Virology **265,** 296–307 (1999)
Article ID viro.1999.0057, available online at http://www.idealibrary.com on IDEAL®

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Identification of a Novel 0.7-kb Polyadenylated Transcript in the LAT Promoter Region of HSV-1 That Is Strain Specific and May Contribute to Virulence

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Received June 23, 1999; returned to author for revision August 9, 1999; accepted October 15, 1999

Herpes Simplex virus expresses latency-associated transcripts (LATs) the function of which remains obscure despite increasing knowledge of their structure and expression. Upstream of the LAT coding region is a region of the genome that is poorly characterized although it lies in an area that is responsible for modulation of reactivation efficiency in two different animal models. Transcript mapping with strains 17, McKrae, KOS, and F has revealed strain differences in this region of the viral genome. Strain 17 and McKrae expressed a novel polyadenylated 0.7-kb transcript that is absent from KOS and F. This transcript is expressed in the LAT direction and has the kinetics of a true late gene during the lytic cycle of infection. A deletion mutant, 17ΔBsa, which does not express the 0.7-kb RNA, is less virulent than the parental strain 17. A rescuant with F sequence (17ΔBsa/RF) shows virulence similar to F, whereas a rescuant with strain 17 sequence (17ΔBsa/R17) is similar to strain 17. Virulence is altered by deletion or substitution in the region encoding the 0.7-kb transcript (*Bsa*I-*Bsa*I); however, reactivation in the mouse explant cocultivation assay or the adrenergically induced rabbit reactivation model remained unchanged. The importance of this region for virulence is discussed. © 1999 Academic Press

INTRODUCTION

Herpes simplex virus (HSV) is a large double-stranded DNA virus with >80 genes encoded by its 152-kb genome (McGeoch, 1989). The most fascinating and mechanistically challenging biological feature of HSV is its ability to establish latent infections from which it can be reactivated in infected animals. During lifelong latent infection in host sensory neurons, the virus can be reactivated periodically by various stress stimuli such as trauma, hormonal, and neuronal injury (for review, see Roizman and Sears, 1995). Viral gene expression during the nonreplicating latent state is limited to a single gene located in the long repeat region of the viral genome (Stroop *et al.,* 1984; Deatly *et al.,* 1987; Stevens *et al.,* 1987; Spivack and Fraser, 1988). Transcripts from this gene are known as latency-associated transcripts (LATs).

Since the discovery of LATs >than 10 years ago, many mutant viruses with disruptions in the LAT locus have been generated and examined in an effort to understand the function of the LAT gene. These studies have demonstrated that LATs are not essential for HSV replication or establishment and maintenance of latency. Nevertheless LATs influence reactivation kinetics (Leib *et al.,* 1989; Steiner *et al.,* 1989). However, although various LAT mutants have been constructed from different strain backgrounds (17, KOS, or McKrae) and tested in different animal models (mouse, rabbit, or guinea pig), the exact role of LATs in HSV pathogenesis remains unclear.

Recent studies from *in vivo* epinephrine-induced reactivation of HSV-1 strain 17 in the rabbit-eye model suggest that, in this model, induced viral reactivation is related to a region corresponding to exon 1 of the LAT mRNA. A mutant virus with a 370-bp deletion between the LAT promoter and 2-kb LAT intron, 17ΔSty, was shown to exhibit a significantly reduced ability to undergo adrenergically induced reactivation (Hill *et al.,* 1996). Nevertheless, 17ΔSty expressed wild-type levels of 2-kb LAT intron and was as replication competent as wild-type virus in lytic infection (Hill *et al.,* 1996). The designation of a low-frequency reactivation phenotype to this region of the viral genome was supported by another mutant virus 17Δ348, which overlapped most of the deletion in 17ΔSty (Bloom *et al.,* 1996). Searching for the genomic region(s) required for efficient viral reactivation has led to the definition of a 437-bp Swa-Not fragment upstream of the LAT promoter (Maggioncalda *et al.,* 1996), which seems to be essential in facilitating epinephrine-induced HSV-1 strain 17 reactivation in the rabbit-eye model (Hill *et al.,* 1997). In the rabbit-eye model,

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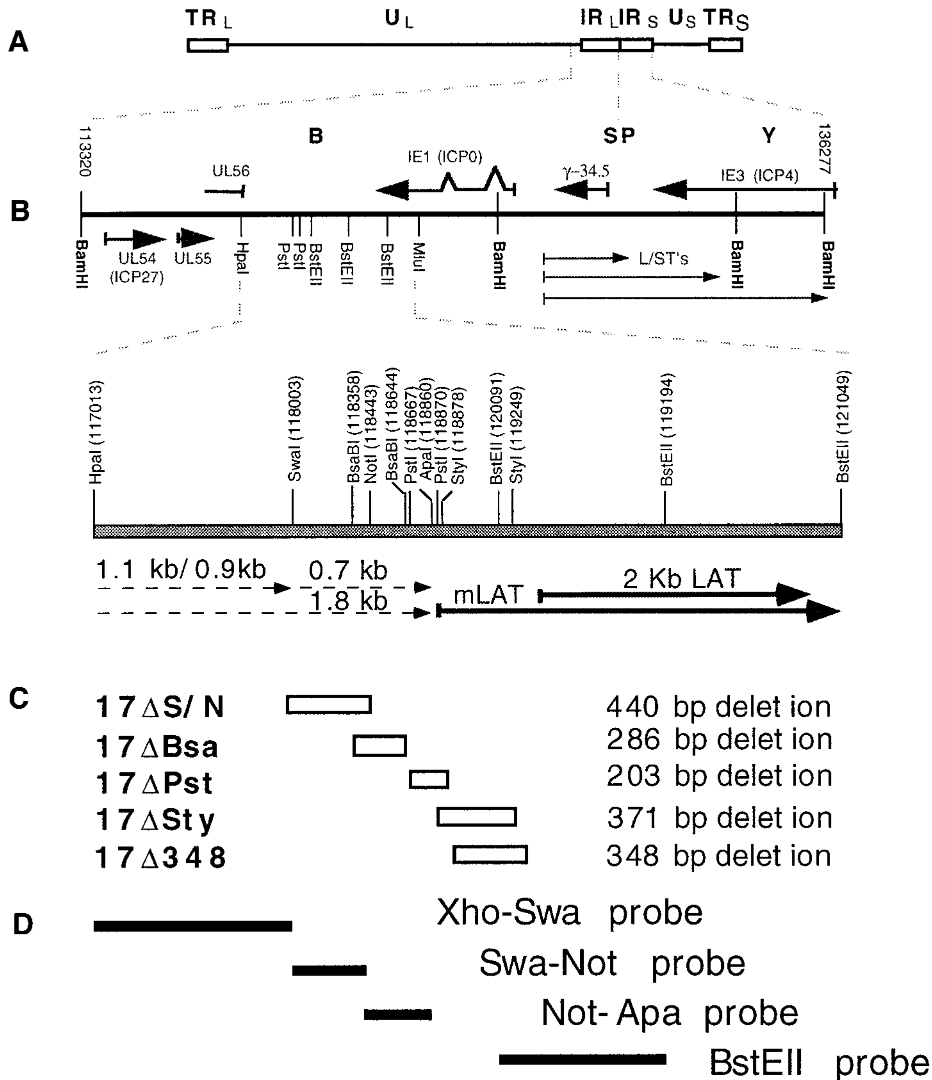

FIG. 1. Schematic representation of genes and transcripts encoded by internal repeat region (IR) of HSV-1 genome. (A) The HSV-1 genome, containing unique long and short (U_L and U_S) regions flanked by the internal repeat (IR) and terminal repeat (TR) regions. (B) Detailed map of the U_L/IR_L (BamB, BamSP, BamY) region and enlargement of the *Hpa*I-*Bst*EII fragment (117,013–121,049) of BamB. The approximate locations of U_L54, U_L55, U_L56, ICP0, γ34.5, and ICP4 mRNAs and L/S transcripts, 2-kb LAT intron, primary mLAT are marked as solid lines with arrows that indicate the direction of transcription. Two low abundance transcripts located in the U_L/IR_L junction region are marked as dashed lines with arrows to indicate the transcriptional direction. Restriction sites used in this study are marked and their positions are labeled by nucleotide coordinates from Perry and McGeoch (1988). (C) Map of HSV-1 deletion mutants 17ΔS/N, 17ΔPst, 17Δsty, and 17Δ348. The locations of the deleted regions in the above four mutants are illustrated by open boxes. The sizes of the deletions are 440 bp (17ΔS/N), 203 bp (17ΔPst), 371 bp (17ΔSty), and 348 bp (17Δ348). (D) The DNA probes used in this study are positioned as solid bars.

this mutant maps the reactivation phenotype between the U_L/R_L junction and the LAT TATA box (Fig. 1). These data suggest that the upstream region (exon 1, or the promoter region), rather than 2-kb LAT intron itself, plays a role in adrenergically induced reactivation.

