JOURNAL OF VIROLOGY, June 1989, p. 2861–2865
0022-538X/89/062861-05$02.00/0

Vol. 63, No. 6

Latency-Associated Transcript but Not Reactivatable Virus Is Present in Sensory Ganglion Neurons after Inoculation of Thymidine Kinase-Negative Mutants of Herpes Simplex Virus Type 1

RICHARD B. TENSER,[1,2]* KATHLEEN A. HAY,[2] AND WADE A. EDRIS[2]

Departments of Medicine (Neurology)[1] and Microbiology,[2] The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Received 24 October 1988/Accepted 14 February 1989

The presence of herpes simplex virus (HSV) latency-associated transcript (LAT) was investigated in sensory ganglion neurons of mice after inoculation with thymidine kinase (TK) mutants of HSV. Ganglion serial sections were examined in order to quantitate numbers of LAT-positive neurons. After inoculation with TK-positive HSV, virus was isolated during latency from explants of most ganglia, and LAT was detected by in situ hybridization in 96% of ganglia. After inoculation with HSV TK mutants, virus was isolated from 0% of ganglia, but LAT was detected in 95 to 100% of ganglia. After inoculation of TK mutants of HSV, therefore, although latent infection as indicated by the isolation of virus from ganglion explants was not detected, the presence of LAT was common. These results suggest that the lack of reactivatable virus after inoculation of HSV TK mutants may be related to a role for HSV TK expression in the reactivation process.

Herpes simplex virus (HSV) readily establishes a latent infection of sensory ganglion neurons in humans (1, 6, 7, 9, 13) and in experimental animals (2, 13, 16, 19, 20). Since neurons are nondividing cells and since HSV thymidine kinase (TK) expression was found to be important for virus replication in nondividing cells in culture (10), we investigated sensory ganglion infection by TK-negative (TK^-) mutants of HSV. TK^- mutants replicated well in vivo in ocular tissues, but the incidence of latency was markedly decreased, and we hypothesized that HSV TK expression was important for the infection of neurons (21). This result has been supported by several other investigators (4, 8, 11, 12). In studies of latent infection with TK^- mutants, the endpoint for the detection of latency has been the isolation of virus from sensory ganglia during the period of latent infection, and inability to isolate virus would not differentiate between defects in the establishment, maintenance, or reactivation of TK^- HSV. Since we recently reported results which indicated that TK^- HSV might be defective for reactivation (22), in the present study we sought to further investigate this possibility by the use of in situ hybridization techniques.

In studies of HSV latency performed by in situ hybridization with HSV DNA probes, cRNA was detected in neurons from ganglia of latently infected humans (7) and experimental animals (20). More recently, the specificity of these results had been enhanced in studies in which hybridization was found to be limited to restriction fragments which encode the viral ICP0 gene but due to hybridization of the DNA strand opposite that of the ICP0 gene (5, 9, 14, 18, 26). RNA detected has been termed latency-associated transcript(s) (LAT) (18, 26). In the present study, in situ hybridization techniques were used on ganglion serial sections to investigate and quantitate LAT expression in sensory ganglia after infection with TK^- HSV mutants. The goal was to determine whether LAT might be present in sensory ganglia despite the relative inability of the virus to reactivate.

