JOURNAL OF VIROLOGY, May 1991, p. 2745–2750
0022-538X/91/052745-06$02.00/0

Vol. 65, No. 5

Expression of Herpes Simplex Virus Type 2 Latency-Associated Transcript in Neurons and Nonneurons

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Received 28 September 1990/Accepted 11 February 1991

The presence of herpes simplex virus type 2 (HSV-2) transcription during in vivo latent infection was investigated by in situ hybridization. Latent infection of mouse dorsal root ganglion was investigated with the *Bam*HI p fragment of HSV-2, which resulted in evidence of ganglion hybridization, and other fragments representing approximately 40% of the genome, which did not result in hybridization. Strand specificity of hybridization was investigated in studies with synthetic oligonucleotides, which supported the conclusion that a latency-associated transcript(s) had been detected. Hybridization was detected with oligonucleotides complementary to the infected-cell polypeptide 0 (ICP0) template strand but not with oligonucleotides synthesized from the ICP0 template strand. Although most hybridization occurred over neurons, in some instances hybridization appeared to occur over nonneuronal ganglion cells, and this was more evident when tissue sections were examined by phase contrast microscopy. Although these results supported the usual neuronal site of HSV-2 latency, latency in nonneuronal cells may be important in considering the pathobiology of HSV-2 infections.

Herpes simplex virus (HSV) latent infection has been well characterized as an infection of neurons, usually of sensory ganglia (3, 9, 13, 17, 26). Several experimental methods have been used to arrive at this conclusion, which is also in keeping with axonal transport of HSV (4, 8, 15). Recent investigations of the presence of HSV latency-associated transcript (LAT) in neurons as detected by in situ hybridization have confirmed the neuronal site of latency (7, 11, 14, 16, 21, 27, 28). Although suggestions have been made that HSV may establish latent infections in vivo in other cell types and other locations, these results have sometimes been questioned.

In most in vivo latency studies done with in situ hybridization techniques, HSV type 1 (HSV-1) has been investigated, and fewer studies have been performed with HSV-2 (9, 24, 26). Recently, HSV-2 LAT was reported; hybridization was detected with one restriction fragment and was negative with other fragments representing the remainder of the HSV-2 genome (18). In the present investigation we extend this report via studies in which hybridization was similarly restricted and hybridization with strand-specific oligonucleotides detected transcripts that were complementary to the strand opposite the infected-cell polypeptide 0 (ICP0) template strand, but did not detect transcripts from the ICP0 template strand. In addition, while HSV-2 LAT was present over many sensory ganglion neurons, hybridization also occurred over occasional nonneuronal cells. Although the present results strongly support the usual neuronal locus of latent HSV-2, latency in even a relatively few nonneuronal cells may alter the way in which the pathobiology of long-term HSV infection is considered.

HSV-2 (strain 333), originally obtained from the American Type Culture Collection and passed multiple times in cell culture, was used. Virus stocks were grown and counted in primary rabbit kidney cells by standard means. HSV-2 latent infection of mouse dorsal root ganglia (DRG) was established by bilateral rear footpad inoculation of CD-1 mice (male and female, 6 to 8 weeks old; Charles River Laboratories, Wilmington, Mass.). Each footpad was inoculated with 10^4 PFU by techniques described previously (27). During the period of latency (28 to 240 days postinoculation), mice were anesthetized and perfused with 3% paraformaldehyde. The fourth and fifth lumbar DRG were removed, and paraffin sections were collected on 3-aminopropyltriethoxysilane-treated slides for in situ hybridization. Hybridization was performed with the 3.9-kb *Bam*HI p fragment, obtained from J. Martin (24), or alternatively with other fragments (D.