Transcripts in the HSV-1 genome upstream of the LAT coding region have been mapped by two groups (Ben-Hur *et al.,* 1989; Singh and Wagner, 1993). Singh and Wagner detected 1.1- and 1.8-kb transcripts, which were 5′ coterminal. From their mapping data, Maggioncalda *et al.* (1996) constructed a *Swa*I-*Not*I deletion mutant that was predicted to transcribed only the 1.1-kb transcript but experimentally did not express either transcript. Thus we set out to reexamine transcripts in this region of the HSV genome.

In the present report, we have identified a novel 0.7-kb polyadenylated transcript mapping just upstream of the LAT TATA box by Northern blot analysis with RNA extracted from HSV-1 strain-17-infected Vero cells and SY5Y neuroblastoma cells and by *in situ* hybridization to trigeminal ganglia sections from mice acutely infected with HSV-1 strain 17. This 0.7-kb transcript is transcribed in the same direction as the LAT and is expressed as a true late (γ2) gene during the lytic cycle of infection.

Furthermore the expression of this transcript is strain specific. It is present only in cells infected with strain 17 and McKrae but absent in cells infected with strain KOS, F, and eight clinical isolates. By generating a deletion mutation and creating 17/F hybrid rescuant viruses, we have shown that the region encoding the 0.7-kb transcript is associated with the higher virulence of HSV-1 strain 17 and McKrae. However, this transcript does not appear to affect reactivation in either the mouse explant cocultivation or in the rabbit-eye model of adrenergically induced reactivation.

RESULTS

Detection of a novel polyadenylated viral transcript upstream of the latency-associated transcription unit in Vero cells infected with HSV-1 strain 17

Vero cells were mock infected, or infected, with HSV-1 strain 17 at m.o.i. = 1 plaque forming unit (pfu) per cell. Total RNA was extracted from cells harvested at 16 h p.i. Polyadenylated RNA was selected by oligo dT cellulose column chromatography (Ambion). After gel electrophoresis using 1.2% agarose and Northern blotting as described under Materials and Methods, filters were hybridized with ^{32}P-labeled 0.4-kb Swa-Not probe (see Fig. 1 for map position). As shown in Fig. 2, a 0.7-kb transcript was detected in infected cells (Fig. 2, lane 2), but not mock-infected cells (Fig. 2, lane 1). The transcript was present in both total and polyadenylated RNA samples. The signal was ninefold stronger (as determined by phosphorimaging) in 1 μg polyadenylated RNA (Fig. 2B, lane 2) than in 10 μg total RNA (Fig. 2A, lane 2), indicating that the 0.7-kb transcript is polyadenylated.

The expression of the 0.7-kb transcript in mouse trigeminal ganglia, 4 days following corneal inoculation with strain 17, was confirmed by *in situ* hybridization using a Swa-Apa probe (data not shown).

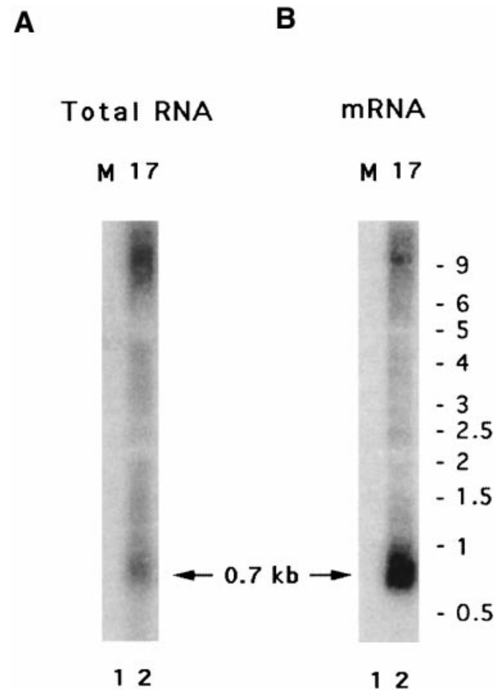

FIG. 2. Northern blot analysis of HSV-1 RNA immediately upstream of the latency-associated transcription unit. Total RNA, or polyadenylated RNA, extracted from mock-infected or HSV-1 strain-17-infected Vero cells 16 h p.i. were hybridized with a α-^{32}P-dCTP-labeled Swa-Not probe (see Fig.1 for location). (A) Total RNA samples (10 μg). (B) Polyadenylated RNA samples (1 μg). The RNA standard markers are labeled in kilobases on the left side. The transcripts detected are marked by arrows.

Fine mapping and kinetics of 0.7-kb RNA expression

To fine map as well as determine to which temporal expression class, the 0.7-kb transcript belongs during productive infection, polyadenylated RNA was extracted from HSV-1 strain-17-infected Vero cells at 0, 4, 8, 13, and 16 h p.i. and hybridized to *Xho*I-*Swa*I, *Swa*I-*Apa*I, and *Bst*EII-*Bst*EII probes (Fig. 3; see Fig. 1 for map positions). Using these three probes it is clear that the 0.7-kb transcript maps to the *Swa*I-*Apa*I region but does not overlap with the *Xho*I-*Swa*I region to the left or the *Bst*EII-*Bst*EII region to the right. Expression of the 0.7-kb transcript could clearly be detected at 8 h p.i., increased dramatically by 13 h p.i., and continued to accumulate at 16 h p.i. (Fig. 3B, lanes 1–5). However, it could not be detected in RNA extracted from Vero cells infected with strain 17 in the presence of 400 μg/ml phosphonoacetic acid (PAA), which inhibits viral DNA synthesis (Fig. 3A, lane 6). Continued expression of U_L23 (TK), an early gene, in the presence of PAA is shown in Fig. 3D. However, consistent with previous findings (Spivack and Fraser, 1988), 2-kb LAT was not detected in the presence of PAA (Fig. 3C, lane 6). These data suggested that the gene encoding the 0.7-kb transcript was a late gene with expression dependant on viral DNA synthesis.

In addition to the 0.7-kb transcript, a 0.9-kb polyadenylated transcript was recognized by the Swa-Apa probe (Fig. 3B). This observation was consistent with the broadband detected by the *Swa*I-*Not*I probe representing two transcripts that are close in size (Fig. 2B). The 0.9-kb RNA was also detected by the *Xho*I-*Swa*I probe and was also sensitive to PAA (Figs. 3A and 3B). These data suggested that the 0.9-kb polyadenylated transcript also belongs to the true late (γ_2) gene family during HSV-1 productive infection.

Previously, two low-abundance 5′-colinear transcripts, 1.1 and 1.8 kb, were mapped to the unique long and repeat long junction regions upstream of the latency-associated transcript (see Fig. 1B) by Singh and Wagner (1993). Because the 0.7- and 0.9-kb transcripts described in this study were also located in this region, we decided to examine the relationship between the transcripts and those observed by Singh and Wagner (1993).

