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Immediate-Early Regulatory Gene Mutants Define Different Stages in the Establishment and Reactivation of Herpes Simplex Virus Latency

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Using nonsense and deletion mutants of herpes simplex virus type 1, we investigated the roles of three immediate-early proteins (ICP4, ICP27 and ICP0) in the establishment and reactivation of ganglionic latency in a mouse ocular model. DNA hybridization, superinfection-rescue, and cocultivation techniques provided quantitative data that distinguished between the failure of a virus to establish latency in the ganglion and its failure to reactivate. Null mutants with lesions in the genes for ICP4 and ICP27 did not replicate in the eye or in ganglia and failed to establish reactivatable latent infections. Three ICP0 deletion mutants which could replicate in the eye and ganglia varied in their ability to establish and reactivate from the latent state, demonstrating that ICP0 plays a role both in the establishment and the reactivation of latency. The use of viral mutants and a variety of stage-specific assays allowed us to better define the stages in the establishment and reactivation of herpes simplex virus type 1 latency.

Observations from animal models and clinical studies of herpes simplex virus type 1 (HSV-1) infections in humans have led to the classical theory of herpesvirus latency which describes the stages characteristic of this lifelong neurotropic infection (48). Following entry into the host, virus undergoes primary replication in the skin or mucosae. The virus then gains access to the distal axon terminals of sensory neurons and travels by axonal transport to neuronal cell bodies in sensory ganglia, where limited replication may occur and viral gene expression is repressed, leading to the latent state. No free infectious virus can be detected in ganglia during latency, although the controls responsible for maintaining the latent state may break down such that infectious virus is again produced to initiate a reactivation event.

During the initial replicative phase of infection, all, or nearly all, viral genes are expressed. The immediate-early (or α) genes of HSV-1 are expressed in the absence of prior viral protein synthesis, and their expression is required for the expression of the early (or β) and late (or γ) classes of viral genes (5, 9, 15, 16, 36). By contrast, during latency in neurons, viral gene expression is almost completely repressed. HSV-1, therefore, exhibits two very different modes of gene expression during the acute and latent phases of its life cycle within the host. The mechanisms involved at the molecular level in the repression of viral gene expression during the establishment and maintenance of latency and the derepression of viral gene expression that signals reactivation are not understood.

To identify viral factors that play a role in the establishment and reactivation of latency, we focused on the immediate-early proteins of HSV-1. These proteins are the first to be synthesized in the viral replicative cycle and three of them (infected cell proteins [ICPs] ICP4, ICP0, and ICP27) have been shown to modulate gene expression during productive infection. ICP4 performs an essential role in virus replication (9, 28) and has at least two regulatory activities: the *trans*-activation of early and late gene expression and the repression of expression of ICP4 and possibly other immediate-early genes (6, 8–10, 12, 23, 24, 31). Although ICP0 is a potent *trans*-activator of all three classes of HSV-1 genes (immediate-early, early, and late) in transient expression assays and likely plays a major role at some point in the viral life cycle (10, 12, 24, 31, 40), the gene for ICP0 can be deleted with only minor effects on productive infection in cell culture (34, 40). The observation that ICP4 can inhibit the activities of immediate-early HSV-1 promoters, whereas ICP0 can induce these activities in transient expression assays, led O'Hare and Hayward (25) to propose that ICP4 and ICP0 together may mediate the switch between the lytic and latent modes of infection. Studies of temperature-sensitive and deletion mutants in the gene for ICP27 have demonstrated that this protein, like ICP4, plays an essential role in virus replication (23a, 34). In transient expression assays, ICP27 is able to further down- or upregulate the expression of specific early and late genes whose expression is induced by ICP4, ICP0, or both (11, 31a, 35a; L. Su and D. M. Knipe, manuscript in preparation). The demonstrated roles of immediate-early proteins in the regulation of HSV-1 gene expression and the availability of a series of well-characterized nonsense and deletion mutants in the genes for these three proteins prompted the present investigation of their roles in latency.

A major problem in previous studies of latency has been the inability to differentiate between the failure of a virus to establish latency and its failure to reactivate from the latent state. In the present study we define the establishment of latency by the demonstration of viral DNA in individual latently infected ganglia. Based on this definition, we used two approaches to examine and quantify levels of viral DNA in infected ganglia. The first approach is slot-blot hybridization, and the second involves superinfection of dissociated

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ganglionic neurons with a replication-incompetent mutant. The ability of superinfection to generate progeny virus would demonstrate not only that viral DNA was present in the latently infected ganglion but also that it was retrievable and capable of biological activity.

We used these two approaches, in combination with conventional virus assay and cocultivation techniques, to examine the behavior of wild-type HSV-1 strain KOS and selected isogenic deletion and nonsense mutants in immediate-early HSV-1 genes in a mouse eye model to assess the roles of their protein products in latency and to begin to delineate the stages in the establishment and reactivation of ganglionic latency. The mouse model is useful because spontaneous activation of the lytic cycle in vivo is rare and because there are certain similarities to latent infections in humans (for a review, see reference 1). We describe the behavior of mutants with lesions in the genes for ICP4, ICP0, and ICP27. These mutants are blocked at pivotal stages in the latency process: replication at the peripheral site of inoculation (ICP4 and ICP27) and efficient establishment and reactivation of latency (ICP0). These mutants thus distinguish between events that occur at the earliest and latest stages of latency at the molecular level, providing an initial framework for further dissection of the latency process.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero), E5, 3-3, and 0-28 cells were propagated as described previously (33). E5 cells, which express complementing levels of ICP4, were made from Vero cells as described previously (7). Cell lines 3-3 and 0-28 were also derived from Vero cells and express complementing levels of ICP27 (23a) and ICP0 (34), respectively.

Procedures for the growth and assay of the KOS strain of HSV-1 have been described previously (35). The ICP4 mutants ΔAT, *n*6, *n*12, and *n*18 were kindly provided by N. DeLuca (Dana-Farber Cancer Institute, Boston, Mass.) and propagated as described by DeLuca and Schaffer (7). The ICP27 deletion mutant 5*dl*1.2 was constructed and grown as described by McCarthy et al. (23a). The origin and propagation of the two KOS ICP0 deletion mutants *dl*x0.7 and *dl*x3.1 have been described elsewhere (34). HSV-1 strain 17 and *dl*1403 were kindly provided by N. Stow and G. Clements (Medical Research Council Virology Unit, Glasgow, and University of Glasgow, Glasgow, Scotland) and have been described elsewhere (40).

