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Disruption of the 5′ and 3′ splice sites flanking the major latency-associated transcripts of herpes simplex virus type 1: evidence for alternate splicing in lytic and latent infections

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The herpes simplex virus type 1 (HSV-1) latency-associated transcripts (LATs) are the only viral gene products expressed within latently infected neurones. The most abundant (major) LATs consist of two collinear nuclear polyA RNAs of 2 kb and 1·5 kb which it has been suggested represent stable introns derived from a less abundant primary transcript (minor LAT). Consistent with this proposition is the identification of consensus splice donor and acceptor sites flanking major LATs which are conserved between HSV types 1 and 2. Here we test the functionality of the predicted splice sites within the context of the virus genome during productive infection *in vitro* and latent infection *in vivo*. To this end viruses in which the LAT splicing signals were disrupted by site-directed mutagenesis were constructed. We report that mutation of the splice acceptor site abrogates 2 kb major LAT generation during productive infection but does not significantly influence major LAT synthesis during neuronal latency. Similarly, mutation of the splice donor site significantly reduces levels of 2 kb major LAT during productive infection but has no detectable effect on the generation of 2 kb major LAT during neuronal latency as assessed by Northern and *in situ* hybridization analyses of latently infected neuronal tissue. From these data it can be concluded that the proposed splice sites flanking the major LAT region are dispensable for 2 kb major LAT production in neurones latently infected with HSV-1 but constitute functional splicing signals in productively infected non-neuronal cells.

Introduction

Herpes simplex virus type 1 (HSV-1) establishes latency within sensory neurones innervating the site of primary infection (reviewed by Wildy *et al.*, 1982). During latent infection the viral genome is maintained in a non-replicative state and transcription is limited to a single region within the virus repeats giving rise to the latency-associated transcripts (LATs) (reviewed in Fraser *et al.*, 1992; Ho, 1992). There is no evidence for the production of a LAT-encoded protein during latency and LAT expression is not essential for the establishment or maintenance of latency (Javier *et al.*, 1988; Sedarati *et al.*, 1989). However, recent studies indicate that LATs are important for reactivation of virus from latency (Krause *et al.*, 1995) and that this function resides within a 350 bp region within LAT (Bloom *et al.*, 1996). The most abundant (i.e. major) LATs consist of two collinear, predominantly nuclear polyA$^-$ RNAs of 2 kb and 1·5 kb which overlap and are partially complementary to the mRNA for immediate early protein 110 (IE110) (Spivack & Fraser, 1987; Stevens *et al.*, 1987). The major LATs appear to share 5′ and 3′ termini, differing only in the excision of a small intron with the unusual use of GC instead of the consensus GU at the 5′ cleavage site (Wagner *et al.*, 1988; Spivack *et al.*, 1991). During lytic infection in tissue culture only the 2 kb LAT is produced, indicating that recognition of the internal splice sites is neurone-specific.

The mechanism by which major LATs are generated is unclear. It has been proposed that major LATs are stable, non-linear introns derived from a less abundant primary transcript (Dobson *et al.*, 1989; Farrell *et al.*, 1991). Consistent with this hypothesis, the 5′ end of major LAT maps to nucleotide 119461 within a putative splice donor site located 0·7 kb downstream of the latency-associated promoter LAP1 and a potential splice acceptor 1·95 kb downstream of the 5′ end (Perry & McGeoch, 1988; Wagner *et al.*, 1988; Farrell *et al.*, 1991). These processing sites are conserved between HSV types 1 and 2 (McGeoch *et al.*, 1991) and excision of 2 kb LAT from a reporter gene using the predicted splice sites has been

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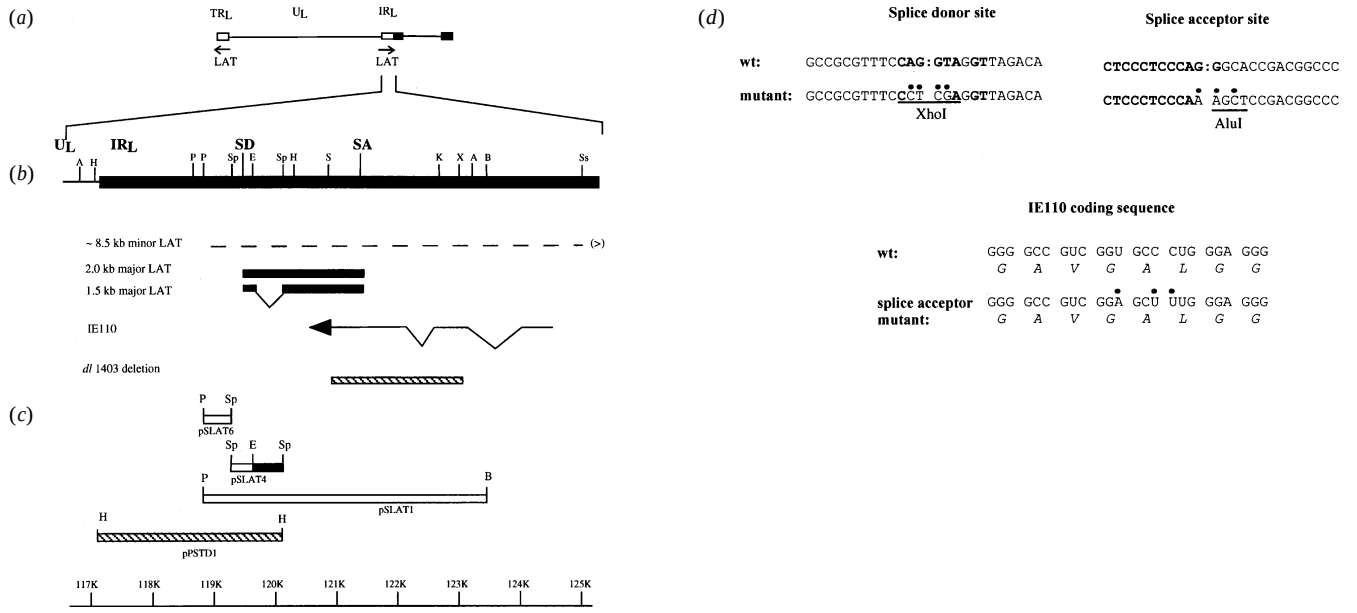