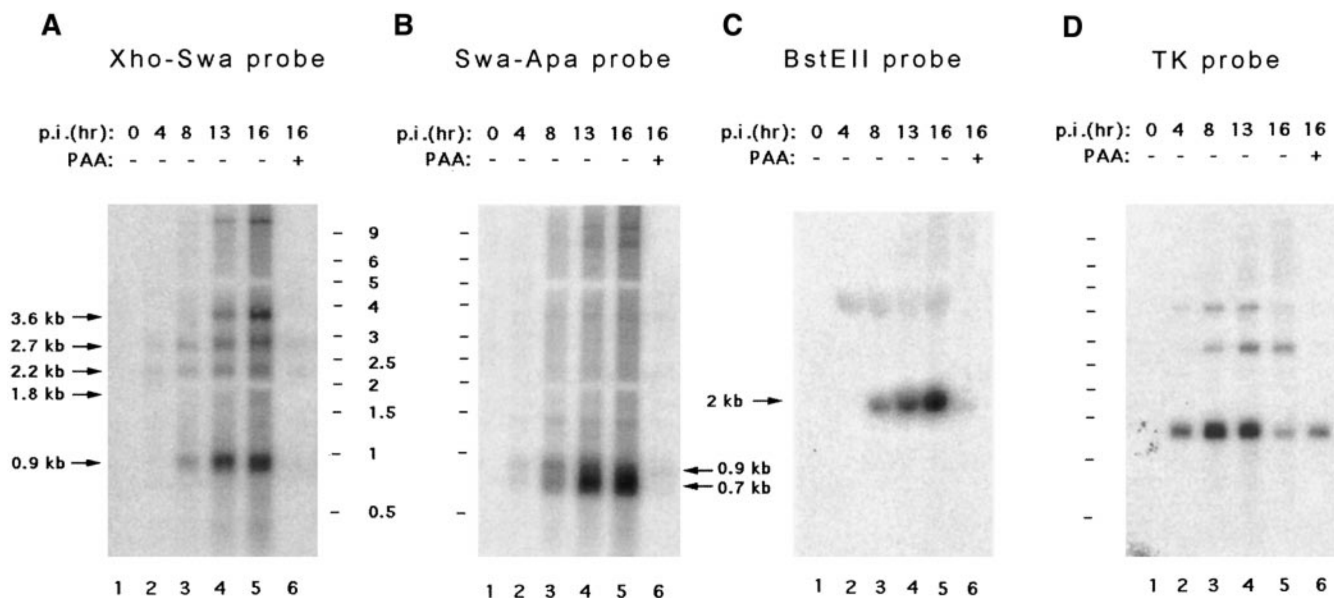

FIG. 3. Northern blot analysis of the temporal expression pattern of HSV-1 strain 17. Total RNA or polyadenylated RNA was extracted from Vero cells infected with HSV-1 strain 17 at 0, 4, 8, 13, and 16 h p.i. in the absence of (lanes 1–5) or presence of (lane 6) phosphonoacetic acid (PAA, 400 μg/ml). PAA was added 60 min before infection and was continuously present until RNA extraction. (A) Polyadenylated RNA (1 μg) hybridized with a 1.0-kb *Xho*I-*Swa*I probe. (B) Polyadenylated RNA (1 μg) hybridized with a 0.85-kb *Swa*I-*Apa*I probe. (C) Total RNA (10 μg) hybridized with 0.9-kb *Bst*EII-*Bst*EII. (D) Polyadenylated RNA (1 μg) hybridized with a 1.8-kb *Pvu*II-*Bgl*II probe from the *Bam*HI TK coding fragment. RNA markers are labeled in kilobase. The position of the 0.7-kb RNA, 2-kb LAT, and 0.9-kb RNA are marked by arrows.

Using the Xho-Swa and Swa-Apa probes in Figs. 3A and 3B, we detected a major 0.9-kb and a minor 1.8-kb band, but not a 1.1-kb band. The 0.9-kb RNA may correspond to the RNA previously designated as 1.1 kb (Singh and Wagner, 1993). The size difference may be due to the use of different RNA markers or separation conditions. Consistent with its previously reported low abundance (Singh and Wagner, 1993), the 1.8-kb transcript was very faint. Three additional bands of 2.2, 2.7, and 3.6 kb, were also detected. These may be transcripts extending from the unique long into the internal repeat region of the viral genome (for example extended forms of ICP27 or U_L55). However, they do not extend to the right of the Swa site at 118,003 nt because they were detected in Fig. 3A but not 3B. The precise mapping of the 2.2-, 2.7-, 3.6-kb transcripts is beyond the scope of this study. Nevertheless the 0.7-kb RNA was not detected by the Xho-Swa probe, indicating that the 0.7-kb RNA was distinct from the previously identified 1.1-kb transcript and confirming that the 0.7-kb transcript has not been previously characterized.

Direction of transcription of the 0.7-kb polyadenylated transcript

To determine the direction of transcription, the 857-bp *Swa*I-*Apa*I fragment was cloned into a pGem7zf vector, which has T7 and SP6 promoters flanking the cloning site in opposite directions, at the *Sma*I and *Apa*I sites (Fig. 4A). Because the 0.7-kb RNA could be detected by both Swa-Not and Not-Apa DNA probes (data not shown), the template DNA was linearized by *Not*I restriction enzyme digestion. The α-^{32}P-UTP-labeled single-strand RNA synthesized by SP6 RNA polymerase was transcribed in the LAT sense direction, whereas single-strand RNA synthesized by T7 RNA polymerase was transcribed in the LAT antisense direction. In Fig. 4B, the T7-driven RNA probe but not the SP6-driven RNA probe hybridized to the 0.7-kb transcript, indicating that the T7-driven single-strand RNA coded for the complementary sequence of the 0.7-kb transcript. This result was further confirmed by a ribonuclease protection assay (data not shown). In the probe/sample RNA mixture, only the probe produced by the T7 promoter was protected from ribonuclease digestion (data not shown). Thus the transcriptional direction of the 0.7-kb RNA was determined to be that of the latency-associated transcript.

Strain-specific expression of the 0.7-kb polyadenylated transcript

To further characterize the 0.7-kb transcript, we examined its expression from four commonly used lab strains, 17^+, McKrae, F, and KOS. Following infection of neuroblastoma SY5Y cells, no expression of the 0.7-kb transcript was detectable with strain F and KOS, whereas comparable amounts of the transcript were detected in RNA extracted from 17^+-and McKrae-infected cells (Fig. 5B). This strain-specific expression was also observed in Vero cells (data not shown). In contrast, the 0.9-kb transcript was expressed by all four strains (Fig. 5). The 2.2- and 2.7-kb transcripts also appeared equally present in all four isolates (Fig. 5A).

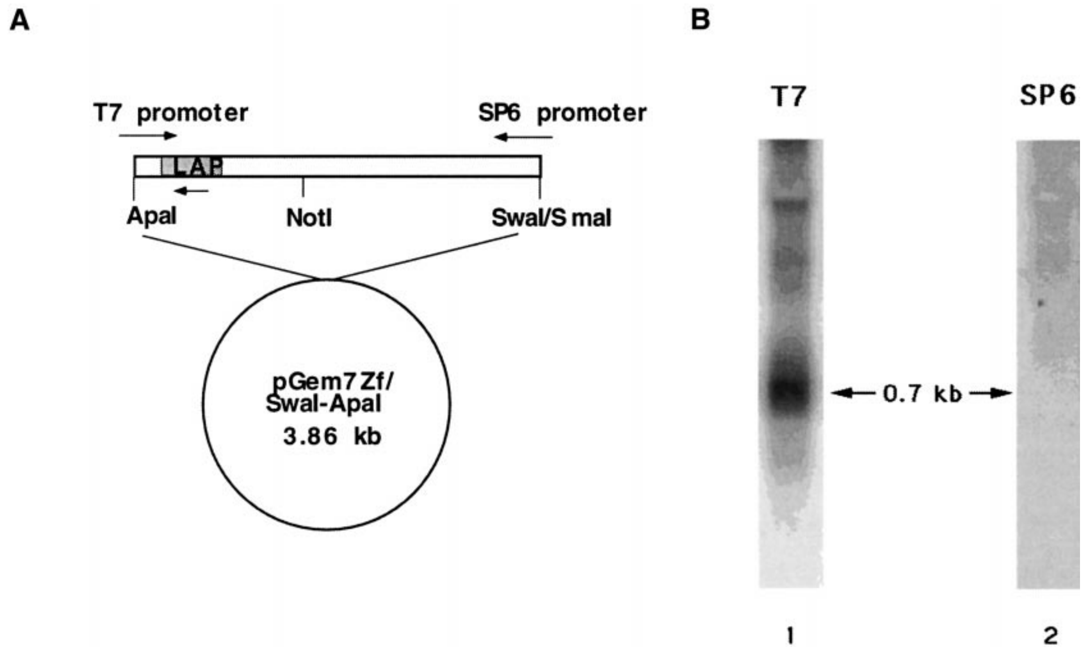

FIG. 4. Transcriptional direction of the 0.7-kb RNA. (A) A 857-bp *Swa*I-*Apa*I fragment was cloned into pGem7zf vector at the *Sma*I/*Apa*I sites to construct plasmid pSwa-Apa. The DNA was linearized by digestion with *Not*I restriction enzyme before performing an *in vitro* transcription assay using RNA polymerase T7 or SP6. (B) Polyadenylated RNA (1 μg) extracted from Vero cells infected with HSV-1 strain 17 was analyzed by Northern blot hybridization using a α-^{32}P-UTP-labeled single-strand RNA probe. Lane1: A T7-driven single-strand RNA probe, antisense to the LAT direction; lane 2: SP6-driven single-strand RNA probe, sense to the LAT direction. The position of the 0.7-kb RNA are marked with an arrow.

