JOURNAL OF VIROLOGY, Oct. 1989, p. 4455–4458
0022-538X/89/104455-04$02.00/0

Vol. 63, No. 10

Herpes Simplex Virus Type 1 Latency-Associated Transcription Plays No Role in Establishment or Maintenance of a Latent Infection in Murine Sensory Neurons

FARHAD SEDARATI,[1] KENNETH M. IZUMI,[1] EDWARD K. WAGNER,[2] AND JACK G. STEVENS[1]*

Department of Microbiology and Immunology, University of California at Los Angeles, Los Angeles, California 90024-1747,[1] and the Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California 92717[2]

Received 24 April 1989/Accepted 29 June 1989

Using herpes simplex viruses deleted and restored for the latency-associated transcripts (LATs), we have quantitatively assessed the role of the transcripts in establishment and maintenance of latent infection. Determination of the number of neurons latently infected and the copy number of viral genomes per latently infected ganglion indicated that there is no difference between viruses expressing and not expressing the transcripts. In addition, the amount of viral DNA present in ganglia latently infected with the LAT-negative virus KOS 8117 did not differ from the value for LAT$^+$ counterparts over an 11-month period of analysis. From these results we conclude that LATs play no role in establishment or maintenance of a latent infection with herpes simplex virus type 1. If these transcripts play a role in latency, they must function during the reactivation step.

Latency-associated transcripts (LATs) are unique herpes simplex virus type 1 (HSV-1) transcripts selectively present in latently infected sensory neurons of mice (6, 9, 14, 18), rabbits (10, 19), and humans (1, 2, 4, 16, 17, 20). Since the discovery of these RNAs, there has been growing interest in defining any role they might play in latency and reactivation. Our recent studies with X10-13 (6), a deletion mutant not expressing the LATs, showed that the transcripts were not absolutely essential for either establishment of latency in dorsal root ganglia of mice or reactivation of virus in vitro. These results were later confirmed by others (15), who showed that LATs were dispensable for the establishment of the latent infection in murine trigeminal ganglia. In these studies, quantitative aspects of the phenomena were not investigated and a potential role for the RNAs in in vivo reactivation were not studied. Here we report that an engineered HSV-1 which does not express the LATs is established and maintained in a latent state in mice as efficiently as a marker-rescued variant of this agent which expresses the transcripts.

The viruses used were KOS(M); KOS 8117, an LAT-negative deletion mutant of KOS(M); and KOS D362, a marker-rescued derivative of this mutant. Details regarding construction and the genetic structure of these agents are presented elsewhere (K. M. Izumi, A. B. McKelvey, G. Devi-Rao, E. K. Wagner, and J. G. Stevens, Microb. Pathog. in press). The overall experimental approach was to quantitate the amount of viral DNA present in murine spinal ganglia at various times after infection with these agents. If there was less DNA present in ganglia harboring the LAT-negative virus, then LAT RNAs would be functioning in the establishment or maintenance of the latent infection.

Mice were infected on the abraded rear footpads with 10^7 PFU of each virus as described elsewhere (12). At 0, 1, 2, 3, 4, 5, 7, 9, and 30 days postinfection, lumbosacral ganglia (six ganglia per side per animal) from the right or left side of three mice were removed, and ganglia from the two sides were pooled separately and stored at −70°C. First, the replicative capacities of the three viruses were compared by homogenizing one set of tissues and titrating for infectious virus according to a procedure previously described (11). The replicative capacities of KOS 8117 are virtually identical to those of KOS(M) and the LAT-restored KOS D362 (Fig. 1A). In each case, a significant amount of virus was detected in ganglia by day 2 postinfection and it reached a peak titer of 4.1×10^5 PFU/g of tissue by day 3 postinfection. The virus titers then gradually declined until no virus was detected in any tissue by day 9 postinfection.

In the second part of this experiment, pools of ganglia taken from the opposite sides of the animals were used as a source of DNA which was quantitated by Southern hybridization methods (13). Frozen tissues were homogenized in ice-cold TES buffer (10 mM Tris hydrochloride [pH 7.4], 200 mM EDTA, 150 mM NaCl). Sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 1% and 0.5 mg/ml, respectively, and samples were incubated overnight at 50°C. The DNA preparations were extracted once with phenol-chloroform-isoamyl alcohol (24:24:1) and twice with chloroform and were then ethanol precipitated. DNA was then suspended in TE buffer (10 mM Tris hydrochloride [pH 8.0], 10 mM EDTA), and RNase A and T1 nuclease (both from Boehringer Mannheim Biochemicals) were added to final concentrations of 100 μg/ml and 10 U/ml, respectively. The samples were digested at 37°C for 2 h, after which proteinase K was added to a final concentration of 0.2 mg/ml and digestion continued for an additional 2 h. These digests were extracted with phenol-chloroform-isoamyl alcohol and chloroform and then ethanol precipitated. DNA concentration was determined by measuring A_{260} in a Beckman DU-7 spectrophotometer.

