JOURNAL OF VIROLOGY, Nov. 1993, p. 6903–6908
0022-538X/93/116903-060$02.00/0

Vol. 67, No. 11

Herpes Simplex Virus Thymidine Kinase and Specific Stages of Latency in Murine Trigeminal Ganglia

JENNIE G. JACOBSON,[1,2]† KATHERINE L. RUFFNER,[1] MAGDALENA KOSZ-VNENCHAK,[3]‡ CHARLES B. C. HWANG,[1]§ KRISTIN K. WOBBE,[1]‖ DAVID M. KNIPE,[3] AND DONALD M. COEN[1]*

Department of Biological Chemistry and Molecular Pharmacology,[1] Committee on Virology,[2] and Department of Microbiology and Molecular Genetics,[3] Harvard Medical School, Boston, Massachusetts 02115

Received 25 June 1993/Accepted 16 August 1993

From marker rescue, sequencing, transcript, and latency analyses of the thymidine kinase-negative herpes simplex virus mutant *dl*sactk and studies using the thymidine kinase inhibitor Ro 31-5140, we infer that the virus-encoded thymidine kinase is required in murine trigeminal ganglia for acute replication and lytic gene expression, for increasing the numbers of cells expressing latency-associated transcripts, and for reactivation from latent infection.

Latent infection of a mammalian host by herpes simplex virus (HSV) proceeds via replication at the periphery; access to nerve terminals; replication in ganglia, with full expression of viral lytic genes; and establishment of latency, in which the latency-associated transcripts (LAT) are the predominant viral gene products. The virus can later reactivate from latency, leading to recrudescent disease.

The role of the virus-encoded thymidine kinase (TK) in latent infection has been the subject of considerable debate, even when restricting the discussion to the most commonly used animal model system, the mouse. Several laboratories have reported that TK-negative (*tk*$^-$) mutants fail to replicate within, efficiently express lytic genes in, or reactivate from mouse ganglia (7, 10, 14, 27, 34, 37–39, 43, 53, 54). Nevertheless, ganglia from mice infected with such mutants contain substantial amounts of HSV DNA (22, 34), express LAT (7, 34, 56), and harbor genomes that can complement, recombine with, and/or be rescued by superinfecting virus (7, 10). Accordingly, we and others have concluded that *tk*$^-$ mutants establish latency (7, 10, 34). However, this conclusion has been challenged on various grounds (4, 52, 56, 58).

Additionally, it has not yet been possible to conclude that the latency phenotypes exhibited by *tk*$^-$ mutants were due to mutations affecting TK and only TK. This has been complicated by a lack of agreement on what constitutes a *tk*$^-$ mutant. We define *tk*$^-$ mutants as being completely devoid of TK activity and distinguish them from TK-defective mutants, which, while impaired, express some TK activity. Certain viruses described as *tk*$^-$ have exhibited ganglionic replication and/or reactivation upon explanation in studies as recent as 1992 (43, 53, 55, 57, 58). Moreover, one mutant described as *tk*$^-$ (34) exhibited wild-type numbers of cells expressing LAT in infected ganglia, while two others (7, 56) exhibited diminished numbers. This has raised the possibility that certain of these viruses were not truly *tk*$^-$ and expressed some TK owing to leaky or reverting mutations and thus replicated in and reactivated from ganglia or expressed LAT in wild-type numbers of cells. An alternative possibility, propounded in an influential review (45), is that other *tk*$^-$ mutants contained mutations in additional genes and that these were responsible for the various latency phenotypes. As the *tk* gene overlaps the *UL24* gene (Fig. 1), certain *tk* mutations could also affect it (19). A number of studies investigating the role of TK in pathogenesis and latent infection have, in fact, examined viruses that were mutated in both *tk* and *UL24* (14, 34, 38, 40, 50). Even when care was taken to leave the *UL24* open reading frame intact, deletions that might alter expression of the longer of its two putative transcripts were used (7, 10). To our knowledge, there has been no demonstration via marker rescue that latency phenotypes observed were indeed the result of a mutation within the *tk* gene and not another gene. (The one marker rescue study of which we are aware [34] involved a *tk-UL24* double mutant, and the rescuing fragment was much longer than the *tk* gene.)