However, the 3.7-kb was strongest in strain 17, less strong in strain McKrae, barely detectable in strain KOS, and not detectable in F-strain-infected cells (Fig.5A). Taken together, these data demonstrate that the expression of the 0.7- and 3.7-kb transcript were HSV-1 strain specific. They were detected in RNA from strain 17- and McKrae- but not KOS- or F-infected cells.

Expression from clinical isolates of HSV-1

Because the expression of the 0.7-kb transcript is strain specific in four lab strains, we examined its expression in clinical isolates of HSV-1. Eight isolates: 3981, 5162, 4009, 5824, 4586, 4983, 4984, and 6111, collected from patients who had HSV lesions at either epithelial sites (back) or mucosal surfaces (mouth, lips, and vagina) were used to infect Vero cells. Following gel electrophoresis and Northern blotting, the RNA filter was hybridized with *Xho*I-*Swa*I and *Swa*I-*Not*I probes. Although several transcripts were detected, the 0.7-kb transcript was absent in all eight clinic strains (Fig. 6). The expression of the 0.9-, 2.2-, 2.7-, and 3.7-kb transcripts in clinic isolates was conserved and similar to that of strain 17^{+} except that the size of the 3.7-kb band varied slightly between isolates.

The 0.7-kb RNA is not transcribed from HSV-1 17ΔSty, 17ΔS/N, and 17ΔPst

The mutant virus 17ΔS/N has significantly reduced efficiency in epinephrine-induced reactivation (Hill *et al.*,

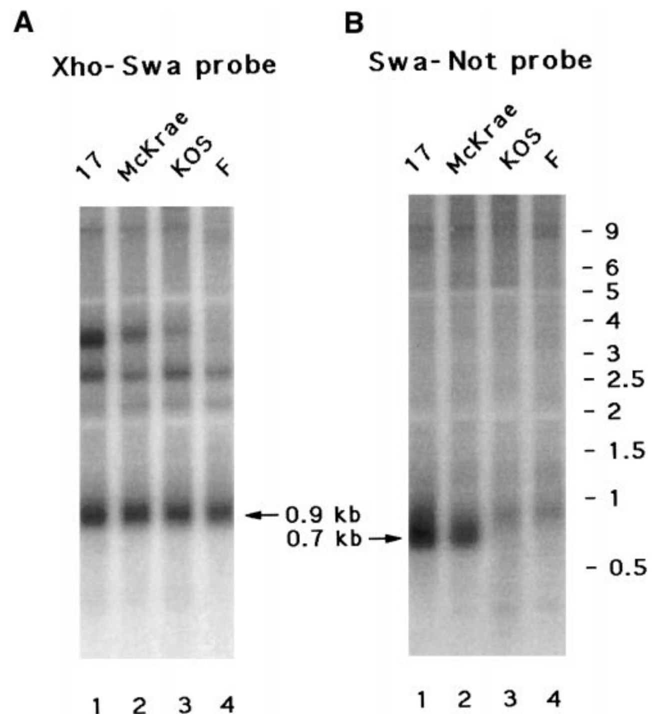

FIG. 5. RNA analysis of various HSV-1 strains. One microgram of polyadenylated RNA extracted from Vero cells infected with strain 17 (lane 1), McKrae (lane 2), KOS (lane 3), and F (lane 4) were separated by agarose gel electrophoresis, blotted onto membranes, and hybridized with a 1-kb Xho-Swa probe (A) or a 0.4-kb Swa-Not probe (B). Millennium RNA markers are marked by kilobase. The position of the 0.7- and 0.9-kb transcripts are labeled with arrows.

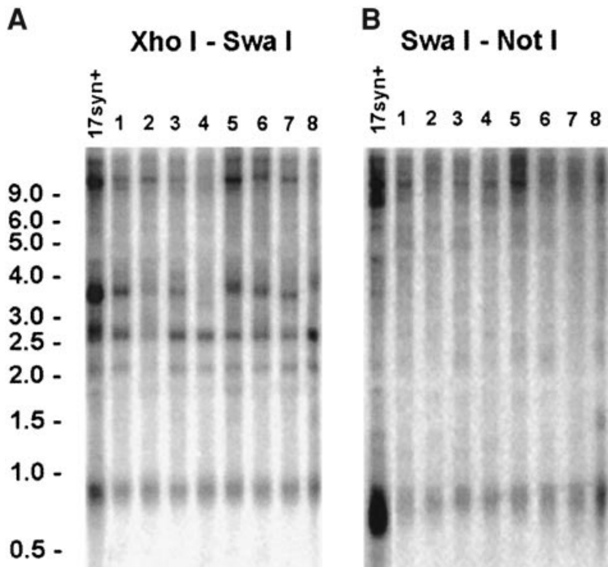

FIG. 6. Clinical isolates do not express detectable amounts of the 0.7-kb RNA. Polyadenylated RNA was extracted from Vero cells infected with strain 17 and eight clinical isolates 16 h p.i. One microgram polyadenylated RNA from each sample was subjected to Northern blot analysis and hybridized with the Xho-Swa probe (A) or the Swa-Not probe (B). RNA standards are marked in kilobase.

1997), suggesting a relationship between transcripts in this region and the aberrant reactivation phenotype. To test this hypothesis, polyadenylated RNA extracted from Vero cells infected with wild-type strain 17 or deletion mutants showing impaired reactivation in the rabbit model 17ΔSty (Maggioncalda *et al.,* 1994), 17ΔS/N (Block *et al.,* 1993), and 17ΔPst (Devi-Rao *et al.,* 1994) were subjected to Northern blotting to detect the 0.7-kb RNA (Fig. 7).

Strikingly, the 0.7-kb polyadenylated RNA present in strain-17-infected Vero cells was diminished in cells infected with mutant 17ΔSty, 17ΔS/N, and 17ΔPst (Fig. 7A), but the 2-kb LAT intron was not diminished (Fig. 7B). Thus the deletions within these mutants affected the expression of the 0.7-kb transcript. Interestingly the 0.9-kb transcript present in 17^{+}, and detected by the *Not*I-*Apa*I probe, is also detected in 17ΔSty, in 17ΔS/N to a diminished extent, and in 17ΔPst not at all. These data support mapping the 0.9-kb RNA to the left of the ΔSty but including at least part of the ΔPst region.

