Journal of NeuroVirology (1996) 2, 268–278

A Herpes simplex virus type 1 mutant with a deletion immediately upstream of the LAT locus establishes latency and reactivates from latently infected mice with normal kinetics

John Maggioncalda[1], Anand Mehta[1], Omar Bagasra[2], Nigel W Fraser[3] and Timothy M Block[1,3]

[1]*Jefferson Cancer Institute, Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania 19107-6799;*
[2]*Department of Medicine, Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania 19107-6799;*
[3]*The Wistar Institute, 36th and Spruce Streets, Philadelphia, Pennsylvania 19101, USA*

The latency associated transcripts (LATs) are the only abundant viral gene products detected during latent herpes simplex virus (HSV) infection of peripheral nerves in animals and people. A LAT promoter has been identified and mutant viruses with lesions removing the promoter and surrounding region have been observed to reactivate slowly from trigeminal ganglia (TG) explanted from latently infected mice. Previous work has shown that most mutants with lesions limited to regions downstream of the LAT promoter reactivate normally. Therefore, to help map the boundaries of the slow reactivation phenotype, a mutant virus with lesions located immediately upstream of the LAT promoter was constructed and called 17ΔS/N. 17ΔS/N contains a 437 nucleotide (nt) deletion 332 nts upstream of the TATAA box of the LAT promoter. In productively infected cells, 17ΔS/N failed to synthesize detectable amounts of the 1.1 and 1.8 kb transcripts which are produced during wild-type infections and are specified by a region just upstream of the LAT promoter. However, 17ΔS/N did produce normal amounts of LAT in tissue culture as well as in neurons derived from latently infected cells, as ascertained by Northern blot and *in situ* hybridization analysis. Moreover, in latently infected mice, 17ΔS/N established and maintained infection in as many neurons as did wild type virus, as determined by *in situ* polymerase chain reaction (PCR) to detect viral DNA. Finally, the virus reactivated from TG derived from latently infected mice with kinetics indistinguishable from those of wild-type virus. Therefore, reactivation from latency, in this model system, does not appear to require function from the viral genomic region located immediately upstream of the LAT promoter.

Keywords: herpes simplex virus; latency; pathogenesis; latent; RNA; mutant

Introduction

Natural infection of animals with herpes simplex viruses is characterized by two discreet phases: lytic and latent (Straus, 1993). Lytic infections, in which the host cell is killed and progeny virus is released, occur at and near the site of viral entry. Following a period of productive infection, latency ensues in the peripheral nervous system, where the virus remains in the host in the absence of clinical manifestations (Stevens and Cook, 1971). However, from time to time, and for unknown reasons, in people and some animal models, the virus may reappear at or near the site of initial infection. This is called reactivation. Lytic infections are studied in tissue culture and have been shown to result in the synthesis of more than 70 different viral gene products. Latent infections have usually been studied in animal models where a more limited gene expression occurs. The host and viral factors that influence the establishment, maintenance and ultimately reactivation, of HSV-1, have been difficult to determine.

During latency no infectious virus can be recovered although a limited number of viral transcripts have been detected in latently infected

Correspondence: TM Block
Received 9 January 1996; revised 5 February 1996; accepted 13 February 1996

cells (Green *et al*, 1981; Kosz-Vneuchak *et al*, 1993; Kramer and Coen, 1995). Of these, the latency associated transcripts (LATs), are the only viral gene products which accumulate to a high enough degree in neurons to be deteced by Northen blot and *in situ* hybridization analyses (Deatly *et al*, 1987; Stevens *et al*, 1987; Wagner *et al*, 1988). The LATs, encoded in the long internal repeats of the viral genome, are actually a family of transcripts. The major LATs accumulate in latently infected neurons and are largely nuclear and non polyadenylated (reviewed in Fraser *et al*, 1992). The 2.0 kb species of the major LATs contains two open reading frames and has been isolated from the cytoplasm of cultured cells (Spivack and Fraser, 1988), although it is present in the nucleus and hypothesized to be an intron (Farrell *et al*, 1991). The splice to form the 2.0 kb LAT is thought to occur in an 8.5 kb precursor transcript which has been detected in tissue culture (Dobson *et al*, 1989; Devi-Rao *et al*, 1991; Wagner *et al*, 1988; Wechsler *et al*, 1988; Zwaagastra *et al*, 1991). The TATAA box of the putative promoter for both the 2.0 and 8.5 kb transcripts is located near nucleotide 118 773 of the viral genome and comprises part the latency associated promoter (LAP1, see Fraser *et al*, 1992). Viruses lacking this promoter fail to accumulate LAT transcripts in neurons (17ΔPst, Devi-Rao *et al*, 1994; KOS 29, Sawtell and Thompson, 1992), although synthesis of a 2.0 kb transcript in cells acutely infected with KOS 29 has been observed (Nicosia *et al*, 1993). Transcripts mapping both upstream and downstream of the major 2.0 kb LAT locus have also been detected in latently infected cells by *in situ* hybridization and have been called 'minor' LATs (Mitchell *et al*, 1990). The minor LATs are believed to be the 8.5 kb precursor transcript of the 2.0 kb LAT.

Since the high abundance in latently infected cells make the LATs unique among viral transcripts, the possibility that they influence HSV pathogenesis has been extensively investigated. To date, all reported LAT mutants replicate in tissue culture and can establish latent infections in mice, although the efficiency of establishment may vary (Fraser *et al*, 1992). With respect to reactivation from ganglia, explanted from latently infected mice, or via *in vivo* challenge of latently infected rabbits and (in the case of HSV-2) guinea pigs (Krause *et al*, 1995), the mutants fall into at least two general categories: (1) those that reactivate with wild-type kinetics and efficiency and (2) those that reactivate aberrantly. Those that reactivate like wild-type virus have mutations limited to the region downstream of the promoter, such as TB1 (Block *et al*, 1990), 17ΔBstE (Deshmane *et al*, 1994; Izumi *et al*, 1989), 17ΔSty (Maggioncalda *et al*, 1994), RH142 (Ho and Mocarski, 1989). Those that reactivate aberrantly are called 'slow reactivators' and have deletions involving the LAT promoter and sequences upstream (to the left), such as 1704 (Steiner *et al*, 1989; Trousdale *et al*, 1991), 17ΔN/H (Block *et al*, 1993), 17ΔPst (Devi-Rao *et al*, 1994) and dl 1.8 (Leib *et al*, 1989) and in HSV-2, 333pLAT- (Krause *et al*, 1995). The mechanism mediating the slow reactivation phenotype is unknown. Some groups believe the nature of the defect lies in the virus' inability to establish, not reactivate from, latency (Sawtell and Thompson, 1992).

