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Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate

(stages of viral pathogenesis/acyclovir-resistance/latency-associated transcript/superinfection rescue/neuronal vectors)

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ABSTRACT **Herpes simplex virus infection of mammalian hosts involves lytic replication at a primary site, such as the cornea, translocation by axonal transport to sensory ganglia and replication, and latent infection at a secondary site, ganglionic neurons. The virus-encoded thymidine kinase, which is a target for antiviral drugs such as acyclovir, is not essential for lytic replication yet evidently is required at the secondary site for replication and some phase of latent infection. To determine the specific stage in viral pathogenesis at which this enzyme is required, we constructed virus deletion mutants that were acyclovir resistant and exhibited no detectable thymidine kinase activity. After corneal inoculation of mice, the mutants replicated to high titers in the eye but were severely impaired for acute replication in trigeminal ganglia and failed to reactivate from ganglia upon cocultivation with permissive cells. Nevertheless, latency-associated transcripts were expressed in neuronal nuclei of ganglia from mutant-infected mice and superinfection of the ganglia with a second virus rescued the latent mutant virus. Thus, contrary to a widely accepted hypothesis, the thymidine kinase-negative mutants established latent infections, implying that neither thymidine kinase activity nor ganglionic replication is necessary for establishment of latency. Rather, thymidine kinase appears to be necessary for reactivation from latency. These results suggest that acyclovir-resistant viruses could establish latent infections in clinical settings and have implications for the use of genetically engineered herpesviruses to deliver foreign genes to neurons.**

Infection of mammalian hosts by herpes simplex virus (HSV) involves several stages. Initially, the virus replicates lytically at a primary site in the skin, cornea, or mucosa. The virus then gains access to nerve terminals and is transported by axonal transport to the secondary site of replication, sensory ganglia. Subsequently, HSV can establish and maintain a latent infection in ganglionic neurons during which infectious virus is not detectable and viral gene expression and DNA replication are severely restricted (1–6). In humans, reactivation of virus from latent infections is responsible for recurrent episodes of disease. Latent infections cannot be cured by currently available antiviral drugs including acyclovir (2–8).

HSV encodes at least 70 proteins (9), of which 30–40 are required for lytic replication in growing mammalian cells (10). The HSV-encoded thymidine kinase (tk), which phosphorylates deoxypyrimidine nucleosides and nucleoside analogs such as acyclovir, is among those viral gene products that are not required for lytic replication (11–13). Furthermore, studies in mice have shown that tk is not required for replication at the primary site of infection, at least when that site is the cornea. These studies have suggested instead that tk is required both for acute ganglionic replication and reactivatable latent ganglionic infections (for review, see refs. 3 and 6). Thus, tk appears to have evolved, at least in part, to function in the interaction of HSV with the ganglionic neuron, which is the site for three stages in HSV pathogenesis—secondary replication, latency, and reactivation.

A widely accepted hypothesis (14–16) for the role of tk in these stages of HSV pathogenesis is that tk is required for the establishment of infection of ganglionic neurons. However, it has not been technically possible until recently to distinguish between a requirement for tk during establishment of infection and a requirement at later stages such as reactivation from latency or a requirement during earlier events such as transport of viral DNA to neuronal nuclei (for review, see ref. 6). Moreover, certain mutant viruses have been described that express little or no tk, yet establish reactivatable latent infections in mice (17–21). Thus, a definitive role for tk in latent infection has not been determined.

The role of tk in viral pathogenesis is of special clinical interest because tk-deficient mutants are resistant to the widely used anti-HSV drug, acyclovir. This resistance stems from the requirement for viral tk to activate acyclovir efficiently (12, 13, 22–25). Such tk-deficient, acyclovir-resistant mutants can arise during treatment of human herpesvirus infections with acyclovir, especially in immunocompromised patients, and increasingly have been associated with poor response to acyclovir therapy (26–35). If tk is not required for the establishment of latent infection, acyclovir-resistant mutants may be able to persist in an infected patient. Moreover, there are anti-HSV drugs under development that are designed to block viral replication in mammalian hosts by inhibition of tk (36, 37) and thus depend upon an essential role for tk during human infections.