The 0.7-kb transcript does not affect viral reactivation kinetics from explant cocultivation

To determine whether the 0.7-kb transcript is involved in viral reactivation in the mouse explant cocultivation model, virus HSV-1 strain 17ΔBsa was constructed (see Materials and Methods). This virus has a 283-bp *Bsa*BI deletion within the Swa-Apa fragment in both the IRL and TRL regions of the viral genome. The deletion corresponds to nucleotides 4832–5115 and 118,363–118,646 in the 17 genome sequence (Perry and McGeoch, 1988). Virus 17ΔBsa did not express 0.7 kb but did express a smaller truncated transcript. Nine mice per group were infected on the cornea with either wild-type 17, mutant 17ΔBsa, or 17ΔPst (Dobson *et al.,* 1989) at doses of 5×10^4 pfu per eye. In mice infected with strain 17^{+}, six mice died before they could establish latent infection, but no death occurred due to infection with either 17ΔBsa or 17ΔPst. Twenty-eight days after inoculation, mice were killed, and trigeminal ganglia were explanted and cocultivated with CV-1 cells. As determined by the appearance of cytopathic effect, >50% of trigeminal ganglia from 17^{+}-infected mice showed viral reactivation by Day 5 with all showing viral reactivation by Day 7. A similar reactivation profile was observed with both 17ΔBsa- and 17ΔPst-infected mouse TG (data not shown). Because it took >20 days for slow-reactivating viruses, such as 1704 (Steiner *et al.,* 1989) and 17ΔN/H (Block *et al.,* 1993), to reached 50% reactivation in this test, we conclude that the mutants, 17ΔBsa or 17ΔPst, reactivate normally in the mouse TG explantation assay. However, the high rate of death in 17^{+}-infected mice compared with the deletion

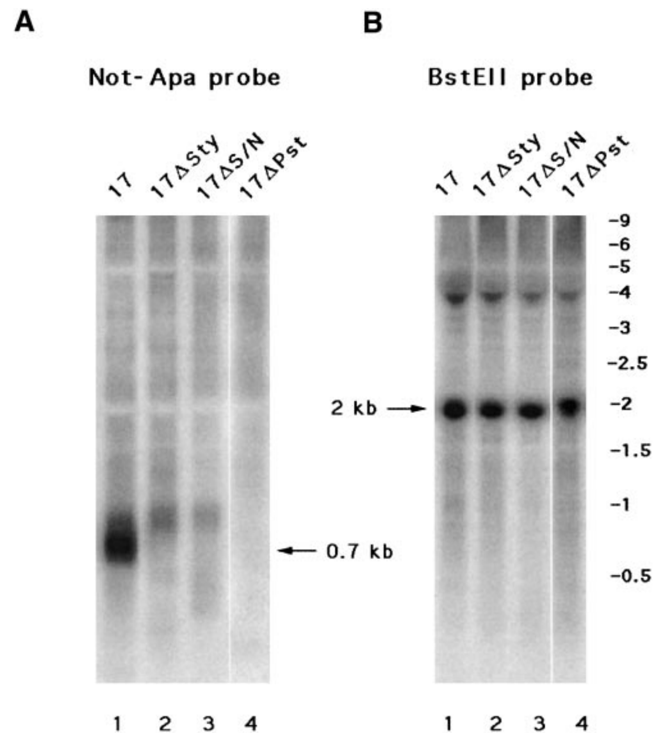

FIG. 7. RNA analysis of HSV-1 strain 17 and derived mutants. Total RNA and polyadenylated RNA were extracted from Vero cells infected with strain 17 (lane 1), 17ΔSty (lane 2), 17ΔS/N (lane 3), and 17ΔPst (lane 4) 16 h p.i. (A) Polyadenylated RNA (1 μg) of each sample was gel electrophoresed, transferred to a membrane, and hybridized with a nick-translated 0.4-kb Not-Apa probe. (B) Total RNA (10 μg) from each sample was electrophoresed, blotted, and hybridized with a 0.9-kb *Bst*EII probe. Ambion's RNA millennium markers (marked in kilobase) were used. The position of the 0.7- and 2-kb transcripts are labeled with arrows.

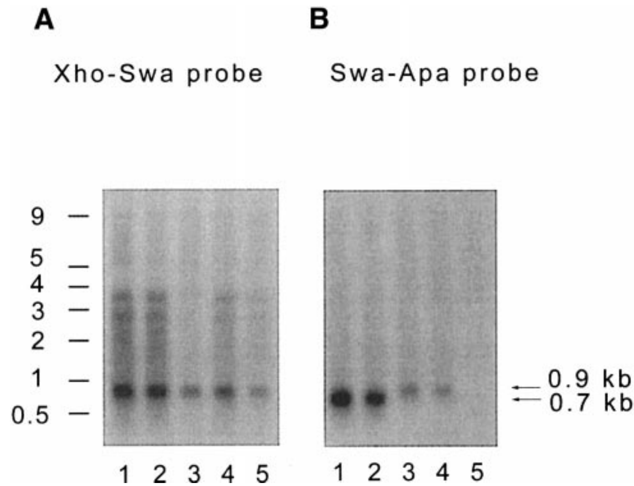

FIG. 8. RNA analysis of 17ΔBsa and revertant viruses. Polyadenylated RNA was extracted from vero cells infected with 17 (lane 1), 17ΔBsa/R17 (lane 2), F (lane 3), 17ΔBsa/RF (lane 4), and 17ΔBsa (lane 5) 16 h p.i. One microgram polyadenylated RNA of each sample was separated by agarose gel electrophoresis, blotted onto membranes and hybridized with the Xho-Swa probe (A) or the Swa-Apa probe (B). RNA markers are marked in kilobase. The positions of the 0.7- and 0.9-kb transcripts are labeled with arrows.

mutants suggested that the deleted region encoding the 0.7-kb transcript plays a role in HSV-1 virulence.

The region of the genome encoding the 0.7-kb transcript alters the virulence of HSV-1 strain 17^+ in Balb/C mice but not in rabbits

To address the question of whether the region encoding the 0.7-kb transcript is involved in the virulence of HSV-1 strain 17, a set of strain-17-based deletion mutant and rescuant viruses were constructed. Wild-type HSV-1 strain17 was used as the parent for 17ΔBsa, and rescuants were made using either strain-F- or -17-based plasmids by homologous recombination (17ΔBsa/RF; 17ΔBsa/R17) The neurovirulence of these viruses was tested in mice and rabbits following corneal inoculation.

The 17ΔBsa deletion mutant lacks a 283-bp *Bsa*BI fragment in both the IRL and TRL region, (bp 4831–5112 and 118,362–118,643 (Perry and McGeoch, 1988). Instead of the 0.7-kb transcript, a smaller truncated transcript was faintly detected from Vero cells infected with this virus (Fig. 8A). The rescuant virus, 17ΔBsa/RF, was constructed by cotransfecting cells with 17ΔBsa viral DNA and plasmid pXho-Sal DNA. This plasmid contains a subfragment of the *Bam*HI E sequence of HSV-1 F strain. Rescuant virus 17ΔBsa/RF was selected by plaque lift assay using probes specific for the deletion region. RNA extracted from 17/RF-infected Vero cells was analyzed in Northern blot, as shown in Fig. 8. Using the *Swa*I-*Apa*I probe, the hybrid mutant expressed undetectable amount of the 0.7-kb transcript, but normal level of both 0.9-kb transcripts as well as the 2.2-, 2.7-, and the 3.7-kb transcript on Northern blotting. The gene expression was similar to that from strain F (Fig. 8). 17ΔBsa/R17 was constructed in a similar manner—the 17ΔBsa mutant was repaired with a 3.3-kb *Hpa*I fragment from the strain 17^+ genomic DNA plasmid pBamB. The 17ΔBsa/R17 had completely restored ability to express the 0.7-kb transcript at a comparable level of wild-type 17.

To compare *in vivo* pathogenicity of these viruses, 4- to 6-week-old Balb/C female mice were infected with 17, F, 17ΔBsa, 17ΔBsa/RF, and 17ΔBsa/R17, at a dose of 5×10^5 pfu per eye. Two independent experiments were performed. The mice infected with wild-type 17 or rescuant 17ΔBsa/R17 developed severe pathological signs and most died by 8 days p.i. However, mice infected with strain F, deletion mutant 17ΔBsa, or F rescued hybrid strain 17ΔBsa/RF, none of which expressed the 0.7-kb transcript in tissue culture (Fig. 8), showed reduced pathological signs, and significant numbers of mice survived the acute viral infection (Fig. 9). In summary, wild-type 17 and the rescuant virus 17ΔBsa/R17, which were capable of expressing a 0.7-kb transcript, exhibited virulence that killed 100% of the infected mice in a short period of time (median survival: ~ 6 days). In contrast, strain F, deletion virus 17ΔBsa, and revertant virus 17ΔBsa/RF, none of which could express the 0.7-kb transcript, showed significantly reduced virulence.

