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The Herpes Simplex Virus Type 1 Immediate-Early Protein ICP0 Is Necessary for the Efficient Establishment of Latent Infection

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The immediate-early protein ICP0 of herpes simplex virus type 1 (HSV-1) is not essential for viral replication. However, ICP0 is important for efficient viral replication during the productive infection and for reactivation of latent HSV-1 in vivo. The in vitro model of HSV-1 latency in dorsal root ganglia neurons was used to examine the role of ICP0 in the individual steps that could lead to the appearance of a decreased reactivation phenotype of ICP0 mutant viruses. After establishment of latent infections in the neuronal cultures, induction of reactivation by nerve growth factor (NGF) deprivation resulted in the production of infectious virus with delayed kinetics and a burst size that was significantly decreased for the ICP0 mutants compared with wild-type HSV-1. The efficiency of establishment of latency with the ICP0 mutants was similarly decreased at least 10-fold, as measured by three criteria: (i) the percentage of neurons expressing the major latency-associated transcript during the latent infection, (ii) the amount of viral DNA detected in the neuronal cultures, and (iii) the percentage of neurons expressing ICP4 immunoreactivity after the induction of reactivation. The most striking finding was that ICP0 supplied by an adenovirus vector significantly restored the ability of an ICP0 mutant to establish latency and reactivation. These results strongly indicate a critical role for ICP0 in the establishment of the latent HSV-1 infection in the in vitro neuronal model.

The immediate-early protein ICP0 of herpes simplex virus type 1 (HSV-1) plays an important regulatory role during viral infection in cultured cells and in mouse latency models. While ICP0 is not essential for normal viral gene expression or replication after productive infection has been initiated, inactivation of ICP0 results in a cell type-, cell cycle-, and multiplicity-dependent defect in the onset of viral gene expression (2, 9, 30, 38). In transfection assays, ICP0 has been shown to increase gene expression nonspecifically by a poorly understood mechanism, which does not discriminate between the viral temporal class or even the source of the target promoter (10, 15, 26). In the mouse latency model, ICP0 mutants reactivate with low efficiency from explanted trigeminal ganglia of mice (1, 3, 17). ICP0 expressed from an adenovirus vector results in reactivation of HSV in an in vitro model of latency in a nonneuronal cell line (16, 44). These data have led to the hypothesis that ICP0 is a critical gene for the induction of reactivation of latent HSV-1 (13).

In the productive infection of cell lines, efficient replication of ICP0 mutants is dependent upon the use of a high multiplicity of infection (MOI). In vivo it is not feasible to manipulate or control the MOI of the virus that results in the latent infections in the ganglia because of virus generated during the productive infection at the site of inoculation and in the ganglia. In vivo, several factors prevent studies of the effects of MOI on the establishment of latency with ICP0 mutants: (i) the physical restrictions of the site of inoculation, (ii) the requirement for a productive infection at the periphery for the establishment of the latent infection, and (iii) the productive infection that occurs in the ganglia, resulting in a lack of temporal control of the infection. Similarly, efficient recovery of the reactivated latent virus from explanted ganglia is dependent on the ability of the virus to replicate.

To determine the role of ICP0 in the establishment of the latent infection and during reactivation, we have analyzed several ICP0 mutants in the in vitro neuronal model of HSV-1 latency. The use of the in vitro neuronal model permits the direct inoculation of neurons with the virus. Infection with wild-type HSV-1 in this model results in the establishment of latency in the absence of a primary infection, achieves high efficiency of latency, and allows reactivation to be induced in a controlled, uniform manner (35, 40–42). Consequently, this model system allowed us to examine the contribution of ICP0 to the individual steps that lead to the establishment of latency and the reactivation of latent HSV-1.

MATERIALS AND METHODS

Viruses and cell culture. HSV-1(17^+) was the wild-type strain used in these studies. The FXE, D8, and D15 mutant viruses with defined deletions in the ICP0 gene have been described previously (9). The relative locations within the HSV-1 genome of the mutation in the ICP0 gene are indicated in Fig. 1. The HSV-1 stocks were grown and quantified by the plaque formation assay on Vero cells (American Type Culture Collection). All virus stocks were also subjected to titer determination on BHK cells (American Type Culture Collection) and produced identical titers as determined on Vero cells.

Replication-defective adenovirus E1A deletion mutants were propagated on a complementing cell line (293) and, the stocks were subjected to titer determination by standard methods. The adenovirus vector expressing ICP0 under control of the adenovirus major late promoter (MLP-0) was generously provided by S. Silverstein (Columbia University, New York, N.Y.) (45). The adenovirus vector expressing β-galactosidase under control of the cytomegalovirus promoter (300_{Bst} β-gal) was generously provided by J. Schaack (University of Colorado Health Sciences Center, Denver, Colo.) (32).

Neuronal culture preparation. Neuronal cultures were prepared from dorsal root ganglia of embryonic day 15 rats as previously described (40, 42). The neuronal cultures were plated on coverslips for in situ hybridization and immunohistochemical studies. For in situ hybridization, immunohistochemical, and reactivation studies, the neuronal cultures contained approximately 10^3 neurons per culture. For Northern blot and DNA analyses, the neuronal cultures contained approximately 10^4 neurons per culture. Latently infected neuronal cultures were prepared as previously described (42). At 14 days after the neuronal cultures were plated, 50 μM acyclovir (Burroughs Wellcome Co., Research

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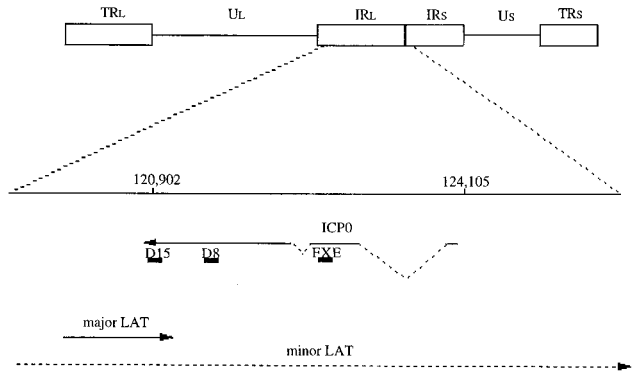

FIG. 1. Schematic representation of the HSV-1 genome. The relative locations of the ICP0 and LAT genes and the regions of the ICP0 gene that were mutated to produce the ICP0 mutant viruses used in this study are indicated.