These data raised the possibility that the slow reactivation phenotype is due to mutations upstream of the putative LAT promoter, rather than within the major LAT coding sequences. Recent experiments have shown that the span of DNA upstream of the LAT promoter encodes a pair of transcripts, 1.1 and 1.8 kb in length (Singh and Wagner, 1993). To determine the role of these transcripts and the upstream region in latency, a mutant virus containing a deletion from the SwaI to the NotI site (118 006–118 443), 332 nucleotides upstream of the putative LAT promoter, was constructed and called 17ΔS/N. 17ΔS/N was tested for its ability to grow in tissue culture, produce LAT region transcripts, and ultimately establish and reactivate from latent infections of mice. In productively infected cells, 17ΔS/N grows normally, but fails to produce the overlapping 1.1 and 1.8 kb transcripts encoded by the region upstream of the LAT promoter (Singh and Wagner, 1993); in spite of the fact that most of the region which specifies the 1.1 kb species is retained in the mutant. In addition, 17ΔS/N produces 2.0 kb LAT in tissue culture and the 1.45–1.5 kb spliced LAT in latently infected cells. Ganglia from mice latently infected with either 17ΔS/N or wild-type virus contain similar numbers of neurons harboring viral DNA, as detected by in situ PCR. Finally, 17ΔS/N reactivated from latent infections of mice with kinetics which were indistinguishable from those observed for wild-type virus, 17syn$^+$. These results suggest that the genomic region responsible for the slow reactivation phenotype, demonstrated by other LAT region mutant viruses, is limited to the span of DNA between the NotI and StyI sites (118 443–118 880), which also encompasses the LAT promoter.

Results

Location of the deletion in the 17ΔS/N genome

The LAT region of the HSV-1 genome is shown in Figure 1A. As depicted, the LAT family of transcripts map to the internal repeats of the viral chromosome and are believed to be transcribed from a pol-2 promoter located between the PstI restriction endonuclease sites (Batchelor and O'Hare, 1990; Wechsler *et al*, 1989; Zwaagastra *et al*, 1991). Viruses with mutations within and downstream the putative LAT promoter have been constructed and tested for their ability to establish and reactivate from latent infections in mice and rabbits (Block *et al*, 1990, 1993; Bloom *et al*, 1994;

Deshmane *et al*, 1994; Ho and Mocarski, 1989; Izumi *et al*, 1989; Krause *et al*, 1995; Maggioncalda *et al*, 1994). Since mutants with deletions surrounding the LAT promoter reactivated slowly from the ganglia of latently infected mice, while almost all mutants with deletions downstream reactivated normally, it was of interest to know if the slow reactivation phenotype actually mapped upstream of the putative promoter.

The region between the SwaI and NotI sites (Figure 1) is located just upstream of the putative LAT promoter. HSV-1 strain 17 mutants bearing deletions of this region were constructed by cotransfecting permissive CV-1 cells with infectious viral DNA and plasmids containing a deletion of the SwaI–NotI region, as in experimental methods. Recombinant mutants were generated, and one isolate called 17ΔS/N was studied in most detail. To determine if 17ΔS/N possessed the intended deletion, viral DNA was isolated and digested with SalI and NotI endonucleases, resolved in agarose gels and Southern blotted. The blots were hybridized with probes specific for the deleted region and for a region adjacent to the deletion (pH-

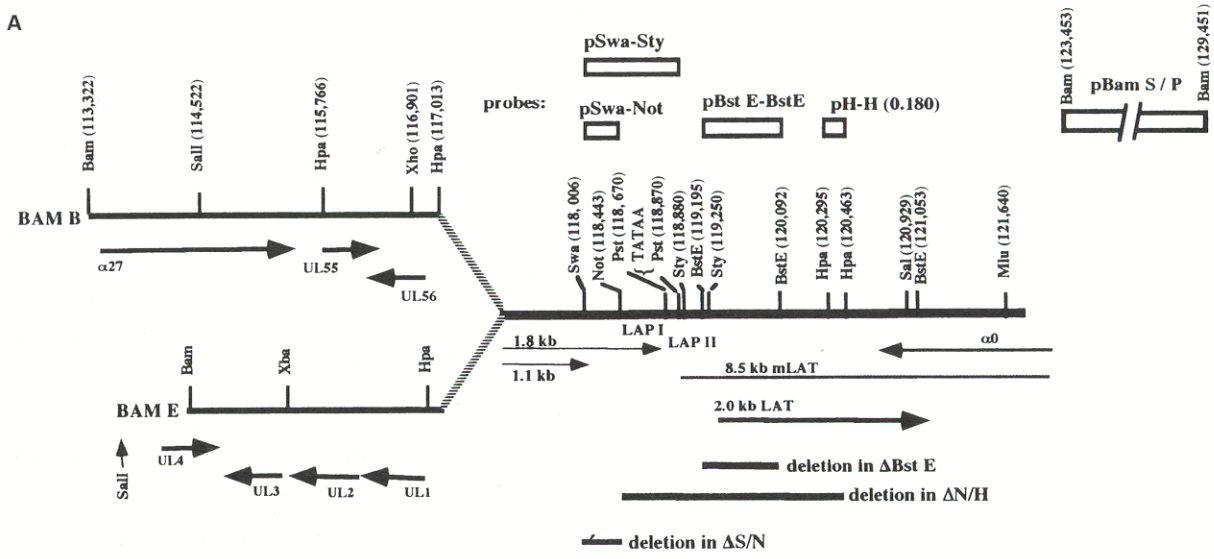

Figure 1 The LAT region of HSV-1 and the deletion in 17ΔS/N. (**A**) Map of deletion mutant 17ΔS/N. The long repeat region of the HSV-1 genome encoding the 2.0 kb major LAT is shown as a bold line which bifurcates into the Bam B and Bam E unique long sequences. The region deleted in 17ΔS/N is shown as a hatched box below the line. The location of other LAT region deletion mutants, 17ΔN/H and 17ΔBst E, is also shown, for comparison. The probe sequences used in this study are shown as open boxes above the map. Restriction endonuclease sites are shown along with the location and direction of the transcripts that map to this region; alpha O, major and minor LATs, and the 1.1–1.8 kb RNA. The SalI site upstream of the UL4 gene in Bam E is depicted off the map since it is beyond the boundary for the Bam E fragment. This SalI site is in UL5. Abbreviations for the restriction endonuclease sites are as follows: Bam (BamHI), Bgl 2 (BglII), BstE (Bst E II), Hpa (HpaI), Mlu (MluI), Not (NotI), Pst (PstI), Sal (SalI), Sty (StyI), Xba (XbaI), Xho (XhoI). The position of the site along the HSV-1 genome is expressed as the nucleotide number along the sequenced HSV-1 genome and is written in parentheses. (**B**) Southern blot of 17ΔS/N DNA. Viral DNA was digested with SalI and NotI, separated through 0.8% agarose gel electrophoresis and blotted on to nylon membranes. These membranes were probed with radioactively labelled pH-H 0.180 (nt. 120 295–120 469). Lane 1, molecular weight markers; Lane 2, wild-type 17syn$^+$ DNA; Lane 3, 17ΔS/N DNA; Lane 4, 17ΔS/N^r DNA. The molecular weight markers from bottom to top are; 2.0, 2.3, 4.3, 6.6, 9.3 and 23.0 kb respectively.

H 0.180, Figure 1B). The pSwa–Not probe hybridized to the expected fragments of wild-type, 17syn$^+$ virus DNA and did not hybridize to any 17ΔS/N sequences (data not shown). The pH-H (0.180) probe hybridized to a single 2.4 kb fragment from wild-type, 17syn$^+$ virus digests (Figure 1B, lane 2). This fragment corresponds to the 2.4 kb span of DNA in the long repeats, internal and terminal, from the SalI site to the NotI site (see Figure 1A). A similar digestion of 17ΔS/N DNA resulted in the appearance of two fragments of 6.0 and 9.0 kb. These two fragments represent SalI digestion products alone, since the NotI site in 17ΔS/N was deleted. The loss of the NotI site (Figure 1B) and the lack of hybridization of the pSwa–NotI probe (data not shown) verified the correct structure of 17ΔS/N. A rescuant virus, with a repaired deletion, was created using the mutant's genomic DNA (see methods). The SalI–NotI digestion product of the rescuant, called 17ΔS/N^r, is also present in Figure 1B (lane 4). The digestion results from 17ΔS/N^r and the wild-type parent, 17syn$^+$, are indistinguishable, suggesting that the deletion in 17ΔS/N had been properly repaired.