In contrast to corneal infection of mice, infection of rabbits showed no difference in pathogenicity among the different viruses except for the lower rate of induced reactivation for parental strain F. Rabbit eyes were inoculated with 5×10^5 plaque-forming units of HSV-1 strain 17, F, 17ΔBsa, 17ΔBsa/R17, or 17ΔBsa/RF. Unlike mouse mortality, rabbit mortality for each virus group was similar. The range of mortality between all of the groups was

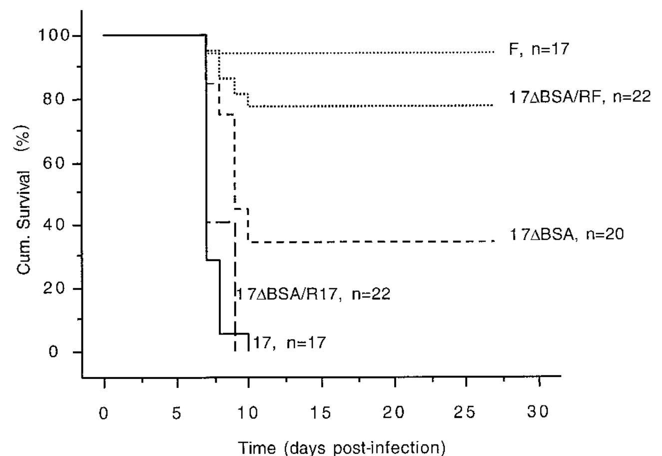

FIG. 9. Kaplan Meier cumulative survival plot for HSV following corneal inoculation of mice. Balb/c mice were inoculated with 5×10^5 pfu of HSV-1 strains 17, F, 17ΔBsa, 17ΔBsa/RF, and 17ΔBsa/R17. Survival of mice was noted and plotted with Stat-View on a Mackintosh computer. There is no significant difference between 17 and 17ΔBsa/R17 ($P = 0.18$); however, there is a significant increase in survival between these viruses and F and 17ΔBsa/RF or 17ΔBsa ($P < 0.005$).

TABLE 1

Induced HSV-1 Reactivation in the Ocular Rabbit Model

HSV-1 strain	Positive rabbits/total rabbits	Positive eyes/total eyes	Positive swabs/total swabs
17^+	6/8 (75.0)	7/13 (53.8)	26/91 (28.6)
17ΔBsa	7/11 (63.6)	11/20 (55.0)	37/140 (26.4)
F	3/6 (50.0)	4/12 (33.3)	6/84 (7.1)
17ΔBsa/R17	5/5 (100)	9/9 (100)	26/63 (41.3)
17ΔBsa/RF	5/6 (83.3)	8/12 (66.7)	32/84 (38.1)

Note. Parentheses enclose percentages.

40–50%. Spontaneous viral shedding in the tear film was minimal or zero for each of the virus groups, ranging from 0 to 5%. Reactivation induced by transcorneal iontophoresis of epinephrine was not significantly different for 17^+, 17ΔBsa, 17ΔBsa/R17, and 17ΔBsa/RF as determined by χ^2 analysis of swabs from sets of two viruses at a time ($P > 0.1$) or by two-tail Fisher's exact test ($P > 0.1$). The number and percentage of Dacron swabs that contained detectable infectious virus per rabbit per day was not significantly different between these four virus groups (Table 1). The number and percentage produced by strain F, however, was significantly lower than the other four virus groups (chi-square, $P = 0.001$; Fisher's exact test, $P = 0.0003$). The tear film swabs taken from the rabbit eyes, rather than whole rabbits or their eyes, were used for the statistical analysis to obtain meaningful results. The numbers and percentages of rabbits and rabbit eyes that produced infectious HSV-1 are included in Table 1 to show that the positive swabs were distributed among rabbits and eyes in each group and are not intended for statistical purposes.

DISCUSSION

The region of the HSV-1 genome upstream of the LAT transcript is important because it codes not only for the LAT promoter but also for several other transcripts. The polypeptides coded by these transcripts are all unknown as are their functions. However, this region of the genome is associated with an inefficient reactivation phenotype in both rabbit *in vivo* and mouse explant models of HSV-1 reactivation .

We have identified a 0.7-kb polyadenylated transcript, expressed with true-late (γ_2) kinetics, in RNA extracted from HSV-1 strain-17^+-infected Vero cells (Fig. 2), and SY5Y neuroblastoma cells by Northern blot analysis; and in mouse trigeminal ganglia acutely infected with 17^+ virus by *in situ* hybridization (data not presented). Northern blots showed that the 0.7-kb RNA is detected by a Swa-Apa probe, which covers ~800 nucleotides just upstream of the LAT promoter, but not by probes specific for sequence either further upstream in the U_L/R_L junction region (Xho-Swa probe) or downstream in the LAT exon 1 region (*Bst*E-*Bst*E probe) (Fig. 3). Therefore we conclude that the 0.7-kb transcript maps in the region of the HSV-1 genome immediately upstream of the LAT TATA box sequence.

Inspection of the HSV-1 strain 17 DNA sequence in the region coding for these transcripts (117,860 to 118,860 nt; Perry and McGeoch, 1988) shows that at the 5′ end of this region, there is one possible promoter and three consensus poly A signals. A TATA sequence occurs at nucleotide 117,950 and three consensus polyadenylated signals are located close to each other (within 30 nucleotides)—one leftward at 117,997 (TTTATT) and two rightward at 118,005 and 118,021 (AATAAA). The 3′ end of this 1-kb region also contains a potential rightward poly A signal (AATAAA) at position 118,711 and the LAT promoter. It is notable that the LAT TATA box has a bipartite feature (TTTATAAA) to which the transcription factor TFIID could bind to either stand of the DNA and initiate transcription. The LAT promoter has been shown to function bidirectionally in transient transcription assays *in vitro* (Batchelor and O'Hare, 1990, 1992). Thus there are consensus promoter elements and polyadenylation signals at both ends of this region (117,860 and 118,860), indicating that RNA transcripts could initiate and terminate in both directions. The novel 0.7-kb polyadenylated transcript detected in this study was mapped within this region. Using a single-stranded RNA probe, the direction of transcription of the 0.7-kb transcript was determined to be in the same direction as the latency-associated transcript. Thus the TATA homology at nucleotide 117,950 and the poly A site at nucleotide 118,711 could function to produce the 0.7-kb polyadenylated transcript. It is interesting to note that despite the presence of a possible 0.8-kb RNA encoded in the opposite strand sequence no transcription in the opposite direction could be detected at this time.

Data from deletion mutants further supported the mapping of the 0.7-kb transcript. The expression of the 0.7 kb was examined in mutant viruses having deletions within or surrounding this region (117,860–118,860). Deletions in 17ΔS/N (118,004–118,440) and in 17ΔPst (118,660–118,863), which knock out the 5′ or 3′ end of the region proposed to encode the 0.7-kb transcript, respectively, completely abolished the expression of the 0.7-kb RNA; while the deletion in 17ΔBsa (118,361–118,643) truncated the transcript to 0.5 kb and reduced its accumulation significantly (visible as a feint band in Fig 8B, lane 5). Interestingly, the 371-bp deletion in 17ΔSty downstream of the LAT promoter diminished the 0.7-kb transcript expression, suggesting that the sequence just 3′ of the polyadenylation site of the 0.7-kb RNA at 118,711 might be important for efficiency of its processing or regulation.

The 0.7-kb transcript is distinct from the previously described 1.1-kb transcript (Singh and Wagner, 1993) based on our observation that the Xho-Swa probe, cor-

responding to the DNA sequence believed to encode the 1.1-kb transcript, failed to detect the 0.7-kb transcript (Fig. 3A). The 1.1-kb, along with a 1.8-kb, RNA were shown by Singh and Wagner (1993) to be a pair of low-abundance, 5′ colinear transcripts mapping between the junction of R_L/U_L and the LAT cap site. Transcription of the 1.1- and 1.8-kb RNAs are believed to initiate just outside the R_L region of the genome and terminate at rightward polyadenylation sites 118,005 and 118,711, respectively (Singh and Wagner, 1993). However, expression of the 0.7-kb transcript most likely occurs from within the R_L region as the putative TATA homology and the poly A signal, are present in both the internal and terminal repeat long segments. Thus there are probably two copies of the 0.7-kb transcript gene, though only one copy of the 1.1- and 1.8-kb transcript gene, present in the strain 17 genome.