Viruses inoculated were TK^+ KOS, TK^- KOS, and the TK^- Glasgow 17 strains of HSV type 1 (HSV-1) used previously (21). Virus stocks were grown and titers were determined by standard means. Footpad inoculation of virus was performed in random-bred male and female CD-1 mice, 6 to 8 weeks old (Charles River Laboratories, Wilmington, Mass.). For the inoculation procedure, mice were anesthetized and approximately 50 μl of virus suspension (10^7 PFU/ml) was placed on each rear footpad. Multiple needle punctures were then made into the footpad through the virus drop. By this procedure more than 90% of mice were found to be latently infected (23), and no mortality was noted (unpublished observation). Mock inoculation was performed similarly in some mice but with medium containing no virus. During the period of HSV latency, 28 to 35 days postfootpad inoculation, mice were sacrificed and the fourth lumbar (L-4) and L-5 dorsal root ganglia (drg) were removed bilaterally. In some mice, drg were explanted for 4 days, and reactivated HSV was isolated as described previously (23). Other drg were fixed in 3% paraformaldehyde, and serial 10-μm paraffin sections were collected on 3-aminopropylthriethoxysilane-treated slides for in situ hybridization. The EC subfragment of the *Bam*HI E fragment of HSV-1 (strain Patton) (17) and multiple other restriction fragments (Fig. 1) were obtained from D. J. Spector and R. W. Hyman, (The Pennsylvania State University College of Medicine) and used to probe latently infected tissues. HSV DNA fragments (Fig. 1) were isolated from plasmid vectors, and 25- to 50-μg portions were nick translated (14°C) with [^{35}S]dCTP (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Specific activities of probes were 1×10^8 to 2×10^8 cpm, and 1 to 3 ng of DNA (approximately 10^5 cpm) was hybridized to sections on each slide. Tissue preparation and hybridization conditions were modified from procedures we used previously (19) and from procedures used by Fraser and coworkers (5, 18). In brief, after tissue sections were deparaf-

* Corresponding author.

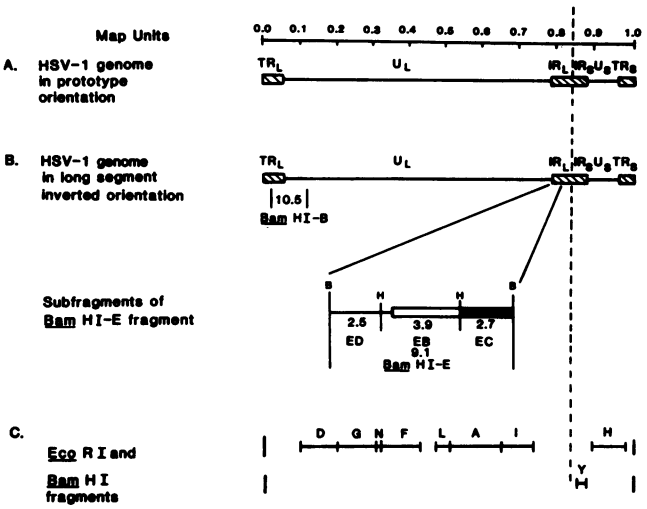

FIG. 1. Map of the HSV-1 genome and the *Eco*RI and *Bam*HI restriction fragments used in in situ hybridization studies. (A) The HSV-1 genome in the prototypic orientation (15). Repeat regions are cross-hatched. The long unique (U_L) and short unique (U_S) regions are bounded by the long terminal repeat (TR_L) and inverted long repeat (IR_L) and by the inverted short repeat (IR_S) and short terminal repeat (TR_S). (B) The HSV-1 genome in the long segment inverted orientation showing the *Bam*HI B fragment. The blow-up shows the *Bam*HI E fragment encompassing the same repeat sequences as in the B fragment. Subfragments of *Bam*HI E showing *Hpa*I sites (H) and *Bam*HI sites (B) are indicated, and sizes of restriction fragments in kilobase pairs are indicated. Diagram is adapted from the work of Spector et al. (17). Positive hybridization of latently infected ganglia was detected with the EC fragment (▬). (C) Restriction fragments used in mixture in hybridization studies which gave negative results when tested on latently infected ganglia.

finized in xylene and rehydrated in graded ethanols, sections were treated sequentially with 0.2 N HCl, proteinase K (5 μg/ml in 10 mM Tris [pH 7.4], 2 M $CaCl_2$), and 0.25% acetic anhydride (in 0.1 M triethanolamine, pH 8.0). Before hybridization, DNA probes were denatured at 100°C for 10 min, quenched on ice, and suspended in 2× sodium chloride-sodium citrate–10 mM Tris (pH 7.4)–1 mM EDTA–Denhardt solution–10% dextran sulfate–45% formamide–100 mg of mouse brain nucleic acid per ml–100 μg of *Micrococcus luteus* DNA per ml. Hybridization was at 46°C for 72 h. After extensive washing, slides were coated with NTB-3 emulsion (Eastman Kodak Co., Rochester, N.Y.), exposed for 4 days at 4°C, developed, and stained with hematoxylin.

