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A Herpes Simplex Virus Type 1 Latency-Associated Transcript Mutant Reactivates with Normal Kinetics from Latent Infection

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The herpes simplex virus type 1 (HSV-1) latency-associated transcripts (LATs) accumulate in neuronal nuclei of latently infected ganglia. Explant reactivation kinetics of LAT deletion mutants in the mouse eye model have suggested a role for the LATs in the reactivation process. This report describes the construction and characterization of an HSV-1 strain HFEM mutant, TB1, disrupted within both copies of the LAT gene. TB1 contains a 440-base-pair segment of bacteriophage lambda DNA in place of a 168-base-pair deletion within the transcribed portion of the LAT gene. The 2.0-kilobase LAT was not produced after infection of tissue culture cells with TB1, but a 0.7- to 0.8-kilobase RNA was expressed. TB1 did establish latent infection after corneal inoculation as efficiently as the parental virus, and its reactivation kinetics from explanted ganglia were similar to those of HFEM. During latent infection with TB1, HSV-1 transcripts were not detectable. Rescuant virus (TB1-R) contained intact LAT genes, synthesized full-length LAT transcripts during productive infection in tissue culture, and reactivated from ganglionic explants of latently infected mice with normal kinetics. Thus, any function these transcripts have in the reactivation process appears to include the region between the putative LAT promoter and the disruption in TB1—a region of approximately 1,600 nucleotides, 800 of which encode the LATs.

Human infection with herpes simplex virus (HSV) is characterized by the persistence of viral genetic information in neurons of the peripheral nervous system for the lifetime of the individual in the absence of overt disease (6, 13, 23). This latent state can be interrupted by reactivation of virus that results in productive infection and recurrent disease.

Mouse models of HSV latency have been extremely informative (reviewed in reference 6) and have revealed that the viral genetic information in trigeminal ganglia exists primarily as an episome (14, 17). Transcription of HSV type 1 (HSV-1) during latency is limited to one region of the viral genome located in the repeat regions IR_L and TR_L (2, 18, 19, 24). Three predominately poly(A)$^-$ transcripts of 2.0, 1.5, and 1.45 kilobases (kb) are present in latently infected neurons (19, 26, 27). The two smaller latency-associated transcripts (LATs) are spliced (27, 28). These LATs (24) map to a region of the inverted repeats that partially overlaps with the alpha gene, ICP0 (alpha 0). However, ICP0 and the LATs are transcribed from different DNA strands of the viral genome (19, 24). Since the LATs are found in the latently infected cell, it is important to know what function(s), if any, they serve in the establishment, maintenance, or reactivation from the latent state.

An HSV-1 × HSV-2 recombinant with predominately HSV-1 genetic material which does not express LATs established latent infections after mouse footpad infection (8). Since mutant and wild-type viruses were recovered from lumbar ganglia of infected mice with equal frequency at similar times after explantation, it was concluded that the LATs are dispensable for the establishment of latent infections. However, studies with an HSV-1 deletion mutant virus (1704), while supporting a nonessential role in establishment of latent infection, have suggested a role for the LATs in the reactivation process (22).

HSV-1 variant 1704 possesses a 3.8-kb deletion in IR_L and adjacent unique DNA sequences and a 1.2-kb deletion in TR_L (11). Variant 1704 does not express LATs in tissue culture or latent infection in mice (22). Although 1704 did establish latent infections in mice, explant reactivation of virus was significantly slower than that of wild-type parental strain 17$^+$. Moreover, Leib et al. (9) have recently reported that a strain KOS-derived LAT$^-$ deletion mutant (*dl* LAT 1.8) reactivates from explants less efficiently than its LAT$^+$ parent. Therefore, the data suggest that the LATs play a role in mediating reactivation.

We constructed a defined mutant containing a single disruption between nucleotides +839 and +1007 (with respect to the proposed RNA start site [29]) of the 1.5- to 2.0-kb LATs to study their role in latency and reactivation. In this communication, we describe the production and characterization of the biochemical and biological properties of an HSV-1 strain HFEM-derived mutant that does not express full-length 1.5- to 2.0-kb LATs because of a replacement mutation. This mutant, TB1, expresses a 0.7- to 0.8-kb LAT region transcript in tissue culture but no detectable transcripts during latency. TB1 established latency in mice after ocular infection as efficiently as its parent and reactivated from explant culture with normal kinetics, which suggests that the first 838 base pairs (bp) of the LATs are involved in their biological function.

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MATERIALS AND METHODS

Cells, media, and virus. CV-1 cells were maintained at 37°C and 5% CO_2 as monolayers in an Eagle medium supplemented with 5% newborn calf serum (1). HSV-1 strain