17ΔS/N fails to produce detectable 1.1 and 1.8 kb LAT upstream transcripts, in tissue culture

At least two, low abundance, polyadenylated transcripts with the same polarity as the major LATs, have been mapped to the HSV genomic region immediately upstream of the LAT promoter (Singh and Wagner, 1993, and see Figure 1). These transcripts, of size 1.1 and 1.8 kb, have been detected during late times of lytic infections. Since, in 17ΔS/N, the poly A processing signal of the 1.1 kb transcript is disrupted along with the 3′ end of the 1.8 kb transcript, it was of interest to know if they were produced in selected mutants lacking upstream sequences. Therefore, total cellular RNA was isolated from CV-1 cells infected with either wild-type strain 17syn$^+$, 17ΔS/N, 17ΔS/N^r (Figure 2A and B), or 17syn$^+$, 17ΔN/H, and 17ΔN/H^r (Figure 2C and D), resolved in agarose gels and Northern blotted, as per Methods. 17ΔN/H (Block *et al*, 1993) contains a deletion that begins downstream of the 3′ end of the 1.1 kb transcript and was expected to be capable of synthesizing this transcript. Blots were hybridized to radioactively labeled probes pSwa–Sty 1 (Figure 2A) or pSwa–NotI (Figure 2C). As shown in both blots (bold arrows) a 1.1 kb transcript is detected in RNA derived from cells infected with wild-type 17syn$^+$ and the mutants' rescuants. The 1.8 kb transcript, however, was difficult to detect. The stippled arrows (Figure 2A and C) identify a heterogeneous migrating set of bands. Based upon molecular weight and hybridization to the pSwa–Sty and pSwa–Not probes these are assumed to correspond to the 1.8 kb transcripts observed by Singh and Wagner (1993). The low abundance and heterogeneity of this set of transcripts was consistent following repeated isolation efforts. The low abundance of this upstream RNA in tissue culture and the differences in the method of RNA isolation (total RNA was used here as opposed to poly(A)$^+$ RNA used in Singh and Wagner [1993])- may explain why the 1.8 kb transcript is more difficult for us to detect. However, it is clear that neither

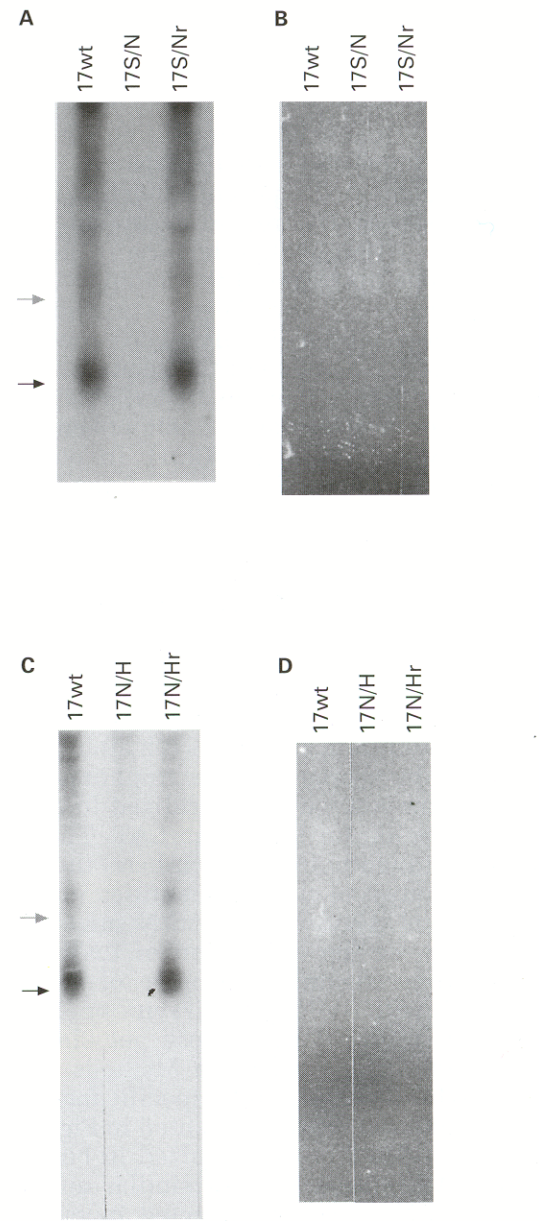

Figure 2 Analysis of the LAT upstream region 1.1 and 1.8 kb transcripts. Total cellular RNA was harvested 8 h post infection with the indicated viruses, separated electrophoretically through 1.2% agarose gels, blotted to nylon membranes and hybridized to double stranded DNA probes pSwa–Sty (A) or pSwa–Not (C) (See Figure 1). (**A**) Northern blot of RNA. (**B**) ethidium bromide stained RNA shown as a loading control for **A**. (**C**) Northern blot of 17syn$^+$, 17ΔN/H, and 17ΔN/H^r total cellular RNA. (**D**) ethidium bromide stained RNA shown as a loading control for **C**. Solid arrows in **A** and **C** indicate 1.1 kb transcript, hatched arrows the 1.8 kb transcript.

17ΔS/N (Figure 2A, lane 2) nor 17ΔN/H (Figure 2C, lane 2) induced detectable levels of either transcript. Photographs of the ethidium bromide stained gels prior to Northern transfer are included as loading controls (Figure 2B and D). Since evaluation of the nucleotide sequence of the 3′ end of the 1.1 kb species suggests a termini is well before the deletion in 17ΔN/H, it was surprising that this transcript was not detected in cells infected with 17ΔN/H. To account for these findings the possibility of a precursor–product relationship between the 1.8 and 1.1 kb transcripts is considered in the discussion.

Major LAT is produced in cells either productively or latently infected with 17ΔS/N

17ΔS/N is the first report of a deletion mutant made exclusively in the region upstream of the LAT promoter. Although the deletion is more than 300 nucleotides upstream of the putative LAT TATAA site, there could have been an impact upon LAT production. Studies involving the promoter of the 2.0 kb LAT have shown that neuronal expression control elements lie upstream of the promoter (Batchelor and O'Hare, 1990; Lokensgard *et al*, 1994; Zwaagastra *et al*, 1991). In particular, the region between the SwaI site and the LAT TATAA was reported to be important for the expression of the LATs during lytic infection of neuroblastoma cells (Morrow and Rixon, 1994). Since the deletion in 17ΔS/N extends into promoter control elements, its ability to produce LATs in cells productively infected in tissue culture, and in neurons latently infected in mice, was determined.

For analysis of tissue culture production, RNA was isolated from CV-1 cells 8 h after infection and resolved by agarose gel electrophoresis. Northern blots of the RNA were hybridized to a radioactive probe specific for the 2.0 kb LAT. The results are shown in Figure 3A. As seen here, the 2.0 kb LAT was produced to similar quantities in either wild-type 17syn$^+$, 17ΔS/N, or 17ΔS/N^r infected cells.