In preliminary studies in which hybridization on serial ganglion sections was evaluated, it was apparent that approximately 25% of hybridization-positive cells were seen on two adjacent sections. Therefore, to eliminate double counting of LAT-positive cells, after all hybridization-positive cells in serial sections of a ganglion were counted, the total was divided by 1.25 to arrive at a corrected figure and eliminate duplicate counts. The absolute count, rather than a corrected count, was used only for ganglia where few hybridization-positive cells were present and where the comparison of positive cells in serial sections eliminated the possibility of duplicate counts.

After inoculation of TK^+ KOS HSV, most mice were found to be latently infected, as determined by the isolation of infectious virus from explanted ganglia (Table 1). This result was similar to results obtained after the corneal or footpad inoculation of larger numbers of mice with TK^+ HSV-1 (21–23). After footpad inoculation of TK^- KOS and TK^- Glasgow virus, latent infections were not detected in any drg, similar to previous results with these viruses after corneal inoculation and testing of trigeminal ganglia (21, 22).

After TK^+ HSV inoculation, neurons positive for hybridization with the *Bam*HI EC fragment (Fig. 2A) were noted in almost all ganglia (Table 1). EC hybridization was also present in almost all ganglia from TK^- Glasgow-inoculated (Fig. 2B) and TK^- KOS-inoculated (Fig. 2C) mice (Table 1). As noted initially in early studies in which probe consisted of purified viral DNA (20) and more recently in studies with cloned restriction endonuclease fragments (5, 9, 15, 18, 26), label was localized to the nuclei of neurons. Hybridization was noted only with the EC subfragment of the *Bam*HI E fragment (Fig. 1), similar to results obtained by others with the *Bam*HI B fragment or subfragments of the B fragment (5, 9, 14, 19). Investigations of hybridization with the *Bam*HI E fragment have been limited (5, 14), and hybridization with E subfragments has not been previously reported. Positive results with the E fragment would be predicted since the same repeat sequences are present in the *Bam*HI B and E fragments and since hybridization has been noted with B subfragments limited to repeat sequences (5, 18). In discussions below we consider EC fragment hybridization to indicate the presence of LAT. Hybridization was not detected when latently infected ganglia were probed with an equimolar mixture of other fragments (0 of 22) (Fig. 1), and hybridization was not noted when tissues from mock-infected and healthy mice were probed with the EC fragment (0 of 20) (data not shown).

Although numbers of LAT-positive neurons were somewhat lower after inoculation with TK^- HSV, the presence of LAT-positive neurons in ganglia from which HSV does not usually reactivate suggests a possible role for HSV TK expression in the reactivation process. It is of interest that D. Coen (D. M. Coen, M. Kosz-Vnenchak, J. G. Jacobson,

TABLE 1. Number of LAT-positive drg neurons after footpad inoculation with TK^+ and TK^- HSV-1[a,b]

Virus inoculated	No. of drg positive/no. tested (%) for:		LAT-positive neurons/positive ganglion	
	Isolation of HSV	LAT	No. positive (mean ± SD [range])	% Positive, mean (range)[c]
KOS TK^+	10/12 (83)	22/23 (96)	23.4 ± 20.2 (4–66)	0.7 (0.1–1.9)
KOS TK^-	0/12 (0)	19/20 (95)	10.7 ± 8.6 (2–22)	0.3 (0.1–0.9)
Glasgow TK^-	0/12 (0)	7/7 (100)	5.9 ± 2.9 (2–10)	0.2 (0.16–0.3)

[a] Data from L-4 and L-5 drg have been pooled.
[b] Latently infected drg, 28 to 35 days after inoculation.
[c] Calculations are based on an estimate of 3,500 neurons per ganglion.