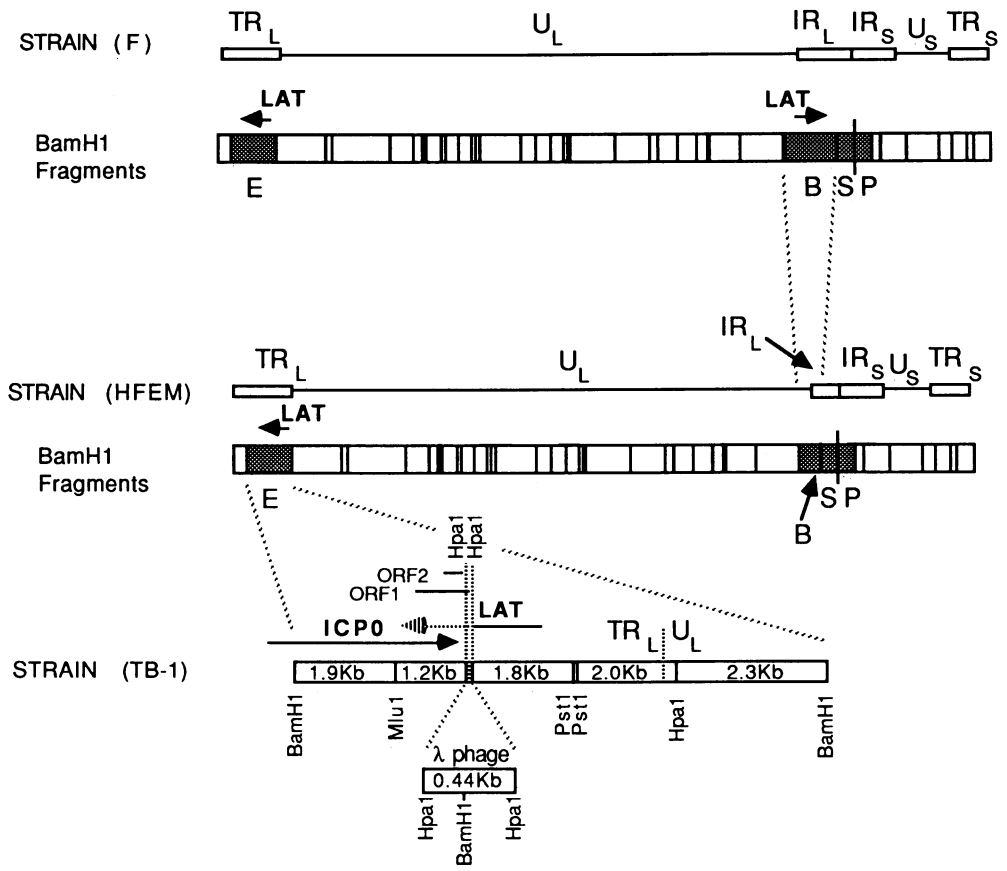

FIG. 1. Map of the HSV genome, the LATs, and the region affected in TB1. Maps of HSV-1 strains F (shown for comparison), HFEM, and TB1 are shown. HFEM contains a deletion within the *Bam*HI B fragment affecting the LATs contained in the long internal repeats (IR_L). TB1, derived from HFEM, contains a replacement mutation within the LAT sequences in the *Bam*HIE fragment. An expansion of the *Bam*HI E fragment is provided (strain TB1), showing the sizes of restriction fragments (numbers in boxes indicate the size of the restriction fragment in kilobases) as well as the locations of the ICP0 and LAT genes, with their directions of transcription (arrows) and the open reading frames described by Wechsler et al. (29). In TB1, a 0.18-kb *HpaI-HpaI* fragment has been replaced by a 0.44-kg λ phage fragment.

HFEM was the parent of all virus mutants. HFEM, derived from strain HF, contains a 4.1-kb deletion within the *Bam*HI B fragment (21). Thus, HFEM contains only one complete copy of the LAT genes (21). Strains 17^+, 1704, and F were also used (22). Plaque purifications were done by inoculating CV-1 cells with dilutions of cell-free virus and overlaying with methylcellulose (2%, 4,000 cP). DNA was isolated from virions as described by Pignatti et al. (16).

Plasmids and DNA transfections, isolation of mutants, and DNA dot-blot analysis. The *tk*-specific probe was pHSV106 (a gift from S. McKnight; described in reference 1). Plasmid PstI-MluI is a pUC18 derivative that contains the 3-kb *PstI-MluI* fragment of the HSV-1 genome spanning the LATs (see reference 21). Plasmid λ-PstI-MluI contains a 440-bp *HpaI* fragment of lambda DNA replacing the 180-bp *HpaI* fragment within *PstI-MluI* (Fig. 1). The 180-bp HSV sequence, deleted in λ-PstI-MluI, was cloned into pUC18 and is called Hpa-Hpa-0.180. The LAT-specific probe was a pUC18 plasmid containing the 1.8-kb HSV sequence between the *PstI* and *HpaI* sites shown in Fig. 1. The alpha 0-specific probe, called MluI-BamHI, is a pUC18 plasmid containing the 1.9-kb *MluI-Bam*HI fragment (Fig. 1). λ-PstI-MluI linearized by *Eco*RI digestion and HFEM virion-derived DNA were transfected to subconfluent monolayers of CV-1 cells by the calcium phosphate precipitation method (1). Four hours after incubation with the precipitate, monolayers were washed and incubated with 20% dimethyl sulfoxide in growth media for 3 min. Monolayers were washed free of dimethyl sulfoxide, overlaid with methylcellulose media, and observed until plaques appeared (usually within 1 week). Plaques were picked and amplified by overnight growth in CV-1 cells. Cell lysates were tested for the presence of lambda DNA by immobilization on nitrocellulose paper and hybridization to a ^{32}P-labeled lambda DNA probe. Positive plaques were subjected to several rounds of plaque purifications before expansion for mass production.

Infection of mice and explant reactivation. After corneal scarification, 4- to 6-week-old female BALB/cBYJ (Jackson Laboratory, Bar Harbor, Maine) mice were infected with approximately 10^6 to 10^7 PFU of HFEM, TB1, TB1-R (rescuant), 17^+, or 1704 per eye. At a minimum of 4 weeks after infection, latently infected mice were killed and the trigeminal ganglia were removed and incubated with monolayers of CV-1 cells. The wells were inspected daily for signs of cytopathic effect. Ganglia were transferred every 4 to 5 days to new cells and observed until reactivation occurred or for 30 days. When virus reactivated, the virus-containing media were removed and saved for DNA extraction. As a reactivation control, ganglia from mice 5 and 7 weeks after infection were titered at the time of explant for infectious virus.

In situ hybridization. Trigeminal ganglia were fixed for 24

to 48 h in fresh paraformaldehyde-lysine-periodate fixative at 4°C, dehydrated in ethanol, and embedded in paraffin (2, 4, 25). Sections 5 to 6 μm thick were mounted on poly-L-lysine-treated slides, deparaffinized with xylenes, and treated with proteinase K. ^{35}S-labeled nick-translated probes were diluted in hybridization mix to contain 1 ng of DNA probe per 5 μl and approximately 10^5 cpm per tissue section. Heat-denatured hybridization mixture (5 μl) was placed on each tissue section and covered with a baked siliconized cover slip and paraffin oil. After hybridization at 50°C for 48 h, the paraffin oil was removed with a chloroform wash and the slides were washed, dehydrated in ethanol, dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.), and exposed for 1 to 2 days at 4°C. After processing with D19 developer (Kodak) and fixer (Kodak), the sections were stained with hematoxylin and eosin. Control ganglia obtained from uninfected BALB/c mice were similarly processed.

Plaque purification. When cytopathic effects were first noted in monolayers during explant reactivation of TB1, fresh media containing human immune serum globulin (Cutter Gamastan; 3 μl/ml) were added to prevent extracellular spread of the virus. After 24 h, isolated plaques were picked to grow viral stocks for DNA extraction as described below.

DNA and RNA analysis. Restriction analysis of virion DNA was done as follows: Virion DNA was prepared (1), digested to completion with restriction endonucleases according to the specifications of the manufacturers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), resolved by electrophoresis through 0.8% agarose gels, and transferred to nitrocellulose paper by the Southern transfer method (12). The presence of lambda (λ)- or LAT-specific DNA within restriction fragments was detected by hybridization to nick-translated DNA probes, washing, and exposure to X-ray film (12).

RNA produced in productively infected cells was analyzed as previously described (19–21). Briefly, CV-1 cells were infected as described in the text. At the indicated times, cells were scraped into a guanidine thiocyanate lysis solution and RNA was sedimented by ultracentrifugation through a CsCl cushion. RNA pellets were suspended in water containing RNasin and resolved by electrophoresis through 1.2% agarose–formaldehyde gels followed by transfer to Gene Screen Plus (Dupont, NEN Research Products, Boston, Mass.). Filters were hybridized to nick-translated probes, washed, and autoradiographed (12).