Triangle Park, N.C.) was added to the culture media so that it was present 12 h prior to and for 7 days following inoculation with HSV-1. At 7 days after viral inoculation, the culture medium was replaced with standard culture medium, which lacks acyclovir. At 14 days after viral inoculation, the neuronal cultures were used in the experiments. The standard neuronal culture medium consists of 10% newborn bovine serum (Gibco, Grand Island, N.Y.) and 100 ng of 2.5S mouse nerve growth factor (NGF) (Harlan Bioproducts) per ml in Dulbecco's minimum essential medium (Gibco/BRL).

To control for variations resulting from the preparation of the neuronal cultures, the data presented are from experiments completed with neuronal cultures prepared at the same time. However, all experiments were repeated to confirm the results.

Reactivation of latent HSV-1. For the induction of reactivation, NGF deprivation was used as previously described (40, 42). NGF deprivation was achieved by adding 1% rabbit anti-mouse NGF serum (titer, >20,000) (40, 42). At intervals after the induction of reactivation, neuronal cultures were harvested and tested for the presence of infectious virus in plaque formation assays with total culture lysates, prepared by three freeze-thaw cycles, subjected to titer determination on Vero indicator cells; alternatively, the cultures were fixed for immunohistochemical studies.

Immunohistochemistry. For immunohistochemical studies, neuronal cultures were fixed with 4% (phosphate-buffered) paraformaldehyde for 2 min and then dehydrated in graded ethanol. For detection of ICP4 by immunohistochemistry, cultures were incubated overnight at 4°C with a 1/1,000 dilution of a rabbit anti-ICP4 serum prepared against a TrpE-ICP4 fusion protein. The clone for generation of the ICP4 fusion protein was a generous gift of K. Wilcox (Medical College of Milwaukee). Indirect immunohistochemistry was performed by the avidin-biotin-peroxidase complex method as specified by the manufacturer (Vectastain; Vector Laboratories, Burlingame, Calif.). The chromogen diaminobenzidine was used as the substrate in the presence of nickel to produce a blue-black precipitate in positively stained cells. Photomicrographs were prepared with a Nikon Optiphot-2 equipped with Hoffman optics.

In situ hybridizations and probes. Neuronal cultures were fixed with 4% (phosphate-buffered) paraformaldehyde for 12 h at 4°C, dehydrated in graded ethanol, and stored at −20°C until used. Detection of the latency-associated transcript (LAT) by in situ hybridization was performed as previously described (36). A riboprobe antisense to the 5′ region of the LAT (pLAT), which has been previously described (36), was used. The pLAT probe corresponds to the nucleotide sequence from 118867 to 120006, based on the sequence data of McGeoch (21). This probe has no overlap with other known genes in the LAT region (22). Photographs of LAT-positive neurons were taken with a Nikon Optiphot-2 equipped with Hoffman optics.

For the determination of the percentage of LAT-positive neurons, cell counts were performed. The frequency of LAT-positive neurons was determined from neuronal cell counts of five random fields (containing 20 to 50 neurons each) per culture from five cultures, using a magnification of ×200 for the counting.

Northern blot analysis. Northern blot analysis was performed by standard methods as described previously (5). Total RNA samples were electrophoresed in formaldehyde-agarose (1%) gels, stained with ethidium bromide to compare relative amounts and determine the location of the rRNA bands, transferred to nylon-based filters (Hybond-N; Amersham), and UV cross-linked. Standard methods were used for the incorporation of [α-^{32}P]dCTP by random primer labeling of the pLAT probe (31). The relative size of the transcripts was determined from comparison with the migration of the rRNA bands. The resulting image was obtained with a Molecular Dynamics PhosphorImager.

Viral DNA analysis. Prior to isolating the DNA, the neuronal cultures were treated with 2 U of DNase I for 10 min at 37°C and then with collagenase (1 mg/ml for 5 min at 37°C) to eliminate viral DNA that may have bound to the collagen or cell surface. DNA was extracted from the neuronal cultures by methods described by Efstathiou et al. (7). For slot blot analyses, the DNA concentration in the samples was determined with a mini-fluorometer (Hoefer Scientific Instruments). An equivalent amount of DNA for each sample was applied, with a slot blot apparatus (Hoefer Scientific Instruments), to nylon-based membrane (Hybond-N; Amersham) by standard methods (27). The viral genome equivalents used for standards were added to Vero cell DNA prior to application to the filter. The filters were probed with the *Bam*HI SP fragment labeled by random primer incorporation of [α-^{32}P]dCTP. The resulting image was obtained with a Molecular Dynamics PhosphorImager and analyzed with NIH Image.

For Southern blot analysis of the viral DNA during the latent infection in the neuronal cultures, the methods used were essentially those described previously (7, 29). After DNA isolation, the samples were digested with *Bam*HI and the fragments were separated in an 0.8% agarose gel. Following transfer to a nylon-based membrane (Hybond-N), the filter was probed as described above for slot blot analysis. The resulting image was obtained with a Molecular Dynamics PhosphorImager and analyzed with NIH Image.

RESULTS

ICP0 mutants reactivate with decreased efficiency from latently infected neuronal cultures. HSV-1 ICP0 mutants were examined after inoculation into primary neuronal cultures to determine the ability of the mutants to reactivate following NGF deprivation. Three well-characterized ICP0 mutants constructed in the strain 17$^+$ background were examined (Fig. 1). The mutant FXE has an in-frame deletion which removes the crucial zinc binding RING finger domain of ICP0 (8, 9). The mutation in D8 removes the nuclear localization signal of ICP0 (8, 9). The mutation in D15 produces a deletion in the C-terminal region of ICP0, which has been implicated in the ability of the protein to multimerize and interact with intranuclear cellular structures (8, 9, 24). The effects of these specific mutations in the ICP0 gene have been well defined in transfection studies (8) and during productive infections with the viral mutants (9). All three of these mutations cause severe impairment of the ability of ICP0 to stimulate gene expression in transfection assays and impair the replication of the mutants in the productive infection (8, 9).

The effects of the MOI used for the establishment of the latent infection on the ability of the mutants to reactivate were determined. Latent infections in neuronal cultures were established with an MOI of either 0.5 or 5 PFU per neuron. After establishment of latency, the ability of the viruses to reactivate was assessed by determining the titers of infectious virus at intervals after NGF deprivation. With all of the ICP0 mutants, there was a marked decrease in the frequency of reactivation compared to that of wild-type HSV-1 (17$^+$) (Table 1). With a low MOI of 0.5 to establish latent infections, the neuronal cultures infected with the ICP0 mutants resulted in the complete absence of reactivated virus. With an MOI of 5 to establish latent infections, evidence of infectious virus after induction of reactivation was both significantly delayed and initially

TABLE 1. Frequency of reactivation of latent wild-type HSV-1 and the ICP0 mutants in neuronal cultures[a]

Virus	% Reactivation frequency at MOI of[b]:	
	0.5	5
17$^+$	40 (4/10)	100 (10/10)
FXE	0 (0/10)	80 (8/10)
D8	0 (0/10)	70 (7/10)
D15	0 (0/10)	80 (8/10)

[a] Sensory neuronal cultures were inoculated with HSV-1(17$^+$) (wild type) or with the ICP0 mutant indicated at a MOI of 0.5 or 5. At 14 days postinoculation, the cultures were treated with anti-NGF to induce reactivation of latent virus. At 5 days after the anti-NGF treatment, the cultures were harvested and tested in plaque formation assays for infectious virus.

