A Novel Class of Transcripts Expressed with Late Kinetics in the Absence of ICP4 Spans the Junction between the Long and Short Segments of the Herpes Simplex Virus Type 1 Genome

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A novel family of transcripts that span the junction between the long and short segments of the herpes simplex virus type 1 genome has been identified. These transcripts, designated L/S junction-spanning transcripts (L/STs), are synthesized in abundance in a variety of cells infected with mutant viruses defective in the gene for ICP4, the major transcriptional regulatory protein of the virus. Transcription of abundant 2.3- and 8.5-kb series of L/STs was shown to initiate within the same sequences as less abundant 4.2-, 7.3-, and >9.5-kb transcripts by Northern (RNA) blot analysis. S1 nuclease analysis revealed a single 5' terminus 28 bp downstream of a TATA box and 6 bp downstream of a consensus ICP4 binding site. The location of the transcriptional start site indicates that the promoter of the L/STs likely corresponds to the bidirectional promoter described by Bohenzky et al. (R. A. Bohenzky, A. G. Papavassiliou, I. H. Gelman, and S. Silverstein, J. Virol. 67:632–642, 1993). The L/STs accumulate with late kinetics in ICP4 mutant-infected cells and are polyadenylated. Mutant viruses encoding forms of ICP4 unable to bind the consensus site, ATCGTC, exhibited abundant expression of the L/STs, whereas mutants encoding forms of ICP4 able to bind this site expressed no detectable L/STs, suggesting that ICP4 plays a critical role in repressing L/ST expression. Their synthesis in ICP4 mutant-infected cells is inhibited by the protein synthesis inhibitor cycloheximide, indicating that they are induced either by an immediate-early viral protein other than ICP4 or by a virus-induced cellular protein. Preliminary evidence indicates that the L/STs are not present in latently infected ganglia. The abundant expression of the L/STs with late kinetics only in the absence of functional ICP4 and the sensitivity of their synthesis to cycloheximide indicate that they are not members of any of the recognized kinetic classes of herpes simplex virus type 1 transcripts but constitute a new class of viral transcript.

The expression of herpes simplex virus (HSV) genes during productive infection has long been recognized to proceed in a coordinate and sequential manner (27, 28; reviewed in references 3 and 32). The classification of HSV genes into broad groups—immediate-early (IE), early (E), delayed early (DE), and late (L)—is based on the kinetics of synthesis of individual viral transcripts and proteins, the effects of various metabolic inhibitors on DNA, RNA, and protein synthesis, and studies of viral mutants.

IE proteins are the first to be synthesized in infected cells and are the major regulatory proteins of the virus. They are required for the synthesis of E, DE, and L proteins and for the repression of their own synthesis (16a, 17, 19, 23, 27, 28, 35, 39a, 41). Transcription of IE genes is activated by a late protein in infecting virions, VP16 (reviewed in reference 24). IE transcripts and proteins are detectable at 1 h postinfection (hpi). Peak synthesis of IE proteins occurs at 3 to 4 hpi and declines rapidly thereafter, although low levels of synthesis are detectable at later times postinfection (27). Productive infection is blocked in the presence of inhibitors of protein synthesis, such that only IE mRNAs are made (27). IE proteins are required to induce the synthesis of E transcripts. Transcription of E genes and synthesis of E proteins begins by 3 hpi, peaks at about 6 hpi, and declines thereafter. E proteins are involved in nucleotide precursor metabolism and viral DNA synthesis. Transcription of DE (but not L) genes occurs at low levels from input genomes prior to the initiation of viral DNA

synthesis and thus does not depend stringently on viral DNA synthesis (26, 57). As viral DNA synthesis begins, high-level expression of DE genes occurs and L gene expression begins. DE and L protein synthesis continues throughout the remainder of the replication cycle. DE and L proteins include envelope glycoproteins, capsid proteins, and other components of mature virus particles.

In contrast to the complex sequence of events that occurs during productive infection, viral gene expression during latency is considerably less complicated. In latently infected cells, viral gene expression is limited to the latency-associated transcripts (LATs), a family of transcripts ranging in size from 2.0 to >8 kb (21). Whether the LATs encode functional proteins and what role they play in the viral life cycle remain to be determined. Although much is known about viral gene expression during productive infection, very little is known about the factors that mediate the switch from productive infection to latency and vice versa. Whatever the mechanism underlying this switch, the regulatory activities of the IE proteins, which orchestrate events during productive infection, must be overridden to establish latency and reinitiated during reactivation from latency.

Physical mapping studies have shown that four of the five IE regulatory genes are located wholly or in part within $b a c$ repeat sequences flanking the unique long (U_L) and unique short (U_S) regions of the genome (12, 36, 38) (Fig. 1), whereas nearly all E, DE, and L genes are located in unique-sequence DNA. One consequence of this arrangement is that IE regulatory genes and other elements located totally within repeat sequences are diploid. In addition to IE regulatory genes, the