RESULTS

Construction and isolation of HSV-1 mutant with replacement mutation within LAT genes. The objective of this study was to begin a functional analysis of the LATs. A strategy was designed to construct an HSV-1 variant with a disruption of all three LATs (19, 26) and removal of the putative splice acceptor sites (27, 28) for the 1.45- to 1.5-kb LATs (19). To construct such a virus, a 440-bp segment of bacteriophage lambda DNA was used to replace the 0.18-kb HSV-1 sequence between the two *Hpa*I sites within the LATs (Fig. 1). The presence of lambda DNA within the mutant genome provides an unambiguous marker of the mutant.

HSV-1 strain HFEM contains a deletion within the *Bam*HI B fragment resulting in the loss of LATs from IR_L. Therefore, HFEM contains only one copy of the LAT gene(s) (21) (Fig. 1). Since HFEM establishes latency in mice (13, 19), it was the target for our mutagenesis study. Genomic HFEM DNA was cotransfected into CV-1 cells with linearized plasmid λ-PstI-MluI. PstI-MluI plasmid contains 3 kb of HSV-1 DNA spanning the LAT gene(s) (Fig. 1). λ-PstI-MluI contains a deletion of 0.18 kb of HSV-1 DNA and an insertion of lambda DNA between the two *Hpa*I sites. More than 200 plaques were picked from the transfection and examined for the presence of lambda DNA within the viral genome by dot-blot hybridization analysis. Figure 2 shows a representative dot-blot profile of 66 plaques occurring after transfection of HFEM genomic and λ-PstI-MluI plasmid. Cell lysates were hybridized with HSV thymidine kinase gene (*tk*)-specific probe to detect HSV DNA (Fig. 2A). Paired spot blots corresponding to those above were hybridized to radioactive lambda DNA (Fig. 2B). Only 1 of the 66 virus isolates in this figure reacted with the lambda probe (Fig. 2B, slot e4). From all the blots performed, four plaques were positive for lambda sequences, and one, called TB1, was selected for further study after five series of plaque purifications.

To isolate rescuants of TB1 that contained intact LAT genes, we cotransfected TB1 genomic DNA into CV-1 cells with linearized PstI-MluI plasmid. Plaques that appeared after transfection of TB1 genomic and plasmid PstI-MluI DNA were isolated from several different wells of a culture tray and expanded for the determination of the presence of the 180-bp LAT sequence deleted in TB1 (Fig. 2C and D). HFEM, TB1 (Fig. 2C, slots B8 and B10, respectively), and all 15 isolates (Fig. 2C, slots A1 to A12 and B1 to B3) were positive with a probe which hybridized to the HSV *tk* gene, thereby revealing the presence of HSV DNA. The Hpa-Hpa-0.180 probe (specific for the 180-bp LAT sequence deleted in TB1) hybridized to HFEM (Fig. 2D, slot B9) but not to TB1 (Fig. 2D, slot B11) isolates, as expected, and confirmed its specificity. Remarkably, 12 of 15 isolates from this marker transfer experiment were positive for the 180-bp LAT sequence (Fig. 2D, slots A1 to A12). Two rescuants, TB1-R3 and -R17, were studied in more detail (see below).

To confirm that the lambda DNA present in TB1 did disrupt the LAT gene(s), genomic DNA from parental HFEM and TB1 was digested with restriction enzymes and analyzed by Southern blots. Hybridization of the HpaI-HpaI 0.180 probe to *Hpa*I digestions of HFEM generated the expected 0.18-kb band that contained the HSV sequence deleted in plasmid λ-PstI-MluI (Fig. 3A, lane 1). The Hpa-Hpa-0.180 probe also hybridized to the expected 9.2-kb fragment corresponding to *Bam*HI-E present in *Bam*HI digests of HFEM (Fig. 3A, lane 2). The Hpa-Hpa-0.180 probe did not hybridize to TB1 DNA (Fig. 3A, lanes 3 and 4). However, a 0.440-kb *Hpa*I fragment derived from TB1 DNA was recognized by the lambda probe (Fig. 3B, lane 3). Moreover, the lambda probe hybridized to 3.2- and 6.3-kb *Bam*HI fragments of TB1 (Fig. 3B, lane 4), as would be expected if the insertion of the altered LATs had occurred within the *Bam*HI E fragment, because the lambda insert contains a *Bam*HI site (Fig. 1). In addition, the *Bam*HI B probe hybridized to the 9.2- and 7-kb *Bam*HI E and B fragments of HFEM, respectively (Fig. 3C, lane 1), and hybridized to the 7-kb *Bam*HI B fragment in the TB1 digest, indicating that this region of the TB1 genome was not altered. The 9.2-kb *Bam*HI E fragment was replaced by two *Bam*HI fragments of 6.3 and 3.2 kb (Fig. 3C, lane 2) which also hybridized to the lambda probe (Fig. 3B). These results show that in TB1 the LAT sequence contained in plasmid Hpa-Hpa-0.180 was replaced with the lambda fragment. This conclusion has been supported by Southern blots with