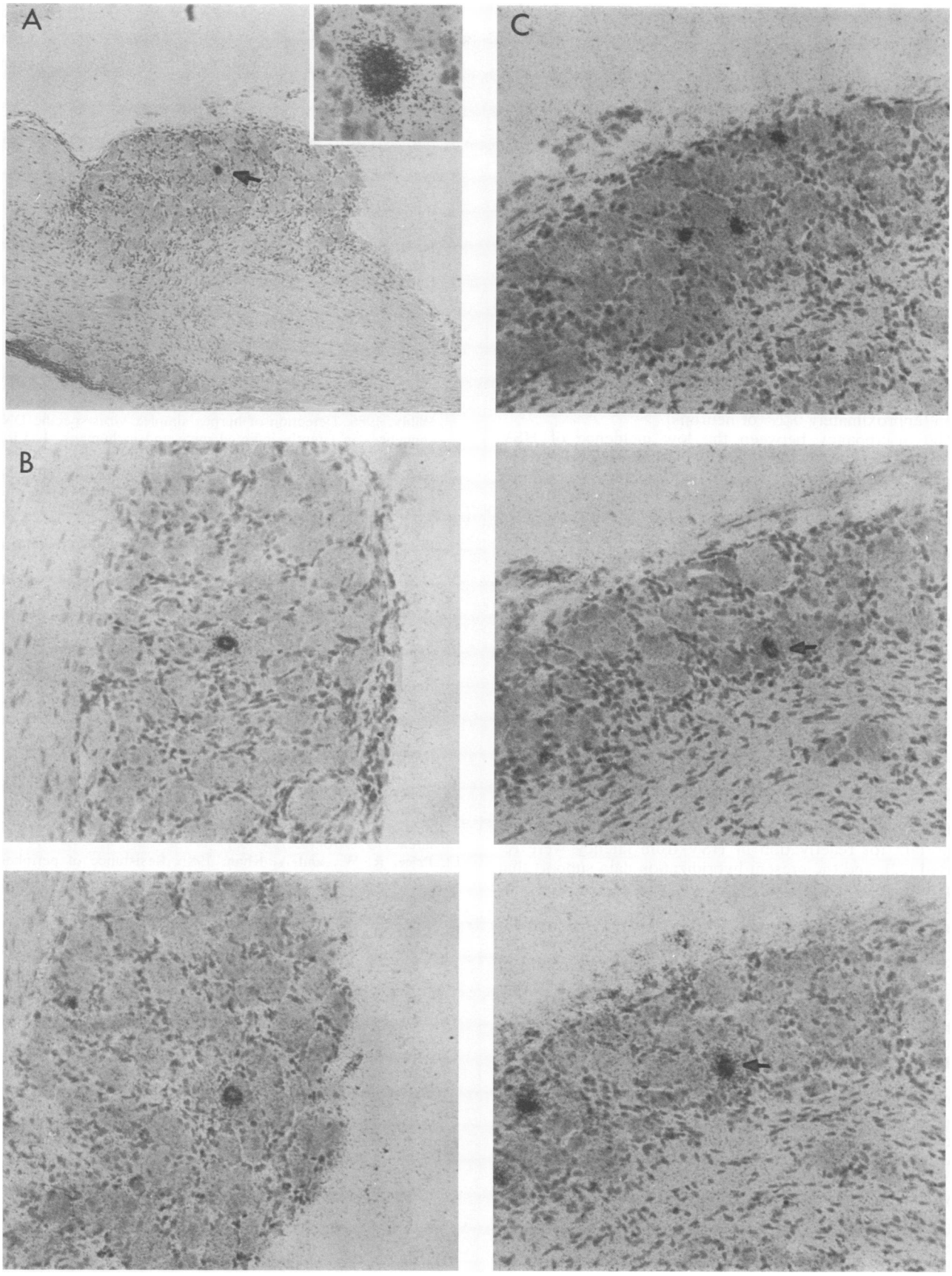

FIG. 2. LAT detection by in situ hybridization. (A) LAT-positive neurons in drg 28 days after footpad inoculation with TK^+ KOS HSV; inset, high magnification of LAT-positive neuron at arrow. (B) LAT-positive neurons in two drg 28 (top) and 35 (bottom) days after footpad inoculation with TK^- Glasgow HSV. (C) LAT-positive neurons in serial sections of drg 35 days after footpad inoculation with TK^- KOS HSV. One LAT-positive cell is seen in two adjacent sections (arrows).

