pW3F were introduced into the genome of 7134 by homologous recombination to yield mutant 171, and the structure of 171 DNA was verified by Southern blot analysis (Fig. 7). By using viral DNA sequences adjacent to the Stul-HpaI fragment containing the ICP0 gene as a probe (Fig. 7B, probe 1), a 3.2-kb SacI-PstI fragment was identified in 171 and 7134 DNA and a 6.2-kb fragment was identified in KOS DNA (Fig. 7). By using probe 2, which is specific for the ICP0 gene (Fig. 7B), fragments of the same sizes were identified (Fig. 7A). In addition, probe 2 identified a 2.9-kb PstI-SacI fragment in 7134 and 171 which reflects the presence of the lacZ gene (Fig. 7). Probe 2 also identified a 5.8-kb PstI fragment in 171 DNA; this fragment demonstrates the presence of the ICP0 gene between the UL26 and UL27 genes. Finally, sequences from the UL26-UL27 region (probe 3) identified the 5.8-kb fragment in 171 DNA and a 1.1-kb PstI fragment in KOS and 7134 DNAs. These restriction patterns are in agreement with the predicted genomic structures of the three viruses (Fig. 7B). Mutant 171 produces uniform blue plaques in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as does its parent virus, 7134, indicating the presence of the lacZ gene in both viruses (data not shown).

**Characterization of mutant 171 during productive infection in vitro.** The wild-type ICP0 genetic information in 171 DNA differs from that in KOS DNA in several respects. First, one...
copy of the ICPO gene is in 171 and two copies are in KOS. Second, although as many ICPO promoter-regulatory sequences as possible were included in the construction of pW3F and 171, there may be unidentified cis-acting elements important for ICPO expression in vivo that are present in the ICPO genes in KOS DNA but not in the ICPO gene in 171 DNA. Third, the ICPO gene in 171 is present in a nonnative location and ICPO expression may depend to some degree on genomic

FIG. 6. Construction of plasmid pW3F. Restriction sites for pW3, pW3F, and pSK are as follows: EcoRI (E), DraI (D), FspI (F), HpaI (H), PstI (P), Sall (S), SacI (Sc), and StuI (St). The stippled box in pW3 is the ST-H fragment containing the ICPO gene used to construct pW3F. The open boxes under plasmid pSK represent sequences used to generate flanking sequences (open boxes) in plasmid pW3F for insertion into the HSV-1 genome by homologous recombination (Fig. 7). Horizontal arrows indicate the direction of transcription and the sites of transcription termination for two groups of coterminal transcripts (UL24, UL25, and UL26 and UL27, UL28, and UL29). The construction of pW3F is described in detail in Materials and Methods.

FIG. 7. Genomic structure of mutant virus 171. (A) Southern blot analysis of KOS, 7134, and 171 DNAs. Viral DNA was digested with PstI and SacI. Fragments were separated on an agarose gel and subjected to Southern blot analysis. Each panel represents the same viral DNA preparation assayed with different probes (probe 1, 2, or 3). For each panel, the left, middle, and right lanes represent KOS, 7134, and 171 DNA, respectively. Molecular size markers are indicated in kilobases in the middle panel on the right. These markers also apply to the other two panels because all three panels were derived from the same gel. (B) Predicted genomic structure of KOS, 7134, and 171. (a) Restriction map (top) and scale (bottom) of regions of the HSV-1 genome from 52 to 55 kb (left) and from 118 to 125 kb (right). Restriction site abbreviations are as indicated in the legend to Fig. 6. (b) Genome structures. The lengths of relevant restriction fragments are shown in kilobases above fragments. The size and direction of transcription of the primary ICPO transcript are represented by arrows. The region of HSV DNA containing ICPO (including flanking sequences) inserted between the UL26 and UL27 genes to construct 171 is depicted by a heavy line. The lacZ coding region originally present in 7134 and in 171 is represented by stippled boxes. (c) Probes used in Southern blot analysis. All three probes were derived from KOS DNA.
location. Finally, insertion of the ICP0 gene may affect the expression of neighboring genes (UL24, UL25, UL26, UL27, UL28, and UL29). Any or all of these factors could affect the growth and gene expression of 171.