Marker rescue of *dl*sactk. To address these issues, we wished to determine whether the latency phenotypes of mutant *dl*sactk (7, 27) were the result of the small deletion engineered at the *Sac*I site in *tk* but not *UL24* coding sequences (7) (Fig. 1). We therefore rescued the *tk* mutation by cotransfecting *dl*sactk infectious DNA with wild-type strain KOS *Eco*RI-N fragment DNA (Fig. 1), as described previously (12). TK-positive progeny were selected by hypoxanthine-thymidine-methotrexate selection in 143 cells (3, 42). One such virus, SacTK$^+$/R/N, was plaque purified twice more and shown by Southern blot hybridization analysis (46) to be restored for the *Sac*I site missing in *dl*sactk. Unlike *dl*sactk (7), SacTK$^+$/R/N exhibited wild-type sensitivity to acyclovir, indicating that marker rescue restored TK activity (46).

Sequencing of *dl*sactk and SacTK$^+$/R/N. To determine the relevant nucleotide differences between *dl*sactk and SacTK$^+$/R/N, regions corresponding to the 2.4-kb *Eco*RI-N fragment used to rescue *dl*sactk were amplified from the two viruses by polymerase chain reaction and sequenced directly without cloning (16) by methods that will be described elsewhere (17). The only difference between the nucleotide sequences of *dl*sactk and SacTK$^+$/R/N within this region was the expected 4-bp deletion at the *Sac*I site in *dl*sactk; this deleted sequence was restored in SacTK$^+$/R/N. Thus, differences in phenotypes

* Corresponding author.
† Present address: Upjohn Laboratories, Kalamazoo, MI 47001.
‡ Permanent address: Institute of Molecular Biology, Jagiellonian University, Cracow, Poland.
§ Present address: Harvard School of Dental Medicine, Boston, MA 02115.
‖ Present address: Waksman Institute, Rutgers University, Piscataway, NJ 08854.