To define more precisely the role of tk in HSV pathogenesis, we constructed tk-negative mutants containing defined lesions in the *tk* gene and tested these viruses in a mouse eye model for acute replication in the eye and trigeminal ganglia and for reactivatable latent infections. The finding that specific RNA species (latency-associated transcripts, LAT) are prominent in latently infected neurons (38–42) and the development of a superinfection–rescue assay (43) allowed us to determine whether the genomes of these mutant viruses can reach trigeminal ganglia and whether they exhibit biological activity characteristic of the latent state. This work has been presented in part.††

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Abbreviations: HSV, herpes simplex virus; tk, thymidine kinase; LAT, latency-associated transcript; PFU, plaque-forming unit(s).

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MATERIALS AND METHODS

Cells and Viruses. African green monkey kidney (Vero) cells, 143 cells, and the KOS strain of HSV were propagated as described (44). The replication-incompetent ICP27 deletion mutant *5dl*1.2 was propagated and assayed on 3.3 cells as described by McCarthy *et al.* (45).

To construct *dl*sptk, a plasmid containing HSV strain KOS sequences from the *Bgl* II site (Fig. 1) to the *Pvu* II site downstream of the *tk* gene was digested to completion with *Pst* I and *Sph* I and treated with T4 DNA polymerase to remove 3′ overhanging sequences. The larger fragment was purified from an agarose gel, recircularized with T4 DNA ligase, and used to transform *Escherichia coli*. Bacterial colonies containing a plasmid with the appropriate deletion were identified by restriction enzyme mapping. One such plasmid was transfected into Vero cells with infectious KOS DNA as described (49). Progeny virus were plated at 37°C in 100 μM acyclovir; plaques resistant to acyclovir were picked and screened for the deletion mutation as described (50) after *Bam*HI digestion. Two independently derived isolates were obtained.

To construct *dl*sactk, pKOS17B2 (44) was digested with *Sst* I (an isoschizomer of *Sac* I), the 4-base 3′ overhanging sequences were removed with T4 DNA polymerase, and the DNA was recircularized with T4 DNA ligase. The ligation mixture was digested again with *Sst* I and used to transform *E. coli*. Bacterial colonies containing an appropriately deleted plasmid were screened by resistance of plasmid DNA to *Sst* I and by digestion with *Alu* I, which recognizes the four central bases recognized by *Sac* I. Transfection, acyclovir selection, and screening were performed as above, except that virion DNAs were screened for the loss of the *Sac* I site, both by digestion with *Alu* I and with *Bam*HI plus *Sst* I. Both deletion plasmids transferred acyclovir resistance to recipient infectious DNAs at frequencies severalfold above those obtained with infectious DNA alone.

Plaque reduction assays to measure susceptibilities to acyclovir were performed as described (51). tk assays were performed as described (22, 23) using 143 cells.

Assays of Acute and Latent Infections in Mice. Acute virus titers in the eye and trigeminal ganglia and reactivatable latent infections in trigeminal ganglia were assayed after corneal inoculation of CD-1 mice (Charles River Breeding Laboratories) as described (21, 43).

***In Situ* Hybridization.** The methods used for *in situ* hybridization have been described (52, 53). The labeled double-stranded DNA probe used in Fig. 3 was plasmid pIPH containing a 1.4-kilobase-pair *Pst* I–*Hpa* I fragment from the LAT coding sequences. Similar results were obtained with labeled purified insert DNA sequences from pIPH.

Superinfection–Rescue Assays. Rescue of virus from latently infected ganglia by superinfection with HSV mutant *5dl*1.2 was performed as described by Leib *et al.* (43). Briefly, ganglia were removed from mice infected 30 days previously, dissociated, and the dissociated cells infected at high multiplicity with *5dl*1.2. Vero cells in suspension were then added and allowed to settle. A methylcellulose overlay was then added. After plaques formed, they were visualized with neutral red, counted, and picked. Viral DNA was obtained from plaque isolates and analyzed by Southern blot hybridization as described (50).

††Jacobson, J., Goldstein, D., Weller, S., Leib, D., Bogard, C., Kosz-Vnenchak, M., Schaffer, P., Knipe, D. & Coen, D., 13th International Herpesvirus Workshop, Aug. 7–13, 1988, University of California, Irvine, CA.

