JOURNAL OF VIROLOGY, Dec. 1993, p. 7276–7283
0022-538X/93/127276-08$02.00/0

Vol. 67, No. 12

Herpes Simplex Virus Type 1 Latency-Associated Transcript (LAT) Promoter Deletion Mutants Can Express a 2-Kilobase Transcript Mapping to the LAT Region

M. NICOSIA, S. L. DESHMANE, J. M. ZABOLOTNY, T. VALYI-NAGY, AND N. W. FRASER*

The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104-4268

Received 22 February 1993/Accepted 17 September 1993

The results of studies in several laboratories suggest that a TATA box-containing promoter located in the herpes simplex virus type 1 internal long repeat and long terminal repeat elements drives expression of the latency-associated transcripts (LATs). In the present study, we show that expression of a 2-kb LAT-related transcript can occur in the absence of this LAT TATA promoter, indicating the existence of a cryptic promoter. By Northern (RNA) blot analysis, we have examined LAT expression by herpes simplex virus type 1 variant strains KOS/29 and 1704, which contain deletions of the LAT promoter region. Our data indicate that KOS/29, despite lacking the 203-bp fragment which contains the LAT TATA box, can express a 2-kb LAT-related transcript during productive infection in tissue culture and in mouse trigeminal ganglia during acute infection and reactivation. Similarly, strain 1704, which contains a larger deletion in this promoter region, also expresses a 2-kb LAT-related transcript during tissue culture infection and reactivation of latently infected trigeminal ganglia. However, LATs are not expressed with either virus during latency. Northern blot analysis with a single-stranded, oligonucleotide probe demonstrates that the 2-kb LAT and LAT-related transcript are colinear and share a large area of sequence similarity. These findings suggest the existence of a second promoter in the LAT gene which can function during lytic infection and reactivation, at least in the absence of the LAT TATA promoter. We propose that this cryptic promoter is located either in a proximal region approximately 300 bp upstream of the start site of the 2-kb LAT or in a distal region starting over 1,226 bp upstream of this site.

Herpes simplex virus type 1 (HSV-1) can remain latent in nuclei of neurons innervating the site of infection (reviewed in reference 12). During latency, the HSV-1 genome persists as a nucleosomal-associated circular or concatameric episome (5, 9, 20, 24, 26, 27). The presence of any latently expressed, virus-encoded polypeptides has yet to be conclusively demonstrated (reviewed in references 11 and 37). However, various groups have shown that the diploid gene encoding the latency-associated transcripts (LATs) is the only actively transcribed gene detected during latency (3, 4, 28, 38, 39). Northern (RNA) blot analyses demonstrated that at least two of three colinear 2-, 1.5-, and 1.45-kb LATs are expressed in latently infected ganglia (17, 32, 36, 38, 42). On the other hand, only a 2-kb LAT was detected in HSV-1 infections of tissue culture cells (17, 32, 33). A larger, transcribed region was detected by in situ hybridization (3, 4, 21, 35) and, extremely rarely, by Northern blotting as an 8- to 9-kb RNA (7, 49) shown to have one promoter (22). Thus, it has been proposed that this large LAT (minor LAT [mLAT]) is transcribed and quickly processed to the more stable, smaller RNA species (reviewed in reference 11).

Initially, the LATs were thought to function in regulating or maintaining the latent state of the virus. However, studies of HSV-1 LAT null mutants indicate that LAT expression is not required for viral replication, establishment, and maintenance of latency in trigeminal ganglia (7, 13–15, 35). Yet, LAT function in affecting reactivation kinetics is suggested by a significant delay in reactivation following explant cocultivation of some HSV-1 LAT deletion mutants (18, 35). The question of whether LATs mediate their function as proteins or RNAs remains unanswered.

Numerous investigators have been studying the transcriptional and posttranscriptional events that regulate LAT expression (reviewed in reference 11). It is generally agreed that the 1.45- and 1.5-kb LATs are splicing products of the 2-kb transcript (34, 41, 44). Confusion remains regarding the synthesis of the mLAT and 2-kb LAT. The nearest, apparently functional TATA box is located almost 700 bp upstream of the 5′ end of the 2-kb LAT (44). This region can drive the expression of the chloramphenicol acetyltransferase gene in transient transfection assays (47–49). Furthermore, Dobson and collaborators (7) showed that this TATA box promotes latent expression of a rabbit β-globin gene engineered in recombinant HSV-1. Thus, the most popular hypothesis suggests that the 2-kb LAT is a stable intron spliced from an mLAT initiated near this TATA box (7, 10). Another hypothesis proposes that the 2-kb LAT is not an intron but has a short leader sequence near the TATA box (34, 49). Thus far, efforts to find such a leader sequence have been unsuccessful. A third hypothesis purports that the LAT gene has alternate transcription start sites, including one located at the 5′ end of the 2-kb LAT (34). Debates continue regarding the validity of these hypotheses.

HSV-1 LAT promoter deletion mutants have been engineered to study LAT expression and function in acute infection, latency, and reactivation. One such mutant is KOS/29 (7). This mutant contains the smallest, most precise deletion of the LAT promoter: removal of the LAT TATA box as a 203-bp *Pst*I-*Pst*I fragment in both copies of the gene. KOS/29 does not express LATs during latency as shown by in situ hybridization and Northern blot analysis (5, 7). Recently, we have shown that KOS/29 reactivates normally in explant cocultivation assays of

* Corresponding author.

latently infected mouse trigeminal ganglia (6). In contrast, a hypothermia-induced reactivation model indicates that KOS/29 reactivates with reduced frequency in trigeminal ganglia and with wild-type frequency in lumbosacral ganglia (29). Another LAT promoter mutant, strain 1704, reactivates with reduced frequency by mouse trigeminal ganglia explant cocultivation assays (35) and by iontophoresis in the rabbit eye model (40). Strain 1704 contains a 0.9-kb deletion in one copy of the LAT TATA promoter region (see Fig. 1) and does not express LATs or mLATs during latency (22, 35).