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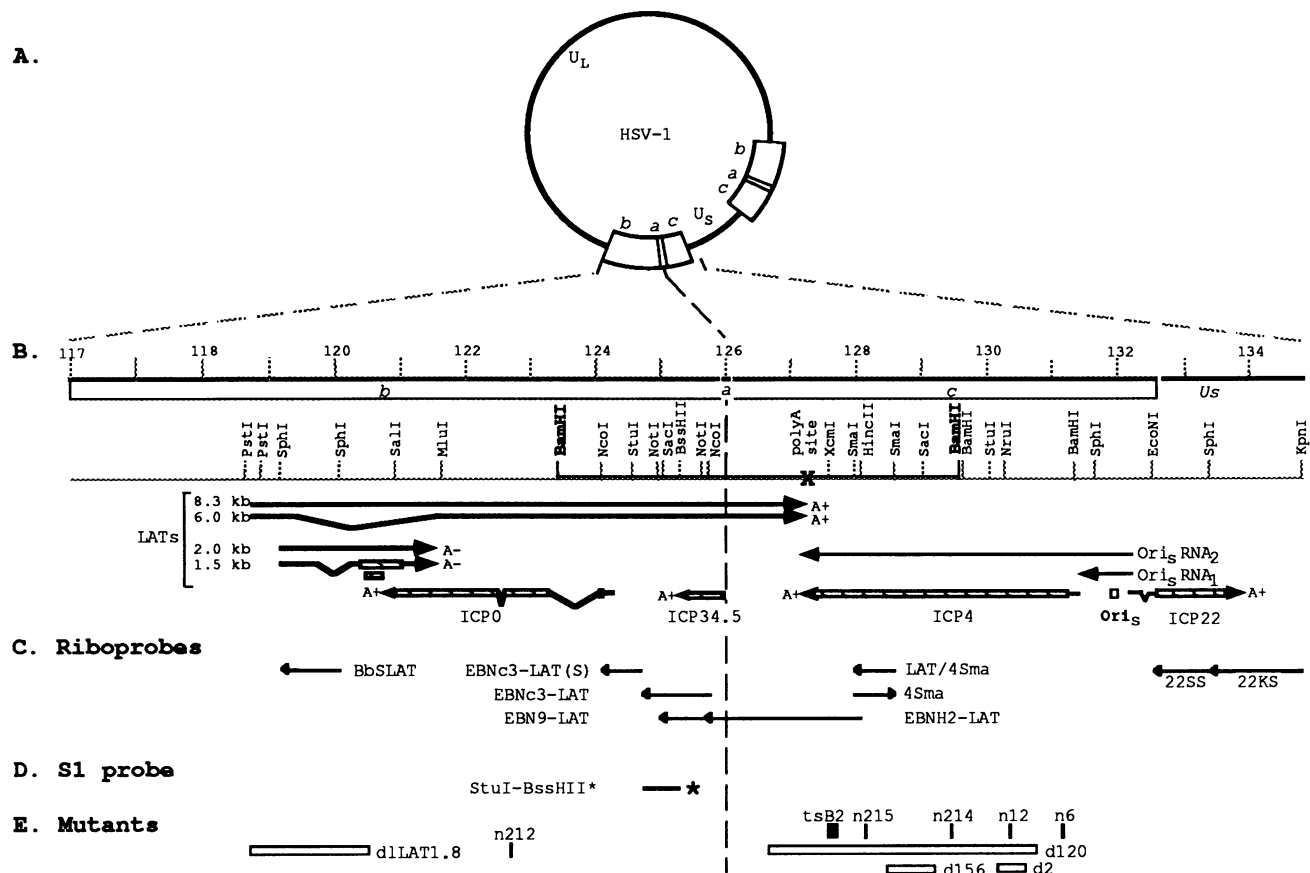
FIG. 1. Physical map of the internal repeat region of HSV-1 DNA. (A) Diagram of the HSV-1 genome. U_L, unique long segment; U_S, unique short segment; b, inverted repeat sequence bracketing U_L; c, inverted repeat sequence bracketing U_S; a, 317-bp sequence between the b and c repeat sequences. Two copies of the b a c repeats exist in circular genomes. (B) Expanded map of internal repeat sequences lying between map units 117 and 134.5 on the physical map of the HSV-1 genome (12, 36, 38). Beneath the scale of kilobase pairs are shown the locations of the b, a, and c sequences and relevant restriction sites in KOS DNA. Beneath the map of restriction sites are shown the locations of the genes and cis-acting elements contained within the sequence from 118 to 134 kb. Specifically, the map shows the locations of sequences specifying the small (1.5- and 2.0-kb) and large (8.3- and putative 6-kb) LATs, the transcripts encoding ICP0, ICP34.5, ICP4, and ICP22, sequences in oriS, and sequences specifying the transcripts designated oriS RNA1 and oriS RNA2. ORFs are shown as hatched bars. (C) DNA sequences specifying the riboprobes (arrows) used in this study. The arrows represent the orientation of these sequences in pGEM vectors as driven by the SP6 promoter. (D) Sequence specifying the DNA probe used for S1 nuclease mapping. The probe was labelled at the BssHII site (asterisk). (E) Locations of the mutations in the mutant viruses used in these studies. n212 contains a nonsense mutation in the ICP0 gene (6). n6, n12, n214, and n215 contain nonsense mutations in the ICP4 gene (20). The open boxes indicate the sequences deleted in dlLAT1.8, which specifies no detectable LATs (34), and d2, d120, and d156 ICP4, which are null mutants (17). The small black box indicates the approximate location of the mutation in tsB2 (23).

b a c repeats contain the junction between the long and short regions of the genome (the L/S junction) as well as other genes and cis-acting elements of fundamental significance to both productive infection and latency. These include (i) the sequences specifying the LATs (2, 21, 33, 37, 51, 56), (ii) the gene encoding a neurovirulence factor, ICP34.5 (8–10), (iii) the a sequence, which contains cis-acting elements involved in circularization, recombination, and packaging of the viral genome (15, 16, 39, 47, 48, 54, 55), and (iv) oriS, an origin of viral DNA replication (49, 52, 53, 55, 58).

During the course of efforts to fine map the LATs within the b a c repeats, we chose to isolate and characterize RNA from cells infected with an ICP4 null mutant in order to enhance LAT expression, as ICP4 has been reported to repress transcription from the LAT promoter (2). Using a series of strand-specific riboprobes in Northern (RNA) blot analysis, we identified a family of previously undescribed 5' coterminal transcripts ranging in size from 2.3 to >9.5 kb that span the L/S

junction. These transcripts, designated L/S junction-spanning transcripts (L/STs), appear to initiate in b sequences immediately to the right of the bidirectional ICP0 promoter described by Bohenzky et al. (4). They cross the L/S junction in the a sequence and terminate in c sequences near the poly(A) addition site at position 127040 (2.3-kb transcript) or beyond (8.5-kb and larger species). Thus, these transcripts are antisense to the genes encoding ICP34.5 and ICP4. Because the DNA template specifying the L/STs includes the L/S junction, it is diploid in circular genomes. Time course studies indicate that the L/STs accumulate in ICP4 mutant-infected cells with late kinetics. The studies described herein demonstrate that expression of the L/STs is repressed by ICP4 and stimulated either by other IE proteins or by virus-induced cellular factors. Because they are not expressed in the presence of cycloheximide, the L/STs are not IE transcripts. The fact that they are expressed at high levels and with late kinetics in the absence of

ICP4, yet are inhibited by cycloheximide, indicates that the L/STs represent a new class of viral transcript.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero; ATCC CCL 81), E5 cells (Vero cells stably transformed with the wild-type gene for ICP4 [17]), 0-28 cells (Vero cells stably transformed with the wild-type gene for ICP0 [42]), and 3-3 cells (Vero cells stably transformed with the wild-type gene for ICP27 [35]) were grown and maintained in Dulbecco's modified Eagle medium (GIBCO Laboratories, Inc., Gaithersburg, Md.) as previously described (41). Mouse neuroblastoma cells (NB41A3; ATCC CCL147) were propagated in F10 medium (GIBCO) supplemented with 2.5% fetal calf serum, 15% horse serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Rat pheochromocytoma (PC12) cells were kindly provided by John Wagner (Cornell University Medical College, New York, N.Y.) and were propagated and maintained as described previously (25).

The KOS wild-type strain of HSV-1 (46) and a series of temperature-sensitive (ts), nonsense, and deletion mutants derived from KOS cells were used in these studies. The ICP4 nonsense and deletion mutants, n6, n12, n214, n215, d2, d120, and d156 (17, 20), were grown and assayed in E5 cells (17). The ICP0 nonsense mutant, n212, was grown in Vero cells and assayed on 0-28 cells (6). The ICP27 deletion mutant, 5dl1.2, was grown and assayed in 3-3 cells (35). KOS, the ICP4 ts mutant, tsB2 (23), the ICP22 nonsense mutant, 22/n199 (1), and the LAT deletion mutant, dlLAT1.8 (34), were grown and assayed in Vero cells (41). The locations of the mutations in these viruses are shown in Fig. 1E.