To examine the growth properties of 171 in vitro, Vero cells were infected with 0.01 or 2.5 PFU of 171, 7134, or KOS per cell. Infected cells were incubated at 37°C for 18 h, and progeny virus was measured by plaque assay (Fig. 8). As previously observed, 7134 produced fewer progeny virus than KOS, and the replication deficiency was more pronounced at the low multiplicity of infection (0.01 PFU per cell) than at the high multiplicity (2.5 PFU per cell) (3). Yields of 171 progeny virus were near the midpoint between those of 7134 and KOS at both multiplicities. Thus, the single ICP0 gene in 171 partially reverses the replication-impairment phenotype of 7134. The failure of 171 to express the wild-type growth phenotype is due more likely to the fact that only one copy of the ICP0 gene was introduced into 7134 DNA or to one or more of the factors mentioned above than to the presence of the mutation in the LAT gene in 171 and 7134. Elimination of LAT expression produced no obvious growth phenotype at either high or low multiplicity during productive infection in vitro (1, 27).

To measure the level of expression of ICP0 and of other viral proteins in 171-infected cells, Vero cells were infected with 171 or KOS, and selected viral proteins were immunoprecipitated and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 9). Like KOS and unlike 7134, which expresses no ICP0 (1), 171 was able to induce the expression of significant amounts of ICP0. The level of ICP0 in 171-infected cells was lower than in KOS-infected cells, however. This was especially clear at the low multiplicity of infection (0.1 PFU per cell) and at early times (3 h) postinfection. At 2.5 PFU per cell the levels of ICP0 and another IE protein, ICP4, in 171-infected cells were comparable to those seen in KOS-infected cells at all times tested. This result was expected because ICP4 expression is not affected by mutations in ICP0 at high multiplicities (3). In contrast, at 0.1 PFU per cell at 3 h postinfection, ICP4 expression in 171-infected cells was reduced relative to that in KOS-infected cells. Expression of gE, an L protein, was reduced in 171-infected cells compared with that in KOS-infected cells at the low multiplicity of infection but less obviously at the high multiplicity. Multiplicity-dependent expression of L (as well as E) proteins by ICP0 mutant viruses has been reported previously (1, 47). These results indicate that 171 is not able to induce wild-type levels of expression of ICP0 and other viral proteins, especially at low multiplicities of infection.

**Figure 8.** Replication of KOS, 7134, and 171 in vitro. Vero cells were seeded at 2.7 × 10^4 cells per 35-mm dish and infected 24 h later at a multiplicity of 0.01 or 2.5 PFU per cell. The titers of viral inocula were determined on 0-28 cells. Progeny virus was harvested 18 h postinfection and quantified by plaque assay on 0-28 cell monolayers.

**Figure 9.** Expression of ICP0, ICP4, and gE in 171- and KOS-infected Vero cells. Vero cells were infected at a multiplicity of 0.1 or 2.5 PFU per cell. Infected cells were pulse-labeled with [35S]methionine for 20 min at 3, 6, or 9 h postinfection, corresponding to lanes 1, 2, and 3, respectively, under each virus heading. Specific proteins were precipitated with a pool consisting of ICP8 antibody (58S, ATCC HB8183) and polyclonal rabbit antibody against ICP0 (J17, prepared by Wendy Sacks), and separated by SDS-polyacrylamide gel electrophoresis as described previously (2, 24). Virus-specific polypeptides (ICP0, ICP4, and gE) are indicated. Monoclonal antibody 58S precipitates ICP4 with little background. J17 antiserum, on the other hand, precipitates a series of polypeptides in addition to ICP0. All bands, except the band designated ICP0, were also precipitated from 7134-infected cells and are thus not ICP0 specific. Among the nonspecific bands, the band designated gE could be chased into an even more diffuse band and was absent from cells infected with a gE deletion mutant.
approximated those in KOS-infected ganglia but were higher than those in 7134-infected ganglia. As for growth in cell culture and growth in eyes and ganglia during acute infection of mice, the frequency of reactivation of 171 from latency was significantly higher than that of 7134 (P = 0.005) and somewhat lower than that of KOS (Table 2). The mutation in dILAT1.8 reduced the frequency of reactivation by approximately 50%, as reported previously (27). Although the LAT mutations in 171 and 7134 also affect the frequency of reactivation, these mutations are identical in both viruses. Consequently, the difference in the frequency of reactivation of these two viruses is most likely due to the presence of the single copy of the ICPO gene in 171.