In the current study, we reexamined LAT expression by HSV-1 variant strains KOS/29 and 1704. We show that both these LAT TATA promoter deletion mutants express a 2-kb LAT-related transcript during acute infection in tissue culture. The LAT-related transcript is very similar and may be identical to the 2-kb LAT. The 2-kb LAT related transcript was also expressed in trigeminal ganglia during acute infection and reactivation of infection by KOS/29. These data indicate the existence of a second promoter in the LAT gene which can express a 2-kb transcript mapping to the LAT region. This second promoter appears to be located either downstream or upstream of the 203-bp *Pst*I-*Pst*I fragment containing the LAT TATA promoter.

MATERIALS AND METHODS

Cell culture and HSV-1 growth and titration. CV-1 cells were grown in Eagle's minimum essential medium supplemented with 5% calf serum at 37°C with 5% CO_2. Subconfluent CV-1 monolayers were infected with HSV-1 KOS(M), KOS/29, strain 17^+, or strain 1704 at 1 PFU per cell. Virus was concentrated from the medium as described previously (3, 4). Virus stock titers were determined for CV-1 and BHK cells.

Lytic infection in tissue culture cells. Depending on the experiment, subconfluent CV-1 monolayers were infected with KOS(M) or KOS/29 (multiplicity of infection of 1) and with strain 17^+ or strain 1704 (multiplicity of infection of 0.3 to 0.4) to examine LAT expression during HSV-1 infection in cell culture. At the times postinfection indicated in the figure legends, cells were washed with phosphate-buffered saline and harvested for RNA processing as described below.

Infection of mice and explant reactivation. Six-week-old female BALB/c mice were infected with approximately 1×10^6 to 2×10^6 PFU of HSV-1 per eye (4, 32, 39). Groups of four or five animals were sacrificed at 3, 4, 5, or 7 days postinfection for acute infection or at least 4 weeks postinfection for latency. Following cervical dislocation, trigeminal ganglia were removed and quickly frozen in liquid nitrogen. For the reactivation experiments, trigeminal ganglia from latently infected animals were incubated at 37°C for 1, 3, 5, or 7 days in tissue culture media supplemented with 5% calf serum and 5% CO_2. At the end of the incubation period, trigeminal ganglia were either immediately processed for RNA extraction or quickly frozen in liquid nitrogen. Tissues were stored at −70°C until used for RNA extraction.

RNA extraction and Northern blot analysis. Pools of 8 to 10 fresh or frozen trigeminal ganglia were homogenized in guanidium solution (4 M guanidium thiocyanate, 0.5% sodium *N*-lauroylsarcosine, 100 mM β-mercaptoethanol, 25 mM sodium citrate [pH 7.0], 0.1% antifoam A; Sigma Chemical Co.) for 20 s with a cell disrupter (Brinkmann Instruments Inc.). Tissue culture cells were harvested in the guanidium solution and homogenized as described for the trigeminal ganglia. From these homogenates, total RNA was prepared by the guanidium thiocyanate extraction-cesium chloride centrifugation procedure of Chirgwin et al. (1) as adapted by Spivack and Fraser (32). Northern blot analysis was performed as previously described (32), with a few modifications. Briefly, 5 or 10 μg per lane of glyoxal-treated RNA was electrophoresed, vacuum blotted (LKB) to a nylon membrane (GeneScreen Plus; NEN), and UV cross-linked by using a Stratalinker (Stratagene). Hybridization was carried out overnight with heat denatured ^{32}P-labeled nick-translated DNA probes (32). Filters underwent a series of two 25-min washes at 65°C, in 1×, 0.5×, and 0.1× SSPE (1× SSPE is 180 mM NaCl, 10 mM monobasic sodium phosphate, and 1 mM EDTA [pH 7.7]) with 1% sodium dodecyl sulfate. Filters hybridized to oligonucleotides were prehybridized for 1 h and hybridized for 4 h at 37°C. Filters were exposed for autoradiography to XAR-5 film (Eastman Kodak) for the times listed in the figure legends.

Preparation of ^{32}P-labeled probes. An HSV-1 strain F *Bam*HI-B-containing plasmid was kindly provided by B. Roizman at the University of Chicago (25). The LAT probe (a 0.9-kb *Bst*EII-*Bst*EII fragment) and the dual LAT-ICP0 probe (a 1.0-kb *Bst*EII-*Bst*EII fragment) were generated by restriction enzyme digestion of *Bam*HI B, gel isolated, and purified with Geneclean II (Bio 101 Inc.) (see Fig. 1B for the position of these fragments in the LAT gene). Purified DNA fragments were labeled by nick translation to a specific activity of 1×10^8 to 5×10^8 cpm/μg of DNA as described previously (32).

A single-stranded 22-mer oligonucleotide (PR9) was also used to probe Northern blots. Primer PR9 hybridizes to nucleotides 119520 to 119544 of the LAT genome, 59 nucleotides downstream of the 5′ end of the 2-kb LAT. This primer was end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP.

RESULTS

KOS/29 expresses a 2-kb LAT-related transcript during tissue culture infection. HSV-1 mutant KOS/29 contains a 203-bp deletion which eliminates the LAT TATA promoter in both copies of the LAT gene (7) (Fig. 1). A ^{32}P-labeled LAT-specific probe (a 0.9-kb *Bst*EII-*Bst*EII fragment) was hybridized by Northern blot to RNA from CV-1 cells harvested at 16 h postinfection with KOS/29 or KOS(M), the parental virus. A 2-kb LAT-related transcript was expressed by KOS/29 at levels comparable to those of the 2-kb LAT made by wild-type virus (Fig. 2). Similar results were observed when we examined LAT gene expression in HSV-1-infected SY5Y cells, a cell line of neuronal origin (data not shown). These surprising results suggest that in tissue culture infection, the LAT gene expresses a LAT-related transcript in the absence of the TATA promoter by a cryptic promoter. This LAT-related transcript appears to be identical to the 2-kb LAT by Northern blot analysis.

We have used a single-strand oligonucleotide probe to further clarify the relationship between the LAT-related transcript and the 2-kb LAT. In Northern blots, both these transcripts are recognized by a 22-mer probe (PR9) that maps to positions 119520 to 119544 of the LAT genome, 59 nucleotides downstream of the 5′ end of the 2-kb LAT (data not shown). Thus, the LAT-related transcript and the 2-kb LAT are transcribed from the same strand and share a large area of sequence similarity.