[b] The numbers in parentheses indicate the number of cultures that were positive for infectious virus per the total number of neuronal cultures tested.

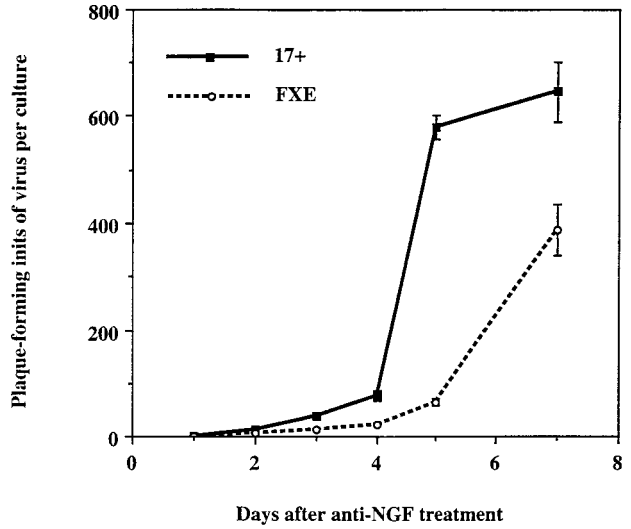

FIG. 2. Comparison of the time course of detection of infectious virus during reactivation after induction with anti-NGF of latent wild-type HSV-1 and the ICP0 mutant FXE. At 2 weeks after establishment of latent infection in neuronal cultures with HSV-1(17^+) (wild-type) or the ICP0 mutant FXE at an MOI of 5, the cultures were treated with anti-NGF to induce reactivation. At the times indicated after anti-NGF treatment, the cultures were harvested and virus titers were determined in plaque formation assays.

decreased in neuronal cultures infected with the ICP0 mutants compared to those infected with strain 17^+. A typical time course of the detection of virus during reactivation is shown for FXE (Fig. 2). These results suggested that the ICP0 mutants either had a reduced ability to reactivate or did not efficiently establish latency.

The percentage of neurons expressing LAT is significantly decreased during the latent infection with the ICP0 mutants. While the functions of the LAT are incompletely understood, these transcripts provide a useful marker of neurons that harbor latent HSV-1 (14). To determine if the ICP0 mutants were capable of expressing LAT, the ICP0 mutants were examined by Northern blot analysis for the expression of LAT during the productive infection in Vero cells. Northern blot analysis showed that all of the ICP0 mutants produced LAT during the productive infection in Vero cells (Fig. 3). In the infection with D15, which is the only one of these viruses with a mutation that alters the major LAT, a broader band corresponding to the LAT species was detected. The apparently higher level of expression of LAT with D15 was variable depending upon the time postinfection when Vero cells were harvested. These results indicate that all the ICP0 mutants were capable of producing LAT during the productive infection.

Under conditions previously shown to result in the establishment of latent infections with wild-type HSV-1 in the majority of neurons (35), neuronal cultures were infected with 17^+, FXE, D8, or D15. By using the LAT-positive in situ signal as the indicator of the presence of latent virus, the efficiency of establishment of latency was examined after inoculation with a MOI of either 0.5 or 5 PFU of virus per neuron (Fig. 4). With all of the mutants, the percentage of neurons positive for LAT detection was significantly decreased compared to that with the wild-type virus (Table 2). Based on the comparison of neuronal cell counts from mock-infected control neuronal cultures, there was no evidence of any significant neuronal cell loss following infection with the ICP0 mutants or wild-type virus when using an MOI of either 0.5 or 5 PFU per neuron. Consequently, neuronal cell death is unlikely to account for the decrease in the number of LAT-positive neurons observed with the ICP0 mutants compared to wild-type HSV-1 infections. Under the conditions defined for the establishment of latency, increasing the MOI to 50 for the inoculation with either wild-type virus or the mutant FXE resulted in death of all the neurons within 3 to 4 days after inoculation. These data suggest that the ICP0 mutants and the wild-type HSV-1 infected neurons with similar efficiency.

Figure 4 shows the typical nucleus-localized LAT signal detected by in situ hybridization from representative fields of neurons latently infected with wild-type HSV-1 or the ICP0 mutant FXE. The D8 mutant-infected cultures demonstrated a similar appearance of in situ hybridization signal for LAT to that of FXE, while D15 mutant-infected cultures had no detectable evidence of LAT-positive in situ signal (data not shown). For D15, interpretation of these results is complicated by the potential effects of the mutation on the 3′ region of the major LAT that may result in a change in the stability or splicing of the major LAT. However, the significantly reduced percentage of LAT-positive neurons detected during the latent infection with FXE and D8 suggests that the establishment of latency with the ICP0 mutants was reduced.

A characteristic of ICP0 mutants is a high particle-to-PFU ratio (9). As a consequence of this characteristic, it was possible that the large number of particles present in the inoculum required to achieve the MOI of 5 PFU per neuron interfered to produce the decreased efficiency in the establishment of latency with the ICP0 mutants. If interference by the particles resulted in the reduced efficiency of establishment of latency with the mutants, this interference would be expected to occur during the infection with the wild-type HSV-1 in the presence of the mutant virus inoculum. To test this possibility, neuronal cultures were coinfected with a 50:50 mixture of the mutant FXE and wild-type HSV-1 (17^+). The percentage of LAT-positive neurons present in the coinfected cultures was compared with that in cultures infected with 17^+ alone. Coinfection with FXE and 17^+ did not alter the frequency of LAT-positive neurons compared to that obtained as a result of infection with 17^+ alone (40% ± 13% and 39% ± 5%, respectively). This suggests that interference from the large number of viral particles in the mutant virus inoculum was not a major factor in deter-

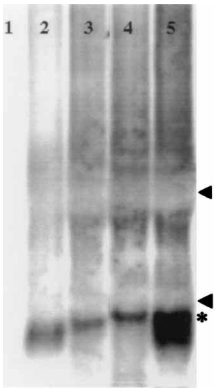

FIG. 3. Detection of LAT by Northern blot analysis during the productive infection in Vero cells after inoculation with wild-type HSV-1 or the ICP0 mutants. Total RNA was isolated from equivalent numbers of Vero cells 24 h after infection with virus at a MOI of 5. For each sample, 20 μg of RNA was resolved on formaldehyde–1% agarose gels, transferred to nitrocellulose, and hybridized with a probe for LAT labeled by random-primer incorporation of [α-^{32}P]dCTP. Lanes: 1, RNA from mock-infected cells; 2, RNA from 17^+-infected cells; 3, RNA from FXE-infected cells; 4, RNA from D8-infected cells; 5, RNA from D15-infected cells. The positions of ribosomal bands are indicated by the arrowheads. The position of the major LAT is indicated by the asterisk.