Mapping of the 0.7-kb transcript has led to the discovery of three novel RNA species in addition to the previously detected 1.1- and 1.8-kb RNA transcribed from the unique long and repeat long junction. They are all polyadenylated, with size of 2.2, 2.7, and 3.7 kb. Because they hybridized to Xho-Swa (but not Swa-Apa and *Bst*E-*Bst*E) probes in Northern blot analysis, they probably map upstream of the 0.7-kb transcript. The presence of the two closely located rightward poly A signals at 118,005 and 118,021 supports the hypothesis that these transcripts may be products extending from the U_L55 and/or U_L54 gene into the internal repeat region. These transcripts were conserved in all HSV-1 strains examined (four commonly used lab strains and eight clinical isolates), yet their structure and function remain to be determined.

The detection of the 0.7-kb and other polyadenylated transcripts upstream of the LAT promoter offers an alternative explanation of 2-kb LAT expression in promoterless LAT mutant viruses. For example in 17ΔPst, the 203-bp Pst-Pst deletion not only removed the LAT TATA box and several transcription regulatory elements, such as the cAMP response element, EGR factor binding site, LPBF site, and ICP4 recognition site, but also removed the putative polyadenylation signal of the upstream 0.7-kb transcript. Therefore the ability of 17ΔPst virus to express the 2-kb LAT during acute but not latent infection, may simply be the result of the 2-kb LAT intron splicing from a run-through transcript of the 0.7-kb gene. This possibility was proposed previously (Nicosia *et al.,* 1993). An alternate possibility of a second latency promoter (TATA-less promoter) at the 5′ end of the LAT intron in exon 1, which functioned exclusively during productive infection, has also been proposed (Chen *et al.,* 1995).

The most interesting feature of the 0.7-kb transcript is its strain-specific character. We have shown that the 0.7-kb RNA was expressed in strain-17- and McKrae-infected cells, but not in cells infected with KOS, F, or eight clinical HSV-1 isolates. However, a slightly larger band of 0.9 kb, which was codetected by the Swa-Apa probe, was present in all strains tested in this study. Sequence comparison reveals >95% identity among strain 17, KOS, and McKrae, in the 600 bp upstream of the LAT cap site (Strelow *et al.,* 1994). The 3′ polyadenylation signal (AATAAA) is conserved in all three strains. However, the available sequencing data does not cover the 5′ portion of the 0.7-kb transcript, including the putative promoter region for strain KOS and McKrae. Thus it is possible that minor changes in the basal promoter or transcription regulatory elements may result in lack of expression of the 0.7-kb transcript. Knowledge of the complete DNA sequence of various strains of HSV-1 other than 17 would be most useful in these and other functional and pathogenic studies.

Interestingly the two strains expressing the 0.7-kb transcript are related. Strain McKrae was isolated from a Scottish patient in the United States (H. E. Kaufman, L.S.U. Eye Center, New Orleans, LA, personal communication), and HSV strain 17 was isolated from a child in Ruchhill Hospital in Glasgow, Scotland, in the mid 1960s from a cold sore on his cheek (Brown *et al.,* 1973).

HSV strains have been shown to vary in their ability to cause disease in animal models and humans. Strain 17 and McKrae have been shown to be much more virulent than KOS and F. During ocular or footpad infection, 10^4 pfu of 17^+ leads to >50% mortality in mouse. In contrast, KOS and F strains are not lethal at dose of 10^7 pfu (Thompson *et al.,* 1986). A correlation between strain virulence and the 0.7-kb transcript is suggested by our animal data. First, the expression of the 0.7-kb transcript exhibited a strain-specific character being detected only in RNA extracted from cells infected with virulent strains 17^+ and McKrae but not with nonvirulent strains KOS and F. Interestingly, the eight clinic isolates that caused recurrent lesions at epithelial and mucosal surface in human but not severe CNS disease, such as encephalitis, were not capable of expressing the 0.7-kb transcript. Second, deletion mutations that abolished the 0.7-kb transcript expression (17ΔBsa and 17ΔPst) decreased the neuropathologic signs in infected mice and increased their survival rate dramatically. A 67% mortality rate in 17^+-infected mice to 0% rate in 17ΔBsa- or 17ΔPst-infected mice at a dose of 5 x 10^4 PFU was seen (data not shown). Third, although 17ΔBsa/17R and 17ΔBsa/RF, two rescuant viruses, differed only in the region coding for the 0.7-kb transcript in Northern blot analysis, they displayed completely different phenomenon in viral acute infection in mouse. 17ΔBsa/17R, in which the deletion was repaired by the corresponding fragment from 17 strain, showed wild-type virus 17 pathogenicity. At a dose of 5 x 10^5 pfu per eye (100-fold higher than the LD_{50} of strain 17) 17ΔBsa/17R killed 100% of the infected mice in 10 days (Fig. 9). In contrast, the rescued virus 17ΔBsa/RF, in which the repairing sequence came from strain F, behaved more like F strain. Nearly 80% of

infected mice survived the same high dose of virus and established latency in trigeminal ganglia. Although the molecular mechanism behind these observation remains to be addressed, our studies suggest that the 0.7-kb transcript (or some other transcript encoded in this region) is involved in the superior virulence of strain 17 and McKrae.

Previous studies in a rabbit adrenergically induced reactivation model have mapped an inefficient reactivation phenotype to a region between the *Swa*I (118,004 bp) and *Sty*I (118,878 bp) sites, the region of the genome that covers the 0.7-kb transcript gene (Hill *et al.,*1997; Loutsch *et al.,* 1999). Our data indicated that the 0.7-kb transcript may not be involved in HSV-1 reactivation as measured by the adrenergically induced rabbit reactivation model, despite the fact that three deletion mutants (17ΔS/N, 17ΔPst and 17ΔSty), which had reduced ability to undergo reactivation upon epinephrine induction, failed to express the 0.7-kb transcript in tissue culture. The deletion mutant, 17ΔBsa, and the intertypic recombinant, 17ΔBsa/RF, which both lack 0.7-kb transcript expression, exhibit an efficient reactivation phenotype comparable to the wild-type strain 17 and revertant 17ΔBsa/17R. Because the exchange of F strain sequence in 17ΔBsa/RF only affected the production of the 0.7-kb transcript but not the other surrounding transcripts, as detected by Xho-Swa and *Bst*E-*Bst*E probes, we concluded that the absence of the 0.7-kb transcript alone did not cause aberrant reactivation phenotype. Other defects of gene expression caused by deletions in mutant viruses, 17ΔS/N, 17ΔPst, and 17ΔSty, might contribute to their abnormal reactivation phenotype in rabbit.

In the mouse explant cocultivation reactivation model, the emerging picture of mutations causing the slow reactivation phenotype is somewhat different from that of the rabbit model. Many deletions have been introduced into the LAT region of HSV-1 strain 17 and tested in the mouse explantation model. The reactivation pattern reveals that the slow reactivators, such as 17ΔN/H, possess large deletions covering the upstream LAT promoter, downstream exon1, and 2-kb LAT sequence. Deletion mutations that affect small portions of the LAT region, such as in 17ΔS/N, 17ΔBsa, 17ΔPst, 17ΔSty, and 17ΔBstE, had no significant impact on reactivation, regardless of the positions of their deletion relative to the LAT promoter. These observations lead us to the speculation that a *cis* effect such as chromatin modification or special chromatin structure, present at this locus, plays an important role in the mouse explant reactivation model.

MATERIALS AND METHODS

Cells and viruses

Vero (African green monkey) cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. SY5Y (SK-N-SH; human neuroblastoma) cells were cultured in RPMI medium supplemented with 10% fetal calf serum. Wild-type HSV-1 strains 17, F, KOS, and McKrae as well as mutant viruses 17ΔPst, 17ΔSty, and 17ΔS/N were prepared and titered as previously described (Deatly *et al.,* 1987, 1988).