KOS/29 expresses a 2-kb LAT-related transcript in acutely infected trigeminal ganglia but not during latency. LAT expression during acute infection and latency was examined to determine whether this second promoter could function in vivo in mice. Northern blot analysis showed that LAT expression was undetectable in trigeminal ganglia of KOS/29-infected mice during acute infection and latency (Fig. 3). As a positive

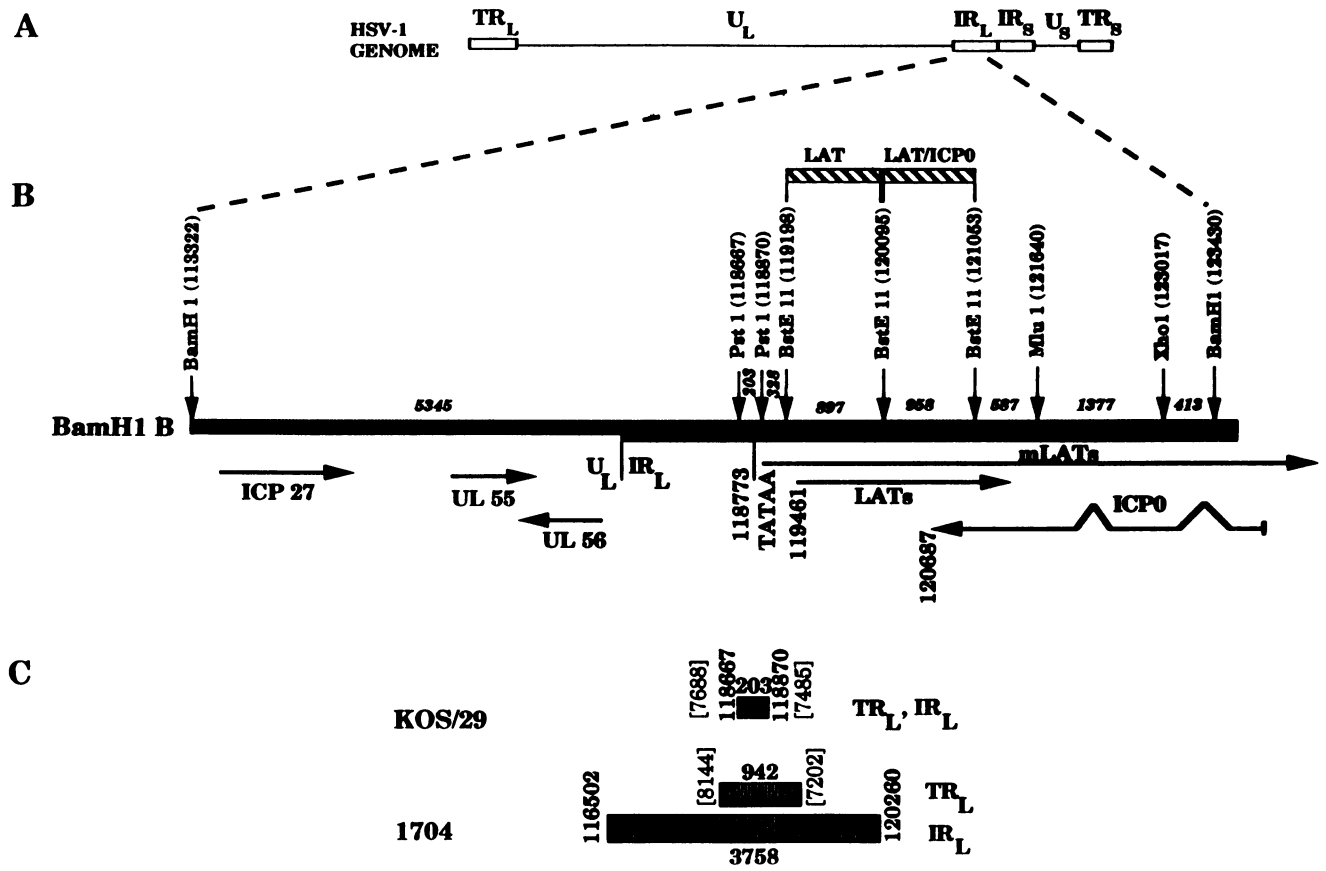

FIG. 1. LAT region of HSV-1. (A) A linear representation of the HSV-1 genome, which consists of long and short terminal repeat elements (TR_L and TR_S, respectively), unique long and short elements (U_L and U_S, respectively), and internal long and short repeat elements (IR_L and IR_S, respectively). The diploid gene encoding the LATs maps to the TR_L and IR_L. (B) *Bam*HI B fragment encompassing part of the LAT gene in IR_L. The positions of neighboring (ICP 27, UL55, UL56) and overlapping (ICP0) genes are marked. The LAT promoter maps at position 118773 within a 203-bp *Pst*I-*Pst*I fragment. This promoter is thought to initiate transcription of the mLAT, a large, unstable, 8- to 10-kb RNA, which is quickly processed to more stable 2-, 1.5-, and 1.45-kb LATs. The 5′ end of these stable LATs is thought to map at position 119460 (23, 45). The diagonally striped boxes indicate the restriction fragments used as probes in our Northern blot analysis. The LAT probe is a 0.9-kb *Bst*EII-*Bst*EII fragment (nucleotide positions 119198 to 120095). The LAT-ICP0 probe is a 1.0-kb *Bst*EII-*Bst*EII fragment (nucleotide positions 120095 to 121053). (C) Deletions in the LAT regions of HSV-1 mutants KOS/29 and strain 1704. Bracketed numbers indicate the nucleotide positions of corresponding deletions made in the TR_L (*Bam*HI E). KOS/29 contains a 203-bp deletion in both copies of the LAT gene. Strain 1704 has a 942-bp deletion in *Bam*HI E and a 3,758-bp deletion in *Bam*HI B.

control, we show that the LAT probe detects the accumulation of the 2-kb LAT in trigeminal ganglia of mice infected with KOS(M). ICP0 mRNA remained undetected during the course of acute infection by either virus even though the LAT-ICP0 probe was shown to label both transcripts in other experiments (see Fig. 5 and 6). Both 2- and 1.5-kb LATs were expressed in the trigeminal ganglia of animals latently infected with KOS(M). LATs and mLATs were not detected by in situ hybridization of trigeminal ganglia latently infected with KOS/29 (data not shown).