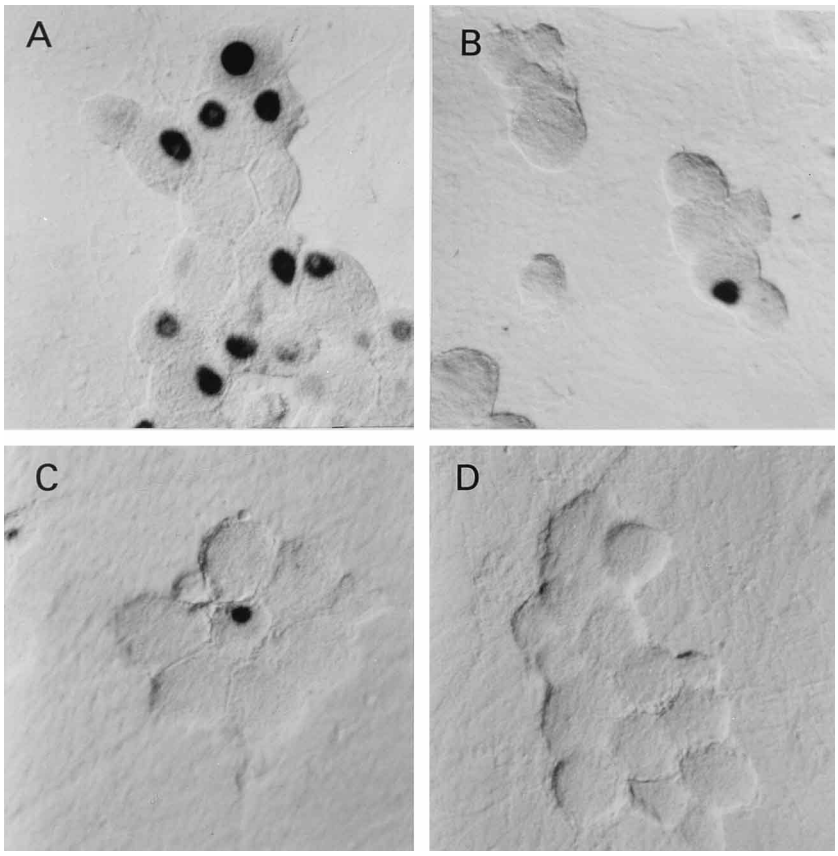

FIG. 4. Detection of LAT by in situ hybridization in neurons during the latent infection with wild-type HSV-1 or the ICP0 mutant FXE. In situ hybridization with a LAT-specific digoxigenin-labeled riboprobe was used to examine neuronal cultures for LAT expression 2 weeks after inoculation with either HSV-1(17^+) (wild-type) (A and C) or the ICP0 mutant FXE (B and D). Cultures were infected at an MOI of 5 (A and B) or 0.5 (C and D). No signal was detected with the sense strand control riboprobe (data not shown).

mining the reduced percentage of neurons expressing LAT following the establishment of the latent infections.

Efficient establishment of latency with an ICP4-minus mutant. The decreased establishment of latency with the ICP0 mutants could result from a decreased ability to replicate. If HSV-1 replication is critical for the establishment of latency, a virus incapable of replication would be predicted to inefficiently establish latency. To examine this possibility, neurons in culture were infected with d120, a virus that has a mutation in the essential ICP4 gene (4). Latent infections of neuronal cultures were established with either d120 or the parental wild-type strain, KOS. Figure 5 shows representative results from in situ hybridization experiments to detect LAT in the neuronal cultures 2 weeks after inoculation. A higher percentage of the neurons following inoculation with d120 were LAT positive (82%) than neurons inoculated with the wild-type HSV-1 KOS (51%). These results indicate that a virus with a mutation in an essential gene was able to efficiently establish latency in neurons in culture. These results indicate that viral replication is not necessary for the efficient establishment of latency. The failure of ICP0 mutants to efficiently establish latency appears unrelated to a direct affect of the reduced replicative efficiency of these mutants.

Mutation in ICP0 results in a reduced abundance of viral DNA in neuronal cultures after the establishment of latency. To further investigate the effects of mutations in the ICP0 gene on the establishment of latency, the amount of viral DNA present in the neuronal cultures following the establishment of latency with either the mutants or the wild-type virus was compared. To determine if the amount of viral DNA differs for the ICP0 mutant FXE compared to the parental strain 17^+, DNA was harvested from neuronal cultures 2 weeks after establishment of latent infection with 17^+ or FXE. As shown in Fig. 6, the amount of viral DNA detected during the latent infection was decreased by more than 10-fold in the cultures latently

TABLE 2. Percentage of neurons expressing LAT during the latent infection with the ICP0 mutant viruses compared to wild-type HSV-1[a]

Virus	% of LAT-positive neurons at MOI of[b]:	
	0.5	5
17^+	2 ± 1	58 ± 8
FXE	0[c]	4 ± 1
D8	ND[d]	8 ± 3
D15	ND	0

[a] Sensory neuronal cultures were examined during the latent infection (14 days postinoculation) for LAT by in situ hybridization methods after inoculation with HSV-1(17^+) (wild type) or the ICP0 mutants indicated at a MOI of 0.5 or 5. The frequency of LAT-positive neurons was determined from cell counts of five random fields (containing 20 to 50 neurons each) per culture.

[b] Results are expressed as the mean percentage of LAT-positive neurons from five cultures and standard error of the mean.

[c] *, one or no LAT-positive neurons were observed in the five cultures examined.

[d] ND, not determined.