Riboprobes. The riboprobes used in this study are shown in Fig. 1C. The BamHI K fragment containing the L/S junction and part of the b a c repeats from pSG28 (44) was cloned into pGEM3Zf(+) (Promega, Madison, Wis.). The 1,750-bp NcoI fragment from pBamK (map units 124 to 125.8) was subcloned into the NcoI site in pGEM5Zf(+) to yield pNco. To generate riboprobe EBNc3-LAT, pNco was linearized at the StuI site, and the StuI-NcoI sequence was transcribed as instructed by the manufacturer (Promega). To generate EBNc3-LAT(S), pNco was cleaved with StuI and HincII (HincII is in the polylinker beyond the right-hand NcoI site) and religated such that only the NcoI-StuI fragment remained. EBNc3-LAT(S) was transcribed from this sequence. A plasmid containing the NotI subfragment (map units 124.9 to 125.7) from pNco cloned into the NotI site of pGEM5Zf(+) was transcribed to yield EBN9-LAT. Riboprobe EBNH2-LAT was derived from the NotI-HincII fragment from pBamK, and riboprobe LAT/4Sma was derived from the SmaI fragment from pn11 (19), which contains the wild-type ICP4 gene. Riboprobe 4Sma, used to detect the ICP4 transcript, was derived from the same SmaI fragment from pn11 but cloned in the opposite direction. Riboprobe BbSLAT was derived from the 786-bp SphI fragment from the BamHI B fragment of KOS DNA, which contains the sequences specifying the 2-kb LATs. Riboprobes 22SS and 22KS were derived from the EcoNI-SphI and SphI-KpnI fragments, respectively, containing most of the ICP22 gene and some downstream sequences. The sizes and orientation of cloned fragments were verified by restriction enzyme analysis. Riboprobes were transcribed from the SP6 promoter in the vector as instructed by the manufacturer (Promega).

Northern blot analysis. Approximately 4 × 10⁶ NB41A3 or 2 × 10⁶ Vero or E5 cells were seeded in 100-mm-diameter petri dishes 24 h prior to infection. Cells were infected at a multiplicity of 10 PFU per cell in 0.5 ml of inoculum. After absorption for 1 h at 37°C, medium was added to infected cells, and incubation was continued at 37°C for the indicated times. Total cell RNA was harvested as described previously (29). Briefly, monolayers were washed twice with cold phosphate-buffered saline and scraped into 0.5 ml of GIT buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate, 100 mM β-mercaptoethanol). The volume was adjusted to 3.0 ml with GIT buffer, and the cell suspension was subjected to Vortex mixing for 15 s to shear DNA. The GIT-RNA solution was loaded onto a 2-ml cesium chloride cushion (5.7 M cesium chloride, 25 mM sodium acetate), and the sample was centrifuged at 35,000 rpm in an SWi50.1 or SWi55.1 rotor at 20°C for 18 h. The RNA pellet was resuspended in diethyl pyrocarbonate-treated water, ethanol precipitated, and resuspended again in 100 μl of diethyl pyrocarbonate-treated water. RNA concentrations were determined by optical density at 260 nm.

For Northern blot analysis, 15 μg of RNA sample was heat denatured in 1× MOPS (20 mM 3-N-[morpholino]propanesulfonic acid, 1 mM sodium acetate, 1 mM EDTA)–50% formamide–17.5% formaldehyde (15 min, 68°C), loaded onto an agarose gel (1% agarose, 16.6% formaldehyde, 1× MOPS), and electrophoresed overnight at 35 V in 1× MOPS buffer as described previously (29). The gel was washed (15 min per wash) once in water and four times in 10× SSC (10× SSC is 1.5 M sodium chloride plus 0.15 M sodium citrate, pH 7.0) before transfer to a Magnagraph nylon membrane (Micron Separations, Inc., Westboro, Mass.) in 10× SSC. The blot was either fixed by baking at 85°C under vacuum for 2 h or exposed to UV light at 1,200 μJ (×100) (UV Stratalinker 2400; Stratagene, La Jolla, Calif.) and prehybridized overnight at 68°C in 50% formamide–5× Denhardt's solution (5 mg of Ficoll [type 400; Pharmacia, Piscataway, N.J.] per ml, 5 mg of polyvinylpyrrolidone per ml, 5 mg of bovine serum albumin [fraction 5; Sigma, St. Louis, Mo.] per ml)–6× SSPE (0.9 M sodium chloride, 60 mM sodium phosphate [monobasic], 6 mM EDTA, pH 7.5)–0.2% sodium dodecyl sulfate (SDS)–100 μg of salmon testis DNA per ml. ³²P-labeled riboprobes were added to the blot in prehybridization buffer for incubation overnight at 68°C. The blot was rinsed once in 2× SSC–1% SDS and washed twice for 15 min in 2× SSC–1% SDS at room temperature, twice for 15 min in 0.1× SSC–0.1% SDS at 68°C, and once for 15 min in 0.1× SSC–0.1% SDS at 85°C. Blots were exposed to XAR-5 film (Kodak, Rochester, N.Y.), and bands were visualized by autoradiography.

S1 nuclease analysis. The S1 nuclease mapping procedure used in these studies has been described elsewhere (30). To map the 5' end of the L/STs, 50 μg of plasmid pNco was digested with BssHII, end labeled with ³²P, and digested with StuI to yield a 443-bp double-stranded DNA probe labeled at the BssHII site (Fig. 1D). The probe and 5 μg of total RNA were denatured at 85°C, hybridized at 65°C overnight, digested with 1,000 U of S1 nuclease (GIBCO) at 40°C for 40 min, and electrophoresed on a 5% polyacrylamide–8 M urea sequencing gel together with a sequence ladder of the same DNA. Sequencing was performed by the Sanger method (45), using Sequenase version 2.0 reagents (United States Biochemical, Cleveland, Ohio). The primer sequence was 5'-CGCGC CGCGGCTCGTGGG-3', of which the 5'-terminal nucleotide corresponds to the labeled nucleotide of the S1 probe.