To confirm the results from the acute infection experiments, a separate group of animals was infected and LAT levels were assayed in the trigeminal ganglia of mice sacrificed on the fourth or seventh day postinfection (Fig. 4). In this case, a faint 2-kb LAT-related transcript was visible on day 4 following infection with KOS/29. This low-level RNA expression was probably not detected in the previous experiment because of the shorter exposure time. These RNAs were assayed in three separate Northern blots to ascertain that there was no cross-contamination of the samples. Furthermore, LAT-probe-pos-

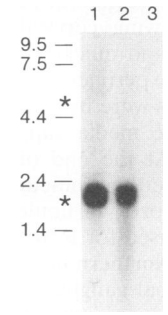

FIG. 2. LAT expression in CV-1 cells productively infected with KOS(M) or KOS/29. Total RNA was prepared from CV-1 cells harvested at 16 h postinfection. Lanes: 1, 5 μg of RNA from KOS/29-infected cells; 2, 5 μg of RNA from KOS(M) (the parental wild-type virus)-infected cells; 3, 5 μg of RNA from mock-infected cells. This blot was probed with the ^{32}P-labeled nick-translated LAT probe (Fig. 1B). The autoradiograph was exposed for 7.5 h. The locations of size markers (in kilobases) are indicated on the left. The positions of 28S and 18S rRNA are marked by asterisks.

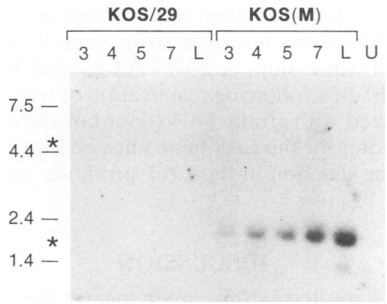

FIG. 3. LAT expression during acute infection and latency in mouse trigeminal ganglia. Mice were infected with HSV-1 KOS(M) or KOS/29 after corneal scarification. RNA was prepared from trigeminal ganglia of uninfected mice and mice sacrificed on 3, 4, 5, or 7 days and 6 weeks (latent) postinfection, as indicated above each lane. Each lane contains 10 μg of total RNA. The Northern blot was hybridized to the ^{32}P-labeled LAT-ICP0 probe. This autoradiograph was exposed for 3 days at −70°C. The locations of size markers (in kilobases) are noted on the left. The positions of 28S and 18S rRNA are marked by asterisks.

itive cells were detected in trigeminal ganglia at 4 days postinfection by KOS/29 by in situ hybridization (data not shown). Figure 4 also shows that trigeminal ganglia infected with KOS(M) accumulated both 2- and 1.5-kb LATs during the course of infection. In these experiments, we were unable to detect ICP0 mRNA during acute infection by both wild-type and mutant viruses. Deshmane et al. (6) have shown that KOS/29 infection produced virus titers similar to those of KOS(M) during acute infection of mouse trigeminal ganglia. Thus, our observed differences in the levels of 2-kb transcripts are not due to decreased viral replication by KOS/29 relative to the parental virus.

A 2-kb LAT-related transcript is expressed by KOS/29 during reactivation of latently infected trigeminal ganglia. Figure 5 compares LAT expression by KOS/29 and KOS(M) during reactivation of latently infected trigeminal ganglia. In these experiments, trigeminal ganglia were incubated in media without a cell monolayer (as is usually done for explant cocultivation). We noted that acridine orange staining of the

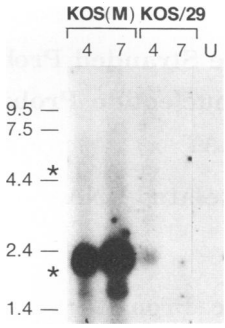

FIG. 4. LAT expression during acute infection in mouse trigeminal ganglia. Mice were infected with KOS(M) or KOS/29 following corneal scarification. RNA was prepared from trigeminal ganglia of uninfected mice and mice sacrificed 4 or 7 days postinfection, as indicated above each lane. Approximately 10 μg of RNA isolated from these trigeminal ganglia was processed for Northern blot analysis and probed with the ^{32}P-labeled LAT-ICP0 probe. This autoradiograph was exposed for 10 days at −70°C. The locations of size markers (in kilobases) are indicated on the left in kilobases. The positions of 28S and 18S rRNA are marked by asterisks.

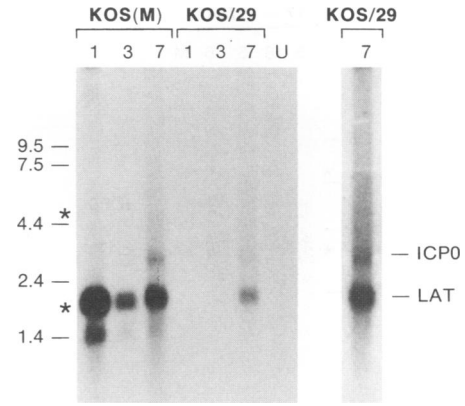

FIG. 5. LAT expression during reactivation following explant cultivation of latently infected mouse trigeminal ganglia. This northern blot illustrates what happens to LAT expression during reactivation. Trigeminal ganglia from mice latently infected with KOS(M) or KOS/29 were explanted and incubated in tissue culture media at 37°C for 1, 3, or 7 days, as indicated above each lane. Approximately 10 μg of RNA isolated from these trigeminal ganglia was processed for Northern blot analysis and probed with the ^{32}P-labeled LAT-ICP0 fragment. It was noted that acridine orange staining showed that lane 3 had a little more RNA than the other lanes (data not shown). The right lane is a 7-day exposure of the day 7 lane. The autoradiographs shown in the other lanes were exposed overnight at −70°C. The locations of size markers (in kilobases) are indicated on the left. The positions of 28S and 18S rRNA are marked by asterisks.