To test for the expression of LATs in latency, RNA from trigeminal ganglia of mice latently infected with wild-type 17syn$^+$ or 17ΔS/N was isolated, resolved by agarose gel electrophoresis and Northern blotted as in Methods. These blots were hybridized to a probe specific for the LATs, as before, and the results presented in Figure 3B show that the LATs were synthesized in both wild-type and 17ΔS/N infections in similar amounts. LAT specific RNA was not detected in mock infected tissues. The low intensity of the hybridization signals seen here can be attributed to the small number of ganglia used to harvest the latent RNA and not a reduction in LAT expression. Furthermore, this blot was developed on film for 7 days which accounts for build up in the nonspecific background seen. These data show that the mutant 17ΔS/N is capable of expressing the major LATs characteristic of productive and latent infections.

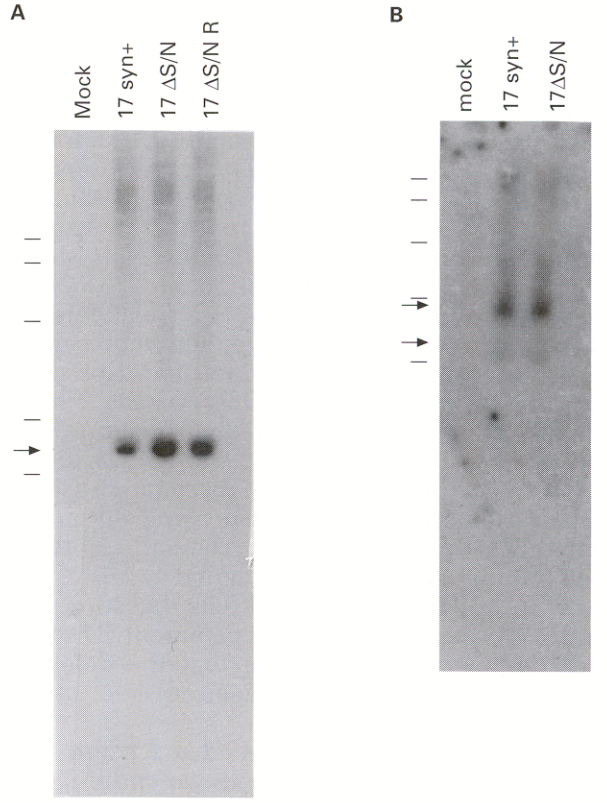

Figure 3 Analysis of latent and productive LAT transcripts. Northern blot analysis of major LAT production in lytically infected cells (**A**) and in latently infected ganglia (**B**). RNA was resolved by agarose gel electrophoresis, Northern blotted, and hybridized to probe pH-H 0.180 (See Figure 1). (**A**) RNA is from CV-1 cells harvested 8 h post infection with an MOI of 10.0. Lane 1, molecular weight markers (bottom to top, 1.3, 2.3, 4.4, 7.4, 9.4); lane 2, mock infected; lane 3, wild-type 17syn$^+$; lane 4, 17ΔS/N; lane 5, 17ΔS/N^r. (**B**) RNA, isolated from four, separate, trigeminal ganglia for each virus, was treated as above. Lane 1, molecular weight markers (see above); lane 2, mock infected ganglia; lane 3, wild-type 17syn$^+$; lane 4, 17ΔS/N. The blot was developed for 5 days. Arrows indicate the 2.0 kb LAT in (**A**) and the 1.45–2.0 kb LATs in (**B**).

Distribution of LAT expressing cells in trigeminal ganglia from mice infected with 17ΔS/N

Although Northern blot analysis of RNA derived from latently infected mice suggested that 17ΔS/N produced near normal levels of major 1.5–2.0 kb LAT, the distribution of these transcripts within the ganglia was not determined. That is, compared with the wild-type virus, the LATs detected by Northern analysis of 17ΔS/N latently infected ganglia could have been accumulating in far fewer cells. Therefore, the distribution of LAT signal within the trigeminal ganglia was determined by *in situ* hybridization experiments. Trigeminal ganglia from mice latently infected with wildtype 17syn$^+$, 17ΔS/N and 17ΔS/N^r were removed, sectioned and hybridized to LAT specific probe as in (Methods). The results are shown in Figure 4. LATs were detected as dense hybridization signals over ap-

proximately 1–2% of the neurons prepared from mice latently infected with wild-type strain 17 or 17ΔS/N (Table 1). 17ΔS/N^r also behaved as wild-type.

A similar pattern was observed in sections tested for the expression of mLAT. The mLAT is thought to extend 8.5 kb downstream of the LAT promoter through a polyadenylation addition signal (Zwaagastra *et al*, 1991). Using a probe specific for the mLAT (pBam S/P, see Methods), *in situ* hybridization of tissue derived from mice latently infected with either in 17syn$^+$, 17ΔS/N or 17ΔS/N^r revealed that an average of 1–2% of all the neurons counted were found to be positive (Figure 5, see Table 1). Again, a dense hybridization signal over a neuron was recorded as a positive.

17ΔS/N DNA is in as many neurons as wild type virus in latently infected mice

The neurons detected by *in situ* hybridization for major and minor LAT are apparently only a minority of the total number of HSV infected cells (Mehta *et al*, 1995; Slobedman *et al*, 1994). Therefore, although 17ΔS/N and wild-type virus induced

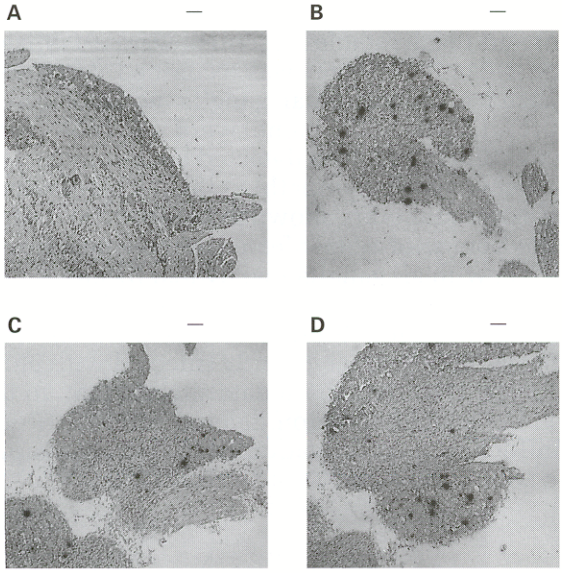

Figure 4 Major LAT expressing neurons in latently infected ganglia detected by *in situ* hybridization. Trigeminal ganglia were hybridized, *in situ*, to radioactively labeled probe pBst E-Bst E (see Figure 1). The ganglia were derived from mice mock infected (**A**), or latently infected with wild-type 17syn$^+$ (**B**), 17ΔS/N (**C**), 17ΔS/N^r (**D**). Hybridization signals can be seen over positive neurons as dark spots. The solid bar indicates a size of 50 μm.

