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A Novel Herpes Simplex Virus Type 1 Transcript (AL-RNA) Antisense to the 5′ End of the Latency-Associated Transcript Produces a Protein in Infected Rabbits

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Following primary ocular infection, herpes simplex virus type 1 (HSV-1) establishes a lifelong latent infection in sensory neurons of the trigeminal ganglia. Latency-associated transcript (LAT), the only known viral gene abundantly transcribed during HSV-1 neuronal latency, is required for high levels of reactivation. Recently we showed that three different mutants that do not alter the LAT promoter but contain deletions within the 5′ end of the primary LAT transcript affect viral virulence (G. C. Perng et al., J. Virol. 75:9018-9028, 2001). In contrast, in LAT-null mutants viral virulence appears unaltered (T. M. Block et al., Virology 192:618-630, 1993; D. C. Bloom et al., J. Virol. 68:1283-1292, 1994; J. M. Hill et al., Virology 174:117-125, 1990; G. C. Perng et al., J. Virol. 68:8045-8055, 1994; F. Sedarati, K. M. Izumi, E. K. Wagner, and J. G. Stevens, J. Virol. 63:4455-4458, 1989). We therefore hypothesized that the 5′ end of LAT and/or an as yet unidentified gene that overlaps part of this region is involved in viral virulence. We report here on the discovery and initial characterization of a novel HSV-1 RNA consistent with such a putative gene. The novel RNA was antisense to the 5′ end of LAT and was designated AL-RNA (anti-LAT sense RNA). The AL-RNA overlapped the core LAT promoter and the first 158 nucleotides of the 5′ end of the primary LAT transcript. AL-RNA was detected in extracts from neuron-like cells (PC-12) infected with wild-type HSV-1 but not in cells infected with a mutant with the AL region deleted. The deletions in each of the above three mutants with altered virulence encompass the 5′ end of the AL-RNA, and these mutants cannot transcribe AL. This supports the hypothesis that the AL gene may play a role in viral virulence. Based on comparison to the corresponding genomic sequence, the AL-RNA did not appear to be spliced. The AL-RNA was polyadenylated and contained an open reading frame capable of encoding a protein 56 amino acids in length with a predicted molecular mass of 6.8 kDa. Sera from three of three rabbits infected with wild-type HSV-1 but not sera from any of three rabbits infected with a mutant with the AL-RNA region deleted recognized the *Escherichia coli* recombinantly expressed AL open reading frame on Western blots. In addition, four of six rabbits infected with wild-type virus developed enzyme-linked immunosorbent assay titers against one or more AL synthetic peptides. These results suggest that an AL protein is produced in vivo.

Herpes simplex virus type 1 (HSV-1) is a large (152-kbp) double-stranded neurotropic DNA virus. The majority of adults in the United States are HSV-1 seropositive. HSV-1 infects mucosal tissues, usually the mouth, nose, eye, or genital tract. Following ocular infection, HSV-1 travels up nerves and establishes latent infection in sensory neurons of the trigeminal ganglia. Although it is well known that the latent virus can reactivate at various times throughout the life of an individual and produce recurrent disease, the mechanism(s) by which this occurs remains to be fully elucidated. Recurrent ocular HSV-1 infection can produce corneal scarring leading to loss of vision. As a result, HSV-1 is one of the most common infectious causes of corneal blindness in the developed world.

Latency-associated transcript (LAT) is the only abundantly transcribed viral gene during neuronal latency (28, 35). The primary LAT transcript is 8.3 to 8.5 kb long (9, 43). A very stable intron, the 2-kb LAT, is spliced from the primary transcript (11). This 2-kb LAT intron is the major LAT RNA detected during latency (9, 32, 34, 38, 39, 42). The LAT gene is located in the viral long repeats and is thus present in two identical copies. As such, the LAT region accounts for over 16 kb of the 152-kb HSV-1 genome. Having one gene occupy over 10% of the genome of a virus that contains over 80 genes suggests that there is high selective pressure to maintain the sequence of the LAT region. Not surprisingly, there are also other genes in the LAT region. LAT overlaps the viral genes for ICP0 and ICP34.5 in an antisense direction (28, 35).

Studies with various LAT mutants have shown that LAT increases the induced and spontaneous reactivation phenotypes in the rabbit ocular model (14, 20) and the induced reactivation phenotype in mice (3, 8, 16, 23, 29, 33). This may be the result of LAT enhancing establishment of latency, LAT being directly involved in the reactivation stage, or a combina-

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tion of both (19, 25). Support for a role for LAT in the establishment of latency comes from reports showing that in rabbits and mice more neurons become latently infected with LAT$^+$ virus than with LAT$^-$ virus (25, 29, 36). In addition, LAT has antiapoptosis activity (1, 15, 19). This could protect acutely infected neurons from death and result in increased establishment of latency. It should be noted that these findings do not rule out an additional role for LAT in the reactivation stage. Interestingly, a LAT-null mutant containing the LAT promoter driving the first 1.5 kb of LAT from an ectopic location in the virus (22) and a mutant containing a polyadenylation signal inserted at LAT nucleotide 1499 (5) both have wild-type levels of reactivation. Thus, the major LAT function(s) involved in the latency-reactivation cycle appears to reside within the first 1.5 kb of the primary 8.3- to 8.5-kb transcript.