D. A. Leib, C. L. Bogard, P. A. Schaffer, K. L. Tyler, and D. M. Knipe, Proc. Natl. Acad. Sci. USA, in press) have independently also noted evidence of LAT by in situ hybridization after inoculation of mice with other TK^- HSV mutants, and J. Stevens has obtained similar results (personal communication).

After TK^+ KOS virus inoculation, the presence of LAT was detected in 96% of L-4 and L-5 drg. An average of 23.4 LAT-positive neurons was detected in drg positive for LAT, and the range was 4 to 66. There are approximately 3,500 neurons in mouse L-4 and L-5 drg (unpublished results). Therefore, approximately 0.7% of neurons were LAT positive (0.2 to 1.9%). After inoculation with TK^- KOS virus, 95% of ganglia were LAT positive, and an average of 10.7 neurons per ganglion were positive (approximately 0.3% of neurons). After inoculation with TK^- Glasgow HSV, 100% of ganglia were LAT positive, although the average number of positive neurons was decreased to 5.9 per positive ganglion (approximately 0.2% of neurons).

The discrepancy between the low incidence of HSV isolation from drg after footpad inoculation of TK^- HSV and the ready detection of LAT in such ganglia was striking. Although numbers of LAT-expressing neurons were somewhat lower after inoculation with TK^- HSV, proportions of ganglia positive for LAT were similar. Rates of HSV reactivation, however, were very different.

If the expression of LAT by neurons is equated with the presence of latent HSV infection, the present results suggest that the TK^- viruses established latent infections but that reactivation was generally defective. Less likely, it might be speculated that LAT-positive neurons are not the neurons from which HSV reactivates and that TK^- viruses did not establish latent infections. There is presently no basis for this conclusion, and it is thought more likely that the present results support a role for HSV TK expression in reactivation from latency. Although defects in addition to that in TK expression may exist for the TK^- viruses studied, obtaining similar results for these independently isolated mutants supports a role for HSV TK expression. In a previous study, data which suggest that HSV TK expression might be important for reactivation of HSV from latency were reported (22), and the present hybridization data support that hypothesis.

The present report raises the issue of definitions of latency, since LAT was present after TK^- HSV inoculation, although reactivatable virus was uncommon. In some ways this result was similar to that obtained in studies of latency of central nervous system tissues, since reactivation of latent HSV from that tissue has generally been at low frequency (2, 24) or not detected (5, 25), despite the ready detection of LAT (5). In addition, the reported limited reactivation of an ICP0 mutant of HSV which, however, can be rescued by superinfection of ganglia (11a) also suggests reactivation-defective latency. The relative or absolute inability of HSV to reactivate from latency may rest on culture methods used, and it remains possible that with other methods reactivation would occur. At present, however, it appears that several instances of reactivation-defective or incomplete latency have been noted.

The authors thank D. J. Spector and B. Wigdahl for helpful suggestions and K. Rank for secretarial assistance.

This work was supported in part by Public Health Service grant CA 09124 awarded by the National Cancer Institute, by Javits Neuroscience Investigator award NS 20684 (R.B.T.), by Public Health Service grant AI 24010 awarded by the National Institute of Allergy and Infectious Diseases to P.A.S., and by RR 05680 awarded by the Biomedical Research Support Grant Program, National Institutes of Health.