Fig. 1. LAT region of HSV-1. (*a*) Linear representation of the HSV-1 genome showing the positions of the LAT locus (arrows) within the repeat regions (IR_L and TR_L) flanking the unique long sequence (U_L). (*b*) Part of IR_L showing the relative positions of IE110 (shading represents the region deleted from *dl*1403) and LATs. Position of the splice donor (SD) and splice acceptor (SA) sites and restriction enzyme sites utilized in this paper are indicated: A, *Alu*I; B, *Bam*HI; E, *Eco*52I; H, *Hpa*I; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; X, *Xho*I. (*c*) Position of cloned HSV-1 restriction fragments used as templates for DNA and RNA probes for Southern blot, Northern blot and ISH analyses. Major LAT-specific probes for ISH were generated from *Eco*52I (E)-linearized template and correspond to the region shown in black. Scale bar represents nucleotide numbering according to Perry & McGeoch (1988) and McGeoch *et al.* (1991). (*d*) Oligonucleotides used to mutate the splice donor site (nucleotides 119449–119472) and splice acceptor sites (nucleotides 121406–121430) flanking the major LAT region. Nucleotides shown in bold are the components of the consensus splice site. The cleavage site (:) and nucleotides altered by mutagenesis (●) are indicated. The restriction enzyme cleavage sites engineered are underlined. The changes occurring in the IE110 coding sequence as a result of the mutagenesis are shown below. The single letter symbols for the amino acids encoded are shown in italics.

demonstrated in transient transfection assays (Farrell *et al.*, 1991). Furthermore, the 2 kb LAT derived from either productive or latent infections displays structural features consistent with intron lariats (Wu *et al.*, 1996; Rødahl & Haarr, 1997).

In concordance with the intron hypothesis low abundance RNAs, termed minor LATs, corresponding to sequences flanking major LAT have been detected by *in situ* hybridization (ISH) analyses and may represent transcription of an 8·5 kb primary LAT (Dobson *et al.*, 1989; Mitchell *et al.*, 1990; Zwaagstra *et al.*, 1990). The presence of this primary LAT and putative spliced product remains to be confirmed as Northern blot analysis of latently infected tissues has proven problematic (Zwaagstra *et al.*, 1990; Devi-Rao *et al.*, 1991). In more recent mapping studies Spivack *et al.* (1991) sequenced cDNAs generated from latent transcripts and suggested that the splice acceptor site may not be utilized *in vivo*. It also has been suggested that major LAT may represent a separate transcription unit (Chen *et al.*, 1995), raising questions about the precise mechanisms of synthesis and processing of LATs during latency.

In this report we have attempted to test the functionality of the predicted splice sites within the context of the viral genome during productive infection and latent neuronal infection in order to determine whether the 2 kb LAT is produced as an intron in all phases of infection. To this end we have constructed viruses in which the LAT splicing signals have been disrupted. As deletions in this region may destabilize major LAT (Block *et al.*, 1990) and disrupt IE110 function (Everett, 1989) we changed the nucleotide sequence of the splicing signals using site-directed mutagenesis ensuring that the coding sequence of IE110 was not altered. Our studies indicate that mutation of the splice acceptor site abrogates the generation of 2 kb LAT synthesis during productive, but not latent, infection. Disruption of the splice donor site resulted in reduced levels of 2 kb LAT during productive infection but had no major effect on 2 kb LAT generation during latency.

Methods

■ **Plasmids and site-directed mutagenesis.** Plasmid inserts were derived from HSV-1 *Bam*HI B fragment and nucleotide numbers in parentheses are based on the strain 17 sequence (Perry & McGeoch, 1988; McGeoch *et al.*, 1991).

pSLAT1 contains a 4·6 kb *Pst*I–*Bam*HI fragment (nucleotides 118867–123460) from HSV-1 SC16 in pBluescribe M13⁻ (pBS). pSLAT4,