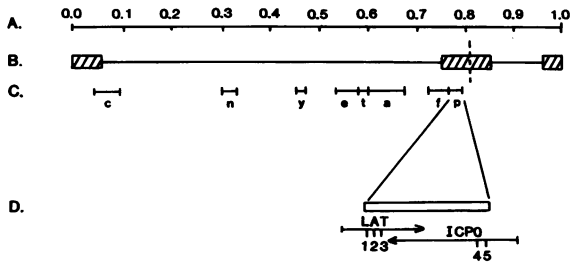

FIG. 1. Map of the HSV-2 genome and the DNA probes used in in situ hybridization studies. (A) Map units. (B) The HSV-2 genome in the prototypic orientation (20). Repeat regions are cross-hatched, and the joint is shown by a vertical dashed line. (C) *Bam*HI fragments according to Wilkie et al. (29). Positive hybridization was achieved only with the *Bam*HI p fragment from the long internal repeat. (D) HSV-2 *Bam*HI p fragment and LAT and ICP0 transcripts according to Mitchell et al. (18). The numbers 1, 2, and 3 indicate oligonucleotides complementary to the ICP0 template strand (antisense to LAT) (1, LAT1-30; 2, LAT291-320; 3, LAT681-710) and the numbers 4 and 5 indicate oligonucleotides synthesized from the ICP0 template strand (antisense to ICP0) (4, ICP03,591-3,220; 5, ICP03,111-3,140). Hybridization was detected with oligonucleotides 1 and 2. Oligonucleotide sequences are from the sequence of *Bam*HI-p from D. McGeoch (16a).

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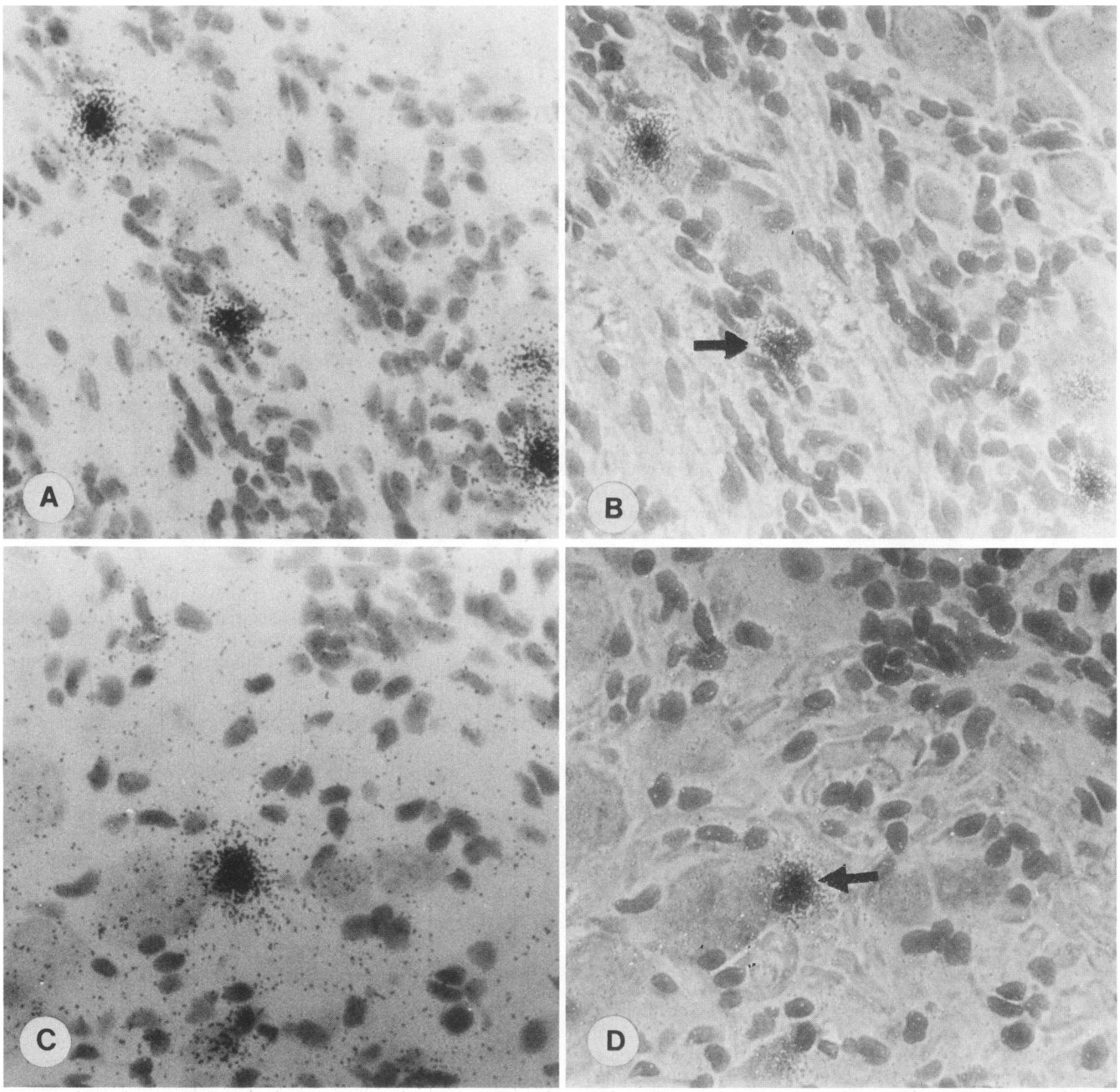

