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Characterization of Herpes Simplex Virus Type 2 Transcription during Latent Infection of Mouse Trigeminal Ganglia

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Using a cornea trigeminal ganglion model, we have investigated transcription by herpes simplex virus type 2 (HSV-2) during latency in mice. Latency was verified 2 months postinoculation by reactivation of HSV-2 after explant cocultivation of trigeminal ganglia from the majority of mice (83%). Transcription during latent HSV-2 infection was limited to the repeat regions of the viral genome as determined by in situ hybridization using restriction fragment probes representing 100% of the HSV-2 genome. Further mapping of the positively hybridizing region by using subfragments showed that transcription occurred from approximately 11.5 kb of contiguous DNA fragments. A 1.0-kb *Pvu*I-*Bam*HI fragment within the *Bam*HI F fragment and a 0.3-kb *Bam*HI-*Sal*I fragment and a 3.4-kb *Sal*I-*Bam*HI fragment within the *Bam*HI P fragment hybridized more strongly than other subfragments in in situ hybridization experiments. All positive signals were confined to the nucleus. The RNA that hybridized to the 3.4-kb *Sal*I-*Bam*HI DNA fragment probe by in situ hybridization corresponded to a 2.3-kb transcript on Northern (RNA) blots. Under our conditions for Northern blot hybridization, the 3.4-kb *Sal*I-*Bam*HI probe of HSV-2 hybridized to a limited degree with the latency-associated transcripts of HSV-1. Shorter spliced species of latency-associated transcript RNA, which are seen during HSV-1 latency, have not been detected in latent HSV-2 RNA. However, viral gene expression during HSV-2 latency appears to be very similar to that during HSV-1 latency.

Herpes simplex virus type 2 (HSV-2) is an important etiological agent in human disease (for a review, see reference 39). A significant proportion of the adult population has antibodies to HSV-2 and may harbor latent HSV-2 infections, and these latent infections may result in acute virus replication in immunocompromised or stressed hosts (39). Most clinical cases of recurrent genital herpesvirus infection are attributed to HSV-2. HSV-2 infection in the newborn, which is usually acquired from a latently infected mother, may cause severe disease with neurologic impairment. In addition, HSV-2 may cause sporadic cases of myelitis, meningitis, and encephalitis.

Numerous studies have been made of the mechanism of latency in HSV-1-infected animals (for a review, see reference 2) and humans (16, 30, 31). In situ hybridization studies of viral RNA expression during latency in mice have shown that there is limited transcription during latency (7, 8, 32) which maps to a region within the long repeat regions of the HSV-1 genome. Northern (RNA) blot analyses have defined three latency-associated transcripts (LAT), 2.0, 1.5, and 1.45 kb in size, which map to the *Pst*I-*Mlu*I restriction fragment within the *Bam*HI B and *Bam*HI E fragments (26, 32). These RNAs are transcribed in a rightward direction (in *Bam*HI-B) on the conventional map of the HSV-1 genome and overlap the 3′ end of the ICP0 gene, which is oriented leftward in *Bam*HI-B (see Fig. 3). In situ hybridization studies have described additional, minor latency-associated transcription which occurs in a rightward direction (in *Bam*HI-B and *Bam*HI-SP) and which corresponds to fragments that are mainly distal to the HSV-1 LAT-encoding fragments (8, 16, 20, 20a). No polypeptides encoded within the LAT have yet been identified in latently infected tissue, although a phenotype of reduced reactivation frequency (17), delayed reactivation (29), or failure to reactivate efficiently in vivo (14) has been associated with mutations in the viral LAT genes.

In contrast, the mechanism of HSV-2 latent infection in ganglionic neurons has received limited investigation. In previous studies, HSV-2 transcription during latency has been detected by in situ hybridization in human thoracic, lumbar, and sacral ganglia (11, 12), in trigeminal ganglia of guinea pigs (34), and in mice (1, 5, 33). The guinea pig models of HSV-2 latency that have been used (25, 27, 28) have proved difficult to use for molecular studies, and there appears to be a possibility of a persistent rather than a latent infection occurring.

In this report, we have established a model for HSV-2 latency in mice that is amenable to molecular studies. Data gained with this model indicate that, as with HSV-1, HSV-2 transcription is confined to the repeat regions of the viral genome. An HSV-2 LAT has been identified and partially mapped by in situ and Northern blot hybridization experiments. The role of the latency-associated transcription in HSV-2 is not known; however, it appears that detectable RNA expression during latency is not essential for establishment, maintenance, or reactivation from latency in HSV-1 (15, 17, 20a, 29). Nevertheless, there are data indicating a possible role of HSV-1 latency-associated transcription in facilitating reactivation (14, 17, 29).