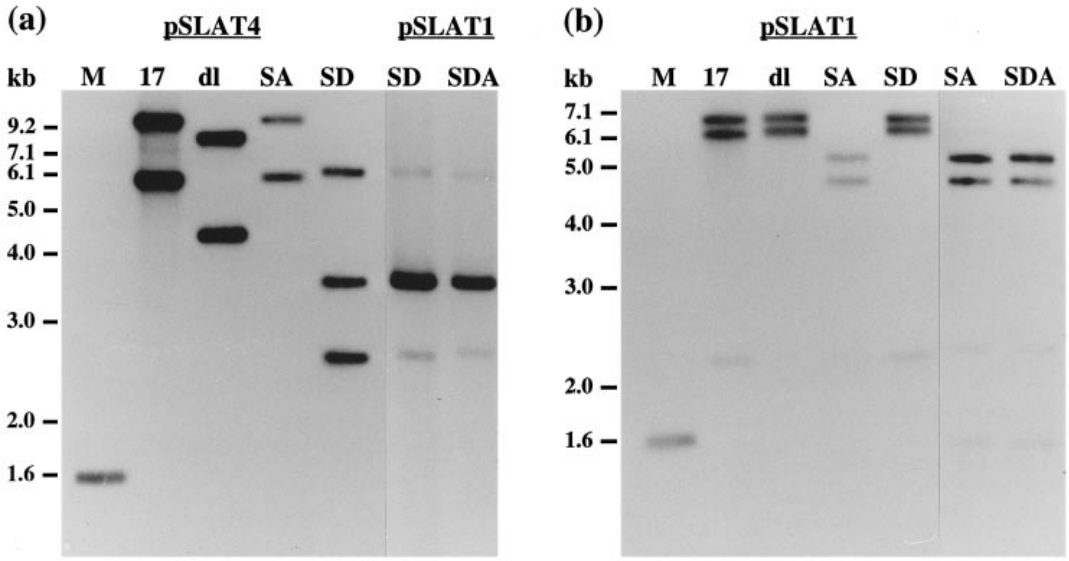

Fig. 2. Southern blot analysis of the genomes of the splice site mutant viruses. Infected cell DNA (2 μg) from HSV-1 strain 17 (17), *dl*1403 (dl), splice acceptor mutant (SA), splice donor mutant (SD) and double splice site mutant (SDA) were digested with *Bam*HI and *Xho*I (*a*) or *Alu*I (*b*) and hybridized with ^{32}P-labelled random primed DNA probe pPSTD1 (*a*) or pSLAT1 (*a* and *b*). DNA band sizes were estimated by comparison with ethidium-stained *Hin*dIII-cut lambda DNA marker track (M). (*a*) The *Bam*HI/*Xho*I fragments generated extend from R_L into U_L, thus for viruses with wild-type splice donor sites (17, SA) pPSTD1 hybridization detects bands of 9·7 kb and 6·09 kb; viruses with mutant splice donor sites (SD, SDA) yield fragments of 6·14 kb, 3·6 kb and 2·5 kb with pPSTD1 and pSLAT1 probes. (*b*) Hybridization of *Alu*I-restricted DNAs with pSLAT1 probes detects fragments of 6·8 kb and 6·2 kb for viruses with wild-type splice acceptor sequences (17, SD), or 5·3 kb and 4·6 kb for viruses carrying the splice acceptor site mutation (SA, SDA).

a 0·78 kb *Sph*I fragment (nucleotides 119292–120078) of HSV-1 strain 17 in pBS. pSLAT6, a 0·42 kb *Pst*I–*Sph*I fragment (nucleotides 118867–119292) of SC16 in pBS. pPSTD1, a 3·3 kb *Hpa*I fragment (nucleotides 117011–120302) of SC16 in pGEM4Z. pJR3, a 6·4 kb *Pst*I–*Sst*I fragment (nucleotides 118664–125067) of strain 17 in pUC9. pingPS, a 2·5 kb *Pst*I–*Sal*I fragment (nucleotides 118867–120903) from pJR3 in ping14.2 (Liu *et al.*, 1991). pingSB, a 2·5 kb *Sal*I–*Bam*HI fragment from pJR3 (nucleotides 120903–123460) in ping14.2.

Oligonucleotide 5′ GCCGCGTTTCCCTCGAGGTTAGACA 3′, encoding a *Xho*I cleavage site, was used to disrupt the splice donor site (Fig. 1*d*) in pingPS, and oligonucleotide 5′ CTCCCTCCCAAAGCT-CCGACGGCCC 3′, encoding an *Alu*I cleavage site, was used to mutate the splice acceptor site (Fig. 1*d*) in pingSB according to the method of Kunkel (1985). Mutagenized progeny (pingSBmu, pingPSmu) were identified by incorporation of the restriction site and the mutations confirmed by sequence analysis. The *Sal*I–*Bam*HI fragment of pJR3 (nucleotides 120903–123460) was replaced with the equivalent fragment from pingSBmu carrying the mutated splice acceptor site, generating pJR3SA. The *Pst*I–*Sal*I fragment of pJR3 (nucleotides 118867–120903) was replaced with the equivalent fragment from pingPSmu containing the mutated splice donor site to generate the splice donor mutant pJR3SD. Similarly, the *Pst*I–*Sal*I fragment of pJR3SA was replaced with the equivalent fragment of pingPSmu, generating pJR3SDA, in which both splice sites were disrupted.

■ **Cells and viruses.** Baby hamster kidney 21 cells (BHK) were grown in Glasgow modified Eagle's medium (GMEM) (Macpherson & Stoker, 1962) with 10% new-born calf serum and 10% tryptose phosphate. The HSV-1 mutant *dl*1403 (a gift from N. Stow, MRC Virology Unit), which has a deletion in both copies of IE110 (Stow & Stow, 1986), was used as the parent virus for recombination. Wild-type virus for these experiments was HSV-1 Glasgow strain 17 syn$^+$ (strain 17). All viruses were propagated on BHK cells.

■ **Transfection of BHK cells and isolation of recombinant viruses.** Virus DNA was prepared from BHK cells infected with *dl*1403 according to the method of Stow & Wilkie (1976). The *Pst*I–*Sst*I fragment of HSV-1 (nucleotides 118664–125067) contained in plasmids pJR3SA, pJR3SD and pJR3SDA spans the deletion in *dl*1403 and thus IE110-competent, recombinant progeny can be enriched by low multiplicity passage (Everett *et al.*, 1991). Infected cell DNA mixed with mutant plasmid DNA (pJR3SA, pJR3SD or pJR3SDA) was transfected into BHK cells by the method of Stow & Wilkie (1976). After 3 days of culture, infected cells were harvested, disrupted by ultrasonic vibration and then passaged twice at low multiplicity (0·001 p.f.u. per cell). Cell monolayers infected at low multiplicity with passaged recombinant stocks were overlaid with 1% agarose in medium as previously described (Forrester *et al.*, 1992). Individual plaques were picked and stocks prepared using BHK cells.

Virus DNA for genome characterization was prepared from infected cells according to Stow *et al.* (1983). Infected cell DNAs (2·5 μg) were digested with either *Alu*I, to check for incorporation of the splice acceptor site mutation, or *Bam*HI and *Xho*I to check mutation of the splice donor site. Restricted DNAs were electrophoresed in 1% agarose–1 × TBE (0·09 M Tris–borate plus 0·001 M EDTA pH 8·0), transferred to nitrocellulose (Southern, 1975) and fixed by baking at 80 °C for 1 h. Filters were hybridized with ^{32}P-labelled random primed probes (Feinberg & Vogelstein, 1984) to the LAT region generated from the 4·6 kb *Pst*I–*Bam*HI fragment of pSLAT1, or the 3·3 kb *Hpa*I fragment of pPSTD1.