FIG. 2. In situ hybridization (*Bam*HI-p) of mouse DRG latently infected with HSV-2, 4-μm sections. For each pair of panels, standard light microscopy is on the left (A, C, E, G) and phase contrast microscopy of the same fields is on the right (B, D, F, H). (A and B) Section showing hybridization over nonneuronal cell (arrow). (C and D) Section with hybridization over a cell likely to be a satellite cell (arrow). (E and F) Section with hybridization over neuron and nonneuron. (G and H) Section with hybridization over cells considered possibly nonneuronal when examined by standard light microscopy but probably neurons when examined by phase contrast microscopy.

Galloway, Fred Hutchinson Cancer Research Center, Seattle, Wash.) representing approximately 40% of the HSV-2 genome (Fig. 1). HSV DNA fragments were nick translated at 14°C with [^{35}S]dCTP (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The specific activities of the probes were 1×10^8 to 2×10^8 cpm/μg, and 1 to 3 ng of DNA (approximately 6×10^5 cpm) was used for each slide. Tissue preparation and hybridization conditions were as described previously (27).

In studies to determine the strand specificity of hybridization synthetic oligonucleotides (model 7500 DNA Synthesizer; Milligen) were tested by in situ hybridization. Oligonucleotides were synthesized according to the unpublished sequence of HSV-2 of D. McGeoch (16a) and kindly made available to us by him. Three 30-mers were used, corresponding to nucleotides 1 to 30, 291 to 320, and 681 to 710 of the *Bam*HI p fragment, which were from the strand opposite to the ICP0 template strand and did not overlap ICP0 (Fig.