MATERIALS AND METHODS

Virus and animal infections. HSV-2 (HG52) (35; obtained from S. M. Brown and J. H. Subak-Sharpe, Institute of Virology, Glasgow, United Kingdom) was grown on CV-1 cells, and virus stocks were prepared as previously described (26). Female BALB/c mice (4 to 6 weeks old) were

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anesthetized and inoculated with 10^6 to 10^7 PFU of HSV-2 in each eye after corneal scarification. Latently infected mice were killed a minimum of 4 weeks after infection. Latently HSV-1 (F)-infected ganglia were derived as previously described (8).

Explant cocultivation. Trigeminal ganglia were removed from latently HSV-2-infected mice (as described above) and placed into culture with monolayers of CV-1 cells. Ganglia were transferred to new cell monolayers after each 4 to 5 days, and the monolayers were observed for cytopathic effects over a total of 4 weeks.

Preparation and labeling of probes. Plasmids containing the *Hin*dIII IK, B, H, E, A, JM, L, and O fragments, the *Bgl*II G fragment, and the *Sal*I A fragment (22) (see Fig. 3) were obtained from G. Hayward. These plasmids were constructed from HSV-2 (333). The *Bam*HI F fragment was derived from HSV-2 (HG52). Plasmid DNA was prepared by standard techniques (19). DNA fragments and subfragments were prepared by restriction digestion and isolation of fragments from agarose gels as described previously (20). The *Pst*I-*Mlu*I fragment of HSV-1 was prepared and labeled as previously described (8). Double-stranded DNA fragment probes were labeled with ^{32}P or ^{35}S by nick translation (19).

In situ hybridizations. Trigeminal ganglia from uninfected, latently HSV-2-infected, and latently HSV-1-infected mice were fixed in PLP (2% paraformaldehyde, 0.1 M lysine hydrochloride, 0.5 M sodium phosphate buffer [pH 7.4], 0.1 mM sodium *m*-periodate), embedded in paraffin, and sectioned at 6 μm. Sections were affixed to poly-L-lysine and 30% Elmer's glue-coated slides. In situ hybridizations were performed as described previously (8, 13) except that the initial wash was at 50°C for 4 h, followed by a 48- to 72-h wash at room temperature. Detection of hybridization was by autoradiography with NTB-2 emulsion. Slides were developed in Kodak D-19 developer after 5 to 6 days of exposure. Slides were stained with hematoxylin and eosin and examined by light microscopy.

Northern blot hybridization. RNA was isolated from trigeminal ganglia of mice latently infected with HSV-2 (HG52) or HSV-1 (F) and from the brains of uninfected mice as described previously (26). Total RNA was denatured, separated by electrophoresis, and transferred to filters (GeneScreen; Dupont, NEN Research Products), and the filters were prehybridized, hybridized, and washed as previously described (20). Hybridization was detected by autoradiography with XAR-5 film and intensifying screens.

Restriction digestion and analysis of viral DNA. Viral DNA from parental HSV-2 (HG52), reactivated HSV-2 (HG52), and HSV-1 (F) was labeled with [^{3}H]thymidine and prepared as previously described (9). The viral DNA was digested with *Bam*HI and separated by electrophoresis. The gels were dried and autoradiographed (9).

RESULTS

Establishment of an HSV-2 model. Infection of 4-week-old mice via the cornea with 10^6 to 10^7 PFU of HSV-2 (HG52) resulted in 0% mortality. These mice were latently infected when tested 2 months after inoculation (see below). In contrast, HSV-2 (333) and (186) resulted in 100% mortality when inoculated via the cornea at similar dosages. Lower dosages of HSV-2 (333) and (186) that did not result in death also did not result in verifiable latent infections by explant cocultivation, in situ hybridization, or DNA spot blot analysis (data not shown).

Explant reactivation. Ganglia dissected from mice latently infected with HSV-2 (HG52) were cocultivated with CV-1 cells as described in Materials and Methods. At least one ganglion from 10 of 12 (83%) of the latently infected mice yielded infectious virus after 9 to 17 days. Virus could be recovered from 50% (12 of 24) of the ganglia from 12 mice that were tested by explant cultures (Fig. 1). In control experiments, all of eight ganglia from four mice latently infected with HSV-1 (similar inoculum dosages) yielded virus after 5 to 7 days in culture. The reactivated HSV-2 (HG52) was verified to be HSV-2 after comparison with the inoculum virus by *Bam*HI restriction enzyme digestion and electrophoretic separation (Fig. 2).