Viruses carrying the mutations in both copies of LAT were plaque-purified twice more and incorporation of the novel restriction site(s) rechecked (as described above) (Fig. 2) before making virus working

stocks. Three viruses were prepared: a splice acceptor mutant, SA virus; a splice donor mutant, SD virus; and SDA virus in which both splice sites are disrupted.

■ **Latently infected tissues.** Female BALB/c mice aged over 8 weeks were infected using the zosteriform model (Simmons & Nash, 1984). Briefly, after depilation a small area of flank skin was scarified with a 27-gauge needle through 10 μl of a virus suspension containing $0{\cdot}5–1 \times 10^5$ p.f.u. Following establishment of latency, pooled, ipsilateral dorsal root ganglia (drg; T6 to L1) were fixed in periodate–lysine–paraformaldehyde (McLean & Nakane, 1974) for 60 min, dehydrated and paraffin-embedded for ISH analysis. Ganglia for RNA isolation were harvested and snap-frozen in liquid nitrogen.

■ **RNA extraction and Northern blot analysis.** BHK cell monolayers were infected with wild-type or mutant viruses at a multiplicity of 10 p.f.u. per cell. Sixteen hours after infection total cellular RNA was prepared (Chomczynski & Sacci, 1987). Total RNA was extracted from latently infected ganglia according to the method of Chomczynski & Sacci (1987).

RNA (5 μg) was electrophoresed through 1% agarose–0·02 M MOPS–2·2 M formaldehyde gels, transferred to nitrocellulose (Sambrook *et al.*, 1989) and fixed by baking at 80 °C for 1 h. Hybridization was carried out overnight at 50 °C with ^{32}P-labelled random primed DNA probes (specific activity $> 3 \times 10^8$ d.p.m./μg). Filters were washed for 40 min in 2× SSC (1× SSC is 0·15 M NaCl plus 0·015 M sodium citrate)–0·1% SDS and again in 0·1× SSC–0·1% SDS at 65 °C. Autoradiographs were generated by exposing filters to XAR5 film (Eastman Kodak).

■ ***In situ* hybridization analyses.** Probes to detect major LATs were transcribed by T7 polymerase from *Eco*52I-linearized pSLAT4 with digoxigenin (DIG)-conjugated rUTP according to manufacturer's recommendations (Boehringher). RNA generated corresponds to HSV-1 strain 17 nucleotides 119686–120078 within the major LAT region. DIG-labelled probes to detect minor LAT-specific sequences (spanning nucleotides 118867–119292) were similarly transcribed from *Eco*RI-linearized pSLAT6 templates. Transcription reactions were ethanol-precipitated then resuspended in 100 μl 10 mM Tris (pH 8)–1 mM dithiothreitol with RNase inhibitor (800 U/ml).

Sections (5 μm) of latently infected ganglia were hybridized with DIG-labelled probes specific for major (pSLAT4/*Eco*52I) or minor (pSLAT6) LAT species as described previously (Arthur *et al.*, 1993). Briefly, hybridizations were carried out overnight at 25 °C below theoretical melting temperature (68 °C) and unbound probe removed by washing in 0·1× SSC–30% deionized formamide–10 mM Tris-HCl (pH 7·5) at 15 °C below melting temperature (70 °C). Bound probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments according to the manufacturer's instructions (Boehringer). Slides were washed in water to stop development, counterstained with rapid haematoxylin for 30 s and water-mounted for photography.

Results

Construction of mutant viruses

To determine if 2 kb major LAT is produced as an intron using the predicted splice sites during productive and latent infections, we analysed 2 kb LAT accumulation by viruses lacking either the splice donor site at nucleotide 119462 (SD virus), splice acceptor site at nucleotide 121416 (SA virus) or both splice sites (SDA virus). These splice sites have been previously shown to constitute functional entities in transient transfection assays (Farrell *et al.*, 1991). The viruses were constructed on the HSV-1 strain 17 background using the deletion mutant *dl*1403 which has a 2 kb deletion within IE110 and major LAT (Stow & Stow, 1986) (Fig. 1*b*). Plasmids containing either the 5′ or 3′ splice site were subjected to site-directed mutagenesis to disrupt site recognition and introduce novel restriction enzyme sites to facilitate screening of mutant progeny. Mutation of the splice donor signal created a *Xho*I cleavage site and mutation of the splice acceptor signal introduced an *Alu*I cleavage site such that the amino acid coding of IE110 was not altered (Fig. 1*d*).

Plasmids containing a disrupted splice acceptor site (pJR3SA), or disrupted splice donor site (pJR3SD), or both splice site mutations (pJR3SDA) within a *Pst*I–*Sst*I fragment were independently cotransfected with *dl*1403-infected cell DNA into BHK cells. The *Pst*I–*Sst*I fragment spans the 2 kb deletion in *dl*1403 and IE110 competent, recombinant progeny were enriched for by low multiplicity passage in culture and then independent recombinant viruses were isolated. Genome integrity and incorporation of splice site mutations into both repeats of the recombinant viruses were assessed by Southern blot hybridization of infected cell DNAs utilizing the novel restriction sites introduced by mutagenesis (Fig. 2). Hybridization of ^{32}P-labelled pPSTD1 probes to *Bam*HI- and *Xho*I-restricted viral DNAs (Fig. 2*a*) detects two bands of 9·7 kb and 6·09 kb in strain 17 and SA viruses which carry the wild-type splice donor sequence. Two bands are also detected in the parent virus *dl*1403, and the size reduction observed corresponds to the deletion within IE110. Incorporation of the *Xho*I site into the splice donor sequence in the SD and SDA viruses results in three bands of 6·14 kb, 3·6 kb and 2·5 kb on hybridization with ^{32}P-labelled pPSTD1 or pSLAT1 probes. Hybridization of ^{32}P-labelled pSLAT1 probes to *Alu*I-restricted viral DNAs (Fig. 2*b*) detects two bands of 6·8 kb and 6·2 kb in strain 17 and SD viruses carrying the wild-type splice acceptor sequence. Incorporation of the novel *Alu*I site by the mutation of the splice acceptor site results in bands of 5·3 kb and 4·6 kb on *Alu*I digestion of SA and SDA viruses.