Animal procedures. Seven-week-old randomly bred CD-1 mice (Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were anesthetized with sodium pentobarbital, their corneas were scarified, and 20 μl of virus at the appropriate concentration per eye was added as described previously (41). At various times postinfection, both eyes of each mouse were swabbed with cotton, and swabs were suspended in 1 ml of cell culture medium. Eye swab material and ganglion homogenates were assessed for infectious virus by standard plaque assay in Vero cells for KOS, in 3-3 cells for 5*dl*1.2, in E5 cells for *n*12 and *n*18, and in 0-28 cells for *dl*x3.1 and *dl*x0.7. Mice were sacrificed at 30 days postinfection, and their trigeminal ganglia were removed. For reactivation assays, ganglia were cut into eight pieces and explanted onto monolayers of the appropriate cell line. After 5 days in culture, explants were frozen, thawed, homogenized, sonicated, and assayed for virus on either Vero or complementing E5, 3-3, or 0-28 cell monolayers. In order to test for infectious virus on day 30, ganglia were frozen, thawed, homogenized, and assayed directly on the appropriate cell type.

Slot-blot hybridization. Individual ganglia were digested with 100 μg of proteinase K in 100 μl of 20 mM Tris hydrochloride (pH 7.5)–20 mM EDTA–0.5% sodium dodecyl sulfate (SDS) overnight at 50°C. The digested material was pipetted gently through a cut-off Eppendorf plastic pipet tip and was mixed gently with phenol. Chloroform was added with gentle mixing, and the aqueous phase was retained following centrifugation. The organic phase was back-extracted with 50 μl of 20 mM Tris hydrochloride (pH 7.5)–20 mM EDTA–0.5% SDS, and the aqueous phases were pooled and extracted once with chloroform. Sodium acetate was added to a concentration of 250 mM, and the DNA was precipitated with ethanol overnight. Following centrifugation, the resulting precipitate was washed once with 70% ethanol, dried, and suspended in 100 μl of 10 mM Tris hydrochloride–1 mM EDTA (pH 7.5). Ganglion DNA was standardized by optical density measurement and by agarose gel electrophoresis alongside standard DNA samples.

For slot-blot hybridization, 1 μg of each DNA sample was applied to a nylon membrane (GeneScreen Plus; Dupont, NEN Research Products, Boston, Mass.) with a slot-blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.) following the recommendations of the manufacturers. The DNA was immobilized on the filter by UV cross-linking (2) and prehybridized for 6 h at 69°C in 2× SSPE (0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.4])–1% SDS–5× Denhardt solution–200 μg of salmon sperm DNA per ml–10% dextran sulfate. The DNA was then hybridized for 20 h with 4 ng of KOS *Eco*RI A, D, I, N, and O fragments per ml and radiolabeled to 1.5×10^9 cpm in the same solution containing 200 μg of denatured salmon sperm DNA per ml. The HSV-1 fragments were chosen because they do not cross-hybridize extensively with mouse sequences (26, 29, 30). The filter was washed 3 times for 30 min each time at room temperature in 2× SSPE–1% SDS with agitation, 3 times for 30 min each time at 72°C in 0.1× SSPE–0.1% SDS, and 2 times for 30 min each time at room temperature in 0.1× SSPE with agitation. The filter was exposed to preflashed film (XAR-5; Eastman Kodak Co., Rochester, N.Y.) (22) with two intensifying screens. To quantitate autoradiographic signals, the film was scanned with a densitometer (Schoeffl).

Superinfection experiments. Ganglia were removed from latently infected mice on day 30 and dissociated with trypsin and collagenase as described by Kennedy et al. (17). Dissociated cells from each ganglion were seeded into individual wells of 6-well plastic plates (Nunc, Roskilde, Denmark) which had been coated with type 1 rat tail collagen (Collaborative Research Inc., Bedford, Mass.) according to the instructions of the manufacturer. One day later, medium was removed and 10^6 PFU of 5*dl*1.2 (yielding a multiplicity of infection greater than 10 PFU per cell) was added to the cells in 0.2 ml and was absorbed for 1 h. Cells were then washed gently 3 times with trypsin diluent, to remove unadsorbed inoculum. Control cultures were treated similarly, but without the addition of virus. Vero cell suspension (1 ml) was then added (10^6 cells per well), and cells were allowed to settle before the medium was removed and a methylcellulose overlay was added. Cultures were stained with neutral red and plaques were counted and picked. Viral DNA was obtained from plaque isolates (5) and analyzed by Southern blot hybridization (38) by using the 4.1-kilobase *Pst*I-*Xho*I subfragment of *Bam*HI-B (all Fig. 3) as the probe for detecting ICP0 sequences.

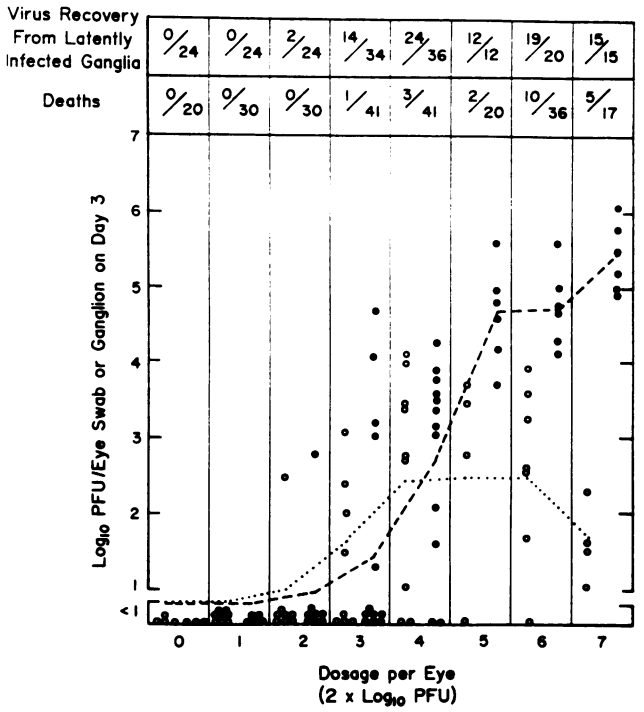

FIG. 1. Effect of variation of KOS dose on acute and latent infection. Three days postinfection, eye swabs (○) and trigeminal ganglia (●) were taken and assayed directly for infectious virus. The results of these assays are shown on the lower portion of the figure. The dotted and dashed lines connect the mean virus titers obtained on day 3 at each dosage for eye swabs (· · · · ·) and ganglion (– – – –) assays, respectively. The number of mice dead on day 30 postinfection as a fraction of the total number of mice inoculated with each virus dose is shown at the top. The efficiency of recovery of virus from latently infected ganglia by explant cocultivation on day 30 is presented as a fraction (number of ganglia from which virus was reactivated/number of ganglia tested).