Although LAT-null mutants (i.e., mutants unable to transcribe any LAT) appear to have virulence properties similar to their wild-type parents (3, 4, 14, 20, 30), it has been proposed elsewhere that the 5′ end of LAT or a gene in this region of the HSV-1 genome may affect viral virulence (21, 41). This is based, at least partially, on the altered viral virulence of some LAT mutants with deletions in the 5′ end of the primary LAT transcript that do not block transcription of the remainder of LAT (21, 24). This suggests that there are two functions, one involved in reactivation and one involved in virulence, that map to the genomic location corresponding to the 5′ end of LAT (i.e., the first 1.5 kb of LAT discussed above). One or both of these functions may reside in LAT or in a gene overlapping the 5′ end of LAT. Several LAT sense transcripts that terminate just prior to the 5′ end of LAT have been reported previously (31, 41), and one of these transcripts has been proposed previously to be involved in virulence (41). However, since this transcript does not overlap any of the LAT RNA it is unlikely to account for the altered virulence seen for mutants containing deletions only within the LAT RNA (21, 24). Consequently, we searched for other possible non-LAT transcripts near the start of LAT transcription.

We report here the discovery of a transcript antisense to LAT (AL-RNA) that overlaps the 5′ end of LAT and the core LAT promoter. AL-RNA was detected by reverse transcription-PCR (RT-PCR) in PC-12 cells acutely infected with wild-type HSV-1 but not in uninfected PC-12 cells or PC-12 cells infected with the LAT-null mutant dLAT2903 (20) (which also has the entire AL region deleted). We mapped the 5′ and 3′ ends of the AL-RNA and found that the transcript is polyadenylated and extends from LAT nucleotide +158 to LAT nucleotide −198 (on the other DNA strand). The deletions within the three LAT mutants with altered viral virulence discussed above each remove the 5′ end of AL and hence result in AL-null mutants. The findings reported here are therefore consistent with the notion that AL is involved in viral virulence. We also found that serum from rabbits infected with wild-type HSV-1 recognized recombinantly expressed AL protein, suggesting that an AL protein is made in vivo.

MATERIALS AND METHODS

Virus and cells. All parental and mutant viruses were triple plaque purified and passaged only one or two times in rabbit skin (RS) cells prior to use. Wild-type McKrae, the McKrae-derived LAT$^-$ dLAT2903, and marker-rescued dLAT2903R viruses have been previously described (20). RS cells were grown in Eagle's minimal essential medium supplemented with 5% fetal calf serum. PC-12 cells were grown in the same medium supplemented with 10% fetal calf serum and 5% horse serum.

Rabbits. Eight- to 10-week-old New Zealand White male rabbits (Irish Farms) were used. Rabbits were treated in accordance with Association for Research in Vision and Ophthalmology, American Association for Laboratory Animal Care, and National Institutes of Health guidelines. Rabbits were bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU of virus into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s as we previously described (28).

RNA isolation. Subconfluent cell monolayers were infected at a multiplicity of infection (MOI) of 5 PFU/cell, total RNA was isolated at various times postinfection (p.i.), and Northern blot analysis was performed according to standard protocols with various ^{32}P-labeled oligonucleotide probes specific for the detection of anti-LAT transcripts in the immediate vicinity of the LAT promoter and the start of LAT transcription.

RT-PCR. Cells were infected at an MOI of 5 PFU/cell or mock infected. Poly(A) mRNA was isolated from total cell extracts with the PolyATract mRNA Isolation System IV (catalogue no. Z5310; Promega, Madison, Wis.), according to the manufacturer's directions. RT was done with the ThermoScript RT-PCR system (catalogue no. 11146-024; Invitrogen Life Technologies, San Diego, Calif.) according to the manufacturer's directions. The primer used for cDNA synthesis was either oligo(dT) or an oligonucleotide corresponding to HSV-1 genomic nucleotides 118661 to 118690 (LAT nucleotides −140 to −111; see Fig. 1E, primer a). All PCRs were done with primer a (see previous sentence) and primer d (corresponding to HSV-1 nucleotides 118910 to 118881 or LAT nucleotides 109 to 80; see Fig. 1E, primer d). The PCR was initiated at 90°C for 5 min. This was followed by 30 cycles of 94°C for 40 s, 60°C for 30 s, and 71°C for 90 s. Extension was at 72°C for 10 min. Southern analysis was performed on the RT-PCR products with a probe corresponding to HSV-1 nucleotides 118811 to 118840 (LAT nucleotides 10 to 39; see Fig. 1E, probe e).

5′ RACE. Poly(A)$^+$ RNA was isolated from PC-12 cells 6 h after infection at an MOI of 5 with a poly(dT) column. 5′ rapid amplification of cDNA ends (RACE) was done according to the protocol suggested by the manufacturer (Invitrogen Life Technologies).