In preliminary analysis of virus growth in BHK cells, the splice site mutant viruses display one-step growth kinetics comparable to strain 17 (data not shown).

LAT production *in vitro*

To determine whether the introduced mutations affect accumulation of 2 kb major LAT during productive infection, total RNA was extracted from BHK cells 16 h after infection with wild-type or mutant viruses. Northern blot analysis of 5 μg of each RNA using a ^{32}P-labelled pSLAT4 probe for major LAT sequences (Fig. 3*a*) indicates that disruption of the splice acceptor site (SA virus) is sufficient to abrogate 2 kb LAT production *in vitro*. Mutation of the splice donor site only (SD virus) results in significant reduction of the levels of 2 kb LAT produced with respect to strain 17. The effect of mutating both

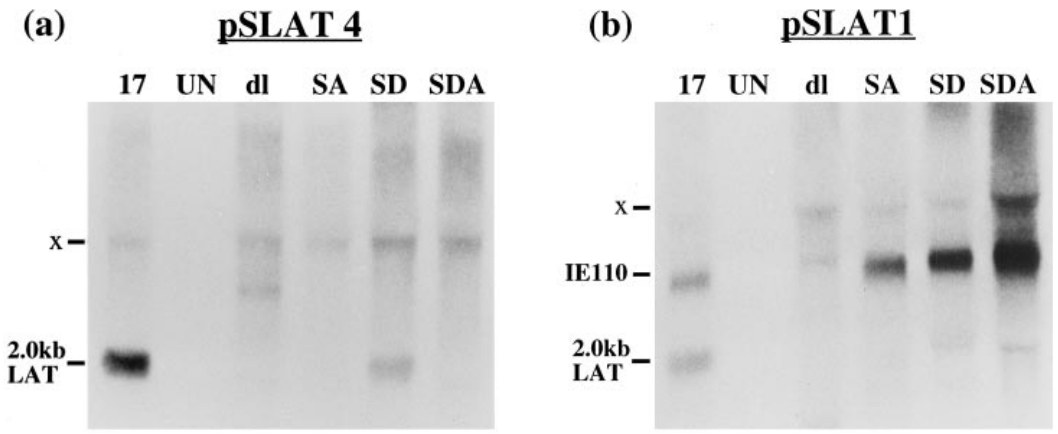

Fig. 3. Northern blot analysis of LAT production in BHK cells. Duplicate Northern blots were made of infected BHK cell RNA harvested 16 h after infection with strain 17, *dl*1403, SA, SD or SDA viruses. Mock infected (UN) and infected BHK RNAs (5 µg) were hybridized to ^{32}P-labelled random primed pSLAT4 (*a*) or pSLAT1 (*b*) DNA probes. The autoradiograph was exposed overnight. (*a*) 2 kb LAT production was observed for strain 17 and SD viruses but not for *dl*1403, SA or SDA viruses. (*b*) pSLAT1 hybridization, which hybridizes to IE110 and major LAT, confirmed only strain 17 and SD virus generate detectable levels of 2 kb LAT. Both probes non-specifically hybridized to an unidentified infected cell RNA (x). Ethidium staining (not shown) and comparison of amounts of the cross-hybridizing species indicate that loading artefacts could not account for the increase in IE110 levels observed in the SA, SD and SDA infections.

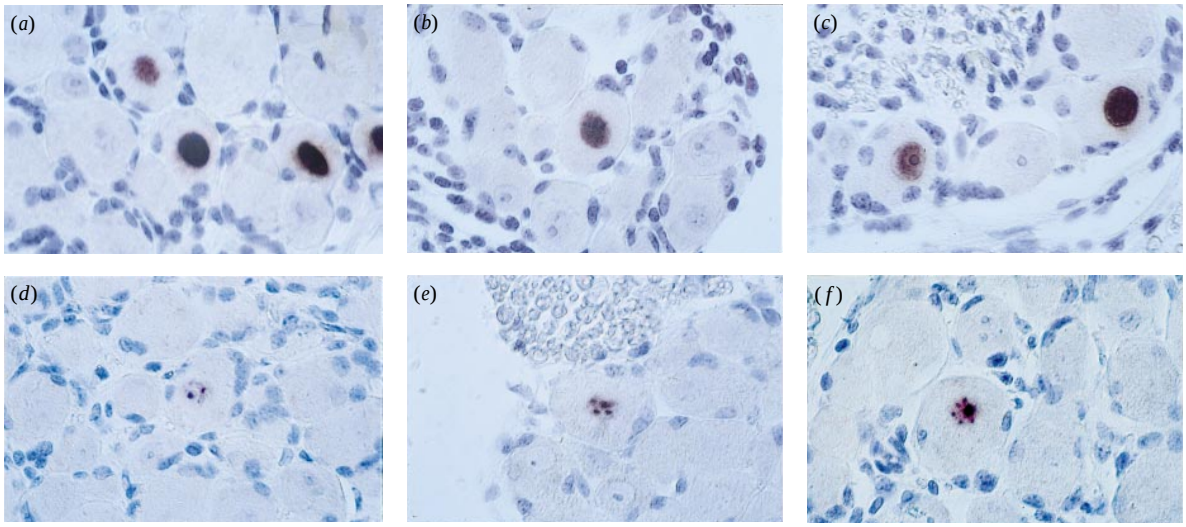

Fig. 4. ISH detection of LATs *in vivo*. Light micrographs of DIG-ISH detection of LATs in 5 µm sections of mouse ganglia 1 month after infection with strain 17 (*a*, *d*); SA virus (*b*, *e*) or SD virus (*c*, *f*). Hybridization with major LAT-specific RNA probes (pSLAT4/E) results in strong, diffuse nucleoplasmic staining (*a*, *b*, *c*). Minor LAT-specific pSLAT6 RNA probes hybridize to discrete subnuclear foci and also weakly stain the infected cell nucleoplasm in all infected tissues (*d*, *e*, *f*). DIG colour development was for 2–5 h. Sections were counterstained with haematoxylin and water-mounted for photography.