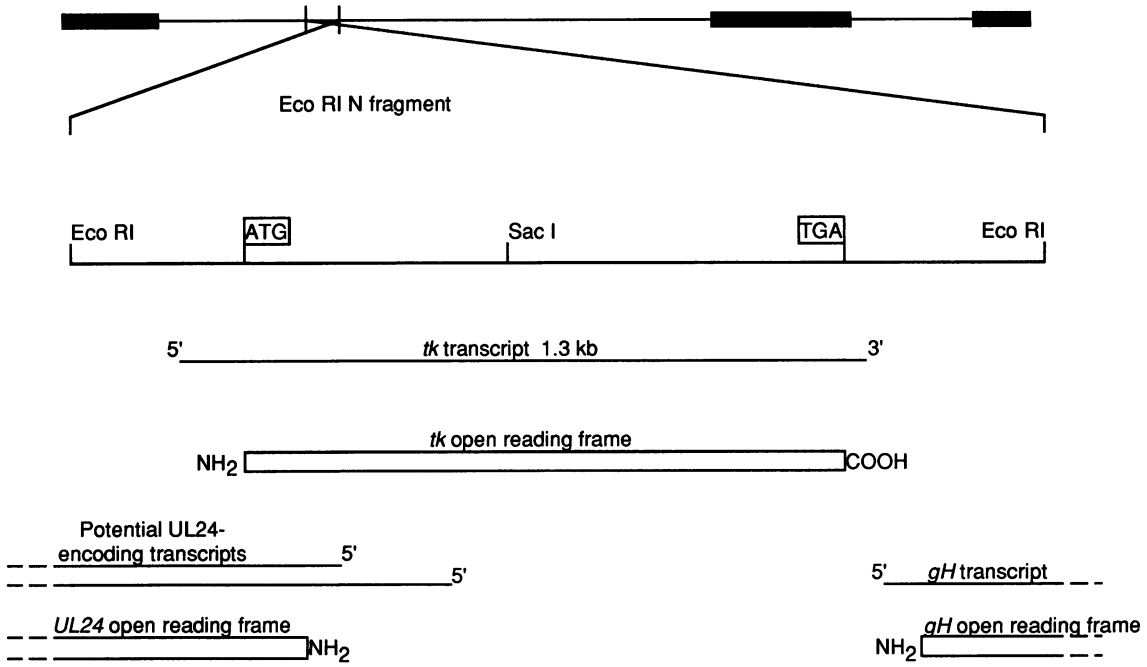

FIG. 1. The HSV *tk* locus. The top line provides a schematic representation of the HSV genome, with the repeat sequences shown as black boxes. The location of the *Eco*RI-N fragment in the prototype arrangement of HSV DNA is indicated. The second line provides an expanded view of the *Eco*RI-N fragment. The locations of the *tk* initiation (ATG) and termination (TGA) codons and restriction sites for *Eco*RI and *Sac*I are indicated. Below this line the map locations for *tk* and *gH* and two potential UL24-encoding transcripts are shown as lines with 5′ and 3′ ends indicated, while the locations of the *tk*, *UL24*, and *gH* open reading frames are shown as open boxes.

between *dl*sactk and SacTK$^+$/R/N can be ascribed to this deletion.

Latency phenotypes: ganglionic replication and lytic gene expression. Following corneal inoculation of mice, *dl*sactk was comparable to the wild-type KOS for acute replication at the site of inoculation but failed to replicate and was severely restricted for lytic gene expression in the ganglia during the first few days postinoculation (7, 27). To determine whether these phenotypes were the result of the engineered 4-base deletion in the *tk* gene of *dl*sactk, we compared acute replication as described previously (6, 7, 30) and lytic gene expression as described previously (27) for KOS, *dl*sactk, and the rescued virus, SacTK$^+$/R/N. All three viruses were present at comparable levels in the mouse eye (Table 1). These titers reflect replication rather than residual inocula, because virus titers at a few hours postinoculation were nearly undetectable, as was shown previously (7). However, whereas *dl*sactk failed to replicate detectably in the ganglia (as described previously [7]), both KOS and SacTK$^+$/R/N replicated to similar titers, more than 3 orders of magnitude greater than that of *dl*sactk (Table 1). Similarly, as reported before (27), although LAT expression 3 days postinoculation was readily detectable by in situ hybridization, performed as described previously (27), in multiple sections from two *dl*sactk-infected ganglia, the expression of lytic transcript *ICP4*, *ICP8*, or *gC* was not detectable. In contrast, the expression of all four transcripts was readily detected in multiple sections from all four ganglia infected with SacTK$^+$/R/N. We conclude that the failure to replicate and to express lytic genes efficiently in ganglia during the first few days following inoculation is the result of the engineered *dl*sactk mutation. A model to explain the effect of the *tk* mutation on lytic gene expression in neurons was discussed in a recent paper (28).

TABLE 1. Virus replication during the acute phase and LAT expression during latency

Virus	Peak virus titer[a]		LAT expression[b]	
	Eye swabs	Ganglia	No. of LAT$^+$ ganglia/ total (%)	Avg no. of LAT$^+$ cells/section
KOS	3.3 ± 0.3	4.0 ± 0.3	38/39 (97)	11.7
*dl*sactk	3.4 ± 0.3	<0.7	4/6 (67)	2.5
SacTK$^+$/R/N	2.9 ± 1.0	4.0 ± 0.2	2/2 (100)	15.0

[a] Log_{10} mean of results from four mice ± standard deviation. Eye swab titers are the result at 1 day postinoculation, and ganglia titers are the result at 3 days postinoculation.

[b] Determined by in situ hybridization using a LAT probe (33).

Numbers of LAT-expressing cells. Ganglia of mice infected with *dl*sactk contain LAT-expressing cells, although fewer such cells were found than in ganglia of wild-type-infected mice (7). As this difference was not observed with another *tk* mutant (34), we wished to determine whether it was the result of the *tk* deletion. We therefore performed in situ hybridization studies of ganglia harvested 30 days postinoculation with KOS, *dl*sactk, and SacTK$^+$/R/N as described previously (27). Ganglia from mice infected with KOS or SacTK$^+$/R/N contained similar numbers of LAT-expressing cells, while ganglia from mice infected with *dl*sactk contained roughly fivefold fewer LAT-expressing cells (Table 1), a result similar to our previous findings (7). Thus, this lower number is the result of the 4-bp deletion in *dl*sactk.