Isolation of mRNA. Polyadenylated mRNA and nonpolyadenylated RNA in total cell RNA were separated by using the PolyATract mRNA isolation system (Promega). Total cell RNA was isolated as described above from NB41A3 cells mock infected or infected with 10 PFU of either n12 or KOS per cell and harvested at 24 hpi.
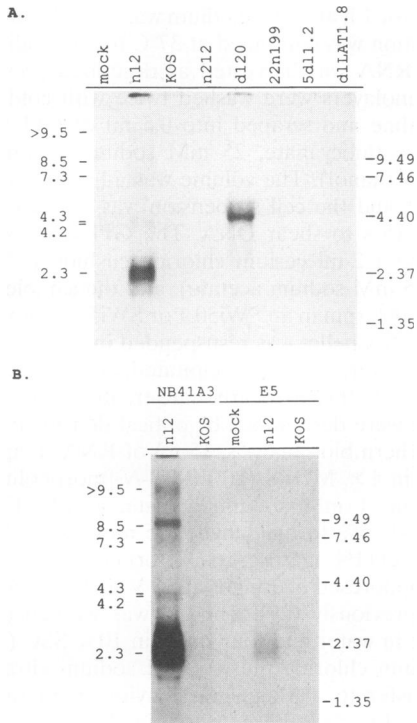
FIG. 2. Northern blot analysis of total RNA from KOS- and mutant virus-infected cells. (A) NB41A3 cells were mock infected or infected with KOS or mutant viruses *n*12 and *d*120 (ICP4), *n*212 (ICP0), 22/*n*199 (ICP22), 5*d*11.2 (ICP27), and *d*/LAT1.2 (LATs) at a multiplicity of 10 PFU per cell. Total RNA was isolated at 18 hpi, separated electrophoretically, and transferred to Magnagraph paper. The viral transcripts were detected by Northern blot analysis using riboprobe EBN9-LAT (Fig. 1C). The locations of RNA size markers are indicated on the right in kilobases; the approximate sizes of the transcripts detected are indicated on the left in kilobases. (B) NB41A3 and E5 cells (ICP4-expressing Vero cells [17]) were mock infected or infected with KOS or *n*12. Total RNA was analyzed by Northern blot hybridization as described above.

RESULTS

A novel family of viral transcripts specified in part by *b* repeat sequences is synthesized in cells infected with ICP4 null mutant viruses. As part of efforts to fine map the LATs expressed from the *b a c* repeat sequences in cells of neural origin, we performed Northern blot analysis of RNA obtained from NB41A3 cells infected with wild-type HSV-1 strain KOS or KOS mutant viruses. ICP4 null mutants *n*12 and *d*120 (Fig. 1E) were used in these experiments in order to increase the levels of detectable LATs, since ICP4 has been shown in transient assays to suppress LAT expression (2). Viruses with mutations in the genes for ICP0 (*n*212), ICP27 (5*d*11.2), and ICP22 (22/*n*199) were also used to determine whether, in the absence of these IE proteins, the LATs might be expressed more efficiently. The LAT deletion mutant *d*/LAT1.8 (Fig. 1E) was used as a LAT-minus control virus.

As shown in Fig. 2A, riboprobe EBN9-LAT detected abundant transcripts of approximately 2.3 kb, and less abundant transcripts of 4.2, 7.3, 8.5, and >9.5 kb, in *n*12-infected cells but not in cells infected with KOS, *n*212, 22/*n*199, 5*d*11.2 or *d*/LAT1.8. In cells infected with the ICP4 deletion mutant *d*120, a single abundant transcript of approximately 4.3 kb was detected. Given the size of the deletion in *d*120 (4.1 kb), the 4.3-kb transcript in *d*120-infected cells may be a stable but
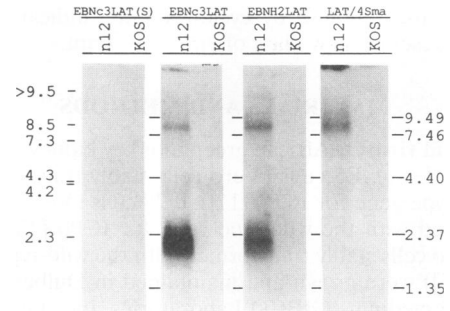

FIG. 3. Physical mapping of viral transcripts present in total cell RNA from KOS- and *n*12-infected NB41A3 cells by Northern blot analysis. NB41A3 cells were mock infected or infected with KOS or *n*12 at 10 PFU per cell. At 18 hpi, total RNA was harvested, separated, and transferred to Magnagraph paper as described in the text. The RNA blot was cut into four strips, and each strip was probed with the riboprobes indicated. No signal was detected by any probe in mock-infected cell RNA (data not shown). RNA size markers are shown on the right in kilobases, and the sizes of the transcripts detected are indicated on the left in kilobases.

deleted form of the larger 8.5-kb species seen in *n*12-infected cells. Close inspection of underexposed gels indicates that the 2.3-kb species synthesized in *n*12-infected cells consists of a series of transcripts differing in size by a uniform unit length. It was not possible to detect the 4.2-, 7.3-, and >9.5-kb transcripts reproducibly or in sufficient quantity to map because of their low abundance in these tests (see also Fig. 3, 5, and 6). Consequently, the relationship of these transcripts to the more abundant 2.3- and 8.5-kb species remains unclear. Transcripts corresponding to the low-abundance 6.3- and 8.0-kb LATs (Fig. 1B) were not detected in these experiments.

The results of initial Northern blot analysis using riboprobe EBN9-LAT as a probe demonstrated that a series of transcripts encoded in part by sequences in the *b* repeats is expressed at high levels in the absence of ICP4, but not in KOS-infected cells or in cells infected with mutants defective in ICP0, ICP22, ICP27, or the LATs. These results also demonstrated that L/ST expression is not dependent on the synthesis of viral DNA, since no viral DNA is synthesized in *n*12-infected cells (20, 23). Identical results were obtained in Vero, HEL, and PC12 cells (data not shown).

Evidence that expression of the L/STs is repressed in the presence of ICP4 was obtained by infecting ICP4-expressing E5 cells with *n*12 and KOS (Fig. 2B). In these tests, low levels of the 2.3-kb transcript were detected in *n*12-infected but not in KOS-infected E5 cells. In contrast, in *n*12-infected NB41A3 (Fig. 2B) and Vero cells (not shown), substantial and approximately equal amounts of the 2.3-kb transcripts were synthesized, whereas no such transcripts were evident in KOS-infected NB41A3 or Vero cells. Because E5 cells express ICP4 only at low levels (i.e., levels that are insufficient to fully complement ICP4 null mutants [18]), synthesis of the 2.3-kb transcripts was not fully suppressed in these cells. Repression of L/ST expression by ICP4 will be considered further below.

Physical mapping of the transcripts. We next used a series of contiguous strand-specific riboprobes to better define the limits of the transcripts in *n*12-infected NB41A3 cells. RNA from KOS-infected cells was used as the negative control. When infected NB41A3 cell RNA was harvested at 18 hpi and examined by Northern blot analysis, the L/STs were detected in *n*12- but not in KOS-infected cells (Fig. 3). In three independent tests, the abundant 2.3-kb transcript was detected

by using probes EBNc3-LAT and EBNH2-LAT; however, probes able to detect upstream and downstream sequences [EBNc3-LAT(S) and LAT/4Sma, respectively] did not detect this transcript. A riboprobe derived from the *Xcm*I-*Hinc*II fragment (map units 126.6 to 128.2) (Fig. 1C) also failed to detect the 2.3-kb transcript (data not shown), indicating that the 3' terminus of the 2.3-kb species is near the *Xcm*I site. The larger, less abundant 8.5-kb transcript was detected with probes EBNc3-LAT, EBNH2-LAT, and LAT/4Sma but not with probe EBNc3-LAT(S) (Fig. 3). The absence of detectable hybridization with EBNc3-LAT(S) suggests that the 2.3- and 8.5-kb transcripts are 5' coterminal near the *Stu*I site (Fig. 1B). The 8.5-kb transcript was also detected with riboprobes 22SS and 22KS (Fig. 1C), which hybridize to the ICP22 transcript (data not shown).