splice sites (SDA virus) was equivalent to mutation of the splice acceptor site alone. Stabilization of a large minor LAT species containing major LAT sequences was not observed for any of the splice site mutant viruses. Comparable levels of a cross-hybridizing viral RNA were observed in each sample, indicating that the inability to detect major LAT was not due to gel loading artefacts.

To ensure that the level of lytic infection in these samples was comparable a duplicate Northern blot was hybridized with a ^{32}P-labelled pSLAT1 probe encompassing IE110 and major LAT regions (Fig. 3*b*). The amount of IE110 RNA observed in cultures infected with splice site mutant viruses was elevated for all three mutants, most significantly for SDA virus (Fig. 3*b*). Comparison of amounts of cross-hybridizing RNA in each track and ethidium staining of a duplicate gel (not shown), indicate that loading artefacts are not responsible for this increase in IE110 RNA. Whether this increase in IE110 RNA results in elevated levels of IE110 protein has yet to be

Table 1. Number of LAT-positive cells in latently infected ganglia

Enumeration of LAT-positive neuronal profiles from ISH analysis of 5 μm sections from mouse dorsal root ganglia latently infected with strain 17 or SA virus as shown in Fig. 4(*a*, *b*, *d* and *e*).

Strain	Ganglionic profiles	Positive neuronal profiles	Positive neurones per ganglion
Major LAT			
17	278	190	0·68
SA	371	208	0·56
Minor LAT			
17	253	81	0·32
SA	202	55	0·27

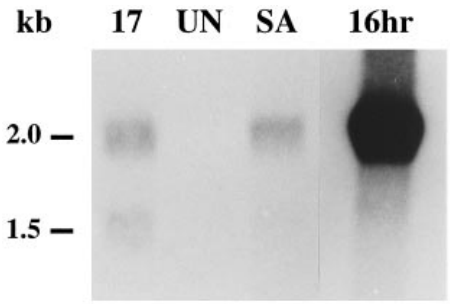

Fig. 5. Northern blot analysis of LAT production *in vivo*. Northern blot analysis of 5 μg of total ganglionic RNA extracted from 7 uninfected (UN) mice or 18 mice 1 month after infection with either strain 17 or SA viruses. Strain 17-infected BHK RNA (2·5 μg) extracted 16 h after infection (16hr) was included as a control for hybridization. Hybridization with ^{32}P-labelled pSLAT4 DNA probe detects major LATs in strain 17- and SA virus-infected ganglia. Autoradiograph exposure was for 8 days.

determined. We conclude from our analysis of LAT biogenesis in productive infection that the splice acceptor site proposed by Farrell *et al*. (1991) is essential for the production of a 2 kb LAT.

LAT production by SA mutant virus *in vivo*

Given that the splice acceptor mutant viruses fail to synthesize LATs during lytic infection we inoculated mice with SA virus to analyse LAT production during latent infection *in vivo*. Two groups of 30 BALB/c mice were infected either with SA or strain 17 viruses and drg harvested 1 month later for Northern or ISH analyses.

ISH analysis of sections (5 μm) of drg latently infected with SA using DIG-labelled pSLAT4/E probes specific for major LAT resulted in strong nucleoplasmic staining (Fig. 4*b*). This staining pattern was indistinguishable from that observed in wild-type virus-infected tissues (Fig. 4*a*). DIG-labelled minor LAT-specific probe pSLAT6 stained discrete foci within neuronal nuclei in SA virus- (Fig. 4*e*) and strain 17-infected tissues (Fig. 4 *d*) as we have previously described (Arthur *et al*., 1993). However, in our recent experiments we detect weak nucleoplasmic staining which we did not previously observe with strain SC16. This was a consistent feature also of strain 17 and not due to the splice site mutations. Indeed we did not discern any difference in the subnuclear distributions of the LAT transcripts generated during latent infection with the strain 17 or SA viruses.

To assess the ability of the SA virus to establish latency in murine tissues the number of LAT-positive neuronal profiles detected by ISH analysis was quantified (Table 1). In animals infected with strain 17, ISH analysis of 278 ganglionic sections with major LAT-specific probe pSLAT4/E detected 190 positive neuronal profiles, which corresponds to 0·68 per ganglionic profile. In animals infected with SA virus, 208 positive neuronal profiles were detected in the 371 ganglionic profiles examined, corresponding to 0·56 per ganglionic profile. ISH analysis of these tissues with minor LAT-specific probe pSLAT6 detected 81 positive neuronal profiles in the 253 ganglionic sections of strain 17-infected tissue examined (0·32 per ganglionic profile). In SA virus-infected tissue, 55 positive neuronal profiles were detected in the 202 ganglionic sections examined (0·27 per ganglionic profile). Thus, in this experiment the SA virus established latency at near wild-type levels.

Animals from the same experimental group were sacrificed and ganglia removed for RNA extraction. RNA (5 μg) from uninfected or latently infected animals was analysed using a ^{32}P-labelled pSLAT4 DNA to probe for major LAT. In contrast to our observations in BHK cells the 2 kb major LAT species was detected at a comparable level in SA virus-infected tissues to that observed in wild-type virus-infected tissue (Fig. 5), corresponding closely to the levels of latency establishment observed by ISH analysis in this experimental group of animals. However, the ratio of the 1·5 kb LAT to the 2·0 kb LAT appears to be reduced in the SA virus-infected sample relative to strain 17.