RESULTS

Construction of Acyclovir-Resistant HSV Mutants with Defined Deletions in the *tk* Gene. Previous studies of the role of tk in HSV pathogenesis mainly have used spontaneously arising tk-deficient mutants (for review, see ref. 6). A capacity to revert and/or the lack of penetrance of *tk* mutations in certain of these mutants may have contributed to their ability to establish reactivatable latent infections in some cases (e.g., refs. 17, 18, 20, and 21). We, therefore, constructed two HSV mutants with specific deletions in the *tk* gene, reasoning that deletion mutations would be more penetrant and less likely to revert. One mutant, *dl*sptk, contains a 360-base-pair deletion removing roughly the middle one third of *tk* coding sequences; the other, *dl*sactk, contains a small deletion that removes a *Sac* I restriction site and should shift the tk translational reading frame (Fig. 1). Neither mutation lies within the open reading frame of the UL24 gene, which overlaps the *tk* gene; mutations in UL24 can decrease HSV growth in cell culture (9, 54). The mutants were selected for resistance to acyclovir after transfection of Vero cells with wild-type HSV strain KOS DNA and plasmids containing the deletion mutations. The presence of the deletions in the viruses was confirmed by restriction enzyme digestion, Southern blotting, and hybridization with radiolabeled *tk* sequences. Isolates containing the deletions were plaque purified three times, after which no undeleted *tk* genes were detectable by Southern blot hybridization.

Both mutants were tested for resistance to acyclovir in plaque reduction assays (51) and proved highly resistant. Doses of about 200 μM acyclovir were required to reduce plaque formation 50%—i.e., about 50-fold higher than that of their wild-type parent. The mutants failed to induce detectable tk activity in tk-deficient human 143 cells (Table 1). Similar results were obtained by measuring thymidine anabolism in infected 143 cells (D.M.C., unpublished results).

Replication and Latency Competence of the tk Mutants in a Mouse Eye Model. We tested the replication and latency competence of these two mutants after corneal inoculation of CD-1 mice. By this route HSV establishes reactivatable latent infections in trigeminal ganglia (14–16). Inoculation of mice with either of the two deletion mutants or wild-type KOS at a dose of 2×10^6 plaque-forming units (PFU) per eye led to comparable titers of infectious virus in the eye 24 hr after infection (Fig. 2; Table 1). These titers represented viral growth rather than residual inocula, because infectious virus was only just at or below the limit of detection 3 hr after infection (Fig. 2). Titers of mutant viruses in the eye declined to lower levels more quickly during the next 3 days than did the titer of wild-type virus (Fig. 2).

In contrast to their replication competence at the primary site of infection, infectious *tk* mutant viruses were nearly undetectable in trigeminal ganglia during the first several days after infection. Only 1 of 22 ganglia from mice infected with the mutants yielded any infectious virus and in that instance only just at the limit of detection (5 PFU per ganglion). Thus, titers of infectious mutant virus in ganglia 3 days after infection were at least four orders of magnitude below those of wild-type KOS (Table 1). The behaviors of the two deletion mutants during the acute phase of infection generally agree with those reported for spontaneously arising tk-negative viruses (14–16), although the more rapid decline in mutant virus titers in the eye has not been reported previously.

To examine the ability of the mutants to reactivate from latent infections, we cultured explanted ganglia with Vero cells. None of 14 ganglia from mice infected 30 days previously with mutant *dl*sptk yielded infectious virus upon cocultivation with Vero cells, although 10/10 ganglia from mice infected with KOS did (Table 1). We attempted to stimulate