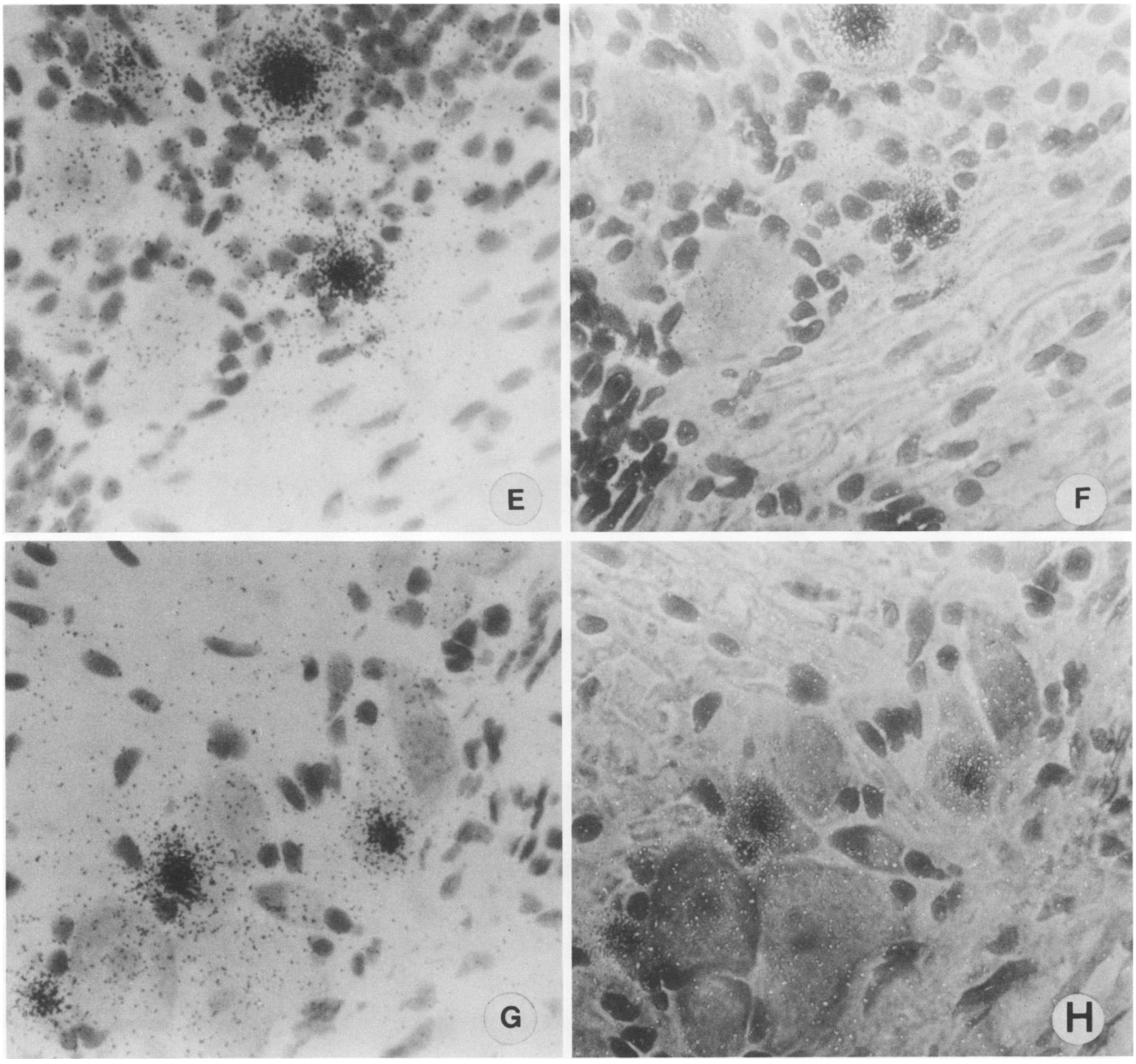

FIG. 2—*Continued.*

1). The three oligonucleotides will be referred to as LAT1-30, LAT291-320, and LAT681-710, respectively. Two oligonucleotides homologous to the ICP0 template strand and within the ICP0 coding sequence were also tested (ICP03111-3140 and ICP03591-3620, of the McGeoch sequence). Oligonucleotides were 3′ end labeled with [^{35}S]dATP (338 Ci/mmol) and used for in situ hybridization (10^9 cpm/μg, approximately 1 ng per slide) as has been done for HSV-1 (25, 28).

Positive results were apparent with hybridization of ganglion sections with the *Bam*HI p fragment (Fig. 2) but not with other cloned fragments (data not shown). Hybridization signal was present over many neurons, usually over the nuclei, as has been seen for HSV-1 (7, 11, 14, 16, 21, 27, 28). Although hybridization signal was usually present in neurons, in some instances signal was present over cells which did not appear to be neurons.

To enhance the discrimination of cell types expressing LAT, ganglion sections of 2 and 4 μm were investigated. Given the relatively large size of neurons and the usually central location of neuronal nuclei, it was thought that thin sections would eliminate the possible problem of overlapping of neurons and nonneurons in a given section. The possibility of hybridization resulting from neuronal overlap of nonneurons was also minimized by the in situ hybridization testing of adjacent serial sections.

Pairs of photomicrographs showing the same fields obtained by standard light microscopy (left) and by phase contrast microscopy (right) are shown in Fig. 2. Hybridiza-