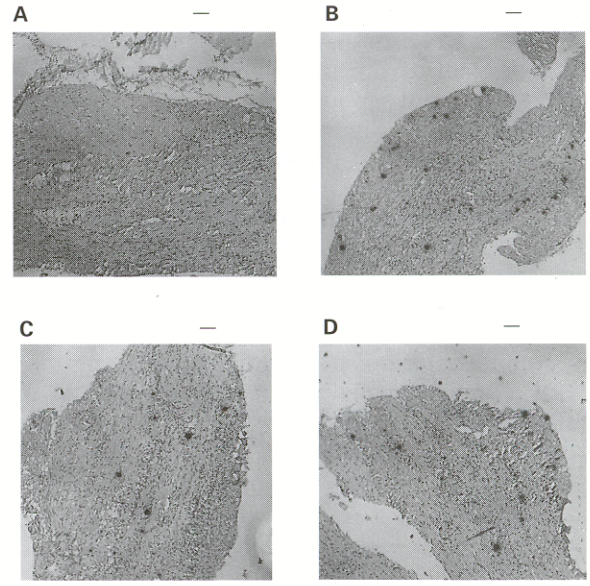

Figure 5 Minor LAT expressing neurons in latently infected ganglia detected by *in situ* hybridization. Trigeminal ganglia were hybridized, *in situ*, to radioactively labelled probe pBam S/P (19). The ganglia were derived from mice mock infected (**A**), or latently infected with wild-type 17syn$^+$ (**B**), 17ΔS/N (**C**), or 17ΔS/N^r (**D**) virus.

Table 1 Number of neurons positive for HSV-1 DNA and major and minor LATs in trigeminal ganglia from latently infected mice[a]

Virus[b]	*No. of neurons counted*[c]	*No. of LAT positive*[d] *neurons (%)*[g]	*No. of mLAT positive*[e] *neurons (%)*	*No. of DNA positive*[f] *neurons (%)*
17 wild-type	>10,000	180 (1.8%)	165 (1.6%)	530 (5.7%)
17 ΔS/N	>10,000	200 (2.0%)	178 (1.7%)	400 (4.0%)
17 ΔS/N R	>5,000	80 (1.6%)	102 (2.0%)	185 (3.7%)

[a]Four trigeminal ganglia were analyzed per virus, each ganglia was derived from a separate mouse.
[b]Mice were infected via corneal scarification with 17 syn$^+$, 17 ΔS/N, 17 ΔS/N^r (see Methods).
[c]Neurons were counted as described in Smoulden *et al* (1983) and Mehta *et al* (1995).
[d]At 28 days post infection trigeminal ganglia were excised and sectioned for *in situ* hybridization to the 2.0 kb LAT using the pBst E-Bst E probe (see Figure 1).
[e]Alternate sections were hybridized to the pBam S/P probe for mLAT hybridization *in situ*.
[f]Trigeminal ganglia analyzed by *in situ* PCR were prepared as in *in situ* hybridization. The PCR primers used were as in Methods. The probe used was specific for the PCR product amplified from the Pol gene of HSV-1.
[g]Number of positive neurons divided by total number of neurons × 100

a similar amount and distribution of LATs, it was still possible that the mutation in 17ΔS/N resulted in a virus which persisted in fewer neurons. Without any detectable marker expressed in the majority of the infected cells, the exact number of latent, LAT-null-expressing neurons is impossible to determine.

The presence of HSV-1 strain 17ΔS/N DNA in latently infected neurons was therefore determined by detection of viral DNA by *in situ* PCR. Briefly, trigeminal ganglia from latently infected mice (inoculated as before) were removed and sectioned as in Methods. Slices of tissue were prepared and incubated with HSV primers in a PCR reaction, followed by hybridization to biotinylated probes as in Methods. Representative results are shown in Figure 6 and are summarized in Table 1. Figure 6A is a negative control in which one set of uninfected tissue was incubated with HSV-1 pol specific primers. There is no detectable signal. Figure 6B and C show examples of positive neurons present in latently infected tissue from wild-type (B), and 17ΔS/N (C) infected ganglia; the color difference between B and C is due to differences in film processing. For each virus, more than 9000 neurons were counted, from four different trigeminal ganglia, derived from four different mice (Table 1). Although there was some variation from ganglia to ganglia, the overall conclusion is clear: viral DNA in mice latently infected with 17ΔS/N or wild-type virus, exists in similar numbers of neurons.

17ΔS/N reactivates from latent infection with normal kinetics

Since downstream mutants such as TB1 (Block *et al*, 1990), and RH142 (Ho and Mocarski, 1989) reactivated normally, in the explant cocultivation assay, yet upstream mutants such as 17ΔN/H (Block *et al*, 1993), dl 1.8 (Leib *et al*, 1989), and 17ΔPst (Devi-Rao *et al*, 1994) reactivated aberrantly, it was important to determine how 17ΔS/N would behave in order to map the LAT region sequences involved in the slow reactivation kinetics.

Balb/c mice were infected via the eye, with wild type strain 17, 17ΔS/N or 17ΔS/N^r, as usual (Methods). Thirty days after inoculation, at a time when infectious virus is not detectable in eye washing or tissue homogenates, the possibility that latent and reactivatable infections were established in the trigeminal nerves was examined. Mice were sacrificed and trigeminal ganglia were placed in culture dishes with monolayers of CV-1 cells. Reactivation of HSV-1 is detected by the appearance of a characteristic cytopathic effect. The results of these experiments are shown in Figure 7. 50% of the ganglia from wild-type infected animals reactivated by day 5, with all ganglia reactivated by day 7. This wild-type profile of reactivation is highly reproducible in our hands. Similar results were observed with ganglia from mice latently infected with either 17ΔS/N or 17ΔS/N^r. These data suggest that, in this assay, the gemomic region deleted in 17ΔS/N does not influence the reactivation rate of herpes simplex virus type I.