DNA sequencing. Sequencing of cloned DNA was done by standard dideoxy sequencing.

Expression of AL protein in *Escherichia coli*. The AL open reading frame (AL ORF) from the ATG codon to the last amino acid before the stop codon was amplified from a cloned full-length RT-PCR product by PCR and fused to a His tag-containing plasmid (Xpress system protein expression pTrcHis; Invitrogen Life Technologies) and expressed in *E. coli* according to the manufacturer's protocol.

Anti-AL ORF peptide antibodies. Two peptides, one representing the putative 5′ end of the AL protein (peptide 1, GRARKVFRTTRDRHGC) and one representing the putative 3′ end of the AL protein (peptide 2, KAEMTQQKQA TAPRGC), were synthesized, and antibody was raised commercially in rabbits (BioSource International, Camarillo, Calif.). The *E. coli*-expressed AL ORF was recognized by both peptide antibodies on Western blots (data not shown), suggesting that these antibodies would be capable of detecting an AL protein produced by HSV-1. In some experiments, numerous AL-related bands were detected in a laddering pattern (data not shown), suggesting that the *E. coli*-expressed AL protein binds tightly to itself or other proteins.

Western blots. The *E. coli*-expressed AL-His tag fusion protein was either used as a crude total cell extract or partially purified with a His-Bind Quick Column (Novagen; catalogue no. 70159-4) according to the manufacturer's directions. The total extract or partially purified protein was run on standard 15% Tricine gels with a wide sample well and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was cut into strips, and individual strips were incubated with serum obtained from rabbits on day 57 p.i. The antibody bound to the blots was visualized by chemiluminescence with secondary antibody conjugated to horseradish peroxidase.

RESULTS

Detection of AL-RNA in tissue culture. For ease of understanding the results presented below, a schematic representation of the relative location of the AL gene, the RNA, and the putative ORF is shown in Fig. 1. Figure 1A shows a schematic presentation of the HSV-1 genome. The terminal and internal inverted long repeats are expanded to show the LAT region in