RESULTS

Latency of HSV-1 wild-type strain KOS in CD-1 mice. To establish the parameters of infection with wild-type virus in the mouse ocular model, mice were inoculated with doses of KOS ranging from 2×10^0 to 2×10^7 PFU per eye (Fig. 1). The minimum dosage of virus required to demonstrate replication in the eye and in ganglia during the acute stage of infection, as measured on day 3 postinfection, was 2×10^2 PFU per eye. The amount of virus found in trigeminal ganglia on day 3 increased as a function of virus dose through 2×10^7 PFU per eye. The peak titer of virus in eye swabs on day 3 was achieved at a dose of 2×10^4 PFU per eye, decreasing at higher dosages, up to 2×10^7 PFU per eye. The decrease in the amount of virus in the eye swabs on day 3 following inoculation with high dosages was possibly caused by the rapid destruction of cells on the surface of the eye which are capable of supporting virus growth.

Notably, the minimum dosage of KOS required for demonstrable replication in the eye and in ganglia was also the lowest dosage at which reactivatable latency was reproducibly demonstrated, as judged by standard ganglion explant procedures on day 30 postinfection. At this time, following even high dosages of KOS, no infectious virus could be detected after homogenization of ganglia and direct assay on Vero cells (data not shown). The dosage required to produce

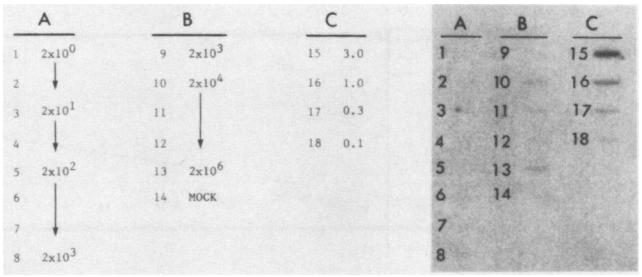

FIG. 2. Viral DNA in ganglia of mice mock infected or infected 30 days previously with various doses (2×10^0 to 2×10^6 PFU) of KOS assayed by slot-blot hybridization. Procedures were as described in the text, except that the DNA was applied to nitrocellulose, immobilized by baking for 2.5 h, prehybridized for 60 min, and hybridized for 14 h. Filters were washed twice for 15 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% SDS, twice for 15 min each time in 1× SSC–0.2% SDS, and twice for 30 min each time in 0.1× SSC–0.1% SDS, all at 68°C. Lane C shows hybridization to 1 μg of uninfected mouse tail DNA containing KOS DNA equivalent to 3, 1, 0.3, and 0.1 viral genome copies per cell.

reactivatable latency in 50% of ganglia (latency $dose_{50}$) in this system was between 2×10^3 and 2×10^4 PFU per eye, whereas reactivatable latency could be detected in all ganglia at doses as low as 2×10^5 PFU per eye.

Less than 30% of mice died following inoculation with 2×10^7 PFU of KOS, indicating that the LD_{50} of HSV-1 strain KOS is greater than 2×10^7 PFU per eye, a dosage that was at least 1,000-fold greater than the latency $dose_{50}$ in our system. Having demonstrated that virus could be reactivated from ganglia of mice inoculated 30 days previously with 2×10^2 to 2×10^7 PFU per eye, we then tested these ganglia for the presence of viral DNA using slot-blot hybridization (Fig. 2). The results of this test showed that viral DNA (ranging from 0.1 to 0.3 genome copy per cell) was detected readily in ganglia of mice that were infected 30 days previously with 2×10^4 and 2×10^6 PFU per eye. To date, 17 of 17 ganglia from mice inoculated with KOS at 2×10^6 PFU per eye have exhibited hybridizable viral DNA ranging from 0.1 to 1 genome copy per cell. Viral DNA in ganglia of mice receiving doses of less than 2×10^4 PFU per eye was more difficult to quantify. Based on these data, we selected 2×10^6 PFU per eye as the dosage for tests of HSV-1 mutants, since this dose of wild-type virus routinely gave reactivatable latency in 100% of ganglia, while it killed less than 30% of the animals, and there was detectable viral DNA in all latently infected ganglia tested. In addition, stocks with sufficiently high titers to achieve this dose could be attained readily with the mutant viruses.

ICP27 and ICP4 are required at the first stage in the establishment of latency: replication at the site of inoculation. To evaluate the role of virus replication and the immediate-early proteins ICP27 and ICP4 in latency, replication-incompetent mutants with lesions in the genes for these proteins were tested for latency.

An ICP27 deletion mutant, *5dl*1.2 (23a) (Fig. 3), was tested in our mouse eye model; and as anticipated it failed to replicate in corneas, as judged when eye swab material yielded no infectious virus when assayed on ICP27-expressing 3-3 cells, which are permissive for growth of *5dl*1.2 (Fig. 4 and Table 1). Likewise, no infectious virus was detected in ganglia on day 3 or 30, and the mutant failed to reactivate from ganglia explanted onto 3-3 cells on day 30 (Fig. 4). In addition, two replication-incompetent viruses