LITERATURE CITED

1. **Baringer, J. R., and P. Swoveland.** 1973. Recovery of herpes-simplex virus from human trigeminal ganglions. N. Engl. J. Med. **288**:646–650.
2. **Cook, M. L., and J. G. Stevens.** 1976. Latent herpetic infections following experimental viraemia. J. Gen. Virol. **31**:75–80.
3. **Croen, K. D., J. M. Ostrove, L. J. Dragovic, J. E. Smialek, and S. E. Straus.** 1987. Latent herpes simplex virus in human trigeminal ganglia. N. Engl. J. Med. **317**:1427–1432.
4. **Darby, G., H. J. Field, and S. A. Salisbury.** 1981. Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature (London) **289**:81–83.
5. **Deatly, A. M., J. G. Spivack, E. Lavi, D. R. O'Boyle II, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome. J. Virol. **62**:749–756.
6. **Efstathiou, S., A. C. Minson, H. J. Field, J. R. Anderson, and P. Wildy.** 1986. Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J. Virol. **57**:446–455.
7. **Galloway, D. A., C. M. Fenoglio, and J. K. McDougall.** 1982. Limited transcription of the herpes simplex virus genome when latent in human sensory ganglia. J. Virol. **41**:689–691.
8. **Gordon, Y. J., D. M. Gilden, and Y. Becker.** 1983. HSV-1 thymidine kinase promotes virulence and latency in the mouse. Invest. Ophthalmol. Vis. Sci. **24**:599–602.
9. **Gordon, Y. J., B. Johnson, E. Romanowski, and T. Araullo-Cruz.** 1988. RNA complementary to herpes simplex virus type 1 ICP0 gene demonstrated in neurons of human trigeminal ganglia. J. Virol. **62**:1832–1835.
10. **Jamieson, A. T., G. A. Gentry, and J. H. Subak-Sharpe.** 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. **24**:465–480.
11. **Klein, R. J., A. E. Friedman-Kien, and E. DeStefano.** 1981. Pathogenesis of experimental skin infections induced by drug-resistant herpes simplex virus mutants. Infect. Immun. **34**: 693–701.

11a. **Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer.** 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. **63**:759–768.

12. **Price, R. W., and A. Khan.** 1981. Resistance of peripheral autonomic neurons to in vivo productive infection by herpes simplex virus mutants deficient in thymidine kinase activity. Infect. Immun. **34**:571–580.
13. **Rock, D. L., and N. W. Fraser.** 1983. Detection of HSV-1 genome in central nervous system of latently infected mice. Nature (London) **302**:523–525.
14. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Org, T. L. Lewis, J. R. Lokensgard, and S. L. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61**:3820–3826.
15. **Roizman, B.** 1979. The cellular organization of the herpes simplex virus genomes. Annu. Rev. Genet. **13**:25–57.
16. **Sekizawa, T., H. Openshaw, C. Wohlenberg, and A. L. Notkins.** 1980. Latency of herpes simplex virus in absence of neutralizing antibody: model for reactivation. Science **210**:1026–1028.
17. **Spector, D. J., T. R. Jones, C. L. Parks, A. M. Deckhut, and R. W. Hyman.** 1987. Hybridization between a repeated region of herpes simplex virus type 1 DNA containing the sequence (GGC)n and heterodisperse cellular DNA and RNA. Virus Res. **7**:69–72.
18. **Spivack, J. G., and N. W. Fraser.** 1988. Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. **62**:1479–1485.
19. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to herpes virus gene

mRNA is promine in latently infected neurons. Science **235:** 1056–1059.

20. **Tenser, R. B., M. Dawson, S. J. Ressel, and M. E. Dunstan.** 1982. Detection of herpes simplex virus mRNA in latently infected trigeminal ganglion neurons by in situ hybridization. Ann. Neurol. **11:**285–291.
21. **Tenser, R. B., and M. E. Dunstan.** 1979. Herpes simplex virus thymidine kinase expression in infection of the trigeminal ganglion. Virology **99:**417–422.
22. **Tenser, R. B., and W. A. Edris.** 1987. Trigeminal ganglion infection by thymidine kinase-negative mutants of herpes simplex virus after in vivo complementation. J. Virol. **61:**2171–2174.
23. **Tenser, R. B., W. A. Edris, and K. A. Hay.** 1988. Herpes simplex virus latent infection: reactivation and elimination of latency after neurectomy. Virology **167:**302–305.
24. **Tenser, R. B., and G. D. Hsiung.** 1977. Pathogenesis of latent herpes simplex virus infection of the trigeminal ganglion in guinea pigs: effects of age, passive immunization and hydrocortisone. Infect. Immun. **16:**69–74.
25. **Tullo, A. B., C. Shimeld, W. A. Blyth, T. J. Hill, and D. L. Easty.** 1982. Spread of virus and distribution of latent infection following ocular herpes simplex in the non-immune and immune mouse. J. Gen. Virol. **63:**95–101.
26. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y.-F. Zhang, W. M. Flanagan, and J. G. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62:**1194–1202.