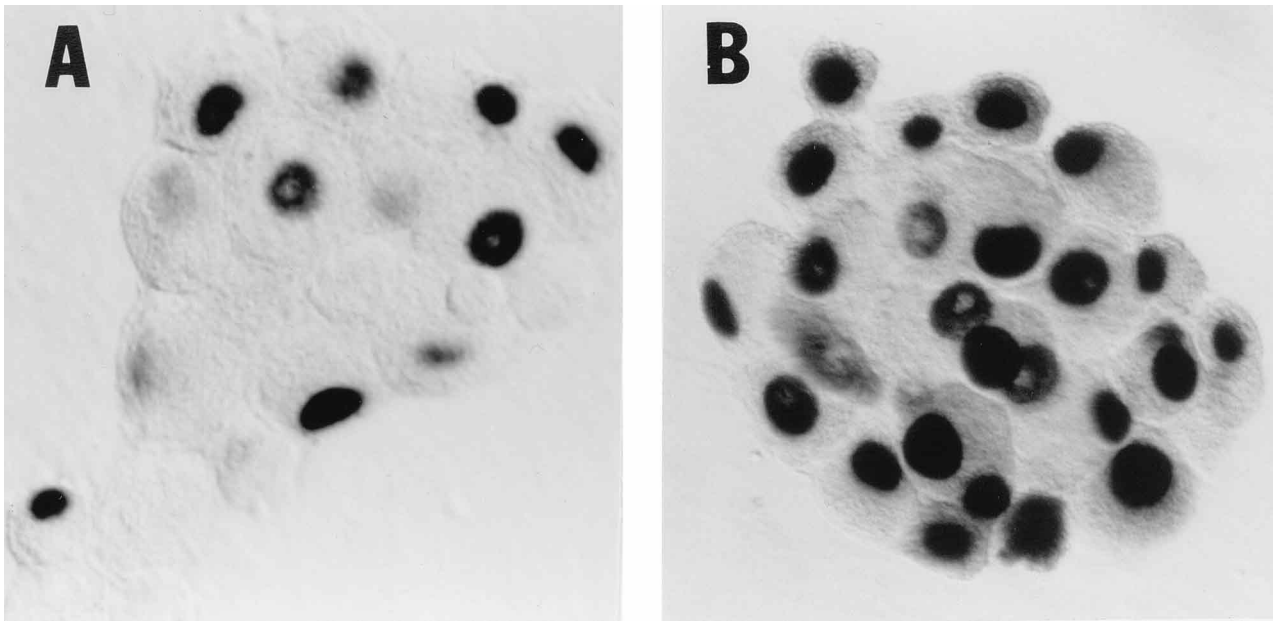

FIG. 5. Detection of LAT-positive neurons during the latent infection with wild-type HSV-1 compared to a replication-incompetent ICP4-negative mutant. In situ hybridization with a LAT-specific digoxigenin-labeled riboprobe was used to detect LAT 2 weeks after inoculation of neuronal cultures with HSV-1(KOS) (wild-type) (A) or the ICP4-negative mutant d120 (B). Cultures were infected at an MOI of 5. No signal was detected with the sense strand control riboprobe (data not shown).

infected with FXE compared to 17^+. The reduced amount of DNA following FXE inoculation was consistent with the reduced frequency of reactivation and the reduced percentage of LAT-positive neurons detected during the latent infection.

To further characterize the amount of viral DNA in the neurons, viral DNA was quantified at several time points during the establishment of the latent infections. Neuronal cultures were infected with either 17^+ or FXE. DNA was harvested at 24 h, 5 days, and 2 weeks after inoculation. The amount of viral DNA was determined by slot blot analysis. Similar amounts of viral DNA were detected in cultures infected with either 17^+ or FXE at 24 h and 5 days postinfection (Fig. 7). However, consistent with previous experiments, the amount of viral DNA detected 2 weeks postinoculation was decreased by more than 10-fold in the cultures latently infected with FXE compared to 17^+. These results suggest that the ICP0 mutant infected the neurons as efficiently as did the wild-type virus but was subsequently lost from the neurons.

Viral genome during latency. During latency in animal models and in the human disease, a characteristic of the viral genome is the loss of detectable ends. This has been interpreted as being most consistent with circularization of the genome. In the neuronal model, a similar result was observed after the establishment of latency with 17^+, as shown in Fig. 8. The viral DNA from ICP0 mutants was present at a level below the limits of detection by these methods. The ratios of the signals from the junction region to those from the terminal fragments were 1.5:1 for the virion DNA (Fig. 8, lane 2) and 10:1 for the latent viral DNA (lanes 3 and 4). During the latent infection with 17^+, the results were not altered by the continuous presence of acyclovir (lane 4). This indicates that the results were not the result of a low level of spontaneous reactivation. These data indicate that the majority of DNA detected in neurons in culture was in an endless form, and they are consistent with data about the viral genome during latency observed in other systems (7, 29).

Mutations in the gene for ICP0 do not decrease the ability of th mutant viruses to initiate viral gene expression during reactivation. ICP0 mutants are reported to reactivate poorly in vivo (1, 3, 17). To determine the ability of ICP0 mutants to initiate immediate-early viral gene expression during reactivation, neurons harboring latent virus were deprived of NGF and examined for ICP4 immunoreactivity. NGF deprivation has been shown to produce uniform reactivation in the neuronal culture latently infected with wild-type HSV-1 (40). At earlier times after the induction of reactivation (12 h after anti-NGF treatment), no evidence of ICP4 expression was detected in cultures infected with either 17^+ or FXE (data not shown). By 24 h after anti-NGF treatment, evidence of ICP4 expression was detected in both the 17^+- and FXE-infected cultures (Fig. 9). While ICP4 was detected with similar times after anti-NGF treatment, the percentage of ICP4-positive neurons was significantly dif-

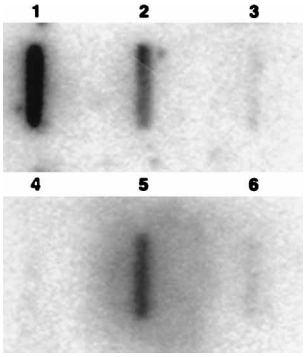

FIG. 6. Quantitation of viral DNA isolated from neuronal cultures during latent infection with the ICP0 mutant FXE compared to wild-type HSV-1. At 2 weeks after establishment of latent infection in sensory neuronal cultures with HSV-1(17^+) (wild-type) or FXE at a MOI of 5, DNA was isolated. The DNA samples were slot blotted and probed with the *Bam*HI SP fragment labeled by random-primer incorporation of [α-^{32}P]dCTP. Slot 1, 15 viral genome equivalents per cell; slot 2, 1.5 viral genome equivalents per cell; slot 3, 0.15 viral genome equivalent per cell; slot 4, 2.5 μg of mock-infected neuronal culture DNA; slot 5, 2.5 μg of 17^+ latently infected neuronal culture DNA; slot 6, 2.5 μg of FXE latently infected neuronal culture DNA.