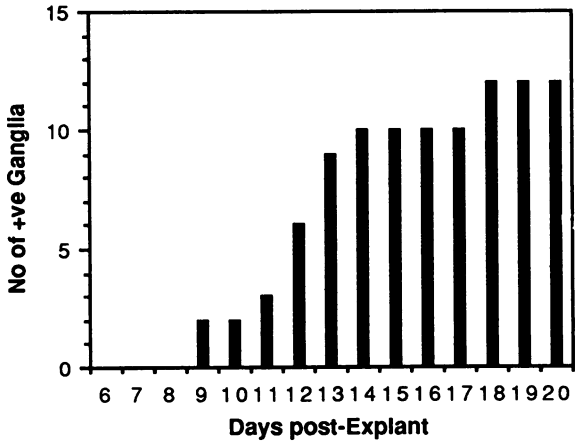

FIG. 1. Reactivation of HSV-2 from latently infected mouse trigeminal ganglia after explant cocultivation. The numbers of positive ganglia are represented on the vertical axis, and the days postexplant at which ganglia become positive are represented on the horizontal axis. Bars represent the cumulative total of positive ganglia at a particular time point. Twenty-four trigeminal ganglia from 12 mice were tested. At least one positive ganglion was present in 10 of 12 mice.

Mapping of LATs. Using in situ hybridization assays, we have mapped transcription from the HSV-2 genome in latently infected mouse trigeminal ganglia. DNA fragments representing 100% of the HSV-2 genome (Fig. 3) were used as probes on sections of latently infected mouse trigeminal ganglia. The *Hin*dIII JM and *Hin*dIII IK fragment probes showed positive signals in in situ hybridization assays (Fig. 3A). Results for *Hin*dIII-B, -H, -E, -A, -O, and -L and *Bgl*II-G probes were negative.

To determine which regions of the positive fragments were responsible for the hybridization signals, we used subfragments of the *Hin*dIII JM and *Bam*HI F fragments as probes (Fig. 3B). Results from the in situ hybridizations on latently HSV-2-infected mouse ganglia with use of these subfragment probes showed that there was a region of strong hybridization that included the *Pvu*I-*Bam*HI fragment of *Bam*HI-F (Fig. 4A), the *Bam*HI-*Sal*I 0.3-kb fragment of *Bam*HI-P, and all or part of the *Sal*I-*Bam*HI 3.4-kb fragment (which includes most of *Bam*HI-P) (Fig. 4C). Sections from uninfected animals showed no positive cells (Fig. 4B and D). In addition, there was a region of weaker hybridization to the right of the strongly hybridizing region (Fig. 4B). This more weakly hybridizing region included all or part of the 5.8-kb *Bam*HI VU fragment (Fig. 4E). Figure 4F shows uninfected ganglia analyzed with this probe. An approximately 1.0-kb *Bam*HI-*Sal*I fragment was very weakly positive in a limited number of animals (Fig. 3B; results not shown). All of these

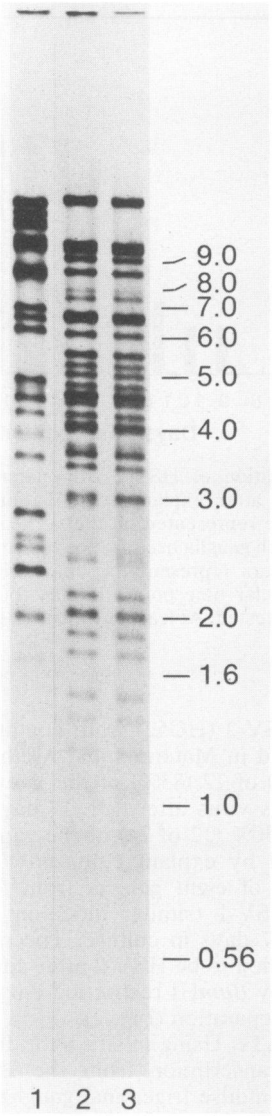

FIG. 2. Restriction endonuclease digestion analysis of reactivated virus. [^{3}H]thymidine-labeled viral DNA was digested with the restriction enzyme *Bam*HI and separated by gel electrophoresis. Lanes: 1, HSV-1 (F) genomic DNA; 2, inoculum virus HSV-2 (HG52) genomic DNA; 3, reactivated virus HSV-2 (HG52) genomic DNA. Numbers to the right are size markers in kilobases.

positively hybridizing DNA fragments are located within the repeat regions of the HSV-2 genome.