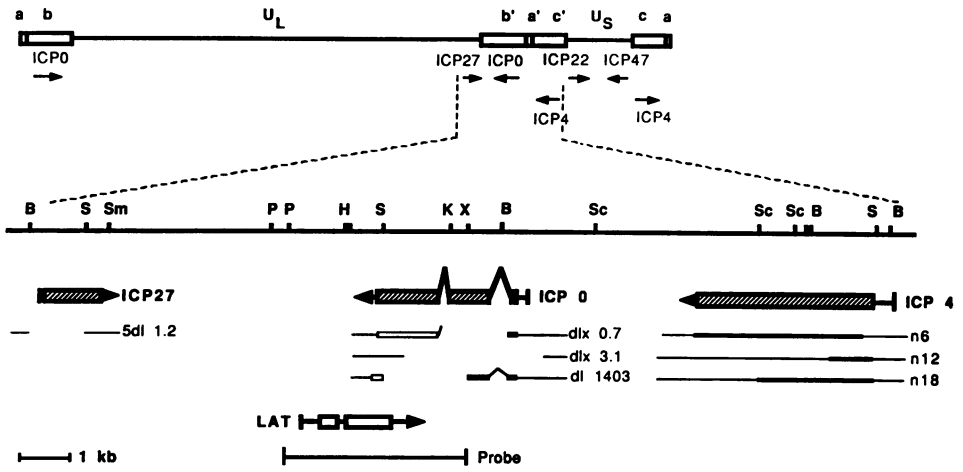

FIG. 3. Physical map of the HSV-1 genome showing the locations of the immediate-early genes, LAT, and the series of mutants used in this study. Beneath the diagram of the HSV-1 genome are shown the locations of the immediate-early genes encoding ICP0, ICP4, ICP22, ICP27, and ICP47. The joint region of the genome from the right-most *Bam*HI site in U_L to the right-most *Bam*HI site in c′ sequences is shown in expanded form. The locations of the transcripts specifying ICP27, ICP0, and ICP4 are shown relative to selected restriction sites: *Bam*HI (B), *Hpa*I (H), *Kpn*I (K), *Pst*I (P), *Sac*I (Sc), *Sal*I (S), *Sma*I (Sm), and *Xho*I (X). Protein-coding sequences are shown as hatched bars. Beneath the transcripts specifying ICP27, ICP0, and ICP4 are shown the structures of the nonsense and deletion mutants used in this study. The wild-type nucleotide sequence is shown as a thin line, and the limits of deleted sequences are shown as deletions in this line. The heavy lines indicate intact coding sequences. The ICP27 mutant 5*dl*1.2 contains a 1,196-base-pair deletion from the *Bam*HI site to the *Sal*I site, including the transcriptional start site, such that no detectable ICP27 is produced (23a). The ICP0 mutant *dl*x0.7 contains a deletion of 798 base pairs and retains sequences encoding 19 amino acids in the first exon of ICP0 (Weizhong Cai, unpublished data). *dl*x3.1 contains a deletion of 2,965 base pairs which includes the transcriptional start site and, hence, fails to specify any detectable ICP0 transcript or peptide. *dl*1403 has a 2,126-base-pair deletion and encodes the N-terminal 105 amino acids of the first and second exons. Sequences in the third exon are not in the same reading frame (open box) (40). The nonsense mutant *n*6 specifies a peptide lacking the N-terminal 90 amino acids of ICP4; *n*12 and *n*18 specify peptides lacking the C-terminal three-quarters and one-third of the ICP4 peptide, respectively (8). Beneath the 3′ end of the ICP0 gene is shown the LAT with its two predicted open reading frames (open boxes) and the position of this transcript relative to the ICP0 gene and its mutants. The last line shows the limits of the probe used in Southern blot analysis. kb, Kilobases.

containing nonsense mutations in the genes for ICP4, *n*12, and *n*18 (Fig. 3) also failed to replicate in the mouse eye and failed to reactivate by conventional cocultivation techniques, even when they were plated on ICP4-expressing E5 cells (Fig. 4 and Table 1).

Densitometric analysis of an autoradiograph (Fig. 5) from slot-blot hybridization of DNA extracted from ganglia of mice infected with *n*12 failed to demonstrate convincingly the presence of viral DNA. Attempts to demonstrate the presence of *n*12 DNA in latently infected ganglia by superinfection of dissociated neurons with the replication-incompetent mutant 5*dl*1.2 (ICP27) were also unsuccessful (Table 1). Taken together, these data suggest that the genomes of 5*dl*1.2 and *n*12, if present in ganglia at all, were present only at very low levels.

We also examined the behavior of two replication-competent ICP4 mutants, *n*6 and ΔAT, as positive controls. The *n*6 mutant specifies a peptide lacking the amino-terminal 90 amino acids of ICP4 (7), and the ΔAT mutant lacks the nucleotides AT from the transcriptional start site of ICP4 (ATCGTC) and overexpresses ICP4 (N. DeLuca, unpublished data). Neither mutant grew to the level of wild-type virus in the eye (10^3 PFU per eye swab sample), although titers in acutely infected ganglia were comparable, at approximately 10^5 PFU per ganglion (Fig. 4). Both *n*6 and ΔAT, however, were reactivated from 10 of 12 and 19 of 22 ganglia, respectively, compared with 77 of 78 ganglia for KOS. Thus, neither deletion of the first 90 amino acids nor overexpression of ICP4 drastically altered the latency competence of HSV-1 in this model.

Taken together, these results indicate that replication at

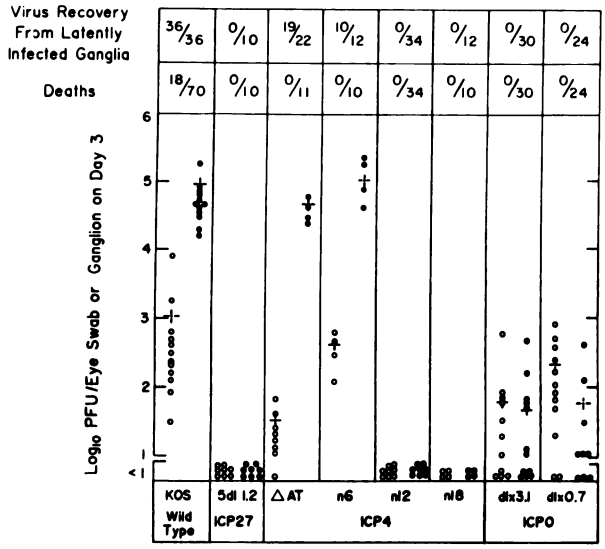

FIG. 4. Acute and latent infection of mice with KOS and mutants in immediate-early genes. Mice were inoculated at a dosage of 2×10^6 PFU per eye with each virus. Virus titers in eye swabs (○) and trigeminal ganglia (●) on day 3 are shown in the lower portion of the figure. Mean titers are indicated by a plus sign. At the top of the figure are shown the number of mice that were dead on day 30 postinfection relative to the total number of mice that were inoculated. The efficiency of recovery of virus from ganglia by explant cocultivation on day 30 is presented as a fraction (number of ganglia from which virus was reactivated/total number of ganglia tested).