To ensure that the LATs examined in this experiment arose from latent SA virus, drg from three mice 1 month after infection were harvested for explant reactivation. Southern blot hybridization analysis of the viruses reactivated from these animals demonstrated that all viruses carried the splice acceptor mutation in both copies of LAT (data not shown).

LAT production by SD virus *in vivo*

Following our observation that the SA virus was able to make 2 kb LAT at near wild-type levels during latent infection a second group of BALB/c mice was infected either with strain 17, SA or SD viruses (40 mice per virus) to examine LAT production by the SD virus. Drg were harvested 1 month after infection for Northern and ISH analyses.

The number of LAT-positive neurones detected by ISH analysis of these tissues was quantified to assess levels of

Table 2. Number of LAT-positive cells in latently infected ganglia

Enumeration of LAT-positive neuronal profiles from ISH analysis of 5 μm sections from mouse dorsal root ganglia latently infected with strain 17 or SD virus as shown in Fig. 4(*a*, *d*, *c* and *f*).

Strain	Ganglionic profiles	Positive neuronal profiles	Positive neurones per ganglion
Major LAT			
17	225	618	2·75
SA	151	344	2·28
SD	232	201	0·87
Minor LAT			
17	225	331	1·47
SA	178	208	1·17
SD	781	212	0·27

latency established by each of the viruses in this experiment (Table 2). Examination of 225 ganglionic profiles of strain 17-infected drg using the major LAT-specific probe pSLAT4/E detected 618 positive neuronal profiles corresponding to 2·75 per ganglionic profile. For SA virus-infected drg, 334 positive neuronal profiles were detected in 151 ganglionic sections examined (2·28 per ganglionic section). In SD virus-infected drg we observed 201 positive neuronal profiles in 232 ganglionic profiles examined (0·87 per ganglionic section). Using minor LAT-specific probe pSLAT6, 331 positive neuronal profiles were detected in 225 ganglionic sections from strain 17-infected drg (1·47 per ganglionic section). In SA virus-infected tissue 208 positive neuronal profiles were observed in 178 ganglionic sections examined (1·17 per ganglionic section) and in SD virus-infected drg, 212 positive neuronal profiles were detected in 781 ganglionic sections examined (0·27 per ganglionic profile). Thus, in this experiment the SD virus established a threefold lower level of latency than strain 17.

To address the issue of whether the SD virus is impaired in its ability to establish latency we quantified the number of LAT-positive neurones by ISH analysis of a second group of latently infected animals infected with either SD virus or strain 17. The level of major LAT-positive neuronal profiles in the strain 17 control tissue was 0·68 per ganglionic profile. For the SD virus-infected drg, ISH analysis of 262 ganglionic sections with major LAT-specific probe pSLAT4/E detected 294 positive neurones, which corresponds to 1·12 per ganglionic section. Thus, in this experiment the SD virus established 1·6-fold greater levels of latency than wild-type. We are, therefore, unable to demonstrate a consistent defect in the establishment of latency with the SD virus.

ISH analysis of SD virus-infected tissues also demonstrated that, as with SA virus, disruption of the predicted splice site did not change the subnuclear distribution of major or minor LATs (Fig. 4*c* and *f*). Thus, we conclude that in the absence of either predicted splice site major LATs are readily generated during latent infection in mice. From these results it may be inferred that different processing sites or pathways of synthesis are utilized for the production of major LATs during productive and latent infections.

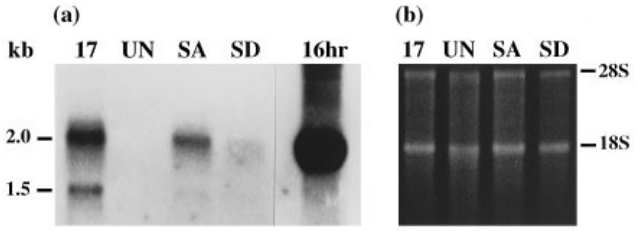

Fig. 6. Northern blot analysis of LAT production *in vivo*. (*a*) ^{32}P-labelled pSLAT4 random primed DNA probes were hybridized to 5 μg of total ganglionic RNA prepared from seven uninfected (UN) mice or 18–20 mice 1 month after infection with strain 17, SA or SD viruses. 2 kb major LATs were detected in all latently infected tissues. Strain 17-infected BHK RNA (2·5 μg) 16 h after infection was included as a control for hybridization (16hr). The autoradiograph was exposed for 6 days. (*b*) Duplicate aliquots of the ganglionic RNA (2·5 μg) ethidium bromide-stained following electrophoresis as an indicator of relative RNA concentrations and loading.

Northern blot hybridization with ^{32}P-labelled major LAT probe revealed that the SD virus produces a major LAT during latency (Fig. 6). The major LAT species detected ran at a lower mobility than the 2 kb major LATs produced by strain 17 and SA viruses, which may be a consequence of differential splice site usage. The amount of 2 kb major LAT observed in SD infection was significantly reduced with respect to wild-type and SA viruses, and the 1·5 kb major LAT species were not visible. As is apparent from our ISH analyses in which the levels of latency established were compared, the frequency of LAT-positive neurones detected in sensory ganglia from SD virus-infected animals was significantly reduced relative to the levels of latency established by both wild-type and SA viruses (Table 2). We therefore consider plausible that the low, but detectable, levels of major LAT observed in our Northern blot analyses of RNA from ganglia latently infected with the SD virus is a result of the low levels of latency established in this experiment, rather than a defect in the synthesis of major LAT.

Discussion

Despite extensive analyses of the HSV-1 LAT locus important questions regarding the synthesis and function of LATs remain unresolved. One such issue is the pathway by which the various collinear RNAs are generated. Current evidence is consistent with the 2 kb and 1·5 kb major LATs representing alternately processed introns derived from an unstable primary transcript. Much of this evidence stems from analyses of 2 kb LATs generated in tissue culture cells which, for the most part, are corroborated by observations *in vivo*.