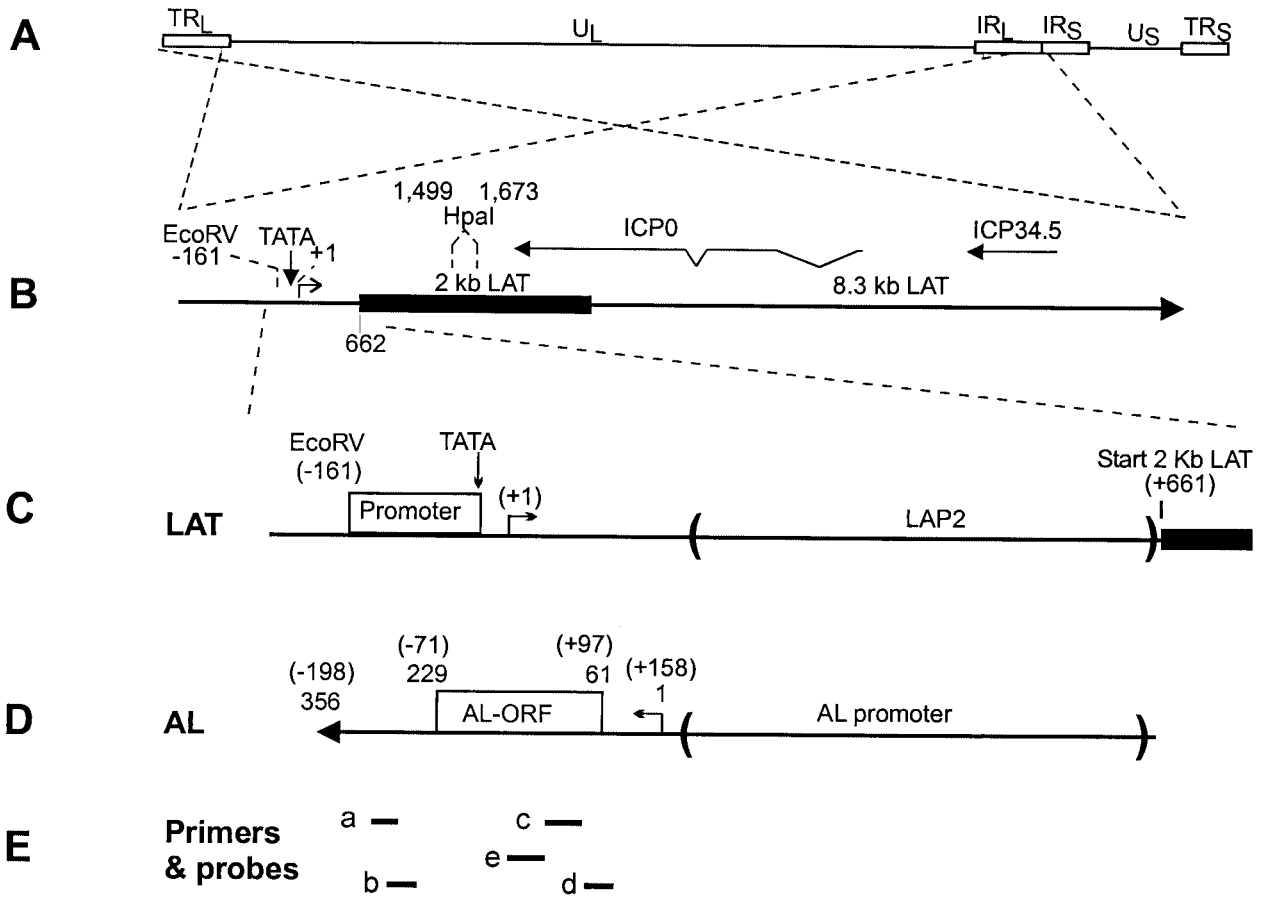

FIG. 1. Relative location of the AL gene. (A) Schematic representation of the wild-type HSV-1 genome. TR_L and IR_L indicate the terminal and inverted long repeats, respectively. IR_S and TR_S indicate the inverted and terminal short repeats, respectively. U_L and U_S indicate the unique long and unique short regions, respectively. The TR_L and IR_L are expanded, and the TR_L is flipped left to right as indicated by the dashed lines, so that both repeats can be represented together in the subsequent panels. (B) Blowup of the long repeats. The primary LAT transcript is indicated by the large arrow. The solid rectangle represents the very stable 2-kb LAT intron. The LAT TATA box is indicated by TATA. The start of LAT transcription is indicated by the arrow at +1 (genomic nucleotide 118801). Several restriction enzyme sites and the relative locations of the ICP0 and ICP34.5 transcripts are shown for reference. (C) Blowup of the 5′ LAT region. The LAT promoter (open rectangle), the start of LAT transcription (arrow, +1), a putative secondary LAT promoter (LAP2), and the beginning of the stable 2-kb LAT are indicated. Nucleotide positions relative to the start of LAT transcription are shown in parentheses. (D) AL gene. The DNA strand opposite that of LAT in panel C is shown. The numbers in parentheses indicate nucleotide positions relative to the start of LAT transcription on the other strand. The remaining numbers indicate nucleotide positions relative to the start of AL transcription. The ORF encoding a putative AL protein is shown as an open rectangle. The AL promoter is located somewhere between the large parentheses in the same region as LAP2. (E) Relative positions of oligonucleotide primers (a to d) and probes (e) used in this report. a, 118661 to 118690; b, 118681 to 118710; c, 118841 to 118870; d, 118881 to 118910; e, 118811 to 118840

more detail (Fig. 1B). The primary 8.3-kb LAT transcript is indicated by the large arrow. The stable 2-kb LAT (an intron) is indicated by the solid rectangle. The relative locations of the ICP0 and ICP34.5 mRNAs are shown for reference. The region near the 5′ end of LAT is expanded to show details of the LAT (Fig. 1C) and AL (Fig. 1D) regions. These two panels are shown at the same scale and are aligned vertically. The AL-RNA (Fig. 1D) is antisense to the LAT RNA (Fig. 1C). In panels C and D, the numbers in parentheses represent nucleotide positions relative to the start of LAT transcription while numbers not in parentheses represent nucleotide positions relative to the start of AL transcription. The locations of primers and probes used in this report are shown relative to AL and LAT in Fig. 1E.

Sequence analysis revealed a potential small ORF downstream of ICP0 in HSV-1 strains 17syn+ and McKrae, the only strains for which this sequence is available. This ORF (Fig. 1D, AL ORF) overlaps part of the LAT promoter and part of the LAT region that is required for spontaneous reactivation (the first 1.5 kb of the primary LAT transcript). Interestingly, the transcript that would encode this novel ORF would be expressed antisense to LAT and would not be contained within the ICP0 mRNA. To look for a potential RNA near the 5′ end of LAT that might encode this ORF, RS, CV-1, and PC-12 cells were infected with wild-type HSV-1 strain McKrae and Northern blot analyses were performed. Probes specific for the detection of anti-LAT transcripts in the immediate vicinity of the LAT promoter and the start of LAT transcription were

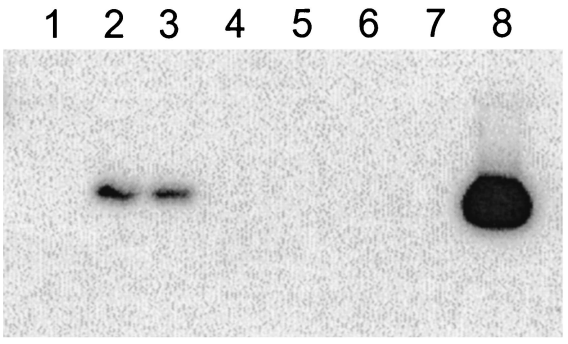

FIG. 2. Detection of AL-RNA by RT-PCR. PC-12 cells were infected at an MOI of 5. Total RNA was isolated 6 h p.i., and RT-PCR was performed as described in Materials and Methods. Lanes: 1, no RT, wild-type McKrae virus-infected PC-12 cells; 2, wild-type McKrae virus; 3, dLAT2903R; 4, dLAT2903; 5, uninfected PC-12; 6, no RT, dLAT2903; 7, no RT, uninfected PC-12; 8, marker, PCR of wild-type McKrae viral DNA.

used to detect this putative transcript. No such RNA was reproducibly detected on Northern blots in any of these cells (data not shown).