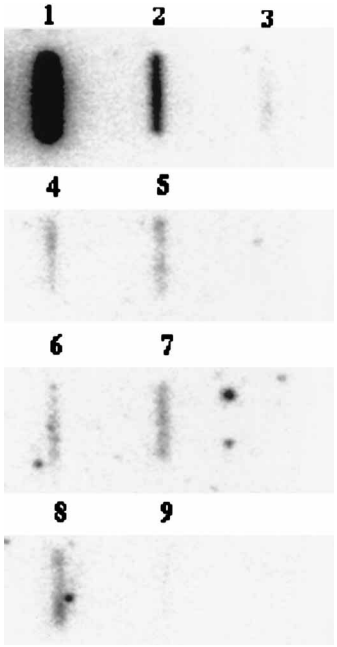

FIG. 7. Quantitation of viral DNA isolated from neuronal cultures during the establishment of latent infection with the ICP0 mutant FXE compared to wild-type HSV-1. At intervals during the establishment of latent infection in sensory neuronal cultures infected with HSV-1(17^+) (wild-type) or FXE at a MOI of 5, DNA was isolated. The DNA samples were slot blotted and probed with the *Bam*HI SP fragment labeled by random-primer incorporation of [α-^{32}P]dCTP. Slot 1, 50 viral genome equivalents per cell; slot 2, 5 viral genome equivalents per cell; slot 3, 0.5 viral genome equivalent per cell; slot 4, 1 μg of neuronal culture DNA 24 h after infection with 17^+; slot 5, 1 μg of neuronal culture DNA 24 h after infection with FXE; slot 6, 1 μg of neuronal culture DNA 5 days after infection with 17^+; slot 7, 1 μg of neuronal culture DNA 5 days after infection with FXE; slot 8, 1 μg of neuronal culture DNA 2 weeks after infection with 17^+; slot 9, 1 μg of neuronal culture DNA 2 weeks after infection with FXE.

ferent for the viruses: 98% of the 17^+-infected neurons but only 9% of the FXE-infected neurons were ICP4-positive. This reduction in the number of ICP4-positive neurons following reactivation of FXE was consistent with a reduced efficiency of the establishment of latency with FXE. These data did not show a further decrease in the efficiency of expression of ICP4 with the ICP0 mutant after reactivation, suggesting that the ICP0 mutant was able to initiate gene expression during reactivation.

ICP0 supplied in *trans* significantly restored the ability of the ICP0 mutant to establish latent infections. We have shown that neurons in culture can be efficiently infected with replication-defective adenovirus vectors for gene transfer (37). To determine if ICP0 would complement the ICP0 mutants, neuronal cultures were coinfected with the ICP0 mutant FXE and a replication-defective adenovirus vector expressing ICP0 under the control of the adenovirus major late promoter. Following establishment of latency, the percentage of neurons expressing LAT was determined by in situ hybridization (Fig. 10). The number of neurons expressing LAT following infection with FXE was increased by 10-fold as the result of coinfection with an adenovirus expressing ICP0 but was unchanged by coinfection with a control, β-galactosidase-expressing adenovirus vector (Table 3). Coinfection with either the ICP0-expressing or the control β-galactosidase-expressing adenovirus vector and wild-type HSV-1 allowed the establishment of latency to occur with no significant effects on the percentage of LAT-positive neurons detected during the latent infection. Upon the induction of reactivation of neuronal cultures harboring latent FXE, the cultures which had been coinfected with the adenovirus vector expressing ICP0 reactivated with frequencies essentially identical to those of cultures infected with wild-type HSV-1 (Table 4). These results further demonstrate a critical role for ICP0 in the establishment of the latent infection.

DISCUSSION

In the in vitro neuronal model, establishment of latency is significantly influenced by mutations in the region of the ICP0 gene in a manner that is similar to the results obtained with in vivo models (1, 17). In the mouse trigeminal model, ICP0 mutants display decreased replication efficiency during the primary infection at the periphery and in the ganglia, decreased abundance of viral DNA during latency, and a decreased ability to reactivate from the explanted ganglia. These observations lead to the conclusion that the ICP0 mutants do not reactivate efficiently (1, 17). In vivo, it has not been possible to determine if a reduction in the establishment of latency with the ICP0 mutants is the result of impaired virus replication during the productive infection or a direct effect of ICP0 on the establishment of latency. Several features of the in vitro latency model permitted experiments to be conducted to further elucidate the complex role of ICP0 in the establishment of latency, which could not be done in the animal models. First, the efficiency of establishment of latency in neurons in vitro is not influenced by a primary infection. Virus is inoculated directly onto the neurons, and viral replication is not required for the establishment of the latent infection (40, 42). Further, both the efficiency of the establishment of latency and the ability to induce a synchronized reactivation are great for wild-type HSV-1 (40, 42). Consequently, these features permit the detection of small amounts of virus produced upon reactivation.

When this model was used to examine individual steps in the HSV-1 life cycle in the neuron, a striking effect of ICP0-region mutations on the ability of the virus to establish latency was observed. All of the experimental approaches indicated that the establishment of latency with the ICP0 mutants was significantly reduced compared to that with wild-type HSV-1: reactivation frequencies and kinetics were reduced, the frequency of LAT-positive neurons was reduced, the amount of viral

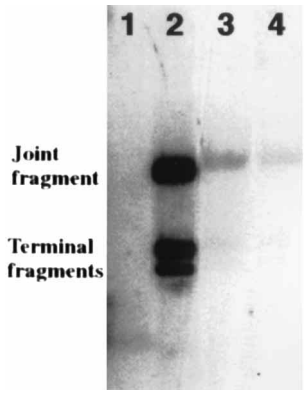

FIG. 8. Analysis of the HSV-1 genome during latent infection in neuronal cultures. After establishment of the latent infection with HSV-1(17^+) at a MOI of 5, DNA was isolated and *Bam*HI digested, and Southern blot analysis was performed with a *Bam*HI SP probe labeled by random-primer incorporation of [α-^{32}P]dCTP. Lanes: 1, 10 μg of DNA from mock-infected neuronal cultures; 2, 0.5 ng of 17^+ virion DNA and 10 μg of Vero cell DNA; 3, 10 μg of DNA from neuronal cultures after 2 weeks of latent infection with 17^+; 4, 10 μg of DNA from neuronal cultures after 2 weeks of latent infection with 17^+, continuously treated with acyclovir.