**DISCUSSION**

**Contribution of mutations in the ICPO and LAT genes to mutant phenotypes during acute infection and reactivation.** Because the ICPO gene is contained wholly within the LAT gene but is encoded by the opposite strand, mutations in the ICPO gene are also present in the LAT gene. The major problem in interpreting data obtained from characterization of these mutants is how to separate the effects of mutations in the ICPO gene from those in the LAT gene. The problem of interpretation becomes especially complex when the data to be interpreted are obtained in animal models rather than in cultured cells. In cultured cells, the defects in ICPO mutants can be complemented in cells that express wild-type ICPO or by transfection of cells with ICPO-expressing plasmids. The ability to provide ICPO in trans in vitro facilitates the assignment of specific phenotypes to mutations in ICPO (3). Unfortunately, other than generating transgenic mice, approaches involving complementation are not possible in animal models. We have therefore utilized two approaches to address this issue. First, we used a series of nonsense mutants that express a spectrum of ICPO transactivating activities and attempted to correlate the growth and reactivation phenotypes of the mutants in mice with the levels of ICPO transactivating activity they express in vitro. In general, we found that the lower the ICPO transactivating activity of a given mutant is, the more severe the growth and reactivation phenotypes are (Fig. 2 and Table 1). These results are consistent with the hypothesis that the impairment in ICPO activity of the ICPO−LAT− double mutants contributes to these phenotypes. Second, we constructed a virus (171) in which the ICPO activity of the null mutant 7134 was partially restored by insertion of a single copy of the ICPO gene between the UL26 and UL27 genes while leaving the double mutations in the ICPO-LAT region unchanged. Only the ICPO activity, not activities that might be associated with the LATs, was restored in this virus. The phenotypes of 171 during productive infection in cultured cells were partially reversed relative to 7134 and the wild-type virus. The failure to achieve total reversal is probably due to expression of ICPO at less-than-wild-type levels by the inserted single ICPO gene or by impaired expression of genes surrounding the insertion site. Whatever the explanation is, 171 also exhibited partial reversal of the phenotypes of the ICPO−LAT− double mutant, 7134, during acute infection, latency, and reactivation in a mouse model. These results demonstrate that the mutation in the ICPO gene contributes substantially to the mutant phenotypes in the mouse model. We also included a LAT-minus mutant (dILAT1.8) as a control in studies with the mouse model. This mutant exhibited no detectable growth phenotype during acute infection in eyes and in ganglia but reactivated with reduced frequency (27). Taken together, these results indicate that during acute infection in the mouse, ICPO (but not the LATs) is important. In contrast, both ICPO and the LATs appear to play a role in reactivation.

**Role of ICPO during acute infection and reactivation from latency.** Available evidence indicates that events that occur during the acute phase of virus infection in animal models strongly influence the efficiency with which the virus establishes latency and is later reactivated. Specifically, virus produced at the site of primary infection (the eye in the mouse ocular model) enters neuronal termini and travels by centrifugal axonal flow to the neuronal cell body. Here, viral DNA reaches the nucleus, and limited viral replication occurs in some neuronal cells. The number of viral genomes present in neuronal nuclei during latency depends in part on (i) the extent of viral replication at the primary site of infection and (ii) the number of viral genomes produced during limited replication in ganglia. It has been hypothesized that the more genomes are present per neuron during latency, the greater is the likelihood of a successful reactivation event. Because ICPO serves to enhance viral replication at the site of inoculation and in ganglia, it could be difficult to define a specific role for ICPO during reactivation.

We think it likely, however, that ICPO does play a specific role in reactivation. If mutations in ICPO reduced the frequency of reactivation solely by an indirect mechanism, i.e., by reducing the number of viral genomes in latently infected cells that are available to be reactivated, then reactivation frequencies should correlate with the number of viral genomes. However, mutant n525, which expresses partial ICPO activity, reactivates with greater efficiency than the ICPO null mutant 7134, although the numbers of genomes of the two viruses in

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**TABLE 2. Frequency of reactivation of KOS, 7134, 171, and dILAT1.8 from latently infected mouse ganglia**

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of ganglia reactivated/ no. tested (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>13/15 (87)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>7134</td>
<td>4/18 (22)</td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>10/15 (67)</td>
<td>0.2</td>
</tr>
<tr>
<td>dILAT1.8</td>
<td>10/18 (50)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* See Table 1, footnotes b and c, for details.
latently infected cells were comparable as judged by PCR analysis. Similarly, the number of genomes in ganglia latently infected with n212 was somewhat greater than that in n525-infected ganglia, yet n525 reactivated more efficiently than did n212. These observations indicate that the role of ICPO in reactivation is to enhance not only the number of genomes in latently infected ganglia but also the level of ICPO transactivating activity. We conclude therefore that in addition to its role in enhancing virus replication during the acute stages of infection, ICPO plays an important role in reactivation.

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