For hybridization, DNA equivalent to that present in two ganglia (4.5 μg per slot) was filtered through a Biotrans nylon membrane (0.2-μm pore size; ICN Pharmaceuticals Inc.) with a slot blot apparatus (Bio-Dot SF blotting apparatus; Bio-Rad Laboratories) (7). Alkali denaturation of DNA in situ was performed by placing membranes on three layers of

* Corresponding author.

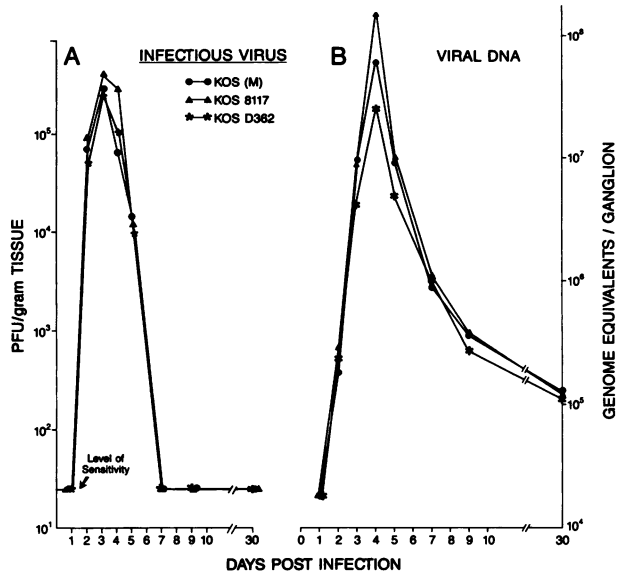

FIG. 1. Appearance of infectious virus and viral DNA in murine spinal ganglia after footpad inoculation of mice with HSV-1 KOS(M), KOS 8117, or KOS D362. Mice were inoculated on both rear footpads with 10^7 PFU of each virus, and at each time indicated, ganglia were removed from right or left side of three mice, pooled separately, and stored at −70°C for later virus titrations or DNA quantitations. (A) Results of virus titrations on one set of ganglia pools, expressed as total PFU per gram of tissue. The level of sensitivity was determined to be 2.5×10^1 PFU/g of tissue. (B) Results of quantitative hybridization experiments analyzing DNA extracted from the ganglia taken from the side of the animals opposite that used for panel A. Values are expressed as HSV-1 genome equivalents per ganglion. Experimental details are presented in the text.

Whatman 3MM filter paper that had been presoaked in 1.5 M NaCl and 0.5 M NaOH for 5 min. Subsequent neutralization of DNA was performed by placing membranes on filter papers that had been presoaked in 3.0 M sodium acetate for 5 min. The membranes were prehybridized for 4 h at 50°C in 20% deionized formamide–0.1% SDS–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1 mM EDTA–5× Denhardt solution–100 μg of denatured mouse brain DNA per ml as carrier. Hybridization was with a ^{32}P-labeled *Hin*dIII N fragment (0.87 to 0.90 map units) of HSV-1 ANG (10^7 cpm/ml) for 4 days at 50°C in 50% deionized formamide–10% dextran sulfate–0.1% SDS–5× SSC–1 mM EDTA–5× Denhardt solution–100 μg of denatured mouse brain DNA per ml used as carrier (8). After hybridization, the membranes were washed in 0.1× SSC–0.1% SDS for 20 min at room temperature (two washes), 2.5 h at 68°C (two washes), and at room temperature (one wash). The membranes were air dried, and autoradiographs were prepared by exposure to Kodak XAR film. The radioactivity present in each slot was determined by counting in a liquid scintillation counter and compared with counts for known quantities of HSV DNA mixed with 4.5 μg of mouse brain DNA and hybridized under identical conditions. Alternatively, DNA quantitations were carried out by densitometric tracing of the autoradiograph with the Bio-Rad model 620 video densitometer according to the instructions of the manufacturer. One genome equivalent of viral DNA was calculated to be 1.66×10^{-4} pg, assuming a mass of 10^8 daltons for HSV-1 DNA.

During the acute phase, in all cases HSV-1 DNA was detected as early as 1 day postinfection and reached a maximum by day 4 postinfection; maximum amounts of 6.0×10^7, 1.5×10^8, and 2.5×10^7 genome equivalents per ganglion for KOS(M), KOS 8117, and KOS D362, respectively, were detected at this time (Fig. 1B). In addition, the viral DNA content of KOS 8117 ganglia during the latent phase (1.3×10^5 genome equivalents per ganglion) was virtually identical to those of KOS(M) and KOS D362 (1.3×10^5 and 1.1×10^5 genome equivalents per ganglion, respectively) (Fig. 1B).