To look for a low-abundance transcript in this region, oligonucleotide primers corresponding to LAT nucleotides −140 to −110 and +109 to +80 (genomic nucleotides 118661 to 118690 and 118910 to 118881, respectively) (primers a and d in Fig. 1E) were used for RT-PCR. Primer a was also used for first-strand synthesis. Thus, the RT-PCR would produce a product only from RNA that was antisense to LAT. PC-12 cells were infected at an MOI of 5 with wild-type strain McKrae, and total RNA was isolated 6 h p.i. An RT-PCR product of the size predicted from the genomic sequence and that hybridized to an internal probe corresponding to LAT nucleotides 80 to 39 (genomic nucleotides 118811 to 118840; probe e in Fig. 1E), was readily detected (Fig. 2, lane 2). The expected size of the RT-PCR product is shown in lane 8, which shows the PCR product generated with wild-type McKrae genomic DNA with the same primers. As expected, no RT-PCR product was detected with RNA from uninfected PC-12 cells (lane 5) or when reverse transcriptase was left out of the reaction mixture (lanes 1, 6, and 7). In addition, no RT-PCR product was detected in PC-12 cells infected with the LAT$^-$ virus dLAT2903 (20) (lane 4). This was expected, since the deletion in this mutant (LAT nucleotides −161 to +1667) encompasses all of the RT-PCR primers. This acts as a further control to confirm that the RT-PCR product originated from the expected location. Also as expected, an RT-PCR product indistinguishable from that of wild-type-infected PC-12 cells was seen in PC-12 cells infected with marker-rescued dLAT2903R (lane 3). These results revealed the presence of an RNA antisense to and overlapping the 5′ end of LAT (AL-RNA).

Mapping the 5′ end of AL-RNA. The 5′ end of the AL-RNA was determined by 5′ RACE as described in Materials and Methods, with an internal primer corresponding to HSV-1 genomic nucleotides 118841 to 118870 (Fig. 1E, primer c). Briefly, PC-12 cells were infected with wild-type McKrae virus at an MOI of 5. Total RNA was isolated 6 h p.i., and poly(A)$^+$ RNA was isolated with a poly(dT) column. 5′ RACE mapped the 5′ end of the AL-RNA to nucleotide 118959 of the HSV-1 genome. This corresponds to LAT nucleotide +158, but on the other DNA strand (Fig. 1C).

Mapping the 3′ end of AL-RNA. The 3′ end of the AL-RNA was mapped by poly(A) selection and RT-PCR. Briefly, RT-PCR was performed on poly(A)$^+$ RNA isolated from PC-12 cells infected with wild-type McKrae virus as described above. Oligo(dT) was used as the first primer, and a synthetic oligonucleotide corresponding to LAT nucleotides −120 to −91 (genomic nucleotides 118710 to 118681; primer b, Fig. 1E) was used as the second primer. The resulting RT-PCR product was cloned and sequenced. Reading from the 5′ toward the 3′ end of the AL-RNA, the sequence was identical to that of the genomic sequence in this region up to genomic nucleotide 118603 (Fig. 3). This corresponds to the nucleotide complementary to LAT nucleotide −198. After this nucleotide, the sequence of the RT-PCR product diverged from the genomic sequence. The divergent sequence corresponded to a 22-nucleotide AU-rich region containing a consensus poly(A) attachment signal (AAUAAA) (40) followed by a 24-nucleotide-long poly(A) stretch.

The 22-nucleotide poly(A) attachment signal found in the AL-RNA was not present at this location in the McKrae genome (Fig. 3). However, this sequence was found on the other DNA strand at a location corresponding to AL nucleotides 227 to 248 (LAT nucleotides −91 to −70). In 17syn+, the only HSV-1 strain that has been completely sequenced (17, 18, 26),

```
                    5′ primer
            |---------------------------→|
118710 RNA TCCTCGTCTG CGGCCTAATG GATTTCCGGG CGCGGTGCCC CTGTCTGCAG
genome     ********** ********** ********** ********** **********


118660 RNA AGCACTTAAC GGATTGATAT CTCGCGGGCA CGCGCGCCCT TAATGGACCG
genome     ********** ********** ********** ********** **********

                                                 3′ primer(oligo-dT)
                                            |←-----------------------
118610 RNA GCGCGGGGAA AATAAAATTA CATCACCTAC AAAAAAAAAA AAAAAAAAAA AAAA
genome     ********
```

FIG. 3. Mapping the 3′ end of the AL-RNA. RT-PCR was performed on poly(A)$^+$ RNA isolated from PC-12 cells infected with wild-type McKrae virus, and the RT-PCR product was cloned and sequenced as described in Materials and Methods. The resulting sequence is labeled RNA. The "genome" sequence is from the same HSV-1 strain (McKrae). The asterisks in the "genome" sequence indicate identity to the RNA sequence. This identity ends after genomic nucleotide 118603. The positions of the 5′ primer and the 3′ oligo(dT) primer are shown.