HSV-2 LAT. RNA from latently HSV-2 (HG52)-infected ganglia was subjected to agarose gel electrophoresis, Northern blot transferred to a filter, and hybridized with the 3.4-kb *Sal*I-*Bam*HI fragment probe. This probe had been previously determined to be a strongly hybridizing region of the HSV-2 genome by in situ hybridization (Fig. 3B). A 2.3-kb RNA band was found to be present in the blot of RNA from latently HSV-2-infected ganglia (Fig. 5A, lane 1). This HSV-2 LAT was slightly larger than the LAT of HSV-1 (F) (Fig. 5A, lane 3). The RNA samples were treated identically as paired samples and separated in adjacent wells on the same gel. The samples were transferred to a filter, which was then divided for hybridization. Hybridized blots were exactly aligned for autoradiography. The 5.8-kb *Bam*HI VU fragment (Fig. 3B), which was positive by in situ hybridization and was adjacent and to the right of the 3.4-kb *Sal*I-*Bam*HI fragment, did not hybridize to any detectable bands on Northern blots of RNA from latently HSV-2-infected ganglia (results not shown).

Homology between HSV-1 and HSV-2 LATs. Nucleic acid probes specific for HSV-1 and HSV-2 would be most useful for hybridization studies of human tissue; therefore, we examined the potential for cross-hybridization between HSV-1 and HSV-2 LAT RNAs. Sections of latently HSV-1-infected ganglia were hybridized with ^{35}S-labeled DNA fragments that map to either the HSV-1 LAT (*Pst*I-*Mlu*I) or the HSV-2 LAT (3.4-kb *Sal*I-*Bam*HI). Sections of latently HSV-2-infected ganglia were hybridized with the same two probes. The in situ hybridization assays for these experiments were performed as described in Materials and Methods. A small percentage of latently HSV-1-infected neurons showed hybridization with the HSV-2 LAT. Similarly, only a few latently HSV-2-infected neurons were positive with the HSV-1 LAT probe (data not shown). In addition, a 0.3-kb *Bam*HI-*Sal*I fragment from within the HSV-2 LAT region (Fig. 3B) that does not overlap with the ICP0 region was used as a probe in in situ hybridization on latently HSV-1- and HSV-2-infected ganglia as described above. This probe also resulted in a weaker hybridization in a smaller number of neurons in the HSV-1-infected ganglia (data not shown).

We detected a weak signal from the HSV-1 LAT RNA when we used the HSV-2 LAT probe (*Sal*I-*Bam*HI 3.4-kb fragment) in Northern blot hybridizations after 14 days of autoradiography (Fig. 5B, lane 3). Thus, there was weak homology between HSV-2 and HSV-1 LAT RNAs when the RNAs were tested with the above-described probes and conditions, in in situ hybridizations, and in Northern blot hybridizations. Northern blot studies of HSV-1 LATs have revealed two minor bands of approximately 1.5 and 1.45 kb (26). These bands were detected for HSV-1 (Fig. 5) but did not appear to be present in HSV-2 (Fig. 5B, lane 1).

DISCUSSION

Although HSV-2 infections are usually associated with genital lesions and HSV-1 is associated with oral lesions, the viruses are interchangeable with regard to site of infection (3, 21, 39). Thus, we are confident that studies of HSV-2 latency in the trigeminal ganglia are applicable to HSV-2 latency at the molecular biological level.

In this study, we have established a model for HSV-2 latency in the trigeminal ganglia of mice which is amenable to molecular biological studies. Several different strains of HSV-2 were examined for their usefulness in mouse latency models. Of the several strains tested, HSV-2 (HG52) provided the best result (50% of ganglia latently infected) with the minimum killing. Suzuki and Martin (33) recently described a mouse HSV-2 latency model, using intracranial inoculation of HSV-2 (MS). This strain was not included in our study.