TABLE 1. HSV-1 transcripts in infected ganglia

Day postexplant	No. of positive neurons/ganglionic section in tissues containing[a]:		
	KOS	KOS 8117	KOS D362
0	0	0	0
1	0.45	0	0.25
2	1.7	0.4	1.0
3	3.0	2.8	3.3

[a] Number positive for transcripts in the HSV-1 *Hin*dIII A region (0.25 to 0.53 map units) in explanted tissues containing the indicated virus.

Although the data show that the amount of viral DNA per ganglion was identical in mice latently infected with each of the three viruses, there is the unlikely possibility that the latent infection established by KOS 8117 does not resemble that established by the other two viruses. Specifically, it is possible that fewer neurons are latently infected with KOS 8117 and that these harbor more viral DNA per neuron. A direct approach to testing this possibility cannot be accomplished, since no viral macromolecules are detectable in cells latently infected with KOS 8117 (the deletion mutant). Therefore, we attempted to address this question indirectly, by scoring neurons positive for HSV transcripts when latently infected ganglia were cocultivated in vitro. Ganglia were removed from mice latently infected with KOS(M), KOS 8117, or KOS D362 and explanted in vitro at 37°C in Eagle minimal essential medium containing 5% fetal calf serum (GIBCO Laboratories) and 5% newborn calf serum (Whittaker M. A. Bioproducts). At 0, 1, 2, and 3 days postexplant, tissues were removed and stored at −70°C. At the end of the experiment, the samples were prepared for in situ hybridization experiments as described before (5) and probed with the restriction fragment *Hin*dIII A (0.25 to 0.53 map units) of HSV-1 17 syn$^+$, and neurons expressing transcripts were scored. HSV-1 transcripts in the *Hin*dIII A region can be detected as early as 1 day postexplant for KOS(M) and KOS D362 tissues (0.45 and 0.25 positive neurons per ganglionic section, respectively) (Table 1). No transcripts were detected in KOS 8117-infected ganglia at this time. However, by 3 days postexplant, when the first virus progeny were detectable (data not shown), a similar number of positive neurons per ganglionic section was scored in the three explant tissues; 3.0, 2.8, and 3.3 positive neurons per ganglionic section were detected for KOS(M), KOS 8117, and KOS D362, respectively. The reason for the observed difference in the kinetics of reactivation in vitro of KOS 8117 compared with KOS(M) or KOS D362 is not clear, but, as is discussed below, it could reflect differences in reactivation capacities of these viruses. In any case, these findings indicate that the same number of neurons are latently infected. We therefore conclude that expression of the LATs plays no role in establishment of latent infections in murine spinal ganglia.

To investigate a possible role for LATs in maintaining the latent infection, we performed quantitative hybridization

TABLE 2. Viral DNA in murine spinal ganglia previously infected with KOS(M), KOS 8117, and KOS D362 over an 11-month period of latent infection[a]

Mo postinfection	Genome equivalents (10^4) of viral DNA/ganglion in tissue infected with:		
	KOS(M)	KOS 8117	KOS D362
1.0	9.9	13.5	11.1
1.5	6.9	13.2	10.0
2.0	4.2	9.0	6.3
2.5	3.0	11.1	3.0
3.0	5.1	10.2	6.0
4.0	5.1	5.1	6.0
6.0	4.2	5.7	5.7
11.0	7.8	6.1	6.2

[a] DNA was extracted from spinal ganglia of latently infected mice at the time indicated. A 4.5-μg portion of each DNA sample was blot transferred to a nylon membrane (Biotrans; ICN) and hybridized to a ^{32}P-labeled *Hin*dIII N fragment of HSV-1 ANG, and an autoradiograph was prepared. Details of methods of DNA extractions, blot transfer, hybridization conditions, and DNA quantitations are given in the text.

experiments, over a period of 11 months, on DNA extracted from ganglia of mice infected with KOS(M), KOS 8117, and KOS D362. Mice were infected as described above, and ganglia were removed (three mice per time point) at 1, 1.5, 2, 2.5, 3, 4, 6, and 11 months postinfection and stored at −70°C. DNA was later extracted and hybridized. The total ganglionic DNA content of mice latently infected with the LAT$^-$ virus, KOS 8117, did not differ significantly from those of LAT$^+$ counterparts, KOS(M) and KOS D362, over the period tested (Table 2). Some fluctuation was observed in the amounts of viral DNA detected in the three virus groups at different time periods. However, this appeared to be a function of the technique used, since similar variations were also observed when three different DNA preparations (three mice each) extracted from one group of mice latently infected with KOS(M) were tested under the same conditions. Here, 8.7×10^4, 6.3×10^4, and 12×10^4 viral genome equivalents were detected per latently infected ganglion. We conclude that the LATs play no measurable role in maintenance of the latent infection, at least over the 11-month time period examined here. Finally, the data also suggest that in all three cases of latent infection the amounts of viral DNA may have decreased over the period studied. We have not studied this aspect further, but it should be pointed out that others (3) reported that the amount of latent HSV-1 DNA remained stable in murine nervous tissues over a 4-month period of study.