Plasmids and probes

pXho-Sal was constructed in pGem7zf$^+$ vector, containing a 4.0-kb *Xba*I-*Sal*I fragment from genomic clone *Bam*HI E of HSV-1 strain F (Wolfe, 1992). The 857-bp *Swa*I-*Apa*I fragment or the 1-kb *Xho*I-*Swa*I fragment from pXho-Sal was then subcloned into pGem7zf$^+$ at the *Sma*I/*Apa*I or at the *Xho*I/*Sma*I site, and designated as pSwa-Apa or pXho-Swa, respectively. Plasmid pΔBsa, which has a 283-bp *Bsa*BI-*Bsa*BI fragment deletion in pXho-Sal, was used to generate recombinant virus 17ΔBsa. Plasmid pBamB contains the HSV-1 strain 17$^+$ genomic sequence from 113,322 to 123,430 bp (Perry and McGeoch, 1988).

Double-stranded DNA probes (*Xho*I-*Swa*I, *Swa*I-*Apa*I, *Swa*I-*Not*I, *Not*I-*Apa*I, *Bst*EII-*Bst*EII, *Pst*I-*Pst*I and *Bsa*BI-*Bsa*BI; see Fig. 1) were radiolabeled with [α-^{32}P]dCTP by a nick-translation system (GIBCO BRL, Gaithersburg, MD) or by a random-primer DNA-labeling system (GIBCO BRL) and were used for Northern blot analysis.

Single-stranded RNA probes labeled with [α-^{32}P]UTP were produced by *in vitro* transcription using MAXIscript kit (Ambion, Austin, TX). Sense (+) and antisense (−) RNA probes transcribed from pSwa-Apa by RNA polymerase SP6 (+) and T7 (−) were used in Northern blot analysis and RNase protection assays.

RNA extraction and Northern analysis

Mock-infected or HSV-1-infected (m.o.i. = 1) Vero cells were harvested at various times postinfection. Total RNA was extracted using TRIZOL reagents, according to the manufacturer (GIBCO BRL). Polyadenylated RNA was then purified from total RNA by using a micropoly(A) kit (Ambion, Austin, TX).

Northern blot analysis was performed as described previously (Spivack, 1987). Briefly, 10 μg aliquots of total RNA, or a 1 μg portion of poly(A)-containing RNA, as well as 2 μg RNA marker (Ambion, Austin, TX), were treated with glyoxal, separated in a 1.2% agarose gel, vacuum blotted to GeneScreen Plus membrane (NEN, Boston, MA), and UV-crosslinked. Blots were prehybridized for 2 h at 50°C in a solution containing 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 1% SDS, 5× SSC, 1 mM EDTA, and 0.1% denatured salmon sperm DNA. Denatured radiolabeled probe were then added to blots in the prehybridization solution and incubated with the blots at 50°C overnight. Blots were washed twice in each of the three wash solutions (1× SSC, 0.5× SSC, 0.1× SSC with 0.1% SDS) for 20 min at 65°C and exposed

overnight on a Phosphor Imager screen (Molecular Dynamics) before analysis.

In situ hybridization

As previously described (Stroop *et al.,* 1984), trigeminal ganglia obtained from uninfected or acutely infected BALB/C mice were fixed at 4°C in 4% paraformadehyde-lysine-periodate solution for a minimum of 4 h, embedded in paraffin wax, and cut into 6-μm sections. After proteinase K treatment, the tissue sections were hybridized with ^{35}S-labeled DNA probe (*Swa*I-*Apa*I or *Bst*EII-*Bst*EII fragment) for 48 h at 50°C. The slides were then washed and exposed in NTB-2 nuclear track emulsion (Kodak, Rochester, NY) for 5 days at 4°C. Following processing in developer and fixer (Kodak), the section were counterstained with hematoxylin and eosin.

Viral constructs

Recombinant virus 17ΔBsa was constructed by deleting 283 bp of the *Bsa*BI-*Bsa*BI region (118,363–118,646 bp) upstream of the LAT TATA box, using homologous recombination. Viral DNA from HSV-1 17ΔPst and plasmid DNA pΔBsa were cotransfected into Vero cells using lipofectamine (GIBCO BRL), as previously described (Zhu and Aurelian, 1997). Recombinant viral plaques were then isolated and purified by plaque-lift assay. Briefly, Vero cells were infected with progeny viruses at low m.o.i. and overlaid with 1% agarose in medium. The viral plaques were then lifted onto BA 85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. After hybridization with ^{32}P-radiolabeled Pst and Bsa fragment probes, plaques were chosen for Pst-positive, but Bsa-negative, phenotype. An additional three rounds of plaque purification were performed to ensure the homogeneity of the recombinant virus, which was named 17ΔBsa. The rescuant virus, 17ΔBsa/R17, was generated by cotransfecting 17ΔBsa viral DNA with a 3.3-kb *Hpa*I-*Hpa*I fragment from pBamB into Vero cells to restore a wild-type 17$^+$ sequence. The 17ΔBsa/RF hybrid virus was created by homologous recombination between 17ΔBsa viral DNA and plasmid DNA pXho-Sal to introduce the corresponding sequence of HSV-1 strain F into the 17 background.

Mouse infection

The corneas of 4- to 6-week old BALB/c female mice (Jackson Laboratory, Bar Harbor, ME), 10–12 mice per each group, were scarified and infected with HSV-1 strain 17$^+$, 17ΔBsa, 17ΔBsa/R17, 17ΔBsa/RF, or HSV-1 strain F, at a dose of $\sim 5 \times 10^5$ pfu per eye for viral virulence experiments. Mice were monitored daily for 28 days following corneal infection.

Explantation cocultivation reactivation assay

Explant cocultivation was performed as previously described (Natarajan *et al.,* 1991). Briefly, 4- to 6-week old BALB/c female mice (Jackson Laboratory) were infected with HSV-1 strain 17$^+$, 17ΔBsa, or 17ΔPst via corneal inoculation at 5×10^4 pfu per eye. At a minimum of 28 days p.i., mice were sacrificed and TG were explanted and cocultivated on CV-1 cells.

Ocular rabbit infection, induced reactivation, and eye swabbing

New Zealand White rabbit (2–3 kg) eyes were anesthetized with one drop each of 0.5% proparacaine hydrochloride, and the corneal surfaces were lightly scarified with a sterile 30-gauge needle. Each eye was inoculated with 5×10^5 plaque-forming units of HSV-1 strain 17$^+$, 17ΔBsa, F, 17ΔBsa/R17, or 17ΔBsa/RF and was rubbed for 30 s to facilitate virus uptake. Slit lamp microscope examination of the eyes was done on post-inoculation Day 3 for verification of acute HSV-1 infection by the appearance of herpetic lesions. Each eye was monitored for spontaneous shedding of infectious virus for 5 days during latency by placing a sterile Dacron swab in the tear duct and then passing it over the conjunctiva and cornea. The swab was immediately placed on a confluent monolayer of primary rabbit kidney (PRK) cells, and the cells were incubated at 37°C and 5% CO_2 for 48 h. Swabs were removed and the cells were monitored every day for cytopathic effect due to infection by HSV-1. Transcorneal epinephrine iontophoresis was done to induce viral reactivation as previously described (Hill *et al.,* 1993). In short, 0.01% epinephrine was delivered through each cornea using 0.8 mA constant voltage for 8 min. Transcorneal epinephrine iontophoresis was done once daily for three consecutive days, and infectious virus was detected by cytopathic effects on PRK cells after tear film swabbing.

ACKNOWLEDGMENTS

We are indebted to Randal Fawl for constructing HSV17ΔBsa and to Dr. Richard Hodinka, Children's Hospital of Philadelphia, for the clinical isolates. We thank Dr. P.A. Schaffer for helpful discussions and acknowledge the excellent secretarial support of Jennifer Driscol in the preparation of this manuscript. This work was supported by Public Health Services Grants NS-33768 and NS-29390 to N.W.F. and EY-06311 to J.M.H. M.E.M. is the recipient of an NRSA Award F32 EY-06948.

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