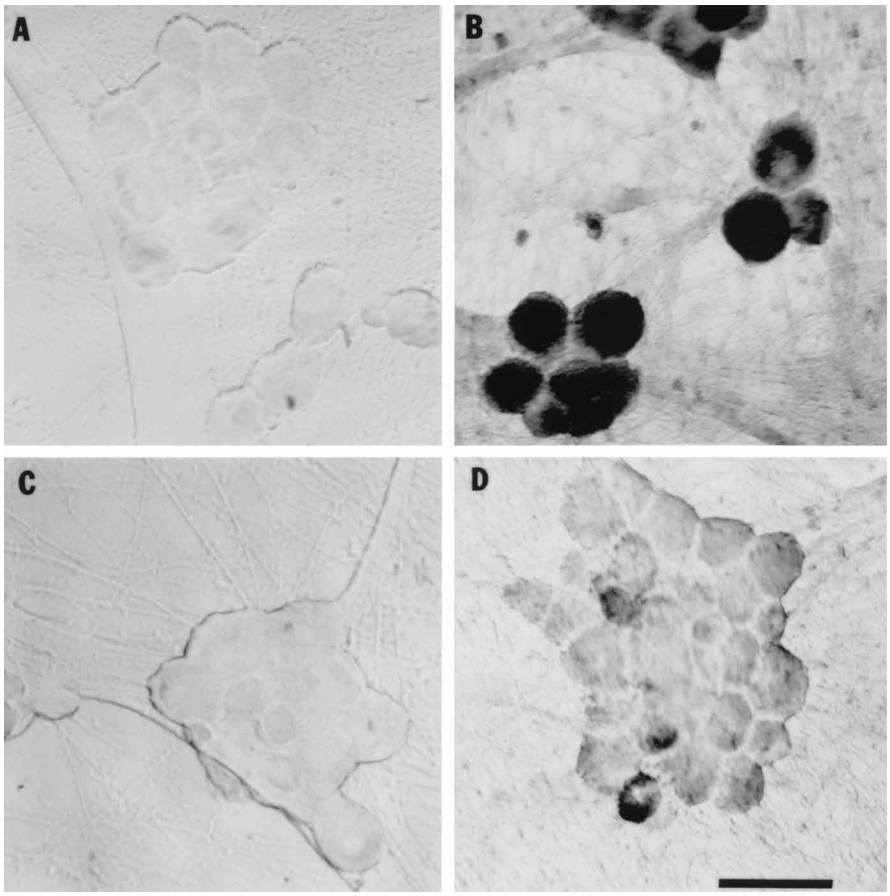

FIG. 9. Immunohistochemical detection of ICP4 in neuronal cultures during reactivation of cultures latently infected with wild-type HSV-1 or the ICP0 mutant FXE. At 2 weeks after establishment of latent infection in sensory neuronal cultures with HSV-1(17^+) (wild-type) or FXE at a MOI of 5, cultures were treated with anti-NGF to induce reactivation. Cultures were fixed at 24 h after anti-NGF treatment and processed for immunohistochemistry to detect ICP4. (A) Latent infection with 17^+; (B) reactivation of latent infection with 17^+; (C) latent infection with FXE; (D) reactivation of latent infection with FXE. The bar in panel D is 50 μm and applies to all panels. Mock-infected cultures showed no evidence of staining (data not shown).

DNA was reduced, and the percentage of neurons expressing ICP4 upon reactivation was similarly reduced. The functional lesion in the mutants was most probably ICP0; this conclusion is supported by the data that all of the ICP0 mutants produced similar results and that ICP0 supplied in *trans* complemented the establishment of latency of the ICP0 mutant.

While the function of LAT remains unclear, the detection of LAT by in situ hybridization is a useful marker for evidence of latent viral genomes. Some evidence suggests that LAT detected by in situ hybridization potentially underrepresents the number of neurons containing viral genomes (23, 28). In addition, evidence indicates that LAT expression is regulated during the establishment of latency in neurons in vitro (35). However, the presence of LAT indicates the presence of HSV-1 DNA. Our results indicate that during the latent infection in the neuronal cultures with the ICP0 mutants, the number of LAT-positive neurons is significantly reduced compared with infection with wild-type virus.

The results from the slot blot analyses of viral DNA indicate that during the latent infection in the neuronal model with wild-type HSV-1, there were approximately 1.5 viral genome equivalents per neuron. In vivo, quantitative studies have detected the equivalent of several hundred copies of the viral genome for each LAT-positive neuron in latently infected ganglia (34). The biological significance of this large amount of viral DNA persisting in the ganglia remains unclear. Studies indicate that the amount of DNA in the ganglia during latency correlates with the extent of productive infection in the ganglia, and the number of LAT-positive neurons is unaffected (34). In the in vitro neuronal model, a productive infection does not occur, because of the use of acyclovir during the initial period after inoculation. The presence of latent ICP4-negative virus indicates that viral replication is not required for the establishment of latency, which is consistent with results obtained with the mouse model (33).

The ICP0 gene is encoded by the same region of the genome that encodes the minor LAT, and the ICP0 gene overlaps with the 3′ end of the major LAT. While the major LAT may have a function during reactivation, a function for the minor LAT has not yet been described (14). Because of the overlapping regions of the ICP0 gene and LAT region transcripts, the analysis of these results must take into consideration the possibility that a product(s) encoded by the opposite strand of DNA is altered by the mutations in the ICP0 gene. The observation that all of the ICP0 mutants, which have mutations in distinct regions of ICP0, produced similar phenotypes in the neuronal cultures makes this possibility less likely. The most convincing evidence for the role of ICP0 in the establishment of latency is that ICP0 expressed from the adenovirus vector provided complementation to the ICP0 mutant FXE. The adenovirus vector expressing ICP0 was not able to completely restore the frequency of LAT detection in the neuronal cul-