TABLE 1. Stages in the process of latency at which mutants with lesions in immediate-early genes are defective

Inoculation → Replication at site of inoculation → Neural spread → Replication in ganglia → Death; Establishment of latency → Reactivation

Gene	Virus	Average virus titers (PFU) found in:		Lethality[a]	Viral DNA in ganglia[b]	Viral genomes rescued from ganglia by superinfection[c]	Reactivation by cocultivation[d]	
		Eye swabs	Ganglia				−DMSO	+DMSO
Wild type	KOS	1×10^3	1×10^5	18/70	0.7	ND[e]	77/78	ND
ICP27	*5dl*1.2	<5[f]	<5	0/10	ND	ND	0/10	ND
ICP4	*n*12	<5[f]	<5	0/30	<0.05	0/14	0/34	0/26
ICP0	*dl*x3.1	8×10^1	7×10^1	0/30	0.5	12/12 (21)	0/46[f]	7/20
	*dl*x0.7	3×10^2	7×10^1	0/24	0.05[g]	5/12 (1)[g]	0/40[f]	0/20
	Mock infection	ND	ND	0/26	<0.05	0/18	ND	ND

[a] Number of mice dying on or before day 30 postinfection per number of mice inoculated.
[b] Assessment of viral DNA in ganglia by slot-blot hybridization. Values represent average genome copy numbers per cell equivalent.
[c] Number of ganglia yielding virus following superinfection per number tested. Values in parentheses indicate the mean number of plaques produced from virus-positive ganglia.
[d] Number of ganglia yielding virus by cocultivation per number of ganglia tested.
[e] ND, Not determined.
[f] Indicates critical stage at which mutant was defective.
[g] Indicates additional stage in which *dl*x0.7 was impaired.

the primary site of infection is important for the establishment of latency.

ICP0 is required for the efficient establishment and reactivation of latency. To assess the role of ICP0 in latency, two ICP0 deletion mutants, *dl*x3.1 and *dl*x0.7 (Fig. 3), that are replication competent in cell culture yet that lack functional ICP0 (as judged by transient expression assays [34]) were tested in our latency model.

The two ICP0 deletion mutants behaved similarly to each other during acute infection. They were both replication competent in the eye and in ganglia, although the levels of infectious virus were 10- to 100-fold lower than those of KOS in the eye and 1,000-fold lower than those of KOS in trigeminal ganglia (Fig. 4 and Table 1). Despite their replication competence, reactivation was never demonstrated from standard explant cultures of ganglia from mice infected with *dl*x0.7 (0 of 40 ganglia) or *dl*x3.1 (0 of 46 ganglia), even when ganglia were explanted onto ICP0-expressing 0-28

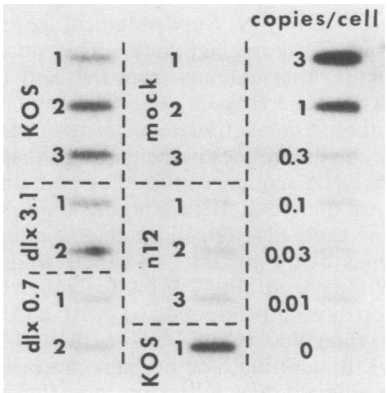

FIG. 5. Viral DNA in trigeminal ganglia of mice inoculated 30 days previously with KOS, *dl*x3.1, *dl*x0.7, and *n*12. Mice were mock infected or infected with a dosage of 2×10^6 PFU of each virus per eye. Ganglia were removed on day 30 and ganglion DNA was subjected to slot-blot hybridization. Hybridization of the probe fragments (*Eco*RI fragments A, D, I, and O) to mouse tail DNA containing 3, 1, 0.3, 0.1, 0.03, 0.01, and 0 viral genomes per cell equivalent are shown in the right-hand column.

cells. The inability of these mutants to reactivate could have been due either to the failure of mutant viral DNA to reach trigeminal ganglia or to the intrinsic properties of the mutants; i.e., they either failed to establish latency appropriately or they lacked the ability to reactivate. To distinguish between these possibilities, we determined whether individual ganglia from mice infected with *dl*x0.7 and *dl*x3.1 contained detectable viral DNA. We used both slot-blot hybridization and the superinfection assay to address this question. Viral DNA was readily detectable by slot-blot hybridization in ganglia from mice that were inoculated 30 days previously with *dl*x3.1 (Fig. 5). These ganglia contained about 0.5 copy of viral DNA per cell compared with 0.5 to 1.0 copy per cell for KOS. Significantly, doses of KOS (2×10^4 to 2×10^5 PFU per eye) that yielded 0.5 copy of viral DNA per cell in ganglia (Fig. 2) yielded reactivatable latency in 75% of ganglia (Fig. 1) compared with <2% for *dl*x3.1 (Fig. 4 and 5). Thus, the failure of *dl*x3.1 to reactivate was not due simply to the absence of viral DNA in trigeminal ganglia.

We next used the superinfection assay to ascertain whether the viral DNA was in a biologically retrievable state. Ganglia from mice that were infected 30 days previously with *dl*x3.1 when dissociated and superinfected with *5dl*1.2 yielded virus in all cases tested (12 of 12), with a mean of 21 plaques produced from each ganglion (Table 1). Southern blot analysis of DNA from individual plaque isolates indicated the presence of both mutant and wild-type ICP0 alleles, demonstrating that reactivated virus resulted from both complementation and recombination following superinfection (Fig. 6). As controls in this study, mock-infected ganglia were infected with *5dl*1.2. These ganglia (0 of 18) yielded no virus. Also, ganglia that were latently infected with *dl*x3.1 were mock superinfected, and again no virus was recovered (0 of 6 ganglia). These results indicate that *dl*x3.1 DNA in trigeminal ganglia is in a biologically retrievable state but that this mutant is defective in its capacity to reactivate from latency.