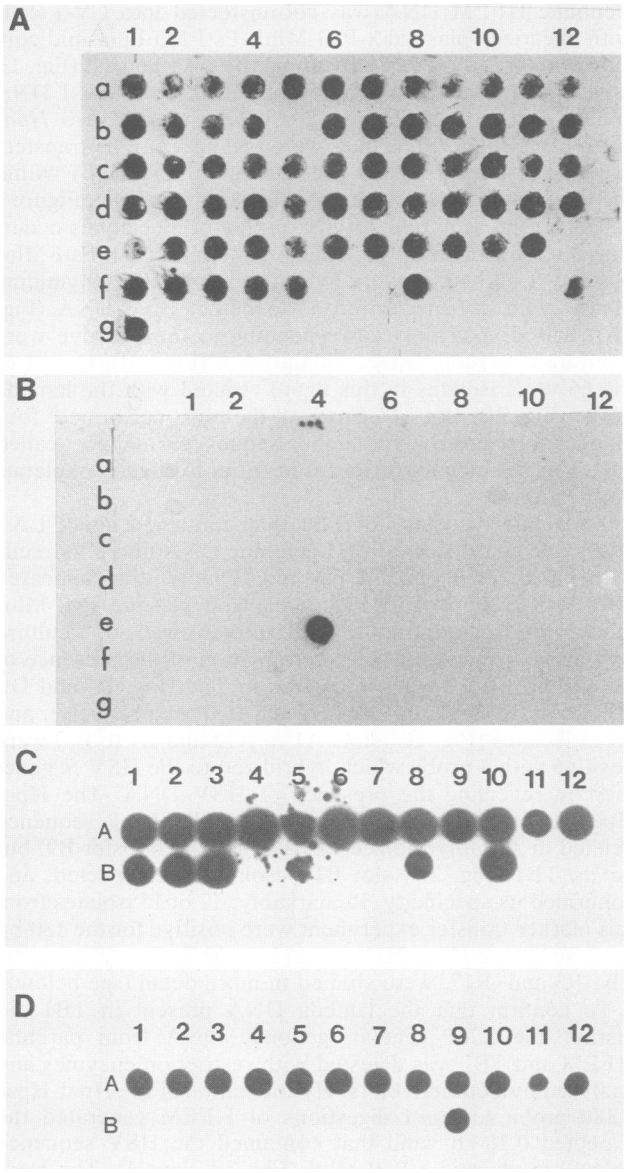

FIG. 2. Dot-blot analysis of plaques after marker transfer of the lambda DNA-disrupted LAT. Plaques appearing after transfection of HFEM DNA and the plasmid containing the lambda-disrupted LAT gene were isolated and passaged in CV-1 cells. The lysates were immobilized on nitrocellulose paper, denatured in situ, and hybridized with radioactively labeled probe that recognizes either the *tk* gene (A, C) or lambda DNA (B). HFEM and uninfected controls are in slots g1 and g3 (A) and g2 and g4 (B), respectively. (C and D) Fifteen plaques appearing after transfection of TB1 virion DNA with PstI-MluI plasmid (LAT-containing plasmid) were isolated and are shown in slots A1 to 12 and B1 to 3. (C) Hybridization to *tk* plasmid is shown to reveal any HSV genetic material. (D) Hybridization to Hpa-Hpa-0.180. HFEM-infected cells are in slots B10 (C) and B9 (D). TB1-infected cells are in slots B8 (C) and B7 (D). Uninfected cells are in slots B12 (C) and B11 (D).

*Bam*HI, *Kpn*I, and EcoRI-digested TB1 DNA (data not shown).

LAT production by HFEM, TB1, and TB1-R in tissue culture. Since TB1 contained a deletion and insertion mutation within the LAT-coding region, it was important to characterize any LAT- and alpha 0-specific transcripts produced in infected cells and compare them with those induced by the parent, HFEM. Figure 4A shows a blot of total infected cell RNA probed with labeled *Pst*I-*Mlu*I DNA. A 2.8-kb alpha 0 transcript was present at early times after HFEM infection (Fig. 4A, lanes 2 and 4, oval). TB1 produced alpha 0 transcripts of normal size and abundance but failed to produce a full-length LAT transcript (Fig. 4A, lanes 1 and 3). TB1-infected tissue culture cells did, however, accumulate a 0.7- to 0.8-kb transcript late in infection (7 h, Fig. 4A, lane 3). Thus, TB1 does not accumulate normal LAT RNA but does accumulate a normal alpha 0 gene product.

Figure 4B shows that the 2.0-kb apparent LAT transcript made by HFEM was recognized by a LAT-specific probe (that excluded alpha 0 coding sequences) (Fig. 4B, lane 1). The 0.7- to 0.8-kb TB1-specific RNA was not abundant at early times after infection (Fig. 4A, lane 1) but was apparent at later times in Fig. 4A, lane 3. No 2-kb LAT transcript was detected in TB1-infected cells with either lambda probe or probe specific for sequences immediately downstream (1.2-kb HpaI-MluI, Fig. 1) of the site of the lambda insertion in TB1 (data not shown). Thus, no detectable full-length LAT is made by TB1.

Transcripts produced by marker-rescued viruses, TB1-R3 and -R17, are shown in Fig. 4B. Since TB1 does not make intact LATs in tissue culture, rescuants TB1-R3 and -R17 were examined for production of LAT transcripts after productive infection. The LAT probe did hybridize to a 2.0-kb transcript present in TB1-R3- and -R17-infected cells, providing evidence that the defect in TB1 has been corrected in both rescuants (Fig. 4B). In addition, the alpha 0 transcript, recognized by an alpha 0-specific probe that excludes LAT sequences, was equally abundant in HFEM- and TB1-infected cells (Fig. 4C). This was important to show since alpha 0-negative mutants exhibit an altered latency phenotype (10).

Growth analysis of TB1 in tissue culture. The growth properties of TB1 and HFEM were compared to determine whether the absence of LATs would compromise virus growth. CV-1 cells were infected with HFEM or TB1 at a multiplicity of infection of 0.01 and 1.0 (Fig. 5A and B, respectively), harvested at various times, and titered for virus yield. Figure 5 demonstrates that the kinetics of virus production of TB1 and HFEM were indistinguishable. Therefore, disruption of the LATs does not alter the efficiency of HSV-1 growth in cell culture.

Infection and reactivation profile of TB1 from latently infected mice. LAT$^-$ deletion mutant 1704 reactivated slowly from explanted trigeminal tissue (22). Also, Leib et al. (9) have reported that a LAT$^-$ virus reactivates from homogenized explanted ganglia of latently infected mice less efficiently than wild-type virus. Therefore, it was important to know whether TB1 had established a latent infection in mice and to evaluate its reactivation profile. Trigeminal ganglia explanted from mice infected with TB1, HFEM, or marker-rescued viruses (TB1-R3) were cocultivated with indicator CV-1 cells and observed for the appearance of cytopathic effects. In mice infected with strain HFEM (12 mice, 18 of 24 ganglia), reactivation was detectable between 9 and 10 days postexplant (Fig. 6A). On day 10, 20 of 31 (65%) of the trigeminal ganglia from TB1-infected mice were reactivation positive (Fig. 6A). By day 15, 79% of the ganglia from TB1-infected mice representing 90% of the mice were reactivation positive. In mice latently infected with a rescued virus, TB1-R3, the virus reactivated with kinetics similar to those of TB1 and HFEM. An analysis of standard error