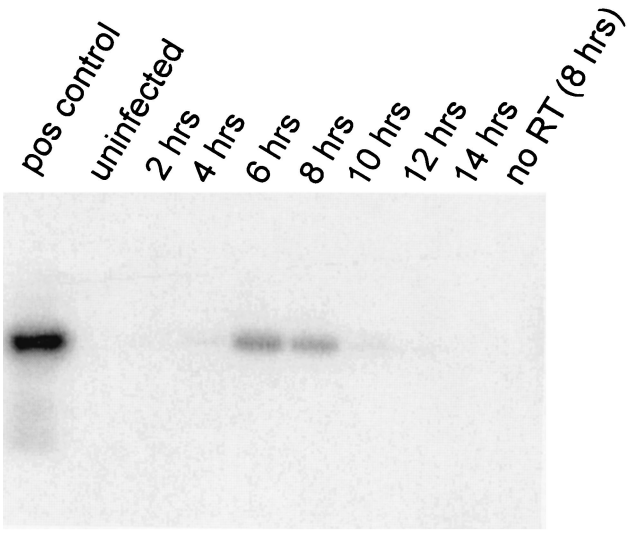

FIG. 4. Kinetics of AL-RNA expression. PC-12 cells were infected at an MOI of 5 with wild-type McKrae virus. Total RNA was isolated at various times p.i., and RT-PCR was performed as described in Materials and Methods. Lanes: 1, positive control (PCR of plasmid AL DNA); 2, uninfected; 3, 2 h p.i.; 4, 4 h p.i.; 5, 6 h p.i.; 6, 8 h p.i.; 7, 10 h p.i.; 8, 12 h p.i.; 9, 14 h p.i.; 10, no RT, 8 h p.i.

this 22-nucleotide sequence is present at the same location as in McKrae and was not found anywhere else in the viral genome. It is unclear how these 22 nucleotides were incorporated into the AL-RNA. We sequenced three independently generated and cloned AL RT-PCR products with identical results. Thus, it is extremely unlikely that the 22-nucleotide poly(A) attachment sequence was due to an artifact. Regardless of these 22 nucleotides, the above sequence information mapped the genomic location of the 3′ end of the AL-RNA to genomic nucleotide 118603.

Potential location of the AL promoter. There is no TATA box located near the 5′ end of the AL-RNA. However, the region of LAT corresponding to the general region in which the AL promoter would be expected to be located (Fig. 1D) has been reported to act as a promoter for the stable 2-kb LAT intron (13) (Fig. 1C) which begins at LAT nucleotide 661. This putative secondary LAT promoter has been termed LAP2 (7, 13). The LAP2 promoter contains several bidirectional elements, such as Sp1 (12), and can enhance transcription in both orientations (2). Thus, it is possible that LAP2 acts as the AL gene promoter.

Kinetics of AL-RNA expression in tissue culture. PC-12 cells were infected as described above. RNA was extracted at different times p.i., and the presence or absence of AL-RNA was determined by RT-PCR as described in Materials and Methods with poly(dT) for first-strand synthesis and AL-specific primers (a and d; Fig. 1E) for the PCR. AL-RNA was detected as early as 4 h p.i., peaked at 6 h p.i., and then declined (Fig. 4). These kinetics were similar to those of the immediate-early gene ICP0 mRNA (data not shown). The kinetics of AL-RNA expression also appeared to be complementary to the kinetics of the expression of LAT RNA, which first became apparent at 6 to 8 h p.i. (data not shown). This is consistent with the possibility of some form of antisense regulation between these two genes.