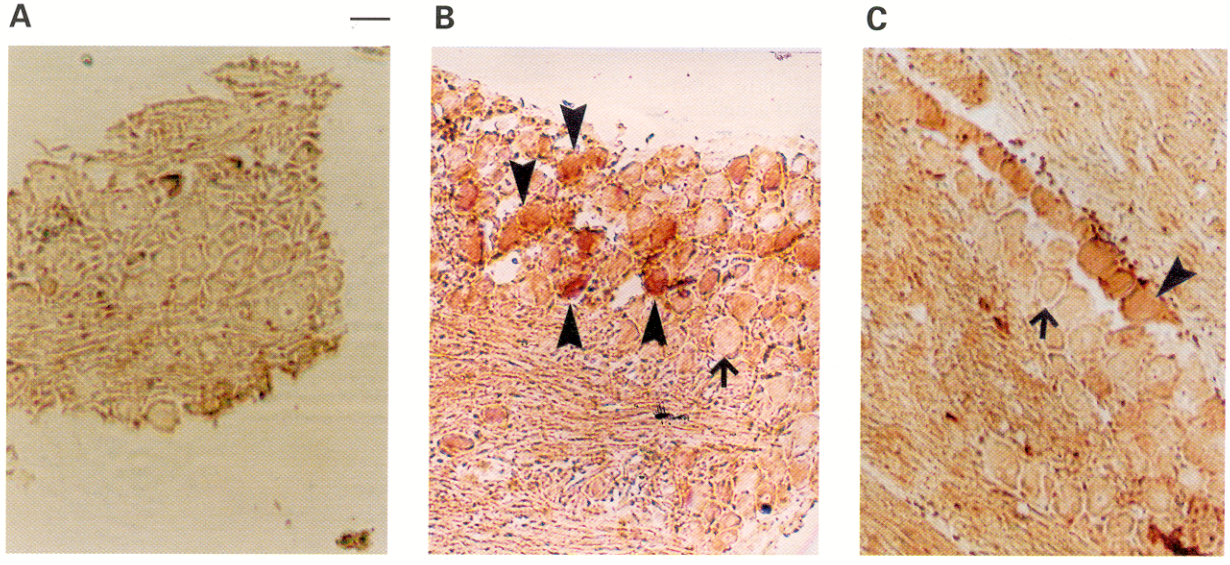

Figure 6 HSV-1 DNA containing neurons in latently infected ganglia detected by *in situ* PCR. Trigeminal ganglia uninfected (**A**), or infected with wild-type 17syn$^+$ (**B**), or 17ΔS/N (**C**), were sectioned and analyzed by *in situ* PCR (see Methods). (**A**) uninfected tissue incubated with HSV pol primers specific for the HSV-1 pol gene (UL 30). (**B**) 17syn$^+$ infected tissue incubated with primers specific for the HSV-1 pol gene. (**C**) 17ΔS/N infected tissue also incubated with primers specific for the HSV-1 pol gene. All the tissues were hybridized to a 5′ biotin labelled probe specific for the HSV pol PCR product (see Methods). Positive neurons are red to brown in color and indicated by the arrow heads, negative neurons are indicated by arrows. Black bar indicates 50 μm.

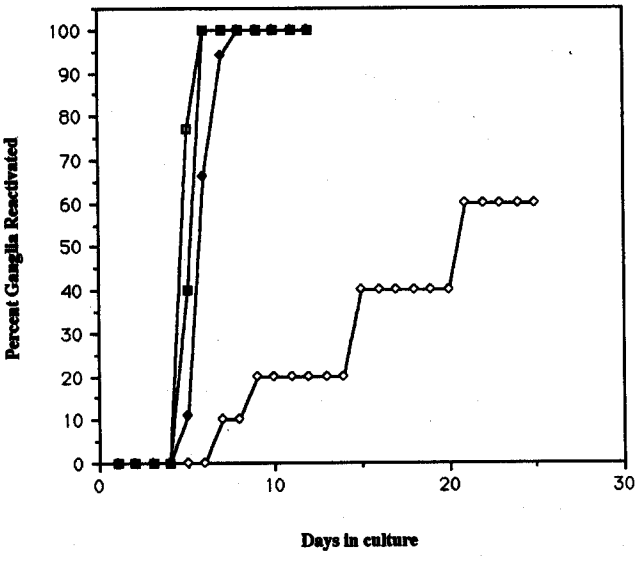

Figure 7 Trigeminal ganglia explant reactivation kinetics of 17ΔS/N. The ganglia were explanted from mice latently infected with either wild-type 17syn$^+$ (open squares), 17ΔS/N (closed diamonds), or 17ΔS/N^r (closed squares), and placed in culture with CV-1 cells. The curve of a known slow reactivator, 17ΔN/H (open diamonds) (3), has been added as a control for slow reactivation kinetics. The x asis shows the time in culture post explant. The y axis shows the percentage of the total number of ganglia that reactivated virus. The number of ganglia used were 10 for 17syn$^+$, 16 for 17ΔS/N, and 7 for 17ΔS/N^r. The assay is described in Methods.

Discussion

The host and viral mechanisms involved in regulating HSV latency and reactivation are central to an understanding of viral pathogenesis. Since the LATs are the only viral gene products detected during latent infection of animal and human peripheral nerves, they have been the object of considerable attention. Results involving LAT region mutants have suggested that the LATs may play a role in reactivation (see Fraser *et al*, 1992). However, there has been some confusion concerning this role, as measured by *in vitro* and *in vivo* reactivation assays. It has been noted that the behavior of the same mutants varies with the animal host used (Hill *et al*, 1990). Also, the nature of certain assays and the differences between the techniques used to perform them can sometimes create discrepancies in the interpretation of reactivation kinetics for a particular mutant (Deshmane *et al*, 1993; Sawtell and Thompson, 1992). Therefore, when comparing the reactivation rates of different mutants it is important to consider the animal host studied, the reactivation assay used and the strain background of the mutants. Overall, however, an emerging pattern has suggested that those mutants which reactivate aberrantly in the explant coculti-vation assay possess lesions both downstream and upstream of the LAT promoter, while those that reactivate normally possess lesions restricted to the downstream region including the 2.0 kb LAT coding sequences. This raised the possibility that the region upstream, and including the LAT promoter, alone, was responsible for the slow reactivation phenotype observed in mice.

It is noted that one downstream mutant, KOS 62, does not conform to this model. KOS 62 possesses a mutation downstream of the LAT promoter and is considered to reactivate aberrantly (Sawtell and Thompson, 1992). However, while the explant reactivation of this mutant is somewhat slower than wild-type KOS, the kinetic difference is subtle compared to that observed for 17ΔPst (Dev-Rao *et al*, 1994) and 17ΔN/H (Block *et al*, 1993) reactivations. The mutation in KOS 62 may actually have more of an impact upon the maintenance of latency rather than reactivation, since fewer neurons appear to be infected with this mutant compared to wild-type virus (Sawtell and Thompson, 1992).

This report therefore, provides important information about the fine mapping of the HSV sequences responsible for mediating the slow reactivation phenotype. To date no deletion mutant has been synthesized exclusively in the region immediately upstream of the LAT promoter. Moreover, the genomic regions necessary for the synthesis and accumulation of a pair of lytic-phase transcripts which map upstream of the LAT promoter was also investigated. The mutant 17ΔS/N has a deletion between the SwaI (118 006) and NotI (118 443) sites of the HSV genome. 17ΔS/N reactivated from explants of the ganglia derived from latently infected mice with normal kinetics. This was surprising since, given the behavior of downstream mutants, it seemed likely that the slow reactivation phenotype would involve genomic sequences upstream of the major LAT promoter. Nevertheless, it is concluded that the viral sequences involved in the slow reactivation rate observed in mice, are confined to the region surrounding the LAT promoter.

The processes controlling explant reactivation and major LAT production were shown to be unrelated since downstream, LAT negative mutants, reactivated normally in the mouse model system (Block *et al*, 1990; Ho and Mocarski, 1989). Attention, therefore, turned to the 1.1 and 1.8 kb transcripts which map immediately upstream of the LAT promoter. The fact that 17ΔN/H (Block *et al*, 1993), a slow reactivator, failed to produce the 1.1 and 1.8 kb transcripts in tissue culture raised the possibility that these RNAs were responsible for controlling the reactivation process. However, since 17ΔS/N produces normal amounts of major and minor LAT, reactivates normally, and fails to accumulate any detectable 1.1 and 1.8 kb transcripts, the dispensability of these upstream RNAs for efficient reactivation is demonstrated. The function, if any, of these upstream transcripts,

which contain small open reading frames, remains a mystery (Singh and Wagner, 1994). In the experiments conducted by others, the 3′ end of both the 1.1 and 1.8 kb RNAs was mapped to separate sites (Singh and Wagner, 1994). However, this work did not clearly define the transcripts as being independent of one another. It is also not clear why 17ΔN/H is unable to produce the 1.1 kb RNA, since all of the coding information necessary for its transcription is retained by the virus. Perhaps the 1.1 kb transcript is processed from the 1.8 kb RNA. In that case, the deletion in 17ΔN/H and 17ΔS/N, at the 3′ end of the 1.8 kb RNA, would adversely affect the synthesis of both transcripts. Clearly, further experimentation on these transcripts will be required to fully characterize their synthesis and role in the HSV-1 infectious cycle.