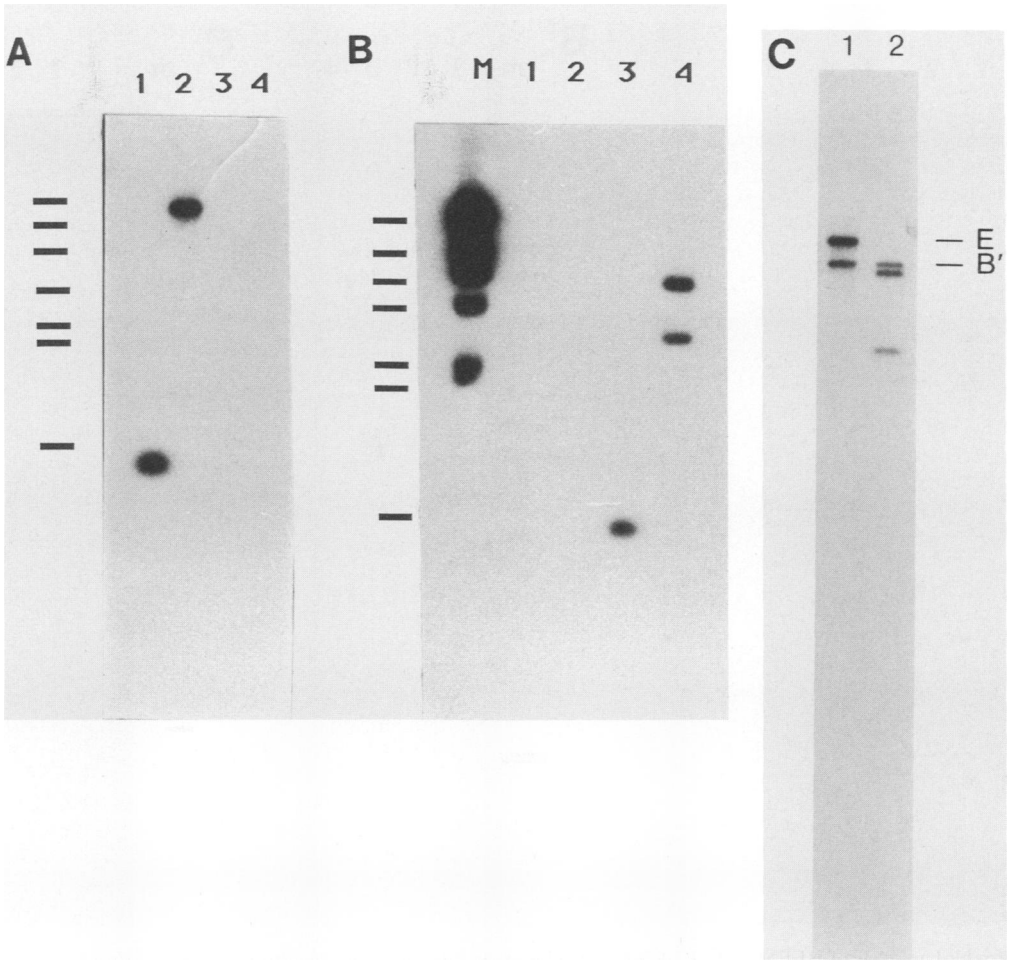

FIG. 3. Southern blot analysis of HFEM and TB1 virus genomes. Virion DNA was digested with the indicated restriction enzyme, resolved by electrophoresis in 0.8% agarose gels, transferred to nitrocellulose, and hybridized with radioactively labeled lambda (B)-, or *Bam*HI-B (C)-specific probes. (A and B) Lanes: 1, *Hpa*I-digested HFEM; 2, *Bam*HI-digested HFEM; 3, *Hpa*I-digested TB1; 4, *Bam*HI-digested TB1; M, *Hin*dIII-digested lambda DNA as molecular weight indicators. Visible bands indicated by the bars are the following molecular sizes (in kilobases): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.55. (C) Southern blot of *Bam*HI-digested HFEM (lane 1) and TB1 (lane 2) DNA. The letters B and E indicate the respective *Bam*HI fragments of the HSV genome (Fig. 1).

indicated that there is no difference between the reactivation profiles of the viruses. Strains 17^+ and 1704 are shown for comparison (Fig. 6B). 1704, a LAT variant unable to produce any LATs, had a much slower reactivation profile than its LAT^+ parent, 17^+.

The study conducted by Leib et al. (9) concluded that only 50% of the mice infected via the eye with the LAT^- mutant yielded virus upon explant of ganglia, compared with 100% of the mice infected with wild-type virus. Since their assay employed homogenization of ganglia 5 days after explantation and the assay used here employed the continuous cocultivation of ganglia with indicator cells, we tested the possibility that assay methods would account for the different behaviors of TB1 and *dl*LAT 1.8. Therefore, 5 days after explant, ganglia from three mice latently infected with TB1 or HFEM were homogenized (six ganglia per virus) and tested for the presence of infectious virus. Similar titers of virus were obtained from HFEM [$(9.7 \pm 6.2) \times 10^3$ PFU] and TB1 [$(2.8 \pm 4.4) \times 10^3$ PFU] ganglia. All ganglia from mice infected with either TB1 or HFEM generated virus at day 5, and the amount of virus was not statistically different. The longer interval required to detect reactivated virus in the explant assay is probably due to the time after reactivation for HSV-1 to be shed from the ganglia and produce cytopathic effects in the indicator monolayer.

Characterization of LAT transcripts in peripheral ganglia. To determine the transcription of TB1 in latently infected mice, we processed ganglia for Northern (RNA) blots or in situ hybridization. Total RNAs were isolated from trigeminal ganglia of latently infected mice, Northern blotted, and hybridized to a *Pst*I-*Mlu*I LAT probe (Fig. 7). While transcripts of 2.0 and 1.5 kb were visible with RNAs extracted from trigeminal ganglia latently infected with F and HFEM (Fig. 7, lanes 1 and 2, respectively), no herpesvirus-specific RNAs were detected in ganglia latently infected with TB1 (Fig. 7, lanes 3 to 5). The 0.7- to 0.8-kb LAT-specific transcript produced by TB1 in productively infected tissue culture cells was not detectable in vivo.

Disruption of the LATs could have resulted in the transcriptional derepression of other regions of the HSV genome in the latently infected cell. To study this possibility and to employ techniques that would permit detection of low amounts of transcripts occurring in individual cells (4), we examined latently infected ganglia by in situ hybridization using probes representing the entire HSV genome as well as a LAT-specific probe. A *Pst*I-*Mlu*I DNA probe hybridized to