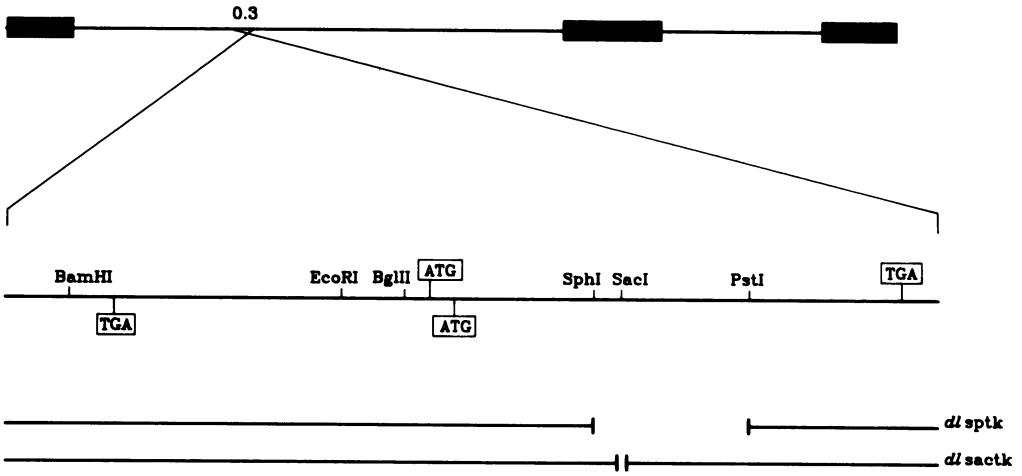

FIG. 1. Locations of deletion mutations in the HSV *tk* gene. (*Upper*) HSV genome in the prototype arrangement with the major repeat sequences shown as solid boxes. The location of the *tk* gene is indicated at map coordinate 0.3. (*Lower*) Diagonal lines connect to an expanded view of this region with selected restriction endonuclease restriction sites indicated. The ATG and TGA marked above the line indicate the beginning and end of the tk coding region (46–48); the ATG and TGA marked below the line indicate the beginning and end of the UL24 open reading frame (9). Locations of the deletion mutations in mutants *dl*sptk and *dl*sactk are shown.

reactivation by co-cultivation in the presence of 200 mM dimethyl sulfoxide, a treatment that greatly enhances the reactivation frequency of certain wild-type strains and mutants from latently infected ganglia (43, 55). However, no virus was recovered from ganglia from mice infected with either of the *tk* mutants reactivated under these conditions (Table 1). Thus, the two deletion mutants were unable to reactivate from latent infections in agreement with most past studies of mutants that do not express detectable levels of tk (for review, see ref. 6).

The *tk* Mutant Viruses Express LAT. The results just described left unanswered the question of whether the *tk* mutants were defective in their ability to reactivate from latent infections or at earlier stages, such as their ability to establish latency. To address this question, we first examined mutant-infected ganglia for LAT expression. Mice were mock-infected or infected with KOS or with either of the *tk* deletion mutants. Thirty days later, ganglia were sectioned and prepared for *in situ* hybridization with a radiolabeled plasmid containing sequences encoding LAT. As shown in Fig. 3, no signal was detected in ganglionic sections from mock-infected mice, whereas silver grains were concentrated over neuronal nuclei in ganglionic sections from mice infected with either KOS or mutant *dl*sactk. Similar hybridization was observed in ganglionic sections from mice infected with mutant *dl*sptk (data not shown). The numbers of grains per nucleus were similar in sections from mice infected with wild-type virus or *tk* deletion mutants. No specific signal was obtained when sections were probed with plasmids containing HSV sequences encoding the major DNA-binding protein, ICP8, or with vector sequences alone as controls for nonspecific hybridization (data not shown).

Table 1. tk activities and acute and latent infections by *tk* mutants and wild-type strain KOS

Virus	% tk activity*	Acute virus titer†		No. of ganglia reactivating/ total no. of ganglia	
		Eye	Ganglia	− DMSO	+ DMSO
KOS	100	5.4 ± 0.3	4.8 ± 0.2	10/10	2/2
*dl*sptk	0.3	5.2 ± 0.3	<0.7	0/14	0/5
*dl*sactk	0.2	5.6 ± 0.1	<0.7	ND	0/5

DMSO, dimethyl sulfoxide at 200 mM during co-cultivation; ND, not done.

*Activities were normalized to those of KOS-infected cell extracts. Mock-infected activity was 0.4% that of KOS.

†Acute virus titers are presented as log titer (mean ± SD). Peak titers in the eye occurred 1 day after infection. Peak titers in ganglia (mean of four samples) occurred 3 days after infection.

Data from several *in situ* hybridization experiments are compiled in Table 2. LAT-positive sections were detected frequently in ganglia from mice infected with the *tk* deletion mutants (12/16). Although the frequencies of positive sections (17/17) and positive cells per section were somewhat (2- to 5-fold) higher in ganglia from wild-type infected mice, the fact that LAT expression was readily detected in most ganglia from mice infected with the *tk* mutants demonstrated that mutant virus DNA reached the neuronal nuclei and was biologically active.