Reactivation. We had previously shown that *dl*sactk fails to reactivate from infected ganglia upon cocultivation of explanted ganglia with susceptible Vero cells (7). We wished to

TABLE 2. Effects of *tk* mutation or TK inhibitor on latency

Virus	No. of ganglia reactivating/no. of ganglia assayed by each protocol[a]		
	Dissociation	Cocultivation	Explant
*dl*sactk	0/18 (0%)	0/6 (0%)	0/8 (0%)
KOS	21/24 (88%)	6/6 (100%)	6/6 (100%)
SacTK$^+$/R/N	11/12 (92%)	ND	ND
KOS + drug[b]	2/6 (33%)	0/5 (0%)	2/6 (33%)

[a] Pooled results of several experiments. See text for description of protocols. ND, not done.
[b] Harvested in 64 μM Ro 31-5140 and then maintained in 100 μM Ro 31-5140.

determine whether it exhibited the same phenotype in two other reactivation protocols: an explant protocol developed for in situ hybridization studies (28), in which ganglia were explanted, bisected, and incubated for 2 days before virus was harvested (5), and a dissociation protocol, in which ganglia were dissociated into individual cells and plated onto a Vero cell monolayer. The latter assay was performed as described previously (31), except that any cultures that did not show cytopathic effect after 9 days were harvested for virus (5) and replated onto Vero cells under a methylcellulose overlay. Those cultures without visible plaques after an additional 10 days were judged to be virus negative. Certain mutants that appear defective for reactivation in the cocultivation protocol can exhibit wild-type reactivation in the dissociation protocol (31, 33), which evidently is more sensitive. *dl*sactk failed to reactivate from ganglia in any of the reactivation protocols, confirming and extending our previous findings (Table 2). Both KOS and SacTK$^+$/R/N reactivated efficiently (Table 2). Thus, rescue of the deletion restored the ability to reactivate from latent infection.

Transcripts of neighboring genes. We then considered whether the *dl*sactk mutation could exert its effects on functions other than *tk* that map nearby. The mutation was not expected to affect gH expression as the *gH* promoter had been mapped to within only 83 bp of the *gH* mRNA 5′ end (51), while the deletion lies nearly 800 bases upstream of this site (Fig. 1). This expected lack of effect was confirmed (see below). Similarly, the mutation should not affect the expression of the shorter of two potential *UL24* transcripts that have been identified because its 5′ end lies nearly 300 bases downstream of the deletion (20, 44, 59). This transcript is of the late kinetic class (20, 44), and all late promoters examined to date map within fewer than 100 bases upstream (11, 15, 21, 51).

However, the 4-bp deletion in *dl*sactk does lie within 50 bp of the apparent transcriptional start site of the longer potential *UL24* transcript (20, 24, 44, 60). We therefore wished to determine whether the deletion had any effect on the expression of this transcript. Cells were infected with *dl*sactk or with PKG7 (8), a control virus whose expression of the longer *UL24* transcript is comparable to that of the wild type (60). Infected-cell RNAs were prepared at 9 h postinfection, as described previously (61), when expression of this transcript was easily detected (60). Primer extension analysis was performed as described previously (61) with a radiolabeled primer specific for the *UL24* transcript and, as a control, a primer that detects *gH* mRNA, whose kinetics resemble those of the *UL24* transcript (60). Under the conditions used, the intensities of the primer extension signals were directly proportional to the amounts of HSV-infected cell RNA added to the reactions (60). Three primer extension products were observed (Fig. 2); one, 116 nucleotides (nt), specific for the *UL24* transcript; one, 55 nt, specific for the *gH* transcript; and one, about 100 nt, specific for a cellular transcript that hybridizes to the *gH* primer (M in Fig. 2). There was no apparent difference in the amount of *UL24* primer extension products relative to *gH* products between *dl*sactk (Fig. 2, lane 3) and the control virus (lane 2). There was also no apparent difference in the amount of either HSV transcript when normalizing to the cellular transcript that hybridizes to the *gH* primer. Thus, *dl*sactk was not discernibly affected for expression of the longer *UL24* transcript or for the *gH* transcript.