Collectively, physical mapping of the 2.3- and 8.5-kb transcripts suggests that a 5' start site is shared by both species and that this site lies in the *b* repeats near the *Stu*I site (Fig. 1B). Both transcripts span the L/S junction, and the 2.3-kb transcript likely terminates in the *c* repeats near the *Xcm*I site. Given its estimated size in gels and assuming a start site near the *Stu*I site in the *b* repeats, the 8.5-kb transcript probably terminates near the *Sph*I site in ICP22 coding sequences in U_S (Fig. 1B). Because these novel transcripts span the L/S junction, they have been designated L/STs.

Mapping of the 5' end of the L/STs. To identify the 5' start site of the L/STs, S1 nuclease mapping was performed. The probe used in these tests was the 443-bp *Stu*I-*Bss*HII fragment, labeled at the *Bss*HII terminus (Fig. 1D). As shown in Fig. 4, a single 5' terminus corresponding to a C residue that lies 28 bp downstream of a TATA box and 6 bp downstream of an ICP4 consensus binding site (ATCGTC) was identified. The identification of a single 5' start site near the center (bp 221) of the 443-bp *Stu*I-*Bss*HII probe and the failure to detect hybridizable RNA by using the EBNc3-LAT(S) riboprobe (Fig. 3) suggest that at least the abundant 2.3- and 8.5-kb species of L/ST begin here.

The L/STs are expressed with late kinetics in ICP4 null mutant virus-infected cells. To examine the kinetics of L/ST expression, a time course experiment was performed with RNA from *n*12-infected NB41A3 cells (Fig. 5). Total RNA was harvested at 6-h intervals through 24 hpi, and Northern blots were probed with riboprobe EBN9-LAT. The 2.3-, 4.2-, and 8.5-kb L/STs were first evident at 6 hpi and accumulated with time through 24 hpi. In these tests, the 7.3-kb species was also visible at 24 hpi. No transcripts were detected in RNA preparations from KOS-infected cells at 6, 12, or 18 hpi, but a very faint band which may correspond to the 2.3-kb species was detected at 24 hpi. The kinetics of accumulation of the 4.2- and 8.5-kb transcripts (and to a lesser extent the 7.3-kb transcript) in parallel with the 2.3-kb transcript likely reflects a common promoter for these transcripts.

The L/STs are polyadenylated. The polyadenylation status of the L/STs was next determined. Total cell RNA was separated into polyadenylated and nonpolyadenylated RNA by using a poly(dT) affinity separation system. RNA species were separated and examined by Northern blot analysis using riboprobe EBN9-LAT (Fig. 1C). As shown in Fig. 6, the L/STs were detected among polyadenylated RNAs (lane 5). A duplicate blot was probed with a combination of riboprobes BbSLAT to detect the LATs (lane 9) and 4Sma to detect the ICP4 transcript (lanes 11 and 12) as controls for poly(A)⁻ and poly(A)⁺ RNAs, respectively.

L/ST expression is repressed by ICP4 peptides able to bind the consensus sequence, ATCGTC. As noted above, the 5' start site of the L/STs is located 6 bp downstream of a consensus
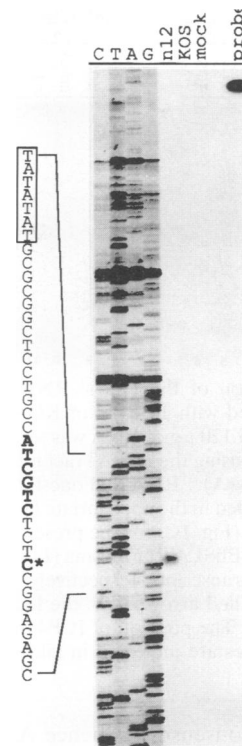

FIG. 4. S1 nuclease analysis of the 5' end of the L/STs. RNA from NB41A3 cells mock infected or infected with KOS or *n*12 was harvested at 18 hpi. Five micrograms of RNA was hybridized to the *Stu*I-*Bss*HII probe (Fig. 1D) and digested with 1,000 U of S1 nuclease. DNA sequencing was performed by the Sanger method (45). The nucleotide to which the *n*12 band corresponds is the C, indicated by the asterisk. The sequence upstream of the transcriptional start site, including a TATA box and a consensus ICP4 binding site (ATCGTC), is shown on the left.

ICP4 binding site, suggesting that L/ST synthesis might be repressed in the presence but not in the absence of ICP4. To test this hypothesis, we made use of the series of KOS-derived ICP4 *ts*, nonsense, and deletion mutant viruses generated in this laboratory (16a, 19, 20, 23) (Fig. 1E). These mutants encode forms of ICP4 that differ in the ability to localize to the
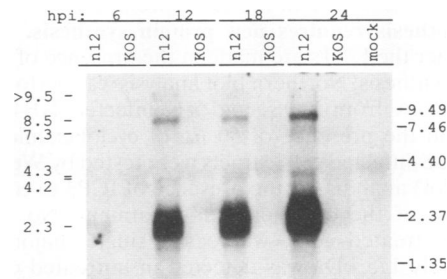

FIG. 5. Kinetics of expression of the L/STs in NB41A3 cells. RNA from NB41A3 cells infected with 10 PFU of KOS or *n*12 per cell was harvested at 6-h intervals through 24 hpi. Mock-infected cells were harvested at 24 hpi. RNA was analyzed by Northern blot hybridization with riboprobe EBN9-LAT (Fig. 1C). The sizes of the four L/STs are indicated on the left in kilobases; RNA size markers are shown on the right in kilobases.
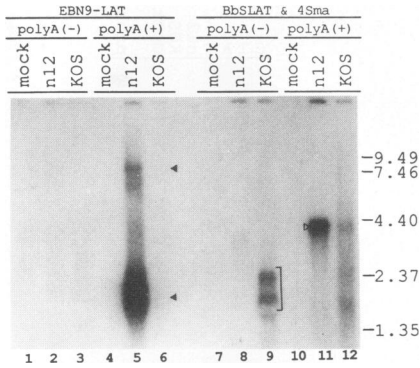
FIG. 6. Polyadenylation of the L/STs. RNA from NB41A3 cells mock infected or infected with 10 PFU of KOS or $n12$ per cell was harvested at 24 hpi. Then 120 μg of RNA was separated into poly(A)$^+$ and poly(A)$^-$ species by using the PolyATract mRNA isolation system (Promega); 15 μg of poly(A)$^-$ RNA and one-fourth the total yield of poly(A)$^+$ RNA were loaded in the appropriate lanes. Lanes 1 to 6 were probed with EBN9-LAT (Fig. 1C) for the presence of the L/STs; lanes 7 to 12 were probed with BbSLAT and 4Sma (Fig. 1C) for the presence of the LATs and ICP4 transcripts, respectively. The locations of the L/STs are indicated by filled arrowheads; the location of the LATs is indicated by the bracket. The position of ICP4 mRNA is indicated by the open arrowhead. Sizes are indicated in kilobase pairs.