RNA gel indicated that lane 3 [KOS(M), day 7] may have had a little more RNA than the other lanes (data not shown). This was considered when comparing LAT and ICP0 RNA levels at different times. This Northern blot shows that the wild-type virus expresses both 2- and 1.5-kb LATs at day 1 of reactivation. The levels of the 1.5-kb LAT appear to have decreased to a greater extent than those of the 2-kb LAT by day 7. On day 7, ICP0 mRNA was also detected, indicating that the cascade of lytic cycle HSV-1 gene expression had been initiated. KOS/29 did not express detectable levels of LATs on days 1 and 3 of reactivation. By day 7, both a 2-kb LAT-related RNA and ICP0 mRNA were detected in RNA from trigeminal ganglia of KOS/29-infected mice. This is most clearly seen in the far right lane, which is a 7-day exposure of reactivation on day 7. A second reactivation experiment indicated that KOS/29 LAT gene expression occurred as early as day 5 postexplant (data not shown). The 1.5-kb LAT was undetectable during reactivation of trigeminal ganglia latently infected with KOS/29. The data for KOS/29 LAT-related RNA expression suggest the presence of a second promoter in the LAT gene which can function in tissue culture infection, at low levels in acute infection in trigeminal ganglia, and during reactivation of latently infected trigeminal ganglia, at least in the absence of the LAT TATA promoter. The 2-kb LAT-related RNA made from this second promoter does not appear to be processed to the 1.5-kb RNA found in ganglia from animals acutely and latently infected with the wild-type virus.

HSV-1 variant strain 1704 expresses a 2-kb LAT-related transcript during acute infection in tissue culture cells. We have reexamined LAT expression by another LAT promotorless mutant, strain 1704, to begin delimiting the possible location of this second LAT promoter. Strain 1704 contains a 3.5-kb deletion in the *Bam*HI-B site which largely disrupts one copy of the LAT promoter and 2-kb LAT (19) (Fig. 1). *Bam*HI-E has a 0.94-kb deletion encompassing the LAT

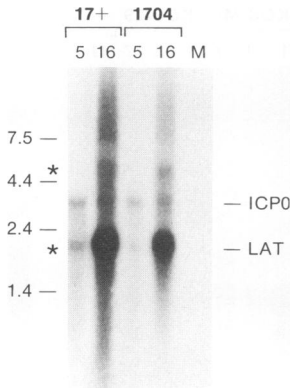

FIG. 6. LAT and ICP0 mRNA expression in CV-1 cells productively infected with strain 17^+ or variant 1704. Total RNA was prepared from CV-1 cells harvested 5 or 16 h postinfection with HSV-1 17^+ or strain 1704 as indicated above each lane. Approximately 10 μg of RNA was loaded in each lane. This Northern blot was probed with the ^{32}P-labeled LAT-ICP0 fragment. The autoradiograph was exposed for 40 min at −70°C. The locations of size markers (in kilobases) are indicated on the left. The positions of 28S and 18S rRNA are marked by asterisks.

TATA promoter and some flanking sequences but not affecting the 5′ end of the 2-kb LAT. The Northern blot in Fig. 6 shows LAT and ICP0 expression in CV-1 cells at 5 and 16 h postinfection. The parental strain of 1704, HSV-1 17^+, was used as a positive control for LAT expression. As expected, following wild-type virus infection, a 2-kb LAT and ICP0 were first visible at 5 h and further accumulated at 16 h. A similar pattern resulted from infection with strain 1704, albeit at a lower level of expression. A 2-kb LAT-related RNA was also made 5 and 10 days following reactivation of trigeminal ganglia latently infected with strain 1704 (data not shown). Thus, the second promoter for the LAT gene must be located outside the 940-nucleotide deletion in the LAT promoter region of strain 1704.

DISCUSSION

The data presented in this paper suggest the existence of a promoter which can function in the absence of the LAT TATA promoter during lytic infection and reactivation to produce a 2-kb transcript mapping to the LAT region. Wechsler et al. have mapped the 5′ end of the 2-kb LAT from latently infected rabbit trigeminal ganglia and human ganglia (43, 44). Wagner et al. (41) used primer extension and S1 nuclease protection experiments to map the 5′ end of the LAT to the position shown in Fig. 7. The LAT-related transcript appears to be indistinguishable by size from the 2-kb LAT in our Northern blot analyses (Fig. 2 and 4 to 6). Furthermore, the LAT-related transcript was recognized by both nonoverlapping probes (LAT and LAT-ICP0) used to detect the 2-kb LAT (Fig. 2 and 4 to 6) (data not shown). This suggests that the 2-kb LAT and LAT-related transcript must be colinear for at least a significant length of sequence (approximately 1,000 bp). The region of overlap is further delimited by the deletion in strain 1704 which pushes the 5′ end of LAT-related transcript closer to that published for the 2-kb LAT (Fig. 7). In addition, Northern blot analyses demonstrate that the LAT-related transcript is recognized by an oligonucleotide that maps 59 bp downstream