One significant exception is the failure to detect sequences at the predicted 3′ end of major LATs generated in neurones. Sequence analysis of LAT cDNAs generated from latently infected tissues indicated that the major LATs may terminate up to 80 nucleotides prior to the splice acceptor sequence (Spivack *et al.*, 1991). In more recent hybridization studies, oligonucleotides complementary to the 20 nucleotides at the predicted 3′ end fail to hybridize to major LATs isolated from latently infected ganglia and productively infected PC12 cells in culture (Wu *et al.*, 1996; Rødahl & Haarr, 1997). Thus, just as there is neurone-specific enhancement of LAP1 activity and splice site usage for the internal intron (Dobson *et al.*, 1989; Spivack *et al.*, 1991), there may also be a neurone-specific pathway for the generation of 2 kb major LATs. The aim of the experiments reported here was to provide formal proof that the splice sites utilized in transfected, non-neuronal cells as reported by Farrell *et al.* (1991) are similarly utilized in productive virus infection and during neuronal latency. To this end we constructed viruses in which the nucleotide sequences of the splice sites flanking the major LAT have been altered by mutagenesis and analysed the synthesis of LATs in productively and latently infected cells.

We initially assessed LAT production by these viruses in BHK cells. 2 kb LAT accumulates late in productive infection (Spivack & Fraser, 1988) and the 5′ end of this RNA maps within a few nucleotides of the 5′ end of the 2 kb LAT in latent tissues (Wagner *et al.*, 1988; Wu *et al.*, 1996). However, in contrast to latent infection, accumulation of 2 kb LAT during productive infection occurs independently of LAP1 function (Nicosia *et al.*, 1993). A region downstream of LAP1 (designated LAP2) which influences maximal LAT expression during productive infection (Goins *et al.*, 1994; Yoshikawa *et al.*, 1996) was predicted to direct independent synthesis of 2 kb LAT (Goins *et al.*, 1994). Analysis of LATs in productively infected BHK cells indicate that the splice acceptor site is essential for 2 kb LAT production in these cells (Fig. 3). Further, loss of the splice donor site significantly reduced 2 kb LAT accumulation with respect to wild-type infection (Fig. 3). Thus, we conclude that during productive infection, 2 kb LAT represents an intron generated using the predicted splice sites, in agreement with the observations of Farrell *et al.* (1991) and is unlikely to represent a product of LAP2-driven transcription. In this study inhibition of splicing did not result in the detection of an 8·5 kb primary minor LAT. It has been proposed that 2 kb LAT may be spliced from large transcripts initiating upstream of LAP1 (Nicosia *et al.*, 1993; Yoshikawa *et al.*, 1996) which may be too large to be resolved on our gels.

A low level of 2 kb LAT was observed in BHK cells infected with the SD virus. It has been noted by others that when a splice site is disrupted through mutation, alternate proximal sites may be utilized. Thus the transcripts observed in SD virus infection may result from processing at alternate sites. Indeed Chen *et al.* (1995) identified two low abundance RNA species from this region with 5′ ends mapping 15 and 180 nucleotides upstream of the SD site which may represent processing at cryptic splice sites. We believe it is most likely the LATs produced in SD virus infection arise through the use of an alternate 5′ splice site. However, until the 5′ end of these transcripts is mapped we cannot rule out the possibility that the mutation of the splice donor site was insufficient to completely disrupt recognition and processing at this site.

In contrast to productive infection, neither splice site disruption abrogated 2 kb major LAT generation in latently infected ganglia. Nor was there any discernible alteration in the subnuclear distribution of major and minor LATs which we predicted would occur if splicing was inhibited. We therefore conclude that the splice sites flanking the major LAT region are dispensable for 2 kb LAT production in latently infected neurones. As the 2 kb LATs generated in latent infections are nuclear, polyA$^-$ RNAs the most obvious possibility is that the RNA is generated through the use of proximal splice sites recognized specifically in neurones. Further transcript mapping using RT–PCR may clarify the sites utilized. Given that the LAT promoter and the recognition of the internal splice sites display neuronal specificity, there is precedent for this proposal. Whether these cryptic sites are utilized in response to the introduced mutations or as part of the normal route of synthesis is not known. Alternately the mutations introduced may not be sufficient to stop site recognition in neuronal cells. Indeed, mutagenesis of the splice donor site leaves intact an adjacent AGGT sequence, nucleotides 119464–119467 (Fig. 1), which may represent a functional donor sequence. The reduction in signal intensity of the 1·5 kb major LAT species with SA and SD viruses may, however, indicate involvement of the conserved splice donor and acceptor signals in the generation of this lower molarity product.

The data presented in this report show that the synthesis of 2 kb LATs during productive infection is dependent on different factors than in latently infected neuronal cells. Conservation of the splice sites between HSV types 1 and 2 suggests a functional role (McGeoch *et al.*, 1991) and given that the effect of site disruption is observed during productive infection, we return to question the role of 2 kb LAT in lytic infection. When it was first determined that major LATs are complementary to part of the IE110 mRNA it was hypothesized that these transcripts initiated and maintained latency by down-regulating IE110 activity through an antisense mechanism (Farrell *et al.*, 1991). This idea lost favour following the isolation of LAT-null mutants which were able to establish and maintain latency. However, a role for LATs in efficient establishment has been proposed (Sawtell & Thompson, 1992; Thompson & Sawtell, 1997) and, moreover, expression of 2 kb LAT has been shown to reduce IE110 transactivation in dual transfection assays (Farrell *et al.*, 1991). Such control of IE110 expression during infection could conceivably enhance latency establishment. It is therefore of interest that elevated levels of IE110 message were observed in BHK cells infected with viruses unable to make 2 kb LAT (Fig. 3*b*). Experiments

are currently in progress to utilize such viruses in an examination of the effect of LAT expression on IE110 transcription and RNA stability during productive infection.

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