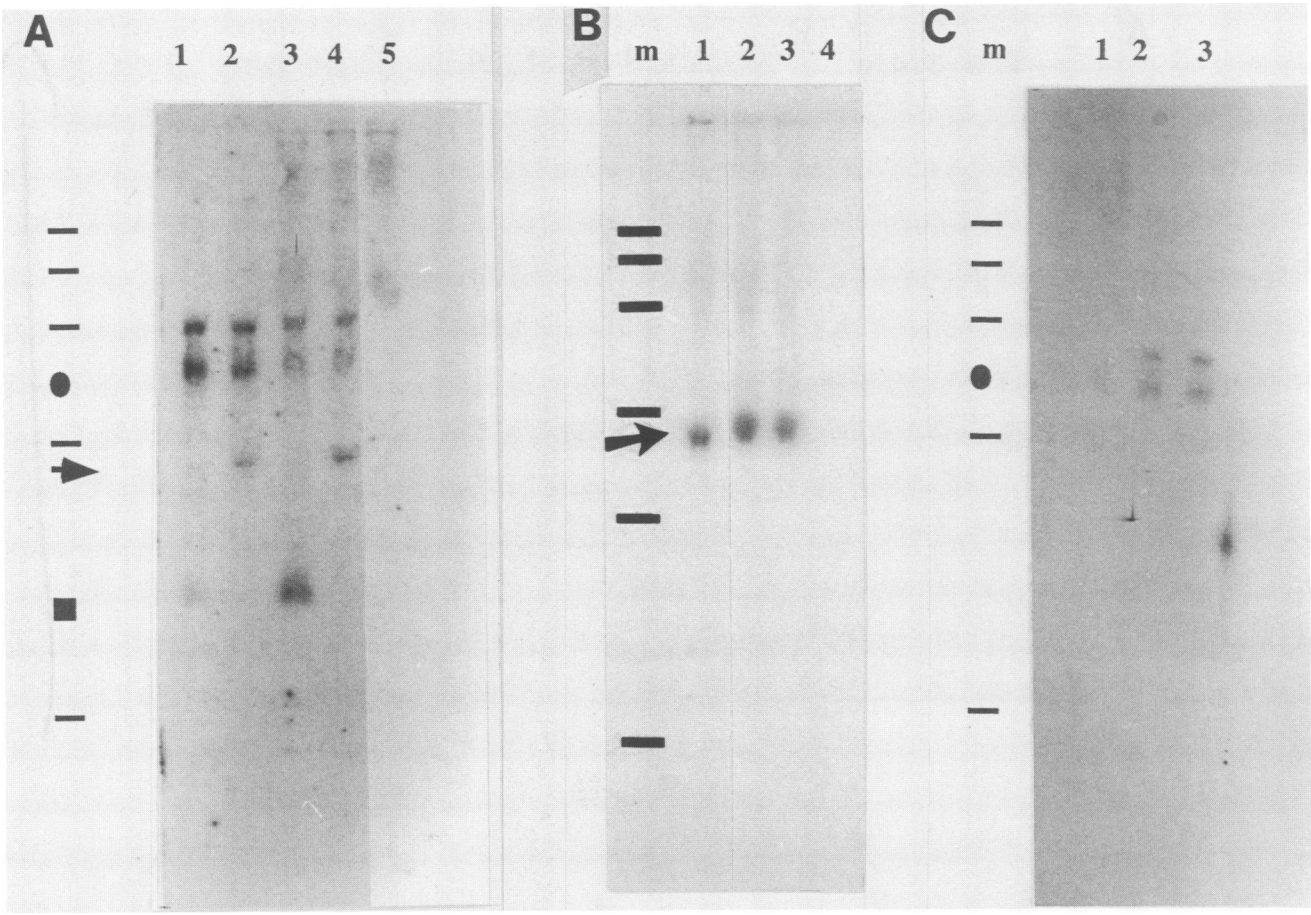

FIG. 4. Northern blot analysis of HFEM and TB1 RNA in productively infected cells. (A) Probe was *Pst*I-*Mlu*I (LAT and alpha 0 specific). Lanes 1 and 3, TB1 RNA isolated at 4 and 7 h after infection, respectively. Lanes 2 and 4, HFEM RNA isolated at 4 and 7 h after infection, respectively. Lane 5, RNA from uninfected cells. (B) Probe was *Pst*I-*Hpa*I (LAT specific). Lane 1, HFEM; lane 2, TB1-R3; lane 3, TB1-R17; lane 4, uninfected cells. A total 4 μg of RNA was loaded per well. For panels B and C, RNA was isolated 5 h after infection. (C) Probe was *Mlu*I-*Bam*HI (alpha 0 specific). Lane 1, Uninfected cell RNA; lane 2, HFEM; lane 3, TB-1. Symbols for all panels: bars under lane m (markers) correspond to molecular size markers (in kilobases) of 9.49, 7.46, 4.40, 2.40, 1.35, and 0.24; ●, alpha 0 transcript, arrow, 2-kb LAT transcript; ■, TB1-specific transcript.

neurons in trigeminal ganglia obtained from mice latently infected with LAT$^+$ strain 17$^+$ (Fig. 8B). In this experiment, strain 17$^+$ was used as a positive control; however, HFEM yields similar results (unpublished observations). No hybridization signal was present in any ganglia from mice latently infected with TB1 (Fig. 8A). A similar pattern of hybridization was obtained by using HSV-1 virion DNA as a probe (Fig. 8C and D). That is, ganglia derived from mice infected with strain 17$^+$ showed clear hybridization to the HSV genomic DNA probe, while no hybridization was seen with TB1 ganglia, even after long periods of exposure. Thus, using the most sensitive techniques available for studying expression, no HSV-1 transcription was detectable in the trigeminal ganglia of mice latently infected with TB1.

Characterization of reactivated virus. To confirm that reactivated TB1 virus remained unchanged during latent infection and reactivation, we recovered virus from reactivated explant tissue. Genomic DNA was prepared, digested with restriction enzymes, and resolved by agarose gel electrophoresis. There was no difference between the profile of TB1 used in the initial inoculum and that of the reactivated virus. Reactivated TB1 retained the lambda sequence in the appropriate position and remained negative for the region of the LAT deletion (data not shown).

DISCUSSION

This report extends previous observations that LAT-defective HSV-1 is capable of establishing a latent infection in mice (7–9, 22). The LAT mutant TB1 established a latent infection and reactivated from explanted trigeminal ganglia of mice with an efficiency and kinetics indistinguishable from those of the parental or revertant strain (Fig. 6). This is in contrast to the altered reactivation of LAT deletion mutants 1704 (22) and *dl*LAT 1.8 (9) as well as the impaired in vivo reactivation profile of the LAT$^-$ variant X10-13 (5). TB1 differs from these null mutants in that it is still capable of making LAT region RNA (Fig. 4), whereas *dl*LAT 1.8 and 1704 are unable to synthesize any LAT (9, 22). Therefore, our data suggest that the role the LATs play in the reactivation process does not require the accumulation of full-length 1.5- to 2.0-kb transcripts in vivo and that the smaller 0.7- to 0.8-kb transcript made by TB1 may be functional during reactivation.