Our data indicate that transcription during latency of HSV-2 is limited to the repeat regions of the viral genome. This transcription consists of (i) a region that is strongly positive upon in situ hybridization (1.0-kb *Pvu*I-*Bam*HI and 3.4-kb *Sal*I-*Bam*HI fragments) and corresponds to a 2.3-kb transcript (HSV-2 LAT) detected on Northern blots (Fig. 5A) and (ii) a region of weaker in situ hybridization (5.8-kb *Bam*HI VU and 1.0-kb *Bam*HI-*Sal*I fragments) adjacent and to the right of the HSV-2 LAT region (Fig. 3 and 4); no transcript is detectable on Northern blots when the *Bam*HI

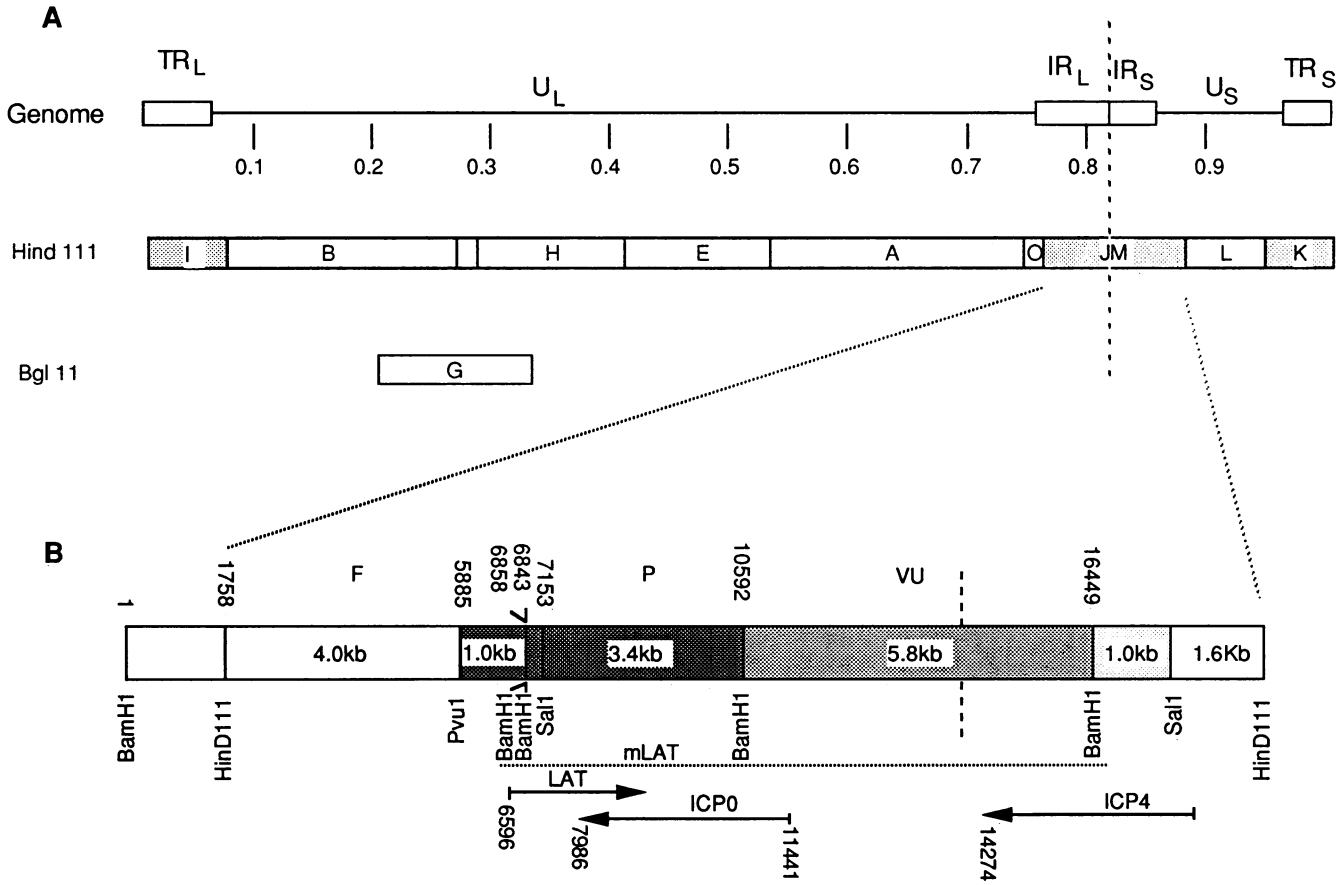

FIG. 3. (A) HSV-2 (HG52) genome showing restriction fragments that were used as probes. Positions of the unique long (U_L), unique short (U_S), long terminal repeat (TR_L), short terminal repeat (TR_S), long internal repeat (IR_L), and short internal repeat (IR_S) regions are indicated, as are the *Hin*dIII fragments (I, B, H, E, A, O, JM, L, and K) and the *Bgl*II G fragment. The fragments positive by in situ hybridization are shaded. (B) Enlargement of the *Hin*dIII JM and *Bam*HI F fragments showing subfragments that were used as probes. The restriction fragments are designated by vertical bars, with the restriction enzyme listed below. The sizes of the fragments, calculated from the sequence, are shown in kilobases. The *Bam*HI-*Sal*I 1.0-kb and *Sal*I-*Hin*dIII 1.6-kb fragment sizes are estimated. The darkly shaded fragments are strongly positive, the lightly shaded fragments are weakly positive, and the open fragments are negative. Sequence numbers are based on determination of the sequence of the HSV-2 (HG52) *Bam*HI F, P, and VU fragments (McGeoch et al., unpublished data). The arrows indicate transcripts; the HSV-2 LAT RNA is drawn in a rightward direction with analogy to the HSV-1 LAT.

VU fragment is used as a probe (data not shown). Furthermore, unlike HSV-1, short species of LAT RNA (1.5 and 1.45 kb) cannot be detected on Northern blots of RNA from latently HSV-2-infected ganglia. However, it cannot be ruled out that these short species accumulate in amounts too small to be detected by Northern blotting.