nucleus, bind to the consensus sequence ATCGTC, and activate expression of viral genes. As shown in Fig. 7, the L/STs were abundant in cells infected with nonsense mutants $n12$ (positive control), $n214$, $d2$, and $d156$ but were not detectable in cells infected with $n6$ or $n215$. In addition, the L/STs were not detected in cells infected with tsB2 incubated at 34°C, the permissive temperature (negative control). In tsB2-infected cells incubated at 39.6°C, the nonpermissive temperature, only low levels of the 2.3-kb L/ST were observed. With the exception of the mutant form of ICP4 synthesized in tsB2-infected cells at 39.6°C, which has not been well characterized, abundant L/STs were present only in cells infected with mutant viruses that specify ICP4 peptides unable to bind the consensus site, ATCGTC (20). Mutant viruses tsB2 (34°C), $n6$, and $n215$, encoding ICP4 peptides able to bind to this site, induced no detectable L/STs. Evidence that the ICP4 genes of all mutants were expressed is shown by the presence of abundant ICP4 transcripts in all lanes but tsB2 (34°C) (Fig. 7B); ICP4 transcripts are not abundant in tsB2-infected cells late in infection at 34°C (23). These findings strongly suggest that ICP4 serves to repress expression of the L/STs through interactions with the ATCGTC sequence in the L/ST promoter.

L/ST synthesis requires new protein synthesis. To determine whether the L/STs are made in the presence of inhibitors of protein synthesis, Northern blot analysis was performed with total cell RNA from KOS- and $n12$-infected NB41A3 cells incubated in the presence of 50 μg of cycloheximide per ml (Fig. 8). KOS-infected cell extracts were tested by Western blot (immunoblot) analysis for the presence of ICP4 to confirm the effectiveness of the cycloheximide treatment. No ICP4 was detected in treated cells, whereas a single major band of approximately 175 kDa was detected in untreated cells (data not shown). As in the Northern blot analyses described above, all five species of L/STs were present in untreated $n12$-infected cells, and no L/STs were detected in RNA from untreated KOS-infected cells. The L/STs were not detected, however, in extracts derived from cycloheximide-treated cells infected with either $n12$ or KOS. In the same experiment, RNA from both KOS- and $n12$-infected cells, treated and untreated, contained
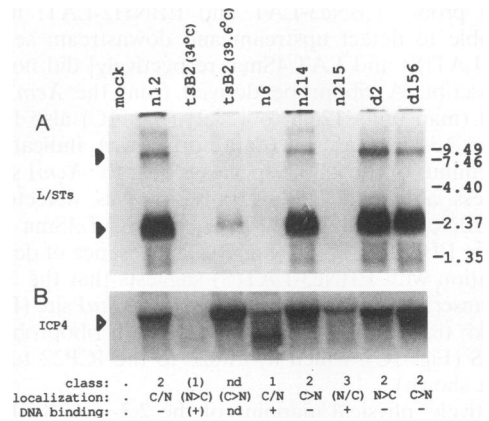

FIG. 7. Northern blot analysis of total RNA from ICP4 mutant virus-infected cells. NB41A3 cells were mock infected or infected with ICP4 mutant virus $n12$, $n6$, $n214$, $n215$, $d2$, $d156$, or tsB2 at a multiplicity of 10 PFU per cell. Cells infected with nonsense and deletion mutants were incubated at 37°C, and cells incubated with tsB2 were incubated at 34 and 39.6°C. Total cell RNA was isolated at 18 hpi, separated electrophoretically, and transferred to Magnagraph paper. In panel A, the L/STs (indicated by arrowheads) were detected by Northern blot analysis using riboprobe EBN9-LAT (Fig. 1C). On a duplicate blot (B), the ICP4 transcripts (indicated by the arrowhead) were detected by using riboprobe 4Sma (Fig. 1C). For each mutant, the class of the ICP4 mutation (1, 2, or 3), cellular localization (C, cytoplasmic; N, nuclear), and DNA binding ability of the mutant ICP4 protein as defined by DeLuca and Schaffer are listed (20). These properties of tsB2 at 34°C are inferred from those of wild-type ICP4. The localization of tsB2 ICP4 at 39.6°C was determined in this laboratory by Zhu (59). The localization of $n215$ has been shown to be both nuclear and cytoplasmic, depending on the multiplicity of infection (20, 59). nd, not determined.

ICP0-specific RNA (data not shown). Together, these findings indicate that expression of the L/STs is dependent upon the de novo synthesis of other viral and/or cellular proteins in $n12$-infected cells.
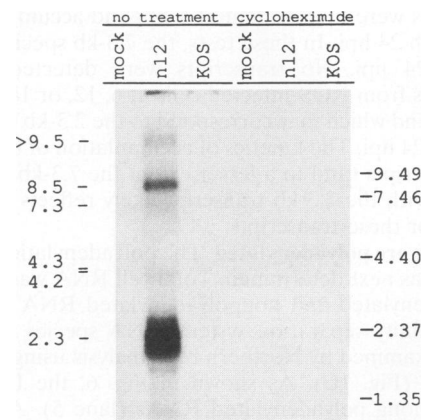