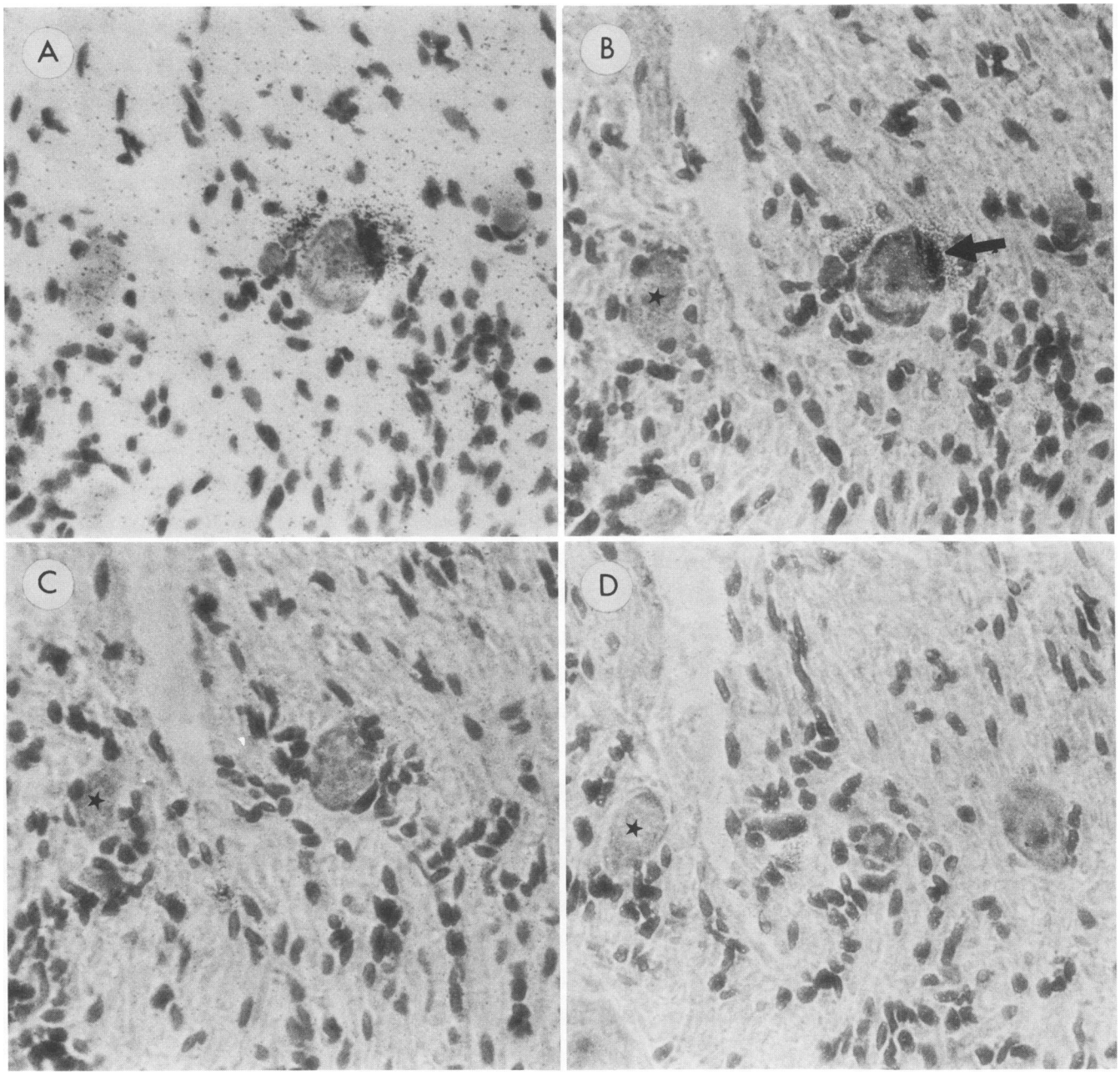

FIG. 3. In situ hybridization (*Bam*HI-p) of mouse DRG latently infected with HSV-2, 4-μm sections. (A) Standard light photomicrograph and (B) phase contrast photomicrograph of the same section, showing evidence of hybridization over a nonneuronal cell (arrow). (C and D) Phase contrast photomicrographs showing adjacent serial sections on either side of the section shown in panels A and B. The lack of label in panels C and D supports the nonneuronal site of hybridization seen in panels A and B. For orientation purposes, a star (★) indicates the same neuron in each section.

tion over nonneuronal cells was most evident when sections were examined by phase contrast microscopy (Fig. 2B and D) and included label of probable satellite cells (Fig. 2C and D). Possibly equally important, labeled cells thought to be neurons when examined by standard light microscopy were seen to be neurons when examined by phase contrast microscopy (Fig. 2G and H). Testing of serial sections further supported the nonneuronal localization of hybridization (Fig. 3). It was estimated that in 10 ganglia examined, 5 to 10% of labeled cells were nonneurons.