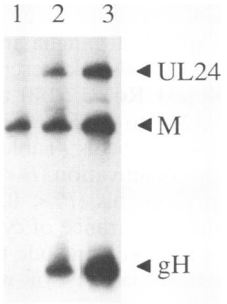

FIG. 2. Expression of the long potential *UL24* transcript. Vero cells were mock infected (lane 1) or infected with PKG7 (lane 2) or *dl*sactk (lane 3). Cytoplasmic RNA was prepared 9 h postinfection and analyzed by primer extension using a radiolabeled primer specific for the *UL24* transcript (CGAGACAATCGCGAACATCTACAC) and, as a control, a primer (GGTCTCGGTGGGGTATCG) that detects *gH* mRNA and a cellular transcript (M). The figure shows the resulting autoradiogram. The positions of extension products specific for the various transcripts are indicated to the right of the figure.

A TK inhibitor greatly decreases reactivation of wild-type virus without diminishing replication in cell culture. Studies investigating the role of TK in latency using inhibitors of this enzyme (1, 2, 23, 25, 32, 41) have generally been interpreted to support the notion that TK is required for reactivation from latency. However, a TK inhibitor diminished the ability of the virus to replicate in cultured mouse cells in the only study that examined such effects (32). As *tk*$^-$ mutants replicate to wild-type titers in these cells (46), these results left open the possibility that the inhibitors may have an effect on some other function in infected cells besides TK.

We therefore used Ro 31-5140, (35, 36), which was kindly provided by J. A. Martin (Roche Research Centre, Welwyn Garden City, United Kingdom), dissolved in dimethyl sulfoxide, and stored at −20°C. Ro 31-5140 inhibits HSV type 1 (HSV-1) TK potently (50% inhibitory concentration, 0.03 μM) but does not inhibit cellular thymidine TKs at concentrations as high as 200 μM (35, 36). We first tested the effect of this compound at 100 μM on HSV replication following infection at a multiplicity of infection of 3 of Vero cells or CD-1 mouse embryo cells (prepared with the kind assistance of C. Bogard, P. Carmillo, and P. Schaffer) in single-cycle growth assays as previously described (49). In Vero cells, the yields at 20 h postinfection for wild-type strain KOS were 210 PFU per cell in the presence of drug and 160 PFU per cell in its absence. In CD-1 mouse embryo cells, the yields were lower than in Vero cells, but unchanged by the presence of drug (2 PFU per cell). The yields reflected virus replication as orders of magnitude less virus were observed at 5 h postinfection during the eclipse phase (46). This concentration of Ro 31-5140 also did not affect KOS plaque formation or plaque size on Vero cells; however, it did strongly antagonize the action of acyclovir (46), as was previously reported (35, 36), indicating that it was entering the cell and inhibiting HSV TK.

We then tested the ability of Ro 31-5140 to inhibit reactivation by KOS from explanted ganglia in the three different protocols used to evaluate *dl*sactk described above. Ganglia were harvested in 64 μM Ro 31-5140 and then maintained subsequently at 100 μM Ro 31-5140. The drug diminished reactivation in all three protocols (Table 2), with statistically significant effects in the cocultivation ($P < 0.005$, Fisher's exact test) and dissociation protocols ($P < 0.025$). The drug also dramatically slowed the appearance of cytopathic effect in the dissociation protocol (18). We conclude that the TK inhibitor inhibits reactivation from latency of wild-type HSV from explanted mouse ganglia.

Is TK required for specific stages of latency? The simplest interpretation of our results is that TK is required for the stages of latent infection altered in *dl*sactk-infected mice. An alternative explanation is that the mutation affects a gene product arising from the *tk* locus other than TK which is responsible for the phenotypes. However, our transcript analyses lend no support to this explanation. There are two other, alternative, explanations. (i) The *gH* and *UL24* promoters in ganglionic neurons are very different from those in Vero cells, or (ii) as yet unknown gene products arise from the *tk* locus. In each case, the 4-bp deletion must affect these promoters or gene products in such a way as to confer the latency phenotypes observed. These alternative explanations are very complicated, and there is no evidence for them. Moreover, the independent approach of using a TK inhibitor supports the idea that TK is necessary for at least one stage of latency, reactivation. We therefore infer that TK is required for the various stages of latency that are impaired in *dl*sactk.