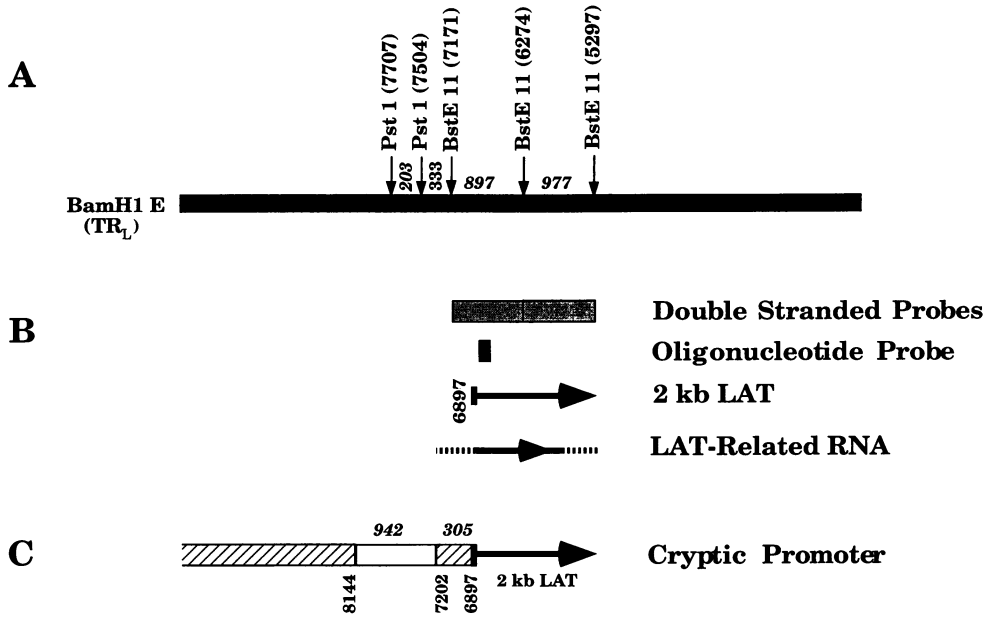

FIG. 7. The location of the 2-kb LAT-related transcript and the cryptic LAT promoter. (A) *Pst*I-*Pst*I deletion in KOS/29 and *Bst*EII sites important in mapping the 2-kb transcript and cryptic promoter from KOS/29 and 1704. (B) Relative positions of double-stranded (nick-translated) probes and a single-stranded (22-mer) oligonucleotide used in detecting the 2-kb LAT and LAT-related transcript by Northern blot analyses. The oligonucleotide (PR9) hybridizes to a sequence starting 59 nucleotides downstream of the 5′ end of the 2-kb LAT. The double-stranded, nick-translated probes are described in the legend to Fig. 1. The fact that all these probes recognize both RNAs indicates that the 2-kb LAT and LAT-related transcript share a large area of sequence identity (marked by the large, bold, colinear arrows). (C) Boxes with diagonal stripes show that the cryptic LAT promoter may be located either in 300 bp remaining upstream of the 2-kb LAT or anywhere in the region starting more than 1,226 bp further upstream of this site. The 5′ end of the 2-kb LAT is positioned at nucleotide 6897.

of the 5′ of the 2-kb LAT (data not shown). These data suggest that in the absence of the LAT TATA box, the LAT gene can express an RNA (LAT-related transcript) which is highly homologous, possibly even identical, to the 2-kb LAT.

Our studies with HSV-1 variant strains KOS/29 indicate that the cryptic promoter in the LAT gene can efficiently drive LAT-related transcript expression during productive infection of tissue culture cells. This cryptic promoter also functions during reactivation by explant cultivation and, at low levels, during acute infection of mouse trigeminal ganglia. In agreement with the findings of Dobson et al. (7) and Deshmane and Fraser (5), we did not detect KOS/29 LAT gene expression during latency. Thus, transcription from this second promoter may be regulated by a mechanism different from that controlling LAT TATA promoter activity. The LAT genes have been placed in a separate HSV-1 gene kinetic class (the λ gene class) because they are the only viral genes transcribed during latency in the absence of other viral gene products (33). Expression of the LAT from the second promoter appears to be regulated similarly to HSV-1 late (γ) genes rather than λ genes because synthesis of the 2-kb LAT-related transcript by KOS/29 and strain 1704 was observed during late times in acute infection and reactivation (Fig. 2 and 4 to 6).

Another interesting property of LAT gene transcription by KOS/29 is the absence of a 1.45- or 1.5-kb LAT. Various groups have shown that these smaller LATs are splice products of the 2.0-kb LAT (34, 42, 44). This splicing activity occurs during latency in mouse trigeminal ganglia and may occur late in infection (33) (Fig. 3 and 4). Our data show that during reactivation of trigeminal ganglia latently infected with KOS/29, a 1.5-kb RNA was undetected despite the significant amounts of the 2-kb LAT-related transcript produced (Fig. 5). One possible explanation is that there may be subtle sequence differences that prevent splicing of the 2-kb LAT-related transcript. Another possibility is that production of 1.5-kb LAT occurs only during latency and, possibly, acute infection but not during reactivation. This hypothesis suggests that during late stages of acute infection by wild-type virus there may be a mixed population of infected cells. One group of cells may be acutely infected, producing only the 2-kb LAT, and another group of cells may be latently infected, accumulating both 2- and 1.5-kb LATs. Thus, the accumulation of the 1.5-kb LAT during acute infection by KOS(M), shown in Fig. 4, may have occurred in a group of cells that reached latency while other cells were undergoing acute infection. This explanation also accounts for the observation that the levels of 1.5-kb LAT drop more rapidly than those of the 2-kb LAT following reactivation of trigeminal ganglia latently infected with wild-type virus (6, 7) (Fig. 5). This may be due to the differential stability of accumulated 2- and 1.5-kb LATs and/or to a decrease in splicing efficiency of newly synthesized 2-kb LAT. Northern blot analysis cannot distinguish between increased rates of transcription and accumulation of a stable transcript.

Our data indicate that the 2-kb LAT-related transcript made during reactivation of trigeminal ganglia latently infected with KOS/29 is newly transcribed since we and others have observed that this virus does not accumulate LATs during latency (6, 7) (Fig. 3) (data not shown). Thus, new transcription seems to cause accumulation of a LAT-related transcript during KOS/29 reactivation of latently infected trigeminal ganglia. This suggests that in the wild-type virus, it is possible that the LAT TATA promoter functions during latency to synthesize both 2- and 1.5-kb LATs and the cryptic LAT promoter acts late in reactivation to produce only the 2-kb LAT. The commonly observed decrease in 1.5-kb LAT expression during reactivation may be due to a switch in transcription between the two promoters, one producing a spliceable 2-kb LAT and the other producing a nonspliceable 2-kb LAT.

We have been localizing the cryptic promoter by examining LAT expression by HSV-1 strain 1704, which has a large spontaneous mutation encompassing the deletion in KOS/29. More specifically, strain 1704 has a 3,758-bp deletion in the unique long (U_L) and internal long (IR_L) repeat elements which disables one copy of the LAT gene and a 942-bp deletion in the long terminal repeat element (TR_L) which removes the LAT TATA promoter (16). This virus has been previously shown not to express LATs or mLATs during latency in mouse trigeminal ganglia (21, 35). Yet, our Northern blot demonstrates that a 2-kb LAT-related transcript is expressed by tissue culture cells infected with strain 1704 to a level approaching LAT expression by the wild-type virus. Furthermore, this LAT-related transcript is also made 5 and 10 days following reactivation of trigeminal ganglia latently infected with strain 1704 (data not shown). Thus, the fact that both KOS/29 and strain 1704 produce LAT-related transcripts despite their very different deletions indicates that the cryptic promoter is not an artifact of the deletion juxtapositioning sequences to create an active promoter element. We suggest that expression of this 2-kb LAT-related RNA is driven by a promoter located either in the approximately 300 bp remaining upstream of the 5′ end of the 2-kb LAT or in a region starting more than 1,226 bp further upstream of this site (Fig. 7). Sequence analysis has shown that there is no additional TATA box located between the LAT TATA promoter and the start site of the 2-kb LAT (45). The first functional TATAA sequences found upstream of the LAT 5′ end are the TATA box for U_L 56 in the *Bam*HI B fragment or U_L1 in *Bam*HI E, both located approximately 2,300 bp upstream of the start site for the 2-kb LAT.