Hybridization was seen with the *Bam*HI p fragment of HSV-2, which includes the 3′ end of the ICP0 gene (18), and was not seen with other HSV-2 fragments, suggesting that hybridization during latency was similar to that for HSV-1 (7, 11, 14, 16, 21, 27, 28). In their recent study, Mitchell et al. performed hybridization with fragments representing the entire HSV-2 genome and found similarly restricted hybridization (18). In the present study, the strand specificity of

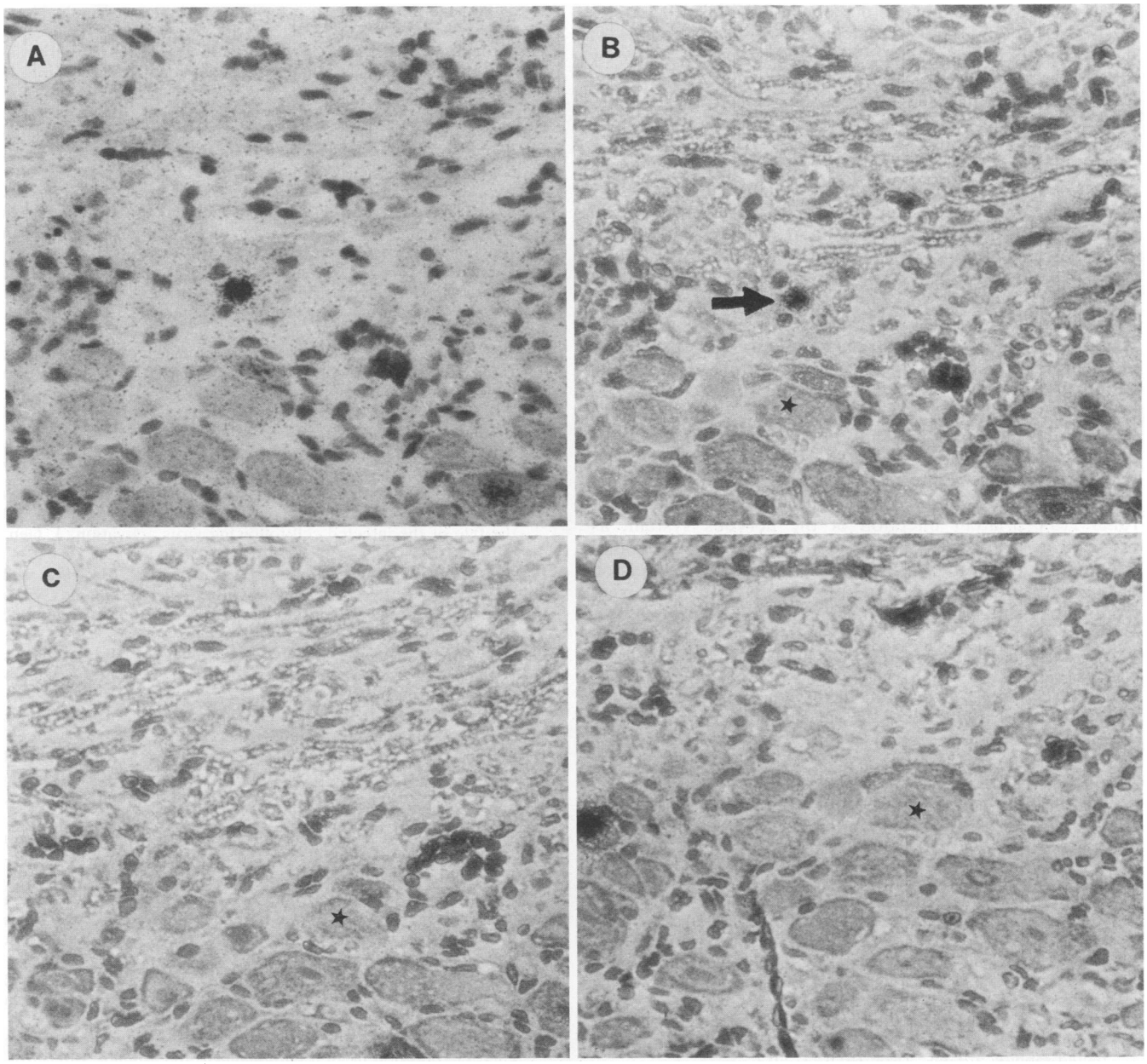

FIG. 4. In situ hybridization (LAT291-320) of mouse DRG latently infected with HSV-2, 2-μm serial sections. (A) Standard light photomicrograph and (B) phase contrast photomicrograph of the same section, showing evidence of hybridization over a nonneuronal cell (arrow). Further evidence of the nonneuronal nature of this hybridization-positive cell is provided in panels C and D, which are phase contrast photomicrographs showing adjacent serial sections on either side of the section shown in panels A and B. For orientation purposes, a star (★) indicates the same neuron in each section.

hybridization was investigated by the use of synthetic oligonucleotides, and the results extend the hybridization results of Mitchell et al. (18), who did not determine strand specificity.

In testing oligonucleotides synthesized from the HSV-2 strand opposite to the ICP0 template strand and not overlapping ICP0 (antisense to LAT), hybridization was detected with LAT1-30 and LAT291-320 but not LAT681-710. Possible reasons for the absence of hybridization with LAT681-710 will be investigated further.

Hybridization over a nonneuronal cell after 2-μm serial sections were tested with LAT291-320 is shown in Fig. 4. Serial section testing excluded the possibility that label was derived from an overlying neuron, and hybridization detected with LAT1-30 and LAT291-320 supported the conclusion that RNA transcribed from the strand opposite to the ICP0 template strand was detected. In addition, hybridization with *Bam*HI p was eliminated by RNase pretreatment of tissue sections (26), and hybridization was not detected with the ICP0-specific oligonucleotides; as would be expected, oligonucleotides synthesized from the ICP0 template strand hybridized to acutely infected cells (data not shown).