Sequence analysis of the AL ORF. The entire sequence of the AL-RNA was determined by cloning and sequencing of overlapping RT-PCR products made against AL-RNA transcribed in wild-type McKrae virus-infected PC-12 cells. Except for the poly(A) addition sequence discussed above, the AL-RNA sequence was identical to that of the genomic sequence

```
AL-RNA 1        ACCCCCGAAA CGGGGAAAAC GAAAAAACAG ACCAGCGGCC GGCCGGCGCT TAGGGGGAGG
genome 118958   ********** ********** ********** ********** ********** **********

                 M  S  P   T  P  L  G   R  P  G   C  R  G   A  R  R  A   A  A  P
AL-RNA 61       ATGTCGCCGA CGCCCCTTGG CCGCCCCGGC TGCAGGGGGG CCCGGAGAGC CGCGGCACCC
genome 118898   ********** ********** ********** ********** ********** **********

                 G  R  A   R  K  V  F   R  T  T   R  D  R   H  G  R  A   P  A  F
AL-RNA 121      GGACGCGCCC GGAAAGTCTT TCGCACCACC CGCGATCGGC ACGGCCGCGC CCCCGCTTTT
genome 118838   ********** ********** ********** ********** ********** **********

                 I  K  A   Q  M  T  Q   Q  K  Q   A  T  A   P  R  G   *
AL-RNA 181      ATAAAGGCTC AGATGACGCA GCAAAAACAG GCCACAGCAC CACGTGGGTA GGTGATGTAA
genome 118778   *********** ********** ********** ********** ********** **********

AL-RNA 241      TTTTATTTTC CTCGTCTGCG GCCTAATGGA TTTCCGGGCG CGGTGCCCCT GTCTGCAGAG
genome 118718   ********** ********** ********** ********** ********** **********

                                                                        |←
AL-RNA 301      CACTTAACGG ATTGATATCT CGCGGGCACG CGCGCCCTTA ATGGACCGGC GCGGGGAAAA
genome 118658   ********** ********** ********** ********** ********** ******

                  poly A signal →||←        poly A        →|
AL-RNA 361      TAAAATTACA TCACCTACAA AAAAAAAAAA AAAAAAAAAA AA
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FIG. 5. Complete sequence of AL-RNA. The sequence was determined as described in the text. AL-RNA indicates the DNA sequence corresponding to the AL-RNA sequence. The genomic sequence is from McKrae (the same HSV-1 strain as the RNA). The AL ORF begins with a methionine (M) at AL nucleotide 61 and ends with a TAG at AL nucleotide 229. The boxed C at genomic nucleotide location 118769 indicates the only nucleotide difference in AL between McKrae and HSV-1 strain 17syn+, which contains a G at this location. The boxed Q indicates that the nucleotide change from C to G changed the predicted amino acid from Q in McKrae to E in 17syn+.

(Fig. 5). Thus, there did not appear to be any obvious splicing of the AL-RNA. Sequence analysis indicated a potential ORF beginning with an ATG at nucleotide 61 of the AL-RNA (with the A corresponding to a T at LAT nucleotide +97). There is an in-frame TAG stop codon at AL-RNA nucleotide 229 (with the T corresponding to an A at LAT nucleotide −71). The total length of the predicted AL ORF is therefore 171 nucleotides (including the stop codon). The AL ORF is capable of coding for a protein of 56 amino acids (168 nucleotides without the stop codon) (Fig. 5). Based on the published genomic sequence for 17syn+ in this region (17, 26), McKrae and 17syn+ both contain this AL ORF, with only a single nucleotide difference. In the McKrae AL, nucleotide 190 of the mRNA (nucleotide 129 of the ORF) is C (Fig. 5, boxed nucleotide), while in the 17syn+ sequence this nucleotide is G. This changes amino acid 43 from glutamine (Fig. 5, boxed amino acid Q) in McKrae to glutamic acid in 17syn+. The high level of conservation (>98% identity) of the putative AL ORF in these two HSV-1 strains supports the likelihood that this is a functional ORF.

The AL ORF is expressed in rabbits infected with wild-type HSV-1. As described in Materials and Methods, antibody was raised against two synthetic AL peptides and against the full-length AL ORF fused to a His tag recombinantly expressed in *E. coli*. These three anti-AL antibodies were used for detection of the putative AL protein in extracts of tissue culture cells infected with wild-type HSV-1. Although AL protein-specific bands appeared to be detected on Western blots, all of the antibodies appeared to react with numerous additional bands. The background was thus too high to obtain conclusive results (data not shown). We therefore took an alternative approach. We looked for the presence of anti-AL antibody in the sera of rabbits infected with wild-type virus. Sera from rabbits infected with dLAT2903 (LAT^-/AL^-) were used as controls.

Rabbits were infected in both eyes with 2×10^5 PFU of wild-type McKrae or dLAT2903 virus per eye, and serum was collected from individual rabbits 57 days p.i., a time at which latency had been well established. Total cell extract from *E. coli* expressing the AL-His tag fusion protein was run on a Tricine gel in a large loading well. The protein was transferred by blotting to PVDF membrane. The membrane was then cut into strips, and the different strips were reacted with different sera (Fig. 6A). Individual sera from three different wild-type-infected rabbits (lanes 1, 2, and 3) recognized a band corresponding in mobility to the AL band recognized by anti-AL ORF peptide antibody (lane M, arrow). In contrast, none of the dLAT2903-infected rabbit sera (lanes 4, 5, and 6) or uninfected rabbit sera (lane U) appeared to recognize this band. In preliminary experiments, we found that antibodies raised against two different synthetic AL peptides reacted with the same band recognized by the anti-His tag antibody and that this band had an apparent molecular weight consistent with that expected for the AL-His tag protein (data not shown).