The HSV-2 LAT (by analogy to HSV-1) is transcribed in a rightward direction (opposite that of ICP0) and is in a map position equivalent to that of the HSV-1 LAT (23, 26, 38). Within the sequence of this region of strain HG52 (D. J. McGeoch, A. Dolan, and C. Cunningham, unpublished data), the sequence GGTAGGTT, to which the 5′ end of the 2-kb LAT RNA of HSV-1 has been mapped (37), is present 6,593 nucleotides from the left *Bam*HI site of *Bam*HI-F (Fig. 3B). The more weakly hybridizing region of HSV-2 maps to a region of the genome that appears to be very similar to the region of minor latency-associated transcription of HSV-1 (8, 20). This minor latency-associated transcription region of HSV-1 has been reported to produce in infected cultured cells an 8.3-kb transcript that overlaps the HSV-1 LAT (10). Therefore, the map positions of RNA transcribed from the HSV-2 genome during latency appear to be very similar to those of the HSV-1 RNA. The significance of the slight difference in size between the HSV-2 LAT (2.3 kb) and the HSV-1 (F) LAT (2.0 kb) is not known. Different strains of HSV-1 produce LATs that differ slightly in mobility (26), suggesting that slight variations in LAT size have little importance and cannot be used to differentiate between HSV-1 and -2.

The map position of HSV-2 latency-associated transcription as defined by our studies differs significantly from the data of Galloway et al. (11, 12). In in situ hybridization studies of human thoracic, lumbar, and sacral dorsal root ganglia, these authors reported that transcription of HSV-2 during latency occurs mainly in the region of 0.07 to 0.32 map units on the genomic DNA. Other areas of hybridization at 0.32 to 0.39, 0.58 to 0.62, and 0.66 to 0.73 map units were also occasionally detected in these studies. These previously reported regions of hybridization during latency are within the long unique region of the HSV-2 genomic DNA. Since studies of HSV-1 transcription during latency in mouse and human neurons have been shown to be very similar (30, 31), it does not seem likely that latency-associated transcription of HSV-2 in human ganglia should differ from that in mouse ganglia. Also, studies of HSV-1 transcription in mouse dorsal root ganglia reflect very closely the results of studies