By defining the reactivation kinetics of mutants upstream and downstream of the LAT promoter, a picture has now emerged which suggests that the only LAT region which contributes to the slow reactivation rate in mice, observed by the *in vitro* explant reactivation assay, is between the NotI and StyI sites (see Figure 1A). This is a region which also contains the LAT promoter. Uncertainty regarding the mechanism by which this region exerts its control remains, since neither the major LAT transcripts nor the transcripts immediately upstream of the promoter are necessary. This span of DNA has recently been found to be part of a region with unusual enhancer-like activity in neuronal cells (Lokensgard *et al*, 1994). Perhaps expression of the 8.5 kb minor LAT from the promoter contained within this region is important for regulating reactivation. It has been noted that 17ΔN/H, a slow reactivator, does not synthesize major or minor LATs, while a downstream, normal reactivating, major LAT negative mutant (17ΔBst E) is still capable of minor LAT expression (Deshmane *et al*, 1994). However, if the minor LAT is important for regulating reactivation, then a large part of the 5′ portion of this transcript is unrelated to its activity since it is also deleted in the downstream mutants 17ΔSty (Maggioncalda *et al*, 1994) and 17ΔBst E (Deshmane *et al*, 1994). Further mutation of the minor LAT will be required to define the importance of this RNA. Also, whether or not the role of the NotI–StyI region in controlling reactivation rate is related to the LAT promoter contained therein, or a structural element, remains to be seen.

Materials and methods

Cells and medium

CV-1 cells were from American Type Culture Collection (Rockville, Md). The cells were maintained at 37°C and 5% CO_2 as monolayers in Eagle medium (MEM; GIBCO, Inc., Bethesda, MD), supplemented with 5% newborn calf serum and 0.35% glucose.

Viruses and plasmids

HSV-1 strain 17syn$^+$ (Spivak *et al*, 1988) was the parent of all viruses used in this study. The plasmid pXhoΔSwa–NotI (pΔSwa–NotI) is a pGEM 7z$^+$ vector plasmid containing the Xho–Xho fragment (116 961–123 017) of the genomic clone fragment BamHI 'e' harboring a deletion between the SwaI site (118 001) and NotIsite (118 443). The viral mutant 17ΔS/N was constructed by cotransfecting wild-type strain 17syn$^+$ genomic and pSwa–Not plasmid DNA into CV-1 cells by the calcium phosphate transfection method, described elsewhere (Block *et al*, 1993). Recombinant viral progeny containing the proper deletion were purified through sequential Southern blot experiments. One such recombinant was chosen for further study and called 17ΔS/N. The rescuant of 17ΔS/N was synthesized using similar transfection techniques, but the mutant genomic DNA was cotransfected with pXho–Xho; which contains the SwaI to NotI region as well as flanking sequences. Recombinants were scored for the reversion of the deletion in 17ΔS/N resulting in a virus which is genetically similar to wildtype. The genomic structure of the rescuant, called 17ΔS/N^r, was verified by Southern blot experiments. Other plasmids used in this study are shown in Figure 1 and were as follows; pH-H 0.180 (120 295–120 469), pSwa–NotI (118 001–118 443), pSwa-Sty (118 001–118 880), pBam S/P (123 464–129 415). All plasmids used were purified either by CsCl or the Wizard miniprep kit (Promega/Fisher Inc). Bacterial cultures were maintained as in (Maniatas *et al*, 1982).

Southern blot anlaysis

Virion DNA was purified using a method which is a modified version of that described elsewhere (Block *et al*, 1990) and digested fully with the desired restriction endonucleases as per manufacture's protocol. Digest products were resolved by agarose gel electrophoresis through 0.8% gels, denatured, neutralized, and transferred to nylon membranes (Southern method) as described elsewhere (Maniatas *et al*, 1982). The membranes were hybridized with double stranded DNA probes labeled with ^{32}P dCTP by the random priming technique (GIBCO BRL, Bethesda, MD). Incubation times and washes were described elsewhere (Maniatas *et al*, 1982).

Northern analysis of RNA

RNA was isolated from infected cells or ganglia as described previously (Spivak and Fraser, 1988) with one exception, for the latent major LAT RNA, five ganglia were harvested per virus instead of 10.

Infection of mice and explant reactivation assay

Four to six week old female BALB/C mice (Jackson Laboratory, Bar Harbor, Maine) were infected by corneal scarification with between 10^5 and 10^6 plaque forming viral units. Four weeks postinfec-

tion the mice were sacrificed and their trigeminal ganglia excised for either embedding and sectioning or whole explant on to CV-1 cells as in (Spivak and Fraser, 1988). The monolayers were checked daily for the appearance of a cytopathic effect. The ganglia were transferred every 5 days to a fresh monolayer.

In situ *RNA hybridization*
Latently infected trigeminal ganglia were excised and placed immediately in cold PBS. The ganglia were then placed into paraffin wax and sliced to 5 μm sections with a microtome cutter. These sections were hybridized to radioactive dsDNA probes labeled with ^{35}S dCTP which bound to the major LAT family and the minor LAT. The details of this protocol appear elsewhere (Mitchell *et al*, 1990).

In situ *PCR for genomic DNA*
The *in situ* PCR DNA amplification of HSV-1 sequences was carried out using the method of Mehta *et al* (1995).

Acknowledgements

We would like to thank Elsa Aglow for her technical assistance in preparing the tissue samples for sectioning. This work was supported by an award from the NIH.

References

Batchelor AH, O'Hare P (1990). Regulation and cell-type-specificity activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. *J Virol* **64:** 3269–3279.

Block TM, Spivack JG, Steiner I, Deshmane S, McIntosh MT, Lirette RP, Fraser NW (1990). A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection. *J Virol* **64:** 3417–3426.

Block TM, Masonis J, Maggioncalda J, Deshmane S, Fraser NW (1993). A herpes simplex virus LAT minus mutant that makes small plaques on confluent CV-1 cells. *Virology* **192:** 618–630.

Bloom DC, Devi-Rao GB, Hill JM, Stevens JG, Wagner EK (1994). Molecular analysis of herpes simplex virus type 1 during epinephrine induced reactivation of latently infected rabbits in vivo. *J Virol* **68:** 1283–1292.

Deatly AM, Spivack JG, Lavi E, Fraser NW (1987). RNA from an immediate early region of the HSV-1 genome is present in the trigeminal ganglia of latently infected mice. *Proc Natl Acad Sci USA* **84:** 3204–3208.

Deshmane SL, Nicosia M, Valyi-Nagy T, Feldman LT, Dilner A, Fraser NW (1993). An HSV-1 mutant lacking the LAT TATA element reactivates normally in explant cocultivation. *Virology* **196:** 868–972.

Deshmane SL, Valyi-Nagy T, Block T, Maggioncalda J, Wolf JH, Fraser NW (1994). An HSV-1 mutant containing the rat b-glucouronidase cDNA inserted within the LAT gene is less efficient than the parental strain at establishing a transcriptionally active state during latency in neurons. *Gene Therapy* **2:** 209–217.