FIG. 8. Effect of cycloheximide on expression of the L/STs. NB41A3 cells were treated with 50 μg of cycloheximide per ml for 1 h prior to mock infection or infection with 10 PFU of KOS or $n12$ per cell. Untreated cells were included as controls. RNA was harvested at 12 hpi and analyzed by Northern blot hybridization using riboprobe EBN9-LAT (Fig. 1C). The sizes of the L/STs are indicated on the left in kilobases; RNA size markers are shown on the right in kilobases.
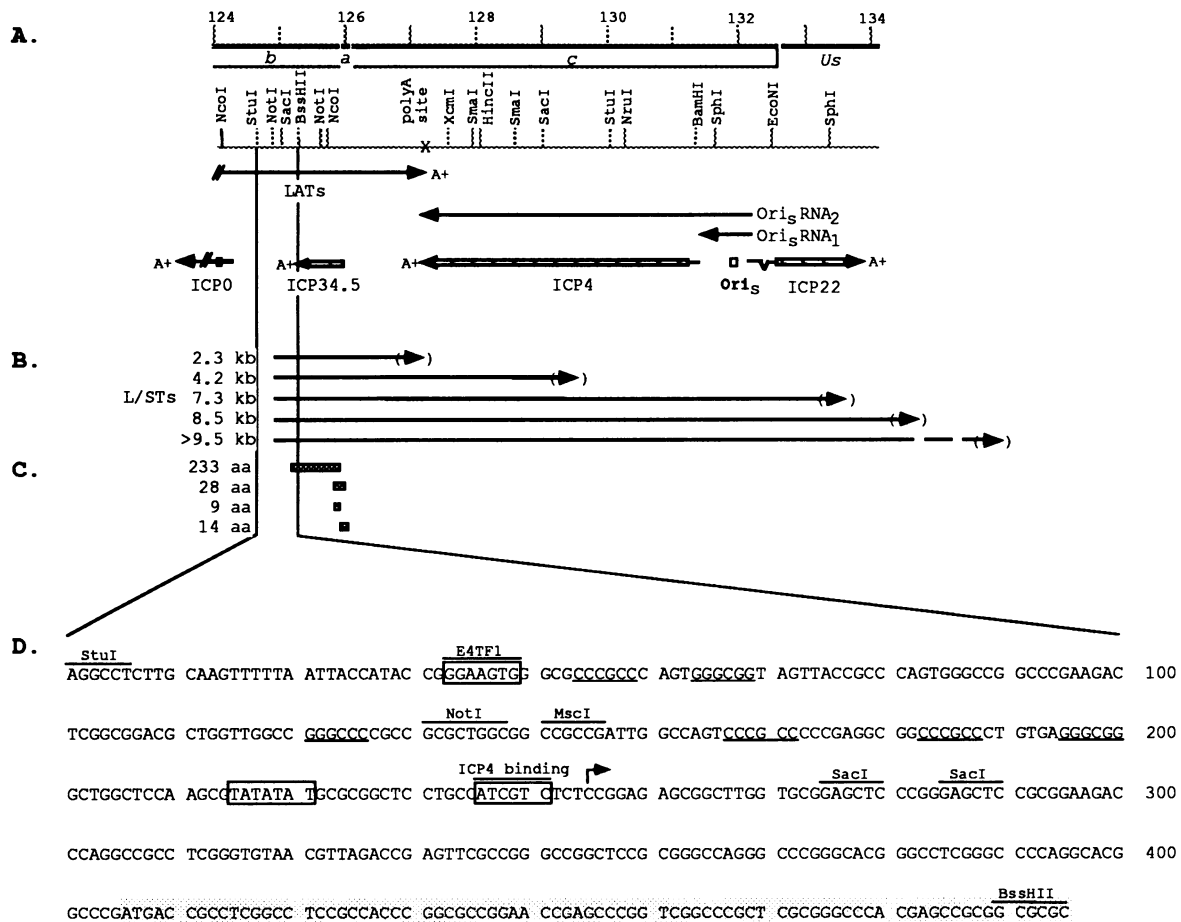
FIG. 9. Expanded physical map of the region of HSV-1 DNA encoding the L/STs. (A) Beneath the scale of kilobase pairs are shown the locations of the *b*, *a*, and *c* sequences and of relevant restriction sites in KOS DNA. Beneath the map of restriction sites are shown the 3' end of the LATs, the 5' end of the ICP0 transcript, and the intact transcripts encoding ICP34.5, ICP4, and ICP22. OriS RNA1 and RNA2 are also shown. OriS is located between the 5' start sites of the ICP4 and ICP22 transcripts. (B) Locations of sequences specifying the L/STs. The direction of transcription is indicated by the arrows. The 5' ends of the transcripts lie between the *Not*I and *Sac*I sites. The 3' ends of the transcripts have not been mapped and are shown in parentheses. (C) The locations of the four potential ORFs within the abundant 2.3-kb L/ST are shown as boxes. aa, amino acids. (D) Nucleotide sequence of HSV DNA between the *Stu*I and *Bss*HII sites. The E4TF1 recognition site (31) and the ICP4 binding site (ATCGTC) are shown as closed boxes beneath lines. The TATA box is shown as a closed box. The sequence specifying the N terminus of the 233-amino-acid ORF is shaded. The transcriptional start site is indicated by an arrow.

DISCUSSION

The L/STs: a new class of HSV-1 transcript. A family of transcripts that span the junction between the L and S components of the HSV-1 genome, the L/STs, has been identified by Northern blot analysis in RNA from cells of neural and nonneural origin infected with ICP4 null mutants (Fig. 9). The abundant expression of the L/STs only in the absence of functional ICP4, their expression with late kinetics, and the sensitivity of their synthesis to cycloheximide in ICP4 mutant-infected cells indicate that they are not members of any of the recognized kinetic classes of HSV-1 transcripts but constitute a new class of viral transcript.

Location and properties of the L/STs. The location of a single 5' start site at nucleotide 125042, together with the failure to detect hybridizable transcripts with riboprobe EBNc3-LAT(S), 710 bp to the left of the *Stu*I site at nucleotide 124818, indicate that the L/STs are 5' coterminal and that their expression is regulated by the bidirectional promoter described by Bohenzky et al. (4). The fact that the L/STs are expressed in abundance only in the absence of ICP4, or in the presence of

ICP4 peptides that are unable to bind ATCGTC, is consistent with the presence of a consensus ICP4 binding site 6 bp upstream of the start site and may explain the difficulties experienced by Bohenzky et al. in identifying and mapping these transcripts in RNA from wild-type virus-infected cells. Although we have not yet mapped the 3' end of the 2.3-kb L/ST species, we estimate that the termination site lies near the polyadenylation sites utilized by ICP4 mRNA and the large LATs (Fig. 9A and B). Notably, the abundant 2.3-kb species appears to consist of a series of transcripts that differ in size by a uniform length. Should these abundant transcripts be both 5' and 3' coterminal, it is possible that these small size differences arise from the presence of serially repeated DNA sequences located to the right of the *a* sequence which vary in copy number among genomes within a given virus preparation as described by Davison and Wilkie (12) and McGeoch et al. (36, 38).

The sequence of the HSV-1 strain 17 genome encoding the 2.3-kb L/STs has been determined by Davison and Wilkie (12) and Murchie and McGeoch et al. (38), and the sequences of

the bidirectional promoter and the *a* repeat have been determined for strain KOS (4, 48). The DNA template specifying the 2.3-kb L/STs contain one long continuous open reading frame (ORF) and three short ORFs (Fig. 9C). The first and longest ORF starts at nucleotide 125180 and extends for 702 bp to nucleotide 125881. It is 233 amino acids in length and extremely arginine rich. A search of the protein data base revealed limited homology between this L/ST ORF, the early protein EP0 of suid herpesvirus 1 (7), and EBNA-1 of Epstein-Barr virus (43). The second ORF consists of only 87 bp (nucleotides 125833 to 125919). The third and fourth ORFs are even smaller, consisting of only 30 and 45 bp, respectively. None of the four ORFs extends into the *a* sequence (125955 to 126373). Notably, ORF1, and indeed the L/STs themselves, do not appear to be essential for virus replication because the mutations in several viable mutants in the ICP34.5 gene, which also disrupt the L/STs and ORF1, have been isolated by Chou et al. (8). On the other hand, because the mutations in the ICP34.5 gene disrupt the L/STs, it is unclear whether the altered virulence of the ICP34.5 mutants in mice is a consequence of mutations in sequences specifying ICP34.5 or the L/STs.