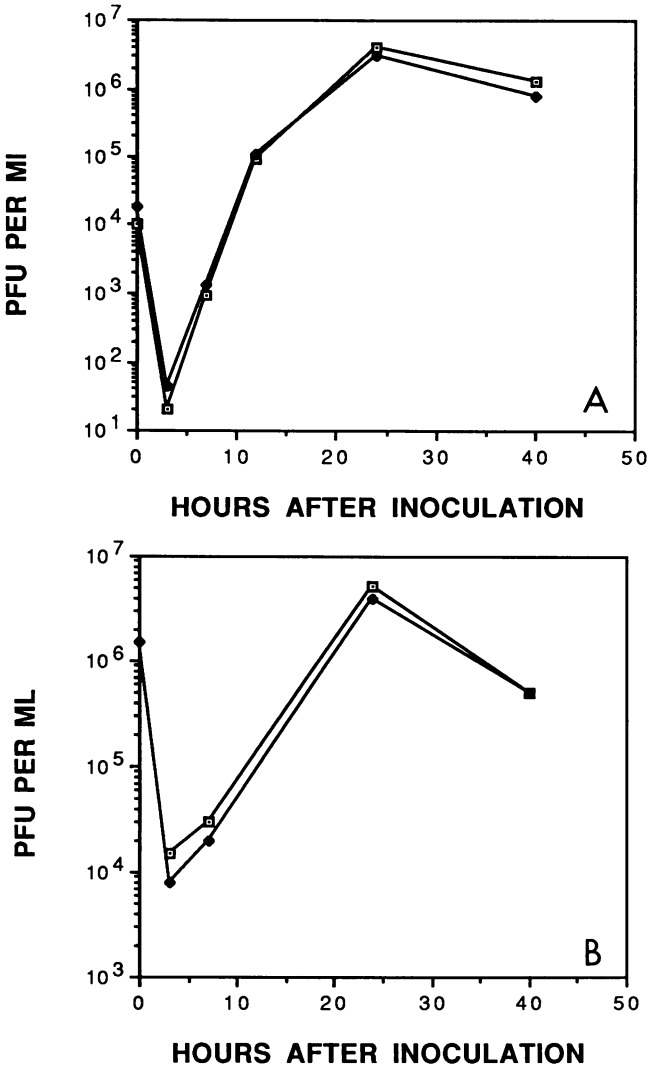

FIG. 5. Growth kinetics of TB1 and HFEM virus. CV-1 cells were inoculated at high (1.0) and low (0.001) multiplicities and harvested at various times after inoculation. The amount of virus present in the lysates was determined by plaque assay. (A) HFEM (◆) and TB1 (□) after low multiplicity of infection. (B) HFEM and TB1 lysates after high multiplicity of infection. Each point is the average number of plaques from three wells and two experiments.

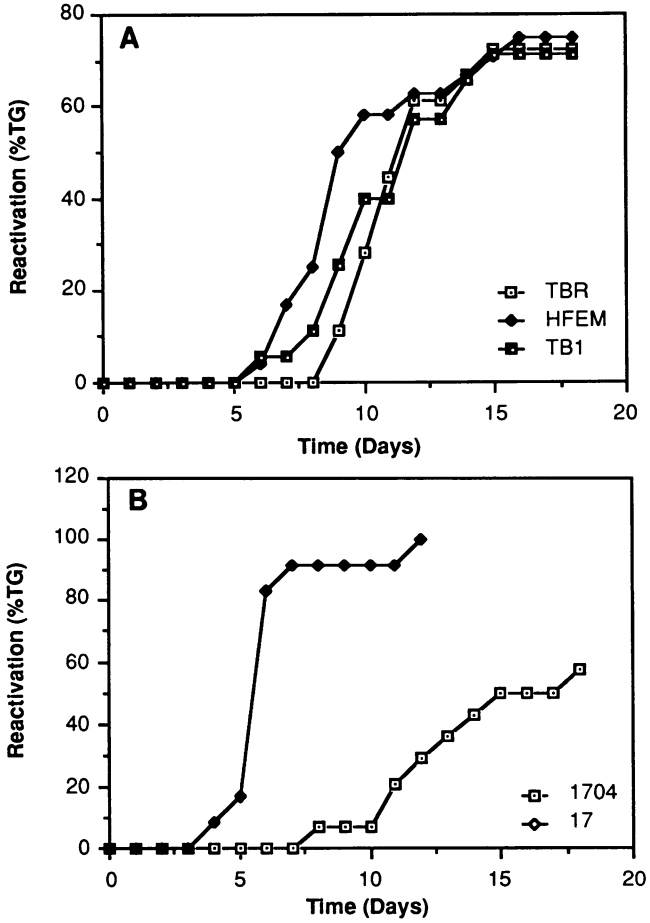

FIG. 6. Explant reactivation time course of HSV-1 strain HFEM and mutant TB1. A trigeminal ganglion (TG) was scored positive for reactivation when cytopathic effect was detected in the CV-1 monolayer. The data are cumulative from four different experiments. (A) HFEM (20 ganglia), TB1 (31 ganglia), and TB1-R3 (18 ganglia). (B) 17^+ (12 ganglia) and 1704 (14 ganglia). Data are plotted as the percentage of reactivated ganglia at each time point.

RNA must have been synthesized from the LAT region but did not accumulate to detectable levels. The results with TB1 and RH142 suggest that (i) the 5′ end of the LATs or its promoter sequence is sufficient to confer biological activity; (ii) a readthrough transcript might include the downstream LAT sequences that are present in both viruses; or (iii) the LATs do not play a role in explant reactivation. We think that the third possibility is unlikely based on the results with 1704 (22) and *dl*LAT 1.8 (9).

As stated, an HSV LAT^- virus, X10-13, established latency and reactivated with normal kinetics from explanted lumbosacral ganglia from mice infected through the footpad (8). X10-13 is an HSV-1 × HSV-2 recombinant containing an HSV-2 insert as well as a deletion within the LAT sequences. Perhaps the HSV-2 sequences compensate for the LAT deletion, or an unstable LAT RNA is produced. Alternatively, there may be a difference between the reactivation processes in lumbosacral and trigeminal ganglia. However, the recent results of Hill et al. (5) showing that X10-13 does exhibit an impaired reactivation profile in rabbits in an eye model support the notion that LATs do play a role in reactivation from the trigeminal ganglia. Even though there are concerns about the relevance of explant reactivation assays, the results of in vivo studies with X10-13 reinforce the value of in vitro explant reactivation assays in predicting the behavior of LAT variants in vivo.

TB1 did not induce any detectable HSV-specific transcripts in ganglia from latently infected mice, showing that the disruption of LATs does not transcriptionally derepress other HSV genes (Fig. 7 and 8). This argues against an antisense function for the LAT, as previously proposed (24). Furthermore, TB1 did not induce full-length LATs in tissue culture (Fig. 4). Instead, a 0.7- to 0.8-kb transcript was produced in productively infected tissue culture cells that hybridized to probes that span the LAT region. These results do not exclude the possibility that a transcript is synthesized during latency that reads through the lambda insert or is located downstream of the insert. Another possibility is that the lambda-specific RNA in the transcript results in its rapid degradation in vivo. Further mapping of the TB1-specific

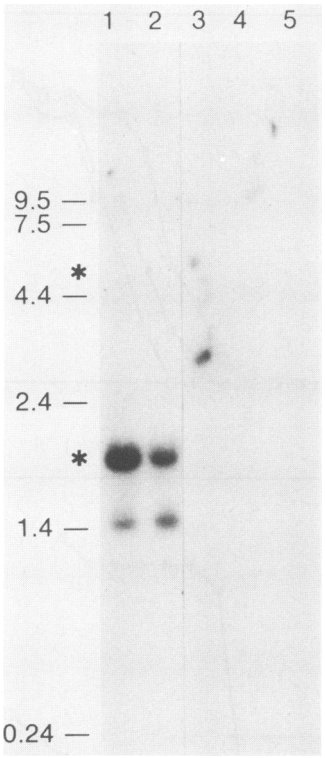

FIG. 7. Detection of HSV-1 transcripts in trigeminal ganglia of latently infected mice by Northern blot analysis. RNA (5 μg) from the trigeminal ganglia of mice were latently infected with HSV-1 strain F (lane 1), strain HFEM (lane 2), mutant TB1 (lanes 3 to 5). Each lane contains RNA from pooled ganglia derived from three mice. The autoradiograph was made with XAR-5 film (Kodak). The positions of RNA markers are labeled in kilobases on the left, and the positions of the 28S and 18S rRNAs are labeled by asterisks. *Pst*I-*Mlu*I probe recognizes the LAT transcripts (Fig. 1).

transcript should help distinguish among these possibilities. It should be emphasized that since the 0.7- to 0.8-kb transcript was not detected in latently infected tissue, it is not known whether it is made but does not accumulate or whether it is not made at all during latency.