Interestingly, late in herpesvirus infections, significant amounts of long viral transcripts accumulate (2). It has been suggested that these transcripts represent long readthrough products of viral genes which are synthesized late in the viral infectious cycle, when transcript processing begins to break down as the cell is dying. If this process is occurring late during HSV infection of neuronal cells, it is possible that readthrough transcripts give rise to the 2-kb LAT-related transcript. However, this hypothesis would require that splicing of the 2-kb LAT-related RNA from a long precursor still be efficient.

The cryptic LAT promoter may be a TATA-less promoter like those commonly found in housekeeping genes. Housekeeping promoters and DNA sequences that may contain a second LAT promoter element share two features: the absence of a canonical TATA box and the presence of G+C-rich regions (reviewed in references 8 and 30). Transcription by many TATA-less promoters is driven by initiator (Inr) elements encompassing 17 bp around the transcription start site (31; reviewed in reference 46). These Inr elements are also found in TATA-containing genes and can function in concert with a TATA box (31). Thus, the second LAT promoter may be an Inr-like element functioning in the presence or absence of the LAT TATA box. We have yet to determine whether the LAT gene contains an Inr element due to the variability in Inr consensus sequences.

The present data suggest the intriguing possibility that our 2-kb LAT-related transcript may be the 2-kb LAT. If this is true, the synthesis of a 2-kb LAT-related transcript by LAT TATA-promoterless strains KOS/29 and 1704 is consistent with the idea that during latency the 2-kb LAT is a stable intron derived from an mLAT, a large primary transcript (7, 10). Various groups have proposed that transcription of this mLAT starts within 30 bp of the LAT TATA box and ends 8 to 10 kb downstream at a polyadenylation site (7, 49) during

latent infection. This theory is supported by experiments with several LAT promoter mutants (7, 21). Supporting this location for the promoter during latency is the synthesis of only globin RNA and not the 2-kb LAT when the globin gene is inserted just downstream of the LAT TATA box (7). Our results with KOS/29 and strain 1704 also support this concept. However, we also show that a cryptic promoter is activated in the LAT gene during nonlatent conditions, such as cell culture or acute infections, or after reactivation in vitro. The location of this promoter is not clear at present, nor is it clear how much of the primary transcript is similar to the primary transcript which is synthesized during latency. It is certainly not identical to the latent primary transcript because of the 5′ deletions in KOS/29 and strain 1704; however, it may share a common 3′ end. At present, we can be certain that the site of transcription initiation is not the same as that used during latency. Thus, in contrast to latent 2-kb LAT, the LAT-related transcript made by KOS/29 and strain 1704 may be spliced from a much larger transcript in these nonlatent conditions or it may arise from a much smaller transcript with or without splicing.

The fact that KOS/29 expresses a 2-kb LAT-related transcript during reactivation does not explain why our laboratory (6) has observed similar rates of reactivation by explant cocultivation of trigeminal ganglia latently infected with KOS/29 and KOS(M). The 2-kb LAT-related transcript does not act in maintaining efficient (wild-type) reactivation kinetics because strain 1704 has a slow reactivation phenotype (35) even though it can express a LAT-related transcript in tissue culture cells and during reactivation of latently infected trigeminal ganglia.

In summary, our data suggest that LAT gene expression may be under the control of multiple promoters which act at different times during the course of infection, latency, and reactivation. Primary transcripts made from these different promoters may give rise to different mature RNA species, which perform distinct functions, and also to a 2-kb LAT with a common function. Examining the effects of mutations in these promoters on LAT expression may help our understanding of the often conflicting data obtained from studies performed with LAT HSV-1 mutants in the past decade.

ACKNOWLEDGMENTS

We are deeply indebted to Larry Feldman (University of California—Los Angeles) for supplying virus KOS/29 and for critical discussion of the manuscript. We also thank M. Brown (Glasgow) for providing virus strain 1704 and Jordan G. Spivack for supplying oligonucleotide PR9. We thank Allan Dillner for excellent technical support. We also greatly appreciate Cheryl McFadden's assistance in manuscript preparation.

This work was supported by Public Health Service grant AI23968 from the National Institutes of Health. M.N. was supported by National Institutes of Health training grant AI07324.