Factors affecting L/ST expression. A distinguishing feature of any gene or *cis*-acting element contained totally within repeat sequences is that it is diploid. For genes encoding *trans*-acting factors, the amplification of the protein product that results from the expression of both genes has significant implications for gene regulation. For example, the expression of two copies of ICP0 rather than one doubles the viral yield and the efficiency of virus reactivation from latency in a mouse model (5). An additional instance of diploidy involves the L/S junction. Any gene containing the L/S junction would be diploid only when the genome is circular, i.e., immediately after the genome enters the nucleus during productive infection, and during latency. In addition, junction-spanning genes would occur in higher copy number in concatemeric molecules generated during viral DNA replication relative to single, linear packaged genomes.

Because the expression of ICP4 is essential for DNA replication but repressive for expression of the L/STs, and because L/ST expression was not detected in KOS-infected cells at the time of peak DNA replication (6 to 12 hpi), the L/STs are not likely expressed from concatemers. Our studies show that the L/STs are synthesized in abundance in cells infected with ICP4 null mutants and at low levels in cells expressing low levels of ICP4 (E5 cells) but are undetectable in cells infected with KOS or ICP0, ICP22, ICP27, and LAT null mutants (all of which express high levels of ICP4). Moreover, L/ST synthesis is completely inhibited in ICP4 null mutant-infected cells in the presence of cycloheximide. Collectively, these observations suggest that ICP4 represses expression of the L/STs and that L/ST expression requires either one or more of the viral proteins synthesized in ICP4 null mutant-infected cells (i.e., ICP0, ICP6, ICP22, ICP27, or ICP47) and/or a cellular protein(s) induced by one of these viral proteins. The proximity to the L/ST start site of an ICP4 binding site long recognized to mediate the repressive effects of ICP4 and the failure of mutant forms of ICP4 unable to bind this site to inhibit L/ST expression support the hypothesis that ICP4 represses L/ST expression.

Among the viral regulatory proteins expressed in ICP4 null mutant-infected cells that may, in theory, be involved in activating L/ST expression, Bohenzky et al. have shown that the L/ST promoter is only modestly activated by ICP0 and weakly activated by VP16. Hence, the probability that either ICP0 or VP16 alone induces L/ST expression at such high levels is not great. ICP22 has recently been shown to repress the transactivating activities of ICP0 and ICP4 (1), and ICP27 is also able to repress ICP0- and ICP4-induced activation of viral genes. By virtue of their ability to repress the transactivating activity (albeit not the synthesis) of ICP4, either of these proteins might play a role in activating L/ST expression. Finally, ICP6 has recently been reported to possess phosphorylating activity in addition to its role as the large subunit of ribonucleotide reductase (11). ICP6 is present in abundance at IE times and may function as a protein kinase to regulate the activities of other IE and cellular proteins.

When do the L/STs function? Generally speaking, the kinetics of expression of individual viral genes parallels the time period during viral replication when the products of these genes function. If one assumes that this is the case for the L/STs, when might they function during replication? Given the observations that the L/STs accumulate in abundance with late kinetics in the absence of ICP4, that they would be expressed at maximum levels from a diploid template in circular genomes, and that they likely require other HSV IE proteins or cellular proteins for their synthesis, when during the life cycle of HSV-1 are the L/STs likely to be synthesized in greatest abundance? Available evidence indicates that ICP4 is not expressed (or is expressed only at low levels) (i) in productive infection prior to IE gene expression, (ii) at late times in the viral replication cycle, and (iii) during latency. Because the L/STs are expressed with late kinetics in the absence of ICP4, it is unlikely that they are synthesized prior to IE gene expression in productive infection. High levels of ICP4 are present at IE and E times, and low levels of ICP4 persist even late in infection. Moreover, the synthesis of low levels of L/STs in *n*12-infected E5 cells which express low levels of ICP4 indicates that they might be synthesized late in infection when levels of ICP4 have dropped. The expression of very low levels of the 2.3-kb L/STs late in KOS infection (Fig. 5) would support this hypothesis. That the L/STs function late in productive infection is plausible but unlikely because (i) virus replication was complete when the L/STs were detected in KOS-infected cells (24 hpi; Fig. 5) and (ii) the extremely low levels of L/STs detected at late times argue against their functioning maximally at this time. On the other hand, it may be that the L/ST gene product functions during the succeeding replication cycle as does VP16 (24). One must also consider the stages of the viral life cycle that include the establishment, maintenance, and reactivation of latency as times when the ICP4 gene is repressed. We have examined mouse ganglia latently infected with wild-type strain KOS for L/ST expression and have detected none. Furthermore, previous studies using L/ST template sequences as probes in in situ hybridization tests did not detect these transcripts in latently infected ganglia (14, 33, 37, 40, 50). Thus, it appears as if the L/STs are not present (at least not at high levels) in latently infected ganglionic neurons during the maintenance of latency.

The absence of L/ST expression in latently infected ganglionic neurons is in sharp contrast to the abundant expression of the LATs in these cells. Indeed, expression of these two families of transcripts appears to be mutually exclusive both in vivo, as just noted, and in vitro, where abundant LATs are detected only in the presence of ICP4, whereas L/STs are detected only in the absence of ICP4. Thus, we attribute the absence of L/ST expression in *dl*LAT1.8-infected cells to the expression of ICP4. The definitive test of the relationship of the LATs and the L/STs will require construction of an L/ST⁻ mutant virus (which should express the LATs), and a LAT⁻ ICP4⁻ mutant virus (which should express the L/STs).

One must also consider the possibility that the L/STs are

expressed only during a brief period of time during HSV-1 infection, such as during the switch from lytic infection to latency, or during the sequence of events that lead to reactivation. ICP4 is known to be actively repressed during the establishment of latency and immediately prior to reactivation (50), yet at both stages of latency, the viral genome is circular and other IE proteins and cellular proteins may be synthesized, facilitating expression of the L/STs. To determine whether this is indeed the case, Northern blot analysis of infected ganglia during the establishment of and reactivation from latency must be conducted.

In this report, we have described a new class of HSV-1 transcript represented by the L/STs. Identification of the specific function(s) of the L/STs will necessitate the isolation and characterization of mutant viruses which fail to synthesize one or more of the L/STs. Whether the L/STs are the only representatives of this new class of transcript or whether additional transcripts of this class are encoded by other regions of the viral genome remain to be determined.

ACKNOWLEDGMENTS

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