The selective expression and abundance of LATs in latently infected neurons suggest a role in the steps of establishment, maintenance, or reactivation of latent infection. The data presented here show that LATs are not involved with the establishment and maintenance phases of the latency. Therefore, in mice, if there is a function for LATs, it must be in the reactivation process. How LATs might function during the reactivation is not immediately obvious. Our studies have clearly shown that two LAT-negative viruses (X10-13 [6] and KOS 8117 [this study]) can be recovered from the latent state in murine ganglia upon explantation in vitro. However, reactivation in vitro may not necessarily mirror the process which occurs in vivo. Therefore, LATs may be postulated to influence reactivation from the latent state in the living animal. It is also possible that the two reactivation processes (in vitro and in the animal) share some features, and the slow kinetics of appearance of lytic phase transcripts in the KOS 8117-explanted tissues (Table 1) and of infectious virus in another system (15) may relate to such a shared function(s). Efforts are now under way to investigate a potential role for the LATs in reactivation from the latent state in vivo.

This research was supported by Public Health Service grant AI-0624 from the National Institutes of Health, grant RG-1647-A-1 from the National Multiple Sclerosis Society, and a grant from the W. M. Keck Foundation. F.S. is a postdoctoral fellow supported by grant FG-776-A-1 from the Multiple Sclerosis Society. K.M.I. is a predoctoral trainee supported by Public Health Service National Research Award GM-07104.

We thank Vivian B. Dissette and Anthony B. McKelvey for technical assistance and June Baumer for help with the densitometric tracing and the computer analysis of the data.

LITERATURE CITED

1. **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. Detection of an immediate early gene "anti sense" transcript by in situ hybridization. N. Engl. J. Med. **317**:1427–1432.
2. **Deatly, A. M., J. G. Spivack, E. Lavi, and N. W. Fraser.** 1987. RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. Proc. Natl. Acad. Sci. USA **84**:3204–3208.
3. **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.
4. **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.
5. **Haase, A., M. Brahic, L. Stowring, and H. Blum.** 1984. Detection of viral nucleic acids by in situ hybridization, p. 189–226. *In* K. Maramorosh and H. Koprowski (ed.), Methods in Virology. Academic Press, Inc., New York.
6. **Javier, R. T., J. G. Stevens, V. B. Dissette, and E. K. Wagner.** 1988. A herpes simplex virus transcript abundant in latently-infected neurons is dispensable for establishment of the latent state. Virology **166**:254–257.
7. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. **7**:1541–1552.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Puga, A., and A. L. Notkins.** 1987. Continued expression of a poly(A$^+$) transcript of herpes simplex virus type 1 in trigeminal ganglia of latently infected mice. J. Virol. **61**:1700–1703.
10. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, 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.
11. **Sedarati, F., R. T. Javier, and J. G. Stevens.** 1988. Pathogenesis of a mixed lethal infection in mice with two nonneuroinvasive herpes simplex virus strains. J. Virol. **62**:3037–3039.
12. **Sedarati, F., and J. G. Stevens.** 1987. Biological basis for virulence of three strains of herpes simplex virus type 1. J. Gen. Virol. **68**:2389–2395.
13. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–517.
14. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61**:3841–3847.
15. **Steiner, I., J. G. Spivack, R. P. Lirette, S. M. Brown, A. R.**

MacLean, J. H. Subak-Sharpe, and N. W. Fraser. 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8**:505–511.

16. **Steiner, I., J. G. Spivack, D. R. O'Boyle II, E. Lavi, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virol. **62**:3493–3496.
17. **Stevens, J. G., L. Haarr, D. D. Porter, M. L. Cook, and E. K. Wagner.** 1988. Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. J. Infect. Dis. **158**:117–122.
18. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235**:1056–1059.
19. **Wagner, E. K., W. M. Flanagan, G. Devi-Rao, Y. F. Zhang, J. M. Hill, K. P. Anderson, and J. G. Stevens.** 1988. The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. **62**:4577–4585.
20. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. Slonina, and H. Ghiasi.** 1988. Fine mapping of the major latency-related RNA of herpes simplex virus type 1 in humans. J. Gen. Virol. **69**: 3101–3106.