The *tk* Mutants Can Be Rescued by a Superinfecting Virus. As a second test for biologically active mutant DNA in

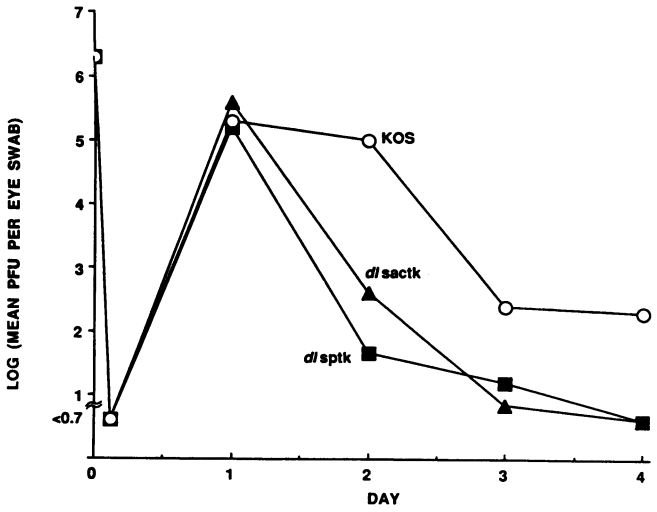

FIG. 2. Growth of *tk* deletion mutants and KOS in the eye. At the times indicated after infection, acute replication in the eye was measured by swabbing with cotton moistened with medium, dipping the swabs in 1 ml of medium, freezing, thawing, and assaying directly for infectious virus on Vero cells. Each point represents the logarithmic mean of infectious virus from at least four eye swabs. The zero time point shown indicates the size of the virus inocula, 2×10^6 PFU. The break in the ordinate indicates the limits of detection, 5 PFU per eyeswab [<0.7 log (PFU)]. Points below this break yielded no detectable infectious virus.

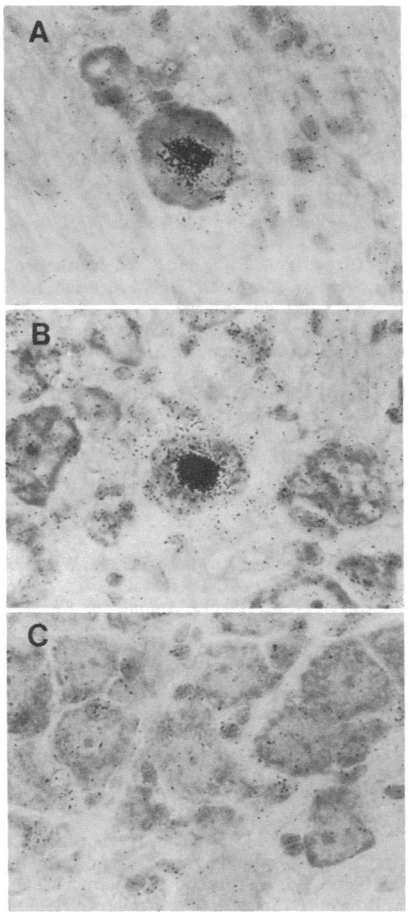

FIG. 3. Detection of LAT in trigeminal ganglion neurons by *in situ* hybridization using a labeled LAT gene double-stranded DNA probe. Shown are sections of ganglia from wild-type virus infection (*A*), *dl*sactk virus infection (*B*), and mock-infection (*C*). (×230.)

ganglia, we performed a superinfection rescue assay (43). Sixteen ganglia from mice that had been infected 30 days previously with *dl*sactk were dissociated and superinfected with a replication-incompetent mutant, 5*dl*1.2. The superinfected ganglion cells were then tested for their ability to give rise to plaques when mixed with Vero cells. All 16 superinfected ganglia yielded HSV plaques, with a mean of 29 plaques per ganglion (Table 3). Southern blot analysis of DNA from six individual plaque isolates indicated the presence of both mutant and undeleted *tk* alleles, demonstrating that the recovered virus resulted from complementation, recombination, or both following superinfection (unpublished results). In contrast, no virus was recovered after mock superinfection of ganglia from mice infected 30 days previously with *dl*sactk or after superinfection with 5*dl*1.2 of ganglia from mice that were mock-infected 30 days previously (Table 3). These results demonstrate that the *tk* mutant

Table 2. LAT expression in ganglia from mice infected with *tk* mutants and wild-type strain KOS

Virus	No. of mice with positive ganglia/ total no. of mice examined	No. of positive ganglia/ total no. of ganglia examined	No. of positive cells/ no. of sections examined
KOS	9/9	17/17	164/21
*dl*sptk	4/5	8/10	35/21
*dl*sactk	3/3	4/6	40/16

Data are from experiments such as those shown in Fig. 3 or experiments using probes including sequences flanking LAT-encoding sequences.