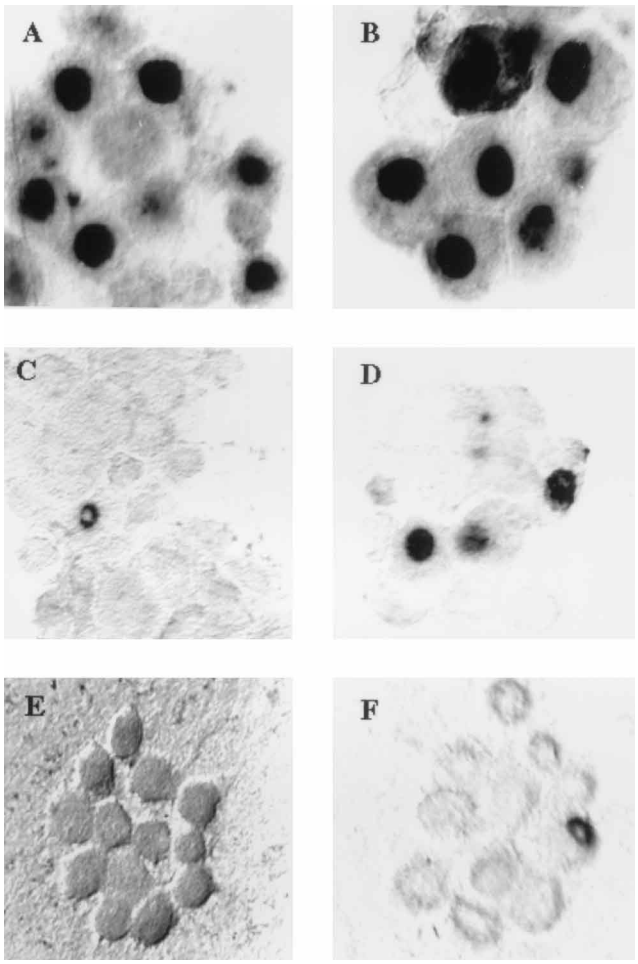

FIG. 10. Detection of LAT-positive neurons following coinfection of HSV-1 with an adenovirus vector. Neuronal cultures were infected for the establishment of latency with the HSV-1 and adenovirus vectors as indicated. The HSV-1 was either the wild-type virus 17^+ or the ICP0 mutant FXE. The recombinant adenovirus vector was either an ICP0-expressing vector (MLP-0) or a control, β-galactosidase-expressing vector (300_{Bst} β-gal). The MOI was 5 for 17^+ and FXE and 50 for the adenovirus vectors. In situ hybridizations show the detection of LAT in neurons during the latent infection with 17^+ alone (A), 17^+ with MLP-0 (B), FXE alone (C), FXE with MLP-0 (D), MLP-0 alone (E), or FXE with 300_{Bst} β-gal (F).

tures to wild-type virus levels. The level of expression or the temporal regulation of expression of ICP0 from the adenovirus vector may not have been optimal for complete replacement of the ICP0 gene product during the establishment of latency with the ICP0 mutant. However, these results show that coinfection of the ICP0-expressing adenovirus vector with FXE increased the number of LAT-positive neurons and restored the reactivation phenotype, strongly supporting the critical role of ICP0 in the establishment of latency.

From experiments in vivo, results similar to those reported here indicated a role for ICP0, not LAT region products, in the establishment of the latent infection. Restoration of a single copy of the ICP0 gene to another region of an ICP0 mutant virus partially restores the reactivation phenotype in the mouse trigeminal model (1). Removal of the ICP0 introns, which could affect the minor LAT, does not alter establishment or reactivation of latency (25).

ICP0 is a potent and nonspecific activator of gene expression in transfection assays and is required for the efficient onset of viral infection in a cell type-, cell cycle-, and multiplicity-dependent manner (2, 9, 30, 38). The mechanisms by which ICP0 achieves these functions are very poorly understood. However, it is becoming increasingly clear that interactions with cellular nuclear structures and proteins are critical for ICP0 activity. In the productive infection, at early times after infection, ICP0 transiently localizes to discrete subnuclear structures, known as ND10 or PODs, and as a consequence the cellular ND10 antigens are dispersed (11, 18–20). The cellular proteins in ND10 are of unknown function, but the ND10 structures can be modified by stress, cytokines, or viral infection (19). In addition, in patients with promyelocytic leukemia, ND10 becomes modified in the leukemic blast cells. This modification arises after a chromosomal translocation has linked the N-terminal portion of the PML protein (a normal constituent of ND10) to the retinoic acid receptor alpha (6, 39). Therefore, the status of ND10 appears to be linked to the physiology of the cell. Mutant ICP0 proteins which are defective in their ability to activate gene expression or stimulate the onset of viral replication also have defective interactions with ND10, either by failing to localize to ND10 in the first place (for example, deletion mutations in the C-terminal region or nuclear localization domain) or by failing to disperse the ND10 antigens (a result of mutations in the RING finger domain). The status of ND10 in neurons and the fate of ND10 during and after the establishment of the latent HSV-1 infection remain to be investigated. However, it is intriguing that the ICP0 mutant viruses used in this study express proteins that are defective in ND10 interactions (12, 18).

Our results indicate that ICP0 is necessary for the efficient

TABLE 3. Effects of coinfection with an ICP0-expressing adenovirus vector on the percentage of LAT-positive neurons detected during the latent infection[a]

Virus	% LAT positive neurons after transduction with[b]:		
	Mock infected	ICP0	β-Galactosidase
FXE	1.5 ± 0.7	16.1 ± 3.1	1.8 ± 0.4
17^+	63.7 ± 4.3	64.4 ± 5.6	ND[c]

[a] Sensory neuronal cultures were examined during the latent infection (14 days postinoculation) for LAT by in situ hybridization methods after inoculation with the ICP0 mutant FXE or the wild-type virus 17^+ at an MOI of 2.5. At the time of inoculation with HSV-1, the neuronal cultures were mock infected or coinfected with an MOI of 50 of either an adenovirus vector encoding ICP0 or β-galactosidase. The frequency of LAT-positive neurons was determined from cell counts of five random fields (containing 20 to 50 neurons each) per culture.
[b] Results are expressed as the mean percentage of LAT-positive neurons from three cultures ± standard error of the mean.
[c] ND, not determined.

TABLE 4. Frequency of reactivation of latent wild-type HSV-1 and the ICP0 mutant FXE with or without coinfection with adenovirus vectors[a]

Virus	% of cultures with infectious HSV-1[b]	
	Mock infected	MLP-0 coinfected
17^+	50 (5/10)	60 (6/10)
FXE	10 (1/10)	50 (5/10)

[a] Sensory neuronal cultures were inoculated with HSV-1(17^+) (wild type) or with the ICP0 mutant FXE at an MOI of 2.5 and either mock infected or coinfected of the adenovirus vector expressing ICP0 (MLP-0) at an MOI of 50. At 14 days postinoculation, the cultures were treated with anti-NGF to induce reactivation of latent virus. At 5 days after anti-NGF treatment, the cultures were harvested and tested in plaque formation assays for infectious virus.
[b] The numbers in parentheses indicate the number of cultures that were positive for infectious virus per the total number of neuronal cultures tested.

establishment of latency. This suggests that during the infection with wild-type HSV-1, ICP0 enters the neuron in the virion or expression of ICP0 is necessary during the establishment of latency. The virion is estimated to contain 100 to 200 molecules of ICP0 (43). Without a functional ICP0 gene, the mutant virus efficiently infected the neurons but was subsequently lost, suggesting that the HSV-1 DNA is unstable in the absence of ICP0. The mechanism of action of ICP0 in the establishment remains to be determined and may provide a unique target for intervention in the establishment of the latent HSV-1 infection.

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