Certain viruses described as tk^- have exhibited detectable ganglionic replication and/or reactivation (43, 53, 55, 57, 58). We suspect that those viruses were not, in fact, completely tk^- but rather that they expressed small amounts of TK activity owing to either leakiness or reversion; indeed, in one case, reversion was documented (43). Even very low levels of TK are sufficient for ganglionic replication and/or reactivation from latency (6, 13, 17, 50, 54). Along these lines, Ro 31-5140 did not completely abolish reactivation, except perhaps in the cocultivation protocol. This may be a result of its failure to inhibit TK activity below the rather low levels that are sufficient for reactivation.

In contrast to our results, Leist et al. (34) found wild-type numbers of LAT^+ cells following inoculation with an engineered *tk* mutant. The engineered mutant used in that study was a *tk-UL24* double mutant in which *tk* protein-coding sequences were retained and, as discussed previously (22), it is possible that this mutant was not absolutely tk^-. The number of LAT^+ cells is frequently taken as a measure of the efficiency of establishment of latency. By this criterion, our results suggest that TK can affect the efficiency of this process. This could be due to the requirement for TK for ganglionic replication, which would increase spread of virus through the ganglion. We have observed a broader distribution of LAT^+ cells in wild-type-infected ganglia than in tk^--infected ganglia (29). Alternatively, the effect could be due to less efficient replication of *dl*sactk in the eye following day 1 postinoculation (7, 18), seeding fewer neurons on subsequent days.

Recently, a mutant described as tk^- was found to reactivate in an in vitro model of latency (58). This result was used to suggest that the failure of *tk* mutants to reactivate in mouse models was due to failure to establish latency in sufficient numbers of cells (4, 58). However, *dl*sactk-infected ganglia contain at least as many LAT^+ cells as another mutant which is reactivation competent (26). Moreover, the mutant used in the in vitro study (58) contained an insertion very near the C terminus of the gene and exhibited ganglionic replication following corneal inoculation of mice, suggesting that it may have expressed low levels of TK. Our result, that Ro31-5140 significantly decreased reactivation from wild-type-infected ganglia, where, by definition, virus established latency at wild-type efficiencies, argues further for a role for TK in reactivation.

We continue to conclude then that tk^- mutants establish latent infections on the basis of the presence in ganglia of sufficient amounts of viral genomes that are biologically active in sufficient numbers of cells (7, 10, 34). However, some investigators (52, 56) have considered such infections to be incomplete latency because of the importance of reactivation as a specific sign of latency. By this consideration, then, those wild-type-infected ganglia that did not reactivate in the presence of Ro 31-5140 were not completely latent. This indicates the difficulties inherent in an operational definition for the establishment of latency that requires that reactivation be part of latency.

Relevance to human infections. In certain animal models other than mice, tk^- mutants can reactivate from latency (38, 40), accentuating the issue of relevance of results in the mouse to human infections. TK-deficient mutants are rarely observed in an immunocompetent human, even under the pressure of acyclovir selection. Even in immunocompromised patients, acyclovir resistance is observed in only about 5% of cases (9). Although there have been examples in patients with AIDS of recurring acyclovir-resistant, TK-deficient HSV (e.g., see reference 48), we are unaware of any case in which the reactivated virus was shown to be a homogeneous population that was truly tk^-. Indeed, there is evidence for mixed populations of acyclovir-resistant virus (e.g., see reference 47) and for viruses that appear by sequence to be tk^- by virtue of frameshift mutations yet synthesize TK via unusual mechanisms (17). These observations are consistent with the idea that TK is important for reactivation in humans, much as it is in mice. Further analysis of recurring acyclovir-resistant clinical isolates will be interesting in this context.

We thank J. A. Martin (Roche Research Centre, Welwyn Garden City, United Kingdom) for kind provision of Ro 31-5140, D. Leib, and P. Schaffer for communication of unpublished results, M. Lachenmann for statistical analyses, P. Digard and J. Scales for enthusiastic figure preparation, J. Brown for help with manuscript preparation, C. Bogard, P. Carmillo, and P. Schaffer for assistance with CD1 cells, and M. Kramer, E. Pelosi, and S. Rundell for help with the mice.

This work was supported by NIH grants PO1 AI 24020 and RO1 AI26126.

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