If it is considered that the presence of HSV LAT denotes latency, it would be concluded that latent infections of nonneuronal cells may be established not infrequently by HSV, at least by HSV-2. It remains to be demonstrated that latent infection of these nonneuronal cells can be reactivated, if it is considered that the definition of latency includes this function (25).

Although HSV latency has been convincingly shown to be primarily an infection of sensory ganglion neurons, HSV-1 latency has been suggested to occur in nonneuronal neural cells (7, 23) and HSV-1 and HSV-2 latency to occur in epithelial cells (1, 2, 5, 19). The possibly nonneuronal locus of HSV latency reported here can also be compared with results obtained with varicella-zoster virus (VZV). A neuronal site of VZV latency determined by in situ hybridization was suggested (10, 12), although in more recent studies nonneuronal hybridization was noted (6). The latter led Straus to offer a model which incorporated differences between possible sites of latency of HSV and VZV to explain differences in the pathobiology of human infections by these viruses (22). Use of phase contrast microscopy and the examination of thin tissue sections may resolve some questions of cell identification. If the occurrence of nonneuronal HSV-2 latent infection of the nervous system, as reported in the present study, is confirmed, it may modify some considerations of HSV latency; latent HSV infection of a cell type(s) which differs functionally and replicatively from neurons may be important in some disease processes.

This study was supported by Public Health Service award NS20684 (R.B.T.).

Helpful discussion with M. Judith Tevethia and the secretarial assistance of Tricia Johnson are appreciated.