The results obtained with TB1 raise several possibilities about the function of the LATs. (i) The small transcript made by TB1 in tissue culture may be sufficient to mediate reactivation. This suggests that the TB1-specific RNA is produced in latently infected cells below detectable limits. (ii) Recently, Wechsler et al. (28, 29) proposed two conserved open reading frames within the LATs based on DNA sequence data from several HSV-1 strains. However, the deletion within TB1 removes the start of open reading frame 1 (36 kilodaltons). Since TB1 reactivates normally, it seems unlikely that the absence of ORF-1 is responsible for the reactivation phenotype of strains 1704 and *dl*LAT 1.8. (iii) It is possible that the 1.5- to 2.0-kb LATs are processing

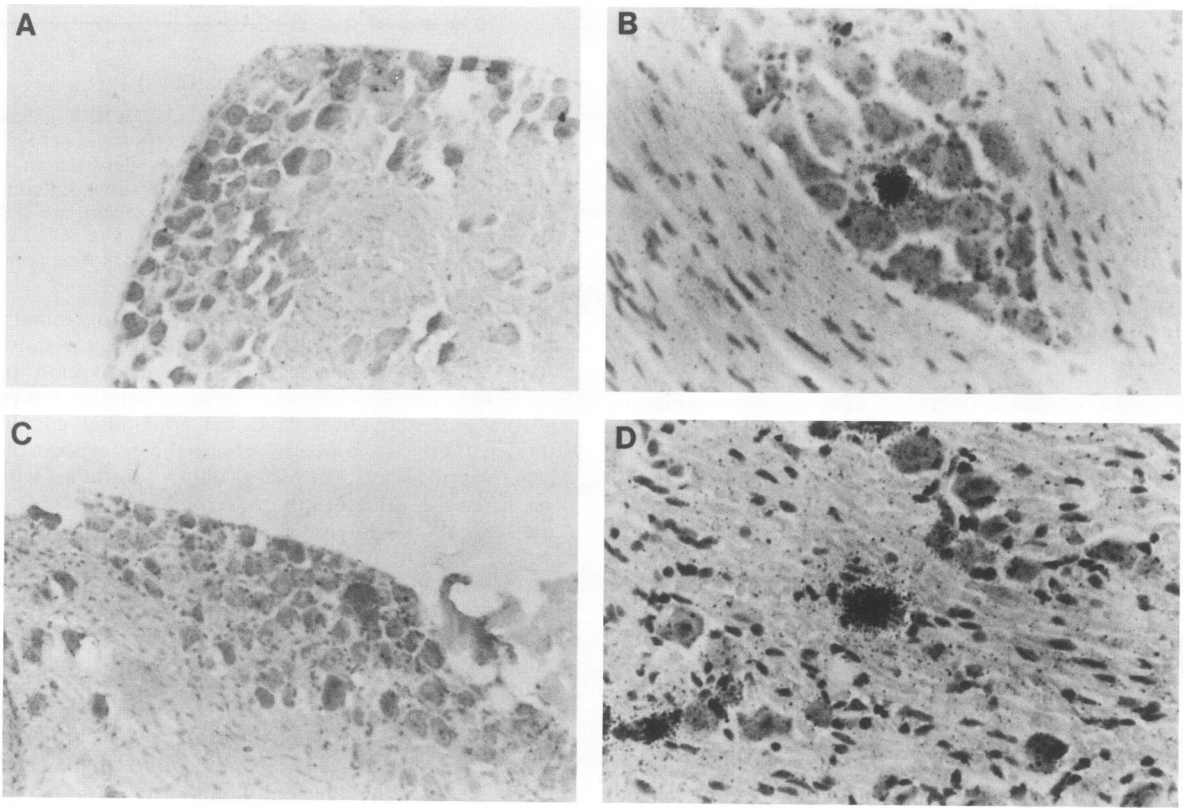

FIG. 8. In situ hybridization of trigeminal ganglia of mice latently infected with HSV-1. The probes used were ^{35}S-labeled nick-translated HSV-1 *Pst*I-*Mlu*I DNA (A and B) and HSV-1 whole virion DNA (C and D). Positive hybridization was seen over nuclei of neurons from trigeminal ganglia of mice latently infected with HSV-1 strain 17$^+$ (panel B, 24 h; panel D, 4 days). No hybridization signal was present in trigeminal ganglion tissue from mice latently infected with TB1 (panel A, 24 h; panel C, 4 days).

products (3) or introns derived from a larger transcript (3, 15). Dobsen et al. (3) have detected a low-abundance 8.5-kb transcript recognized by a LAT-specific probe in infected cells in culture. We have also detected a large-size low-abundance transcript with the LAT probe in productively infected cells (15). Furthermore, we have characterized by in situ hybridization a region of the genome adjacent to the LATs which appears to be weakly transcribed during latency (2, 15). If these minor LATs are the functional LAT species and originate from the LAT promoter (15a), they might be produced at low levels by TB1, since the deletion in that virus is completely within the DNA coding for the 1.5- to 2.0-kb nuclear poly(A)$^-$ transcripts. Strain 1704 contains deletions of the LAT promoter regions, on the other hand, and would be unable to produce the minor LAT (15a). The failure to detect any LAT-specific transcription in TB1 latently infected tissue may be due to the very low abundance of the putative minor LAT.

LAT$^+$ rescuants could be isolated from TB1 after marker transfer of intact LAT sequences at a high frequency (approximately 70%). Under the conditions used in this study, the frequency of recombinant virus resulting from the marker transfer of HSV genes that do not confer any growth advantage is less than 2% (T. Block, unpublished observation). The fact that the majority of the plaques contained intact LAT genes after transfection of TB1 DNA and LAT-positive plasmid suggests that a selection for intact LAT sequences had occurred. The difference between the forward (LAT$^-$ from LAT$^+$ virus) and back (LAT$^+$ from LAT$^-$ virus) marker transfer frequencies is unlikely to be due to differences in the amount of HSV homology present in the rescuing plasmids or to plasmid concentration variability. The λ-PstI-MluI and PstI-MluI plasmids each contain similar amounts of HSV sequence and were used over a range of concentrations in several transfections. Clearly, more work needs to be done to definitively map the reactivation phenotype of LAT deletion mutants such as 1704 and *dl*LAT 1.8.

In conclusion, TB1, a virus that cannot make intact LATs because of a replacement mutation, but does make a small RNA in tissue culture, reactivates from latently infected explanted ganglia in a manner indistinguishable from that of its parental strain, HFEM. The slow reactivation phenotypes associated with null mutants of the LAT gene must therefore be due to lesions that are not present in TB1.

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