Somewhat different results were obtained when *dl*x0.7-infected ganglia were examined for the presence of viral DNA. Slot-blot hybridization frequently indicated the presence of viral DNA in ganglia at low levels, but these were discernibly above the background level of hybridization to

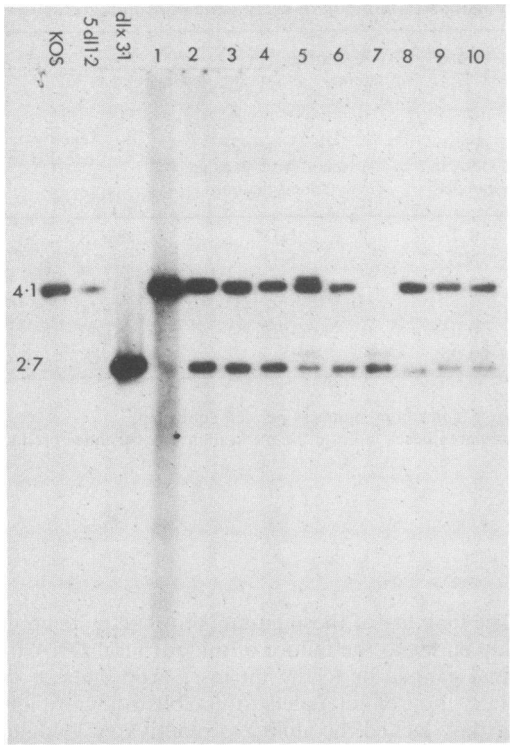

FIG. 6. Southern blot analysis of viral DNA from 10 plaques isolated from ganglia latently infected with *dl*x3.1 following superinfection with 5*dl*1.2. Resulting plaques were picked and amplified, and viral DNA was isolated. The DNA was cleaved with *Pst*I and *Xho*I, and the blot was probed with the *Pst*I-*Xho*I fragment shown in Fig. 3. Viral DNAs from KOS, 5*dl*1.2, and *dl*x3.1 were run as standards. Numbers to the left of the blot are in kilobases.

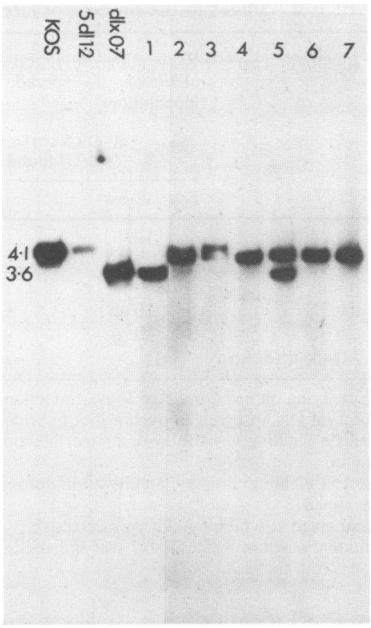

FIG. 7. Southern blot analysis of DNA from 7 plaques isolated from ganglia latently infected with *dl*x0.7 after superinfection with 5*dl*1.2. The DNA that was cleaved with *Pst*I and *Xho*I and the blot was probed with the *Pst*I-*Xho*I fragment shown in Fig. 1. Viral DNAs from KOS, 5*dl*1.2, and *dl*x0.7 were run as standards. Numbers to the left of the blot are in kilobases.

DNA from mock-infected mice (Fig. 5). The amount of viral DNA found in *dl*x0.7-infected ganglia was significantly less than that found in *dl*x3.1-infected ganglia and was 10- to 20-fold less than the amount found in KOS-infected ganglia. This was surprising since the level of *dl*x0.7 replication in eyes and ganglia during the acute stage of infection was roughly equivalent to that of *dl*x3.1. The reduced level of viral DNA in *dl*x0.7-infected ganglia relative to that in *dl*x3.1-infected ganglia was confirmed by superinfection assays in which less than one-half of the ganglia tested (5 of 12) yielded virus and significantly fewer plaques (mean of 1) were produced per ganglion (Table 1). Once again, Southern blot analysis of viral DNA from reactivated plaque isolates revealed the presence of both mutant and wild-type ICP0 alleles (Fig. 7). As with the *dl*x3.1 mutant, no reactivation was demonstrated with dissociated *dl*x0.7-infected ganglia (0 of 6) in the absence of superinfection.

Taken together, these data indicate that ICP0 plays a role in the efficient establishment and reactivation of latency.

ICP0 mutants can be reactivated under special conditions. Certain agents that affect gene expression such as the hypomethylating agent 5-azacytidine and dimethyl sulfoxide (DMSO) have been shown to increase the frequency of reactivation from ganglia latently infected with HSV-1 (46). We therefore attempted to reactivate *dl*x0.7, *dl*x3.1, and *n*12 from latently infected ganglia by using these agents.

The addition of 50 μM 5-azacytidine to the medium of explant cultures failed to reactivate either *dl*x3.1 (0 of 8 ganglia) or *dl*x0.7 (0 of 8 ganglia) from latently infected ganglia. The addition of 22 mM DMSO to explant cultures, however, resulted in reactivation from approximately one-third (7 of 20 ganglia) of *dl*x3.1-infected ganglia, although reactivation was not demonstrated from either *dl*x0.7 (0 of 20 ganglia)- or *n*12 (0 of 25 ganglia)-infected ganglia under these conditions (Table 1). The failure of *dl*x3.1, but not of *dl*x0.7, to reactivate from latency was therefore circumvented by DMSO.

The behavior of the ICP0 deletion mutant *dl*1403 of strain 17. All of our work with ICP0 deletion mutants to this point used *dl*x0.7 and *dl*x3.1, which were generated from wild-type HSV-1 strain KOS. We next examined the behavior of another ICP0 deletion mutant, *dl*1403 (40), of HSV-1 strain 17 (kindly provided by N. Stow, Medical Research Council Virology Unit, Glasgow, Scotland). This mutant has a 2-kilobase deletion that extends from the *Sal*I to *Xho*I sites within the ICP0 gene (Fig. 3). The truncated ICP0 peptide has no detectable *trans*-activating activity in transient assays (40). Despite the differences in the peptides that they encode and the ICP0 DNA sequences that they contain (Fig. 3), the phenotypes of the three ICP0 mutants in cell culture are essentially the same (34, 40). In the mouse model, however, the phenotypes of the mutants and their wild-type parents diverged. Wild-type strain 17 grew to higher titers in eyes and ganglia on day 3 postinfection (1×10^4 and 2×10^5 PFU, respectively) than did strain KOS (1×10^3 and 1×10^5 PFU, respectively). In addition, and perhaps more significantly, 9 of 12 (75%) animals died following inoculation of 2×10^6 PFU of strain 17 per eye, compared with 18 of 70 (26%) animals following inoculation with strain KOS. This indicates that strain 17 is considerably more virulent than KOS and that the usual outcome of corneal infection with 2×10^6 PFU per eye is death and not latency, as was seen with strain KOS. Mutant *dl*1403 grew to slightly higher titers in the eye and ganglia on day 3 postinfection than did *dl*x0.7 and *dl*x3.1