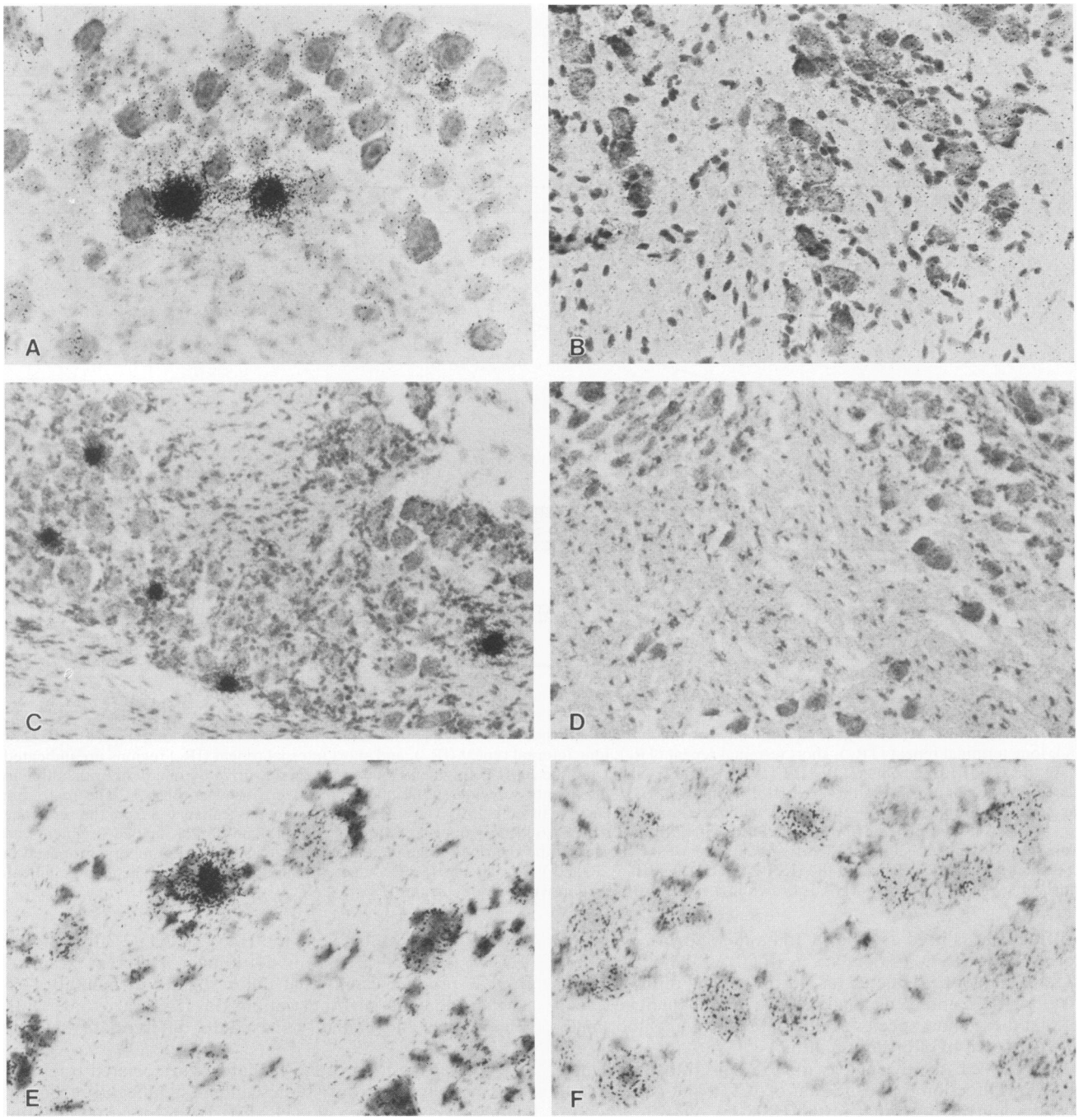

FIG. 4. In situ hybridizations of mouse trigeminal ganglia latently infected with HSV-2. (A, C, and E) Sections from latently infected mouse trigeminal ganglia; (B, D, and F) sections from uninfected mouse trigeminal ganglia. Probes: (A and B) *Pvu*I-*Bam*HI 1.0-kb fragment; (C and D) *Sal*I-*Bam*HI 3.4-kb fragment; (E and F) *Bam*HI-*Bam*HI 5.8-kb fragment.

in latently infected trigeminal ganglia. Therefore, it does not appear that the differences between tissues (trigeminal ganglia in our studies and dorsal root ganglia in the previously described studies [11, 12]) would account for the differences in mapping.

The function and significance of latency-associated transcription from the HSV-2 genome is not known. However, it appears that detectable transcription from the LAT region of the HSV-1 genome is not essential for establishment, maintenance, and reactivation of latency of HSV-1 (15, 17, 20a, 29). It was recently shown that LAT-minus viruses are less efficient (17), are slower to reactivate from latency (29), or fail to reactivate in an in vivo model (14); thus, the putative product of the LAT gene may play a role in reactivation.

Comparative studies of the HSV-1 LAT coding region (37, 38) showed that at least two common open reading frames, ORF1 and ORF2, were present among three different type 1 strains. However, our initial studies of the sequence of the