Devi-Rao GB, Goodart SA, Hect LM, Rochford R, Rice MK, Wagner EK (1991). Relationship between polyadenylated and nonpolyadenylated herpes simplex virus type 1 latency-associated transcripts. *J Virol* **65(5):** 2179–2190.

Devi-Rao GB, Bloom DC, Stevens JG, Wagner EK (1994). Herpes simplex virus type DNA replication and gene expression during explant induced reactivation of latently infected murine sensory ganglia. *J Virol* **68:** 1271–1282.

Dobson AT, Sederati F, Devi-Rao G, Flanagan WM, Farrell MJ, Stevens JG, Wagner EK, Feldman LT (1989). Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. *J Virol* **63 (9):** 3844–3851.

Farrell MJ, Dobson AT, Feldman LT (1991). Herpes simplex virus latency-associated transcript in is a stable intron. *Proc Natl Acad Sci USA* **88:** 790–794.

Fraser NW, Block TM, Spivack JG (1992). The latency associated transcripts of herpes simplex virus; RNA in search of function. *Virol* **191:** 1–8.

Green MT, Courtney RJ, Dunkel EC (1981). Detection of an immediate early herpes simplex virus type 1 polypeptide in trigeminal ganglia from latently infected animals. *Infect and Imm* **34:** 987–992.

Ho DY, Mocarski ES (1989). Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. *Proc Natl Acad Sci USA* **86:** 7596–7600.

Hill JM, Sederati F, Javier RT, Wagner EK, Stevens JG (1990). Herpes simplex virus latent phase transcript facilitates in vivo reactivation. *Virology* **174:** 117–125.

Izumi KM, McKelvey A, Devi-Rao GB, Wagner EK, Stevens JG (1989). Molecular and biological characterizations of a type 1 herpes simplex virus (HSV-1) specifically deleted for expression of the latency associated transcript (LAT). *Microb Pathogen* **7:** 121–134.

Kosz-Vnenchak Magdalena, Jacobsen J, Coen DM, Knipe DM (1993). Evidence for a novel regulatory pathway for herpes simplex virus gene expression in trigeminal ganglion neurons. *J Virol* **67:** 5383–5393.

Kramer MF, Coen DM (1995). Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. *J Virol* **69:** 1389–1399.

Krause PR, Stanberry LR, Bourne N, Connelly B, Kurawadwala J, Patel A, Straus SE (1995). Expression of the herpes simplex virus type 2 latency associated transcript enhances spontaneous reactivation of genital herpes in latently infected guinea pigs. *J Exp Med* **181:** 297–306.

Leib DA, Bogard CL, Kosz-Vnenchak M, Hicks KA, Coen DM, Knipe DM, Schaffer PA (1989). A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent infection with reduced frequency. *J Virol* **63:** 2893–2900.

Lokensgard JR, Bloom, D, Dobson AT, Feldman L (1994). Long term promoter activity during herpes simplex virus latency. *J Virol* **68:** 7148–7158.

Maggioncalda J, Mehta A, Valyi-Nagy T, Fraser NW, Block TM (1994). Analysis of a herpes simplex virus type 1 LAT mutant with a deletion between the putative promoter and 5′ End of the 2.0 kilobase transcript. *J Virol* **68:** 7816–7824.

Maniatas TE, Fritsch EF, Sambrook J (1982). Molecular cloning: a laboratory manual. Cold Spings Harbor Laboratory, Cold Springs Harbor, NY.

Mehta A, Maggioncalda J, Bagasra O, Thikkavarapu S, Saikumari P, Valyi-Nagy T, Fraser NW, Block TM (1995). In situ DNA PCR and RNA hybridization detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice. *Virol* **206:** 633–640.

Mitchell WJ, Lirette RP, Fraser NW (1990). Mapping of low abundance latency associated RNA in the trigeminal ganglia of mice latently infected with herpes simplex virus type 1. *J Gen Virol* **71:** 125–132.

Morrow JA, Rixon FJ (1994). Analysis of sequences important for herpes simplex virus type 1 latency-associated transcript promoter activity during lytic infection of tissue culture cells. *J Gen Virol* **75:** 309–316.

Nicosia M, Deshmane SL, Zablotny JM, Valyi-Nagy T, Fraser NW (1993). Herpes simplex virus type 1 latency-associated transcript promoter deletion mutants can express a 2.0 kilobase transcript mapping to the LAT region. *J Virol* **67:** 7276–7283.

Sawtell N, Thompson RL (1992). Herpes simplex virus latency associated transcription unit promotes anatomical site dependent establishment and reactivation from latency. *J Virol* **66:** 2157–2169.

Singh J, Wagner EK (1993). Transcriptional analysis of the herpes simplex virus type 1 region containing the TRL/UL junction. *Virology* **196:** 220–231.

Slobedman B, Efstathiou S, Simmons A (1994). Quantitative analysis of herpes simplex virus DNA and transcriptional activity in ganglia of mice latently infected with wild-type and thymidine kinase-deficient viral strains. *J of Gen Virol* **75(9):** 2469–2474.

Smoulden A, Wright L, Cunningham T (1983). Neuron numbers in the superior cervical ganglia of the rat: critical comparison of methods for cell counting. *J Neurocytology* **12:** 739–750.

Spivack TG, Fraser NW (1988). Expression of herpes simplex type 1 latency-associated transcript in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. *J Virol* **62:** 1479–1485.

Steiner IJ, Spivack G, Lirette RP, Brown SM, MacLean AR, Subak-Sharpe J, Fraser NW (1989). Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. *EMBO J* **8:** 505–511.

Stevens JG, Cook ML (1971). Latent herpes simplex virus in spinal ganglia of mice. *Science* **173:** 843–845.

Stevens JG, Wagner EK, Devi-Rao GB, Cook ML, Feldman LT (1987). RNA complementary to a herpes virus gene mRNA is prominent in latently infected neurons. *Science* **235:** 1056–1059.

Straus S (1993). Herpes simplex virus and its relatives. In: *Mechanisms of Microbial Disease.* (Schaecter MG, Medoff B, Eisenstein, eds). Williams and Wilkins, Publ., Balt, Md).

Trousdale M, Steiner I, Spivack JG, Deshmane S, Brown S, MacLean A, Subak-Sharpe J, Fraser NW (1991). Evidence that the herpes simplex virus type 1 latency associated transcripts play a role in reactivation of latent infection in vivo. *J Virol* **65:** 6989–6993.

Wagner EK,. Devi-Rao GB, Feldman LT, Dobson AT, Zhang YF, Hill JM, Flanagan WM, Stevens JG (1988). Physical characterization of the herpes simplex virus latency-associated transcript in neurons. *J Virol* **62:** 1194–1202.

Wechsler SL, Nesburn AB, Zwaastra J, Ghiasi H (1989). Sequence of the latency related gene of herpes simplex virus type 1. *Virol* **168:** 168–172.

Wechsler SL, Nesburn AB, Watson R, Slanina SM, Ghiasi H (1988). Fine mapping of the latency-related gene of herpes simplex virus type 1: alternate splicing produces distinct latency-related RNAs containing open reading frames. *Virol* **62:** 4051–4058.

Zwaagstra JC, Ghiasi H, Slanina SM, Nesburn AB, Wheatley SC, Lillycrop K, Wood J, Latchman DS, Patel K, Wechsler SL (1991). Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron derived cells: evidence for neuron specificity and for a large LAT transcript. *J Virol* **64:** 5019–5028.