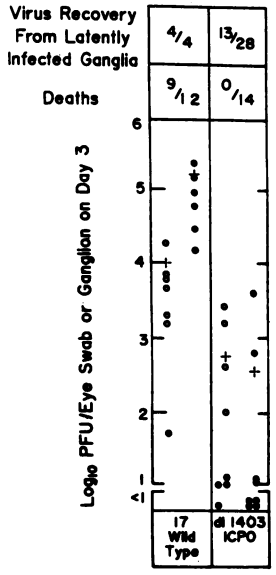

FIG. 8. Acute and latent infection of mice with HSV-1 strain 17 and *dl*1403. Mice were inoculated with a dosage of 2×10^6 PFU of each virus per eye. Virus titers in eye swabs (○) and trigeminal ganglia (●) on day 3 are shown in the lower portion of the figure. Mean titers are indicated by a plus sign. At the top of the figure are shown the number of mice that were dead on day 30 postinfection relative to the total number of mice that were inoculated. The number of ganglia from which virus could be reactivated (in the absence of DMSO) on day 30 relative to the total number of ganglia tested for reactivation is presented as a fraction.

(Fig. 4 and 8). Explant cultures at latently infected ganglia on day 30 postinfection, however, yielded virus in about one-half of the ganglia tested (13 of 28; 46%) in the absence of DMSO or other special treatment. Tests for infectious *dl*1403 virus on day 30 by homogenization and direct assay were negative (0 of 8 ganglia), demonstrating that virus recovered at this time following cocultivation is generated by reactivation. These results indicate that *dl*1403 is less latency competent than its parent virus, strain 17, but is significantly more competent than either *dl*x3.1 or *dl*x0.7 in our latency model.

DISCUSSION

HSV-1 latency involves an ordered series of events which can be dissected by using mutants that are blocked at different stages in the process. In this study we attempted to identify the stages in the establishment and reactivation of ganglionic latency during which HSV-1 immediate-early proteins ICP4, ICP0, and ICP27 function. In previous studies of latency, viral reactivation alone has been used as a measure of latency competence. In this study we showed that the failure of virus to reactivate can be differentiated from its failure to establish latency in neurons.

Wild-type parameters of the mouse eye model. Prior to our testing of mutants in this system, a thorough investigation of HSV-1 strain KOS in the mouse eye model was undertaken in order to standardize this model for our purposes. The mouse eye model has been well documented in the literature for the investigation of HSV-1 latency (20, 37, 43). Our studies of the behavior of wild-type strain KOS in the CD-1 mouse eye model demonstrated that this particular combination of model, virus, and mouse strain is an especially good one for studies of latency. The low neurovirulence of KOS permits us to use a spectrum of dosages with a reasonable guarantee that latency will be established in a high proportion of ganglia and that few animals will die. Our studies with KOS also confirmed the observation of Gordon and Rock (13) that the cocultivation assay is significantly more sensitive than DNA hybridization for the detection of latent virus in ganglia. Although the sensitivity of cocultivation assays can be enhanced by the addition of DMSO or other agents to culture media, the routine use of these agents can mask the reactivation-deficiency of certain viral mutants under normal cocultivation conditions.

Virus replication at the site of inoculation is important for the establishment of latency. The role of virus replication at the site of inoculation in the establishment of latency has been difficult to assess based on the results of previous studies using temperature-sensitive mutants (45), immune serum (18), or antiviral drugs (19) to inhibit viral replication. The interpretation of such studies must include the caveats that temperature-sensitive mutants may leak and that antiviral drugs and immune serum may not completely block viral replication. The use of viruses with deletion and nonsense mutations in essential genes in this study ensured that viral replication could not occur at any stage of the infection process. Any virus that is unable to replicate at the site of inoculation is less likely to gain access to nerve endings than is a virus that is replication competent. A replication-defective virus may be able to enter neurons directly through nerve endings (without the need for prior replication), but such a virus would be unable to leave neurons during reactivation in a conventional cocultivation assay. Indeed, in this study it was found that three nonreplicating mutants in the genes for ICP4 and ICP27 (*n*12, *n*18, and 5*dl*1.2) were not able to reactivate from explanted ganglion cultures, even in the presence of complementing feeder cells. In the case of *n*12, our attempts to rescue the virus from neurons by superinfection with a complementing virus (5*dl*1.2) were also unsuccessful, and any viral DNA in ganglia was below the detection limits of the hybridization techniques. It is likely, therefore, that in the absence of replication at the site of inoculation, the virus is unable to establish reactivatable latency that is demonstrated by the techniques of cocultivation (with or without DMSO) or rescue by superinfection. It should be borne in mind, however, that ICP4 and ICP27 may also play an essential role in later stages of latency.

ICP0 plays an important role in reactivation. The replication competence of ICP0 deletion mutants has made it difficult to assign a function to ICP0 in the infection of cells in culture (34, 40). It appears from the results of this study that ICP0 plays an important role in the reactivation of virus from latent infection since two independent ICP0 mutants of strain KOS, *dl*x0.7 and *dl*x3.1, established latency but did not reactivate in the mouse eye model. For two reasons, the observed reactivation deficiencies of *dl*x3.1 and *dl*x0.7 cannot be attributed simply to their poor growth in cell culture. First, the addition of DMSO to the culture medium of explant cultures allows the reactivation of *dl*x3.1, yet DMSO does not increase the growth of *dl*x3.1 in cell culture (C. Bogard, unpublished data). Second, both *dl*x3.1 and *dl*x0.7 could be reactivated from latently infected ganglia following superinfection with 5*dl*1.2, as described in this report. These two mutants failed to reactivate in an alternative latency model, the mouse footpad model, in the absence of DMSO (G. Clements and N. Stow, personal communication), which is in agreement with our results. The stages of establishment

and reactivation of viral latency are therefore separated by these two mutants.