REFERENCES

1. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Orientation of herpes simplex virus type-1 immediate early mRNAs. Biochemistry **18:**5294–5299.
2. **Deatly, A. M., L. T. Feldman, and T. Ben-Porat.** 1984. The large late virus transcripts synthesized in herpesvirus suis (pseudorabies) virus-infected cells are not precursors of mRNA. Virology **135:**452–465.
3. **Deatly, A. M., J. G. Spivack, E. Lavi, and N. W. Fraser.** 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.
4. **Deatly, A. M., J. G. Spivack, E. Lavi, D. R. O'Boyle, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissue of mice map to similar regions of the viral genome. J. Virol. **62:**749–756.
5. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type-1 DNA is associated with nucleosomes in a chromatin structure. J. Virol. **63:**943–947.
6. **Deshmane, S. L., M. Nicosia, T. Valyi-Nagy, L. T. Feldman, and N. W. Fraser.** 1993. A herpes simplex virus type-1 (HSV-1) lacking the putative LAT TATA elements reactivates normally in explant co-cultivation. Virology **196:**868–872.
7. **Dobson, A. T., F. Sederati, G. Devi-Rao, J. Flanagan, M. J. Farrell, J. G. Stevens, E. K. Wagner, and L. T. Feldman.** 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:**3844–3851.
8. **Dynan, W. S.** 1983. Promoters for housekeeping genes. Trends Genet. **2:**196–197.
9. **Efstathiou, S., C. Minson, H. J. Field, J. R. Anderson, and P. Wildly.** 1986. Detection of herpes simplex virus-specific DNA sequences in latently infected mice and humans. J. Virol. **57:**446–455.
10. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc. Natl. Acad. Sci. USA **88:**790–794.
11. **Fraser, N. W., T. M. Block, and J. G. Spivack.** 1992. The latency-associated transcripts of herpes simplex virus: RNA in search of function. Virology **191:**1–8.
12. **Fraser, N. W., J. G. Spivack, Z. Wroblewska, T. Block, S. L. Deshmane, T. Valyi-Nagy, R. Natarajan, and R. Gesser.** 1991. A review of the molecular mechanism of HSV-1 latency. Curr. Eye Res. **10**(Suppl.):1–13.
13. **Hill, J. M., F. Sederati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology **174:**117–125.
14. **Ho, D. Y., and E. S. Mocarski.** 1989. Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA **86:**7596–7600.
15. **Javier, R. T., J. G. Stevens, V. B. Dissette, and E. K. Wagner.** 1988. A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology **166:**254–257.
16. **Junejo, F., A. R. MacLean, and S. M. Brown.** 1991. Sequence analysis of the herpes simplex virus type 1 strain 17 variants 1704, 1705, and 1706 with respect to their origin and effect on the latency-associated transcript sequence. J. Gen. Virol. **72:**2311–2315.
17. **Krause, P. R., K. D. Croen, S. E. Straus, and J. M. Ostrove.** 1988. Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. J. Virol. **62:**4819–4823.
18. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent infection. J. Virol. **63:**2893–2900.
19. **MacLean, A. R., and S. M. Brown.** 1987. Deletion and duplication variants around the long repeats of herpes simplex virus type 1 strain 17. J. Gen. Virol. **68:**3019–3031.
20. **Mellerick, D. M., and N. W. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology **158:**265–275.
21. **Mitchell, W. J., R. P. Lirette, and N. W. Fraser.** 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.
22. **Mitchell, W. J., I. Steiner, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1990. A herpes simplex virus type 1 variant, deleted in the promoter region of the latency-associated transcripts, does not produce any detectable minor RNA species during latency in the mouse trigeminal ganglion. J. Gen. Virol. **71:**953–957.
23. **Perry, L. J., and D. J. McGeoch.** 1988. The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**2831–2846.

24. **Poffenberg, K. L., and B. Roizman.** 1985. A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkage in packaged genomes and requirements for circularization after infection. J. Virol. **53:**587–595.
25. **Post, L. E., A. J. Conley, E. S. Mocarski, and B. Roizman.** 1980. Cloning of reiterated and nonreiterated herpes simplex virus 1 sequences as *Bam*HI fragments. Proc. Natl. Acad. Sci. USA **77:**4201–4205.
26. **Rock, D. L., and N. W. Fraser.** 1983. Detection of HSV-1 genome in the central nervous system of latently infected mice. Nature (London) **302:**523–525.
27. **Rock, D. L., and N. W. Fraser.** 1985. Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. J. Virol. **55:**849–852.
28. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. M. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61:**3820–3826.
29. **Sawtell, N. M., and R. L. Thompson.** 1992. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivates from latency. J. Virol. **66:**2157–2169.
30. **Sehgal, A., N. Patil, and M. Chao.** 1988. A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol. Cell. Biol. **8:**3160–3167.
31. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57:**103–113.
32. **Spivack, J. G., and N. W. Fraser.** 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61:**3841–3847.
33. **Spivack, J. G., and N. W. Fraser.** 1988. Expression of herpes simplex virus type 1 latency-associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. **62:**1479–1485.
34. **Spivack, J. G., G. M. Woods, and N. W. Fraser.** 1991. Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0-kilobase latency-associated transcript (LAT): translation inhibition of LAT open reading frames by the intron within the 2.0-kilobase LAT. J. Virol. **65:**6800–6810.
35. **Steiner, I., J. G. Spivack, R. P. Lirette, S. M. Brown, A. R. MacLean, J. Subak-Sharpe, and N. W. Fraser.** 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8:**505–511.
36. **Steiner, I., J. G. Spivack, D. R. O'Boyle, E. Lavi, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virol. **62:**3493–3496.
37. **Stevens, J. G.** 1989. Human herpesviruses: a consideration of the latent state. Microbiol. Rev. **53:**318–332.
38. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpes virus gene mRNA is prominent in latently infected neurons. Science **235:** 1056–1059.
39. **Stroop, W. G., D. L. Rock, and N. W. Fraser.** 1984. Localization of herpes simplex virus in the trigeminal and olfactory systems of the mouse central nervous system during acute and latent infections by in situ hybridization. Lab. Invest. **51:**27–38.
40. **Trousdale, M., I. Steiner, J. G. Spivack, S. L. Deshmane, S. M. Brown, A. S. MacLean, J. Subak-Sharpe, and N. W. Fraser.** 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.
41. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y. F. Zhang, J. M. Hill, W. M. Flanagan, and J. G. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62:**1194–1202.
42. **Wagner, E. K., W. M. Flanagan, G. Devi-Rao, Y. F. Zhang, J. M. Hill, K. P. Anderson, and J. G. Stevens.** 1988. The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. **62:**4577–4585.
43. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. Slanina, and H. Ghiasi.** 1988. Fine mapping of the major latency-related RNA of herpes simplex virus type 1 in humans. J. Gen. Virol. **69:**3101–3106.
44. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. M. Slanina, and H. Ghiasi.** 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. J. Virol. **62:**4051–4058.
45. **Wechsler, S. L., J. Nesburn, N. Zaagstra, and H. Ghiasi.** 1989. Sequence of the latency related gene of herpes simplex virus type 1. Virology **168:**168–172.
46. **Weis, L., and D. Reinberg.** 1992. Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. FASEB J. **6:**3300–3309.
47. **Zwaagstra, J., H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1989. *In vitro* promoter activity associated with the latency-associated transcript gene of herpes simplex virus type 1. J. Gen. Virol. **70:**2163–2169.
48. **Zwaagstra, J. C., H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1991. Identification of a major regulatory sequence in the LAT promoter of herpes simplex virus type 1. Virology **182:**287–297.
49. **Zwaagstra, J. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, D. S. Latchman, K. Patel, and S. L. Wechsler.** 1990. 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.