REFERENCES

1. **Al-Saadi, S. A., G. B. Clements, and J. H. Subak-Sharpe.** 1983. Viral genes modify herpes simplex virus latency both in mouse footpad and sensory ganglia. J. Gen. Virol. **64**:1175–1179.
2. **Clements, G. B., and J. H. Subak-Sharpe.** 1988. Herpes simplex virus type 2 establishes latency in the mouse footpad. J. Gen. Virol. **69**:375–383.
3. **Cook, M. L., V. B. Bastone, and J. G. Stevens.** 1974. Evidence that neurons harbor latent herpes simplex virus. Infect. Immun. **9**:946–951.
4. **Cook, M. L., and J. G. Stevens.** 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect. Immun. **7**:272–288.
5. **Cook, S. D., S. K. Batra, and S. M. Brown.** 1987. Recovery of herpes simplex virus from the corneas of experimentally infected rabbits. J. Gen. Virol. **68**:2013–2017.
6. **Croen, K. D., J. M. Ostrove, L. J. Dragovic, and S. E. Straus.** 1988. Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster virus and herpes simplex viruses. Proc. Natl. Acad. Sci. USA **85**:9773–9777.
7. **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.
8. **Dillard, S. H., W. J. Cheatham, and H. L. Moses.** 1972. Electron microscopy of zosteriform herpes simplex infection in the mouse. Lab. Invest. **26**:391–401.
9. **Galloway, D. A., C. Fenoglio, M. Shevchuk, and J. K. McDougall.** 1979. Detection of herpes simplex RNA in human sensory ganglia. Virology **95**:265–268.
10. **Gilden, D. H., Y. Rozenman, R. Murray, M. Devlin, and A. Vafai.** 1987. Detection of varicella-zoster virus nucleic acid in neurons of normal human thoracic ganglia. Ann. Neurol. **22**: 377–380.
11. **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.
12. **Hyman, R. W., J. R. Ecker, and R. B. Tenser.** 1983. Varicella-zoster virus RNA in human trigeminal ganglia. Lancet **ii**:814–816.
13. **Kennedy, P. G. E., S. A. Al-Saadi, and G. B. Clements.** 1983. Reactivation of latent herpes simplex virus from dissociated identified dorsal root ganglion cells in culture. J. Gen. Virol. **64**:1629–1635.
14. **Krause, P. R., K. D. Croen, S. E. Straus, and J. M. Ostrove.** 1988. Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. J. Virol. **62**:4819–4823.
15. **Kristensson, K., A. Valne, A. Persson, and E. Lycke.** 1978. Neural spread of herpes simplex virus types 1 and 2 in mice after corneal or subcutaneous (footpad) inoculation. J. Neurol. Sci. **35**:331–340.
16. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. **63**:2893–2900.

16a. **McGeoch, D. (Institute of Virology, Glasgow, Scotland).** Personal communication.

17. **McLennan, J. L., and G. Darby.** 1980. Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. J. Gen. Virol. **51**:233–243.
18. **Mitchell, W. J., S. L. Deshmane, A. Dolan, D. J. McGeoch, and N. W. Fraser.** 1990. Characterization of herpes simplex virus type 2 transcripts during latent infection of mouse trigeminal ganglion. J. Virol. **64**:5342–5348.
19. **O'Brien, W. J., and J. L. Taylor.** 1989. The isolation of herpes simplex virus from rabbit corneas during latency. Invest. Ophthalmol. Visual Sci. **30**:357–364.
20. **Roizman, B.** 1979. The cellular organization of the herpes simplex virus genomes. Annu. Rev. Genet. **13**:25–57.
21. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpes virus alpha gene mRNA is prominent in latently infected neurons. Science **235**:1056–1059.
22. **Straus, S. E.** 1989. Clinical and biological differences between recurrent herpes simplex virus and varicella-zoster virus infections. JAMA **262**:3455–3458.
23. **Stroop, W. G., D. L. Rock, and N. W. Fraser.** 1984. Localization of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infection by in situ hybridization. Lab. Invest. **51**:27–38.
24. **Suzuki, S., and J. R. Martin.** 1989. Herpes simplex virus-type 2 transcripts in trigeminal ganglia during acute and latent infection of mice. J. Neurol. Sci. **93**:239–251.
25. **Tenser, R. B.** 1991. The role of herpes simplex virus thymidine kinase expression in viral pathogenesis and latency. Intervirology **32**:76–92.
26. **Tenser, R. B., M. Dawson, S. J. Resell, 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.
27. **Tenser, R. B., K. A. Hay, and W. A. Edris.** 1989. 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. J. Virol. **63**:2861–2865.
28. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. M. Slanina, and H. Ghiasi.** 1988. Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-related RNAs containing open reading frames. J. Virol. **62**:4051–4058.
29. **Wilkie, N. M., A. Davison, P. Chartrand, N. D. Stow, V. G. Preston, and M. C. Timbury.** 1978. Recombination of herpes simplex virus: mapping of mutations and analysis of intertypic recombinants. Cold Spring Harbor Symp. Quant. Biol. **43**:827–840.