It is also notable that *dl*x3.1, and especially *dl*x0.7, exhibited reduced levels of viral DNA relative to KOS in latently infected ganglia. This implies that ICP0 may also be important for the efficient establishment as well as reactivation of latency. It is not clear, however, why the *dl*x0.7 mutant exhibited such drastically reduced levels of viral DNA in ganglia relative to *dl*x3.1, because both mutants were equally replication competent in the eye and in ganglia. Further investigation of the nature of these two mutations with respect to ICP0 and the latency-associated transcript may elucidate the nature of this observed difference (Fig. 3; see discussion below). ICP0 has been shown to be important to lytic virus growth in cell culture, with the effect of deleting ICP0 being greater at low multiplicities of infection (34, 40). Because low multiplicities of infection are likely to be encountered during the establishment and reactivation of latency, this could largely explain the latency incompetence of the ICP0 mutants. On the other hand, our data and those of G. Clements and N. Stow (personal communication) with an ICP0 deletion mutant of strain 17 (*dl*1403) indicate that ICP0 may not be absolutely essential either for the establishment or the reactivation of latency. The latency competence of this mutant, however, does appear to be less than that of its parent strain, strain 17.

Two possible explanations exist for the observed differences in the reactivation phenotypes of KOS and strain 17 ICP0 deletion mutants. The first is that strain-specific differences may exist which allow strain 17, but not strain KOS, to reactivate in the absence of a functional ICP0 gene. Major differences in the neurovirulence of strains 17 and KOS have been demonstrated by others (42) and in this study (Fig. 4 and 8). To address this question we have begun to generate a *Xho*I to *Sal*I deletion mutation in the ICP0 gene of KOS, a deletion identical to that found in *dl*1403, so that we can assess strain-specific variables more directly.

A second explanation arises from differences in the sizes and locations of the deletions in ICP0 (Fig. 3). Mutant *dl*x3.1 has a deletion of 2,965 base pairs from residues 1405 to 4370 of IR_L (numbering system of Perry et al. [27]), including the transcriptional start site, and should not express any of the ICP0 transcript or polypeptide, whereas *dl*x0.7 has a deletion of 798 base pairs from residues 2609 to 3407 of IR_L and would specify a peptide which consists of the N-terminal 19 amino acids, which are specified by the first exon (Weizhong Cai, unpublished data). The 3′ limit of the *dl*x0.7 deletion occurs in the second intron, such that the third exon is read out of frame. The *dl*1403 mutant (from strain 17) has a deletion from residues 2939 to 5065 and could specify a polypeptide containing the N-terminal 105 amino acids specified by the first and part of the second exons; this is followed by the carboxy terminus of the third exon, which is out of frame (Fig. 3). It is possible that the 86 amino acids specified by the undeleted portion of the second exon of *dl*1403 are sufficient to render the mutant latency competent in vivo, even though this truncated peptide has no detectable activity in transient expression assays. By contrast, the 19 amino acids specified by the first exon of the *dl*x0.7 mutant are apparently not sufficient to give this mutant the ability to establish or reactivate from latency efficiently.

Of additional interest is the fact that the deletions in these three mutants differ in their positions relative to the recently mapped latency-associated transcript (LAT), which is abundant in the nuclei of latently infected ganglia of mice, rabbits, and humans (3, 32, 39, 44). This transcript derives from the HSV-1 DNA strand opposite to that which encodes ICP0 mRNA and partially overlaps the 3′ end of the ICP0 gene (Fig. 3). Although LAT contains two open reading frames, no LAT-encoded protein has yet been demonstrated. In addition, a second less abundant RNA species, the so-called minor latency-related RNA, has been identified which overlaps the 5′ end of the ICP0 gene (32). This transcript has yet to be fully characterized in terms of its position, size, direction of transcription, and sense.

Although ICP0 is an efficient *trans*-activator of a variety of viral and cellular genes, it is dispensable for productive infection in cell culture (34, 40). It has been suggested that a possible function of LAT is to suppress the expression of ICP0 during latency by antisense repression, i.e., by RNA-RNA hybrid arrest of translation (39). This model for the repression of viral gene expression predicts that HSV-1 mutants lacking ICP0 would be reactivation deficient. The three ICP0 deletion mutants tested were deficient to various degrees in their ability to reactivate. The only ICP0 mutant tested whose deletion did not also delete the 3′ end of LAT was *dl*x0.7, the mutant that was least able to establish and be reactivated from latency (Fig. 3). The *dl*x3.1 mutant, which was slightly more latency competent than *dl*x0.7, may or may not contain the 3′ end of LAT (up to 639 bases), although the extent of LAT sequences deleted cannot be ascertained until the 3′ end of this transcript has been more finely mapped. Mutant *dl*1403, which reactivated with the greatest frequency, lacks at least 1,040 bases of LAT, including the second potential open reading frame of LAT. Based on a comparison of the genotypes and phenotypes of the three ICP0 mutants, it is tempting to speculate that the more LAT sequences removed in the absence of functional ICP0, the more latency competent the virus is, implying that the establishment and reactivation of latency involves the products (i.e., protein or RNA) of both the ICP0 and LAT genes.

Possible roles of cellular factors in latency. The observation that DMSO could induce reactivation of *dl*x3.1 suggests that the block of reactivation of this mutant can be overcome by DMSO. The mode of action of DMSO is unknown, but it has been shown to have an effect on a number of cellular processes (4), to induce recurrent disease in mice latently infected with HSV-1 (14), and to increase the frequency of reactivation from ganglia latently infected with HSV-1 (46). The inability of DMSO to induce the reactivation of *dl*x0.7 is probably caused by the inefficiency with which this mutant establishes latency, based on the low levels of viral DNA detected in ganglia. The precise mechanism by which DMSO induces reactivation of *dl*x3.1 warrants further investigation, with particular reference to the induction of specific viral and cellular genes by this agent.

Indeed, with regard to latency the study of viral factors should not be considered in isolation from host cellular factors such as nerve growth factor (47) and prostaglandins (21), which have been shown to affect the reactivation of HSV-1 from murine ganglia, presumably by perturbation of the physiology of the host cell. Further studies may show how such cellular factors interact with viral gene products such as ICP0 and LAT and elucidate the molecular mechanisms by which HSV-1 can persist for the lifetime of its host.

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