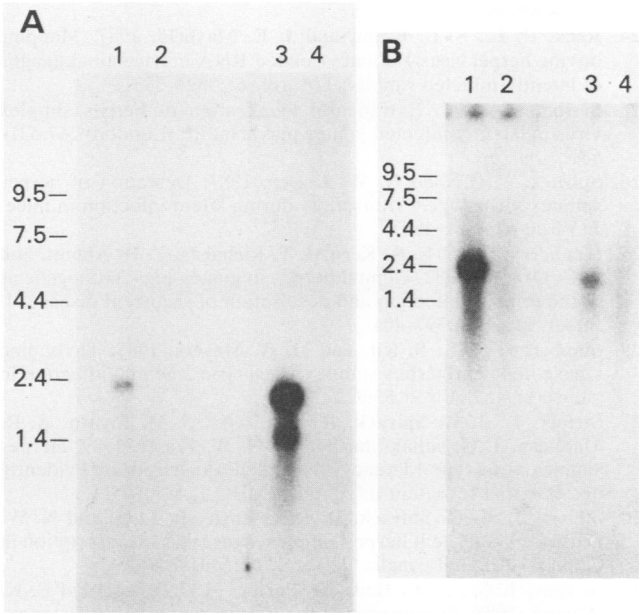

FIG. 5. Northern blot hybridizations showing the size and cross-hybridization with HSV-1 LAT of an HSV-2 LAT. (A) Lanes: 1, 15 μg of latently HSV-2-infected mouse trigeminal ganglion RNA; 2, 15 μg of uninfected mouse brain RNA; 3, 15 μg of latently HSV-1 (F)-infected mouse trigeminal ganglion RNA; 4, 15 μg of uninfected mouse brain RNA. Lanes 1 and 2 were hybridized with the *Sal*I-*Bam*HI fragment of HSV-2; lanes 3 and 4 were hybridized with the *Pst*I-*Mlu*I 3.0-kb HSV-1 DNA probe. Autoradiograph was exposed for 7 days. (B) Lanes: 1, 15 μg of latently HSV-2-infected mouse trigeminal ganglion RNA; 2, 15 μg of uninfected mouse brain RNA; 3, 15 μg of latently HSV-1 (F)-infected mouse trigeminal ganglion RNA; 4, 15 μg of uninfected mouse brain RNA. All lanes were hybridized with the *Sal*I-*Bam*HI fragment of HSV-2.

region of the genome to which the HSV-2 LAT maps indicate that although there is much sequence similarity with HSV-1 in the LAT promoter region, the potential translational open reading frames are different from those of HSV-1 (D. J. McGeoch, A. Dolan, and C. Cunningham, unpublished data). This finding agrees with the lack of common open reading frames found in HSV-1 strains by Lynas et al. (18). Since HSV-1 and HSV-2 are very similar in structure and general biology, these findings make it doubtful that the major LAT RNAs of herpesviruses encode functional proteins of any significant size. It may be that the minor latency-associated RNA encodes a functional protein. Furthermore, it is possible that LATs function through some other mechanism than via a protein; however, as yet there are no experimental data to support this idea.

In addition to the descriptions of HSV-1 and HSV-2 latency-associated transcription, recent data on transcription of pseudorabies virus and bovine herpesvirus type 1 during latent infections of trigeminal ganglia indicate that latency-associated transcription for these neurotropic herpesviruses is limited, partially overlaps an immediate-early gene, and is in a direction opposite that of the immediate-early gene (4, 24). It appears that this limited latency-associated transcription scheme may be common to all herpesviruses that latently infect peripheral ganglia. However, the situation for varicella-zoster virus is not yet clear (6, 36).

We have examined the degree of homology between the HSV-2 and HSV-1 LATs under experimental conditions that would be used in laboratory assays for viral nucleic acids in tissues and shown that there is a low level of homology which is evidenced by weak hybridization of the HSV-2 LAT probe (3.4-kb *Sal*I-*Bam*HI fragment) to HSV-1 LAT RNA (Fig. 5B). In reciprocal experiments, small numbers of latently HSV-2-infected neurons hybridize with the HSV-1 LAT-specific probe (*Pst*I-*Mlu*I fragment), and some latently HSV-1-infected neurons hybridize with the 3.4-kb *Sal*I-*Bam*HI and 0.3-kb *Bam*HI-*Sal*I fragments of HSV-2 in in situ hybridizations. Thus, it seems likely that the use of a DNA fragment from the LAT-encoding region of HSV-1 or HSV-2 as a probe will not easily ensure diagnostic accuracy in Northern blots and in situ hybridizations.

In conclusion, using the techniques of molecular biology, we have shown that HSV-2 (HG52) can form latent infections in the trigeminal ganglia of BALB/c mice and that sufficient neurons are latently infected to allow analysis of HSV-2 latency. Transcription during latency is very similar to that previously reported for HSV-1. There is a 2.3-kb RNA species detected by Northern blotting but apparently no smaller spliced species. As with HSV-1, there is a region of weaker transcription detected mainly downstream of the 2.3-kb transcript.

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