Table 3. Superinfection rescue of *tk* mutants from latently infected ganglia

Infecting virus	Superinfecting virus	No. of ganglia yielding plaques/no. of ganglia superinfected
*dl*sactk	5*dl*1.2	16/16
*dl*sactk	Mock-infected	0/6
Mock-infected	5*dl*1.2	0/4

Ganglia were removed from mice either mock-infected or infected 30 days previously with *dl*sactk and tested for the presence of virus that can be rescued by superinfection with mutant 5*dl*1.2 (43).

DNA was present in ganglia in a biologically retrievable state through 30 days after infection.

DISCUSSION

HSV tk is an example of a viral gene product that has a stage-specific role in pathogenesis. In the mouse eye model, it is required at some point after primary replication at the site of inoculation. We sought to define the role of tk more precisely. Our results demonstrate that after corneal inoculation with tk-negative mutants, viral DNA can reach neuronal nuclei in trigeminal ganglia, be maintained in a biologically retrievable state, and express LAT. We conclude from these results that tk-negative mutants establish latent infections.

We conclude further that tk activity is not required for establishment of latent infections. This conclusion differs from the widely accepted hypothesis (14–16) that tk is necessary for establishment of neuronal infection. Rather, it appears that tk-negative mutants can infect ganglionic neurons but do not multiply within them.

Because tk-negative mutants are severely impaired for acute ganglionic replication yet still establish latency, we conclude that acute ganglionic replication is not required for establishment of latent infections. This conclusion is consistent with suggestions derived from earlier experiments with temperature-sensitive mutants, antiviral drugs, and circulating antibody (for review, see refs. 2–6). However, the finding of larger numbers of LAT-positive neurons in wild-type infected ganglia (Table 2) and detection of more HSV DNA in wild-type infected ganglia than in *tk* mutant-infected ganglia (K. Hicks, D. Yager, J. Katz, and D.M.C., unpublished results) could be interpreted to suggest that acute ganglionic replication serves to increase the number of cells harboring latent virus and/or the number of latent viral genomes. Alternatively, the greater amount of wild-type virus in the eye after day 2 (Fig. 2) may contribute to these differences.

Our results strongly suggest that tk is required specifically for reactivation from latent infections. In the case of *dl*sptk, we infer that the decline in levels of virus in the eye on days 2–4 after infection, the severely diminished titers in ganglia during the same period and failure to reactivate from latent infections are all due to the deletion mutation because indistinguishable results were obtained with two independent isolates of this mutant (unpublished results). A requirement for tk could be explained if there is a requirement for sufficient pools of its enzymatic products, deoxypyrimidine nucleotides, in each of these settings to permit viral DNA replication. We cannot, however, exclude other reasons for a requirement for tk.

Clinical Implications. Acyclovir-resistance is a problem of growing clinical importance as the numbers of immunocompromised patients increase due to AIDS, transplants, and cancer chemotherapy (56). Acyclovir-resistant tk-deficient viruses have been isolated from patients, some of whom had poor responses to acyclovir (26–35). The data presented here indicate that such resistant viruses can establish latent in-

fections. If one can extrapolate from the mouse model to the human setting, it is conceivable that latent acyclovir-resistant virus might be reactivated by superinfection with tk-positive viruses, leading to mixed populations of virus that could retain both pathogenicity and resistance (57, 58) and greater persistence in the affected patient populations.

In addition, efforts have been made to develop drugs that could act against HSV infections in humans by specific inhibition of viral tk activity (36, 37). Our results suggest that such drugs could inhibit HSV reactivation from latency such that they might be useful prophylactically during immunosuppressive treatments. However, based on our results, if such drugs were given during the acute phase of infection they may not prevent the establishment of latency, while removal of drug inhibition might lead to reactivation. We have previously argued that such drugs may also have to be given at relatively high doses, because levels of tk activity less than 10% those of wild-type are sufficient for acute replication and reactivatable latent infections in the mouse eye model (21).

A Potential Vector for Neurons. Based on studies using neuronal cell cultures *in vitro*, HSV has been proposed as a vector to deliver genes to mammalian neurons *in vivo* (59, 60). For this purpose, it would be advantageous for the virus to infect neurons specifically and nondestructively and to express the gene product of interest efficiently and stably. The *tk* deletion mutants described here do not replicate in ganglia, yet express LAT efficiently and stably in mouse neurons *in vivo*. Modified to place the gene of interest under the control of LAT or neuron-specific gene expression signals, perhaps by replacement of *tk* sequences, these mutants may offer all of these advantages.

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