To reduce some of the background bands seen in Fig. 6A, AL-His tag fusion protein was partially purified from *E. coli* extracts with a His tag binding column as described in Materials and Methods. Western blots were then made as described above with wild-type serum 3 and dLAT2903 serum 4 (Fig. 6B). The background was greatly reduced, making it easier to visualize anti-AL antibody in the wild-type sera.

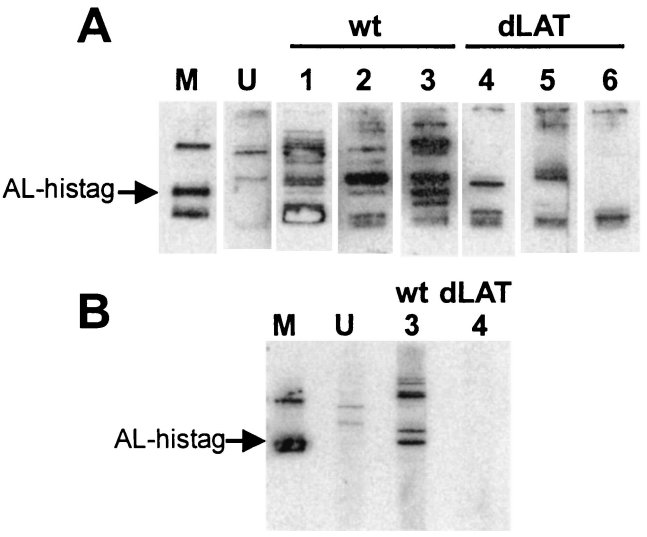

FIG. 6. Detection of *E. coli*-expressed AL protein by sera from infected rabbits. (A) Total extract from *E. coli* expressing the AL-His tag fusion protein was run on a 15% Tricine gel with a single large loading well and transferred to a PVDF membrane. The membrane was cut into strips, and each strip was separately reacted with the indicated antibody. The strips were then reacted with horseradish peroxidase-conjugated secondary antibody for chemifluorescence. The arrow indicates the location of the *E. coli*-expressed AL-His tag fusion protein. Lane M, anti-His tag antibody as marker. Lane U, serum from an uninfected rabbit. Lanes 1 to 3, sera from three different rabbits infected with wild-type virus. Lanes 4 to 6, sera from three different rabbits infected with dLAT2903 (a LAT and AL-null mutant). (B) The *E. coli*-expressed AL-His tag protein was partially purified as described in Materials and Methods and processed as for panel A. Lane M, anti-His tag antibody as marker. Lane U, serum from an uninfected rabbit different from the serum in panel A. Lanes 3 and 4, the same rabbit sera as in lanes 3 and 4 in panel A. wt, wild type.

The sera used in Fig. 6A, along with three additional sera from wild-type-infected rabbits and three additional sera from dLAT2903-infected rabbits, were further examined for the presence of AL antibodies by enzyme-linked immunosorbent assays (ELISAs). ELISA plates were coated with AL synthetic peptide 1 or 2. Sera from four of six wild-type-infected rabbits contained small but significant amounts of immunoglobulin G to either peptide 1 or 2 or both, based on their positive ELISA titers against these peptides. In contrast none of the sera from dLAT2903-infected rabbits produced an ELISA reading above background ($P = 0.03$, single-sided Fisher exact test).

Thus, the AL protein appeared to be expressed following ocular infection of rabbits. The minimal reactivity of the wild-type-infected rabbit serum with the AL-His tag fusion protein and the AL synthetic peptides suggests either that the AL protein is not very immunogenic and/or that only small amounts of AL protein were produced.

DISCUSSION

We report here on the discovery and initial characterization of a novel HSV-1 gene that is antisense to LAT and overlaps 198 nucleotides of the LAT promoter and 158 nucleotides of the 5′ end of the primary 8.3-kb LAT transcript. We have designated this gene AL, for anti-LAT sense. Interestingly, we

were unable to detect an AL transcript by standard Northern blot analysis (data not shown). However, we were able to detect the AL transcript by RT-PCR. This is similar to the UL43 gene of HSV-1, which also could not be detected by Northern blotting but was detected by RT-PCR (6). As suggested elsewhere for UL43 RNA (6), the difficulty in detecting AL RNA may have been due to low-level transcription, high turnover rate, or the fact that AL overlaps LAT in an antisense direction. Double-stranded RNA resulting from complementary AL and LAT RNA could result in rapid degradation by cellular enzymes (37).

The amino acid sequence of the putative AL protein is highly conserved in HSV-1 strains McKrae and 17syn+ (>98% identical). This level of amino acid sequence conservation is similar to that seen with other HSV-1 proteins (10). However, it is in sharp contrast to the much lower amino acid sequence conservation of 10 to 80% in eight potential ORFs within the first 1.5 kb of LAT (located on the other DNA strand opposite AL) (10). Based on the low conservation, it was concluded that none of these potential LAT ORFs encode a protein involved in the HSV-1 reactivation phenotype. In contrast, the high conservation of the putative AL protein amino acid sequence suggests that there is strong selective pressure to maintain the amino acid sequence. This suggests that AL encodes a protein.

We found that rabbits infected with wild-type HSV-1 by the ocular route developed antibodies to the AL protein. This is strong evidence that the AL protein was produced during acute and/or latent infection in rabbits. Combined with the high sequence conservation, it is therefore likely that AL is expressed by the virus in certain cell types and may play a role in the pathogenic potential of HSV-1. The apparently low level of AL antibody and our inability to detect AL protein above the background in infected cells in tissue culture suggested that AL is a low-abundance protein and/or it is produced only in certain cell types or at certain times, such as during latency.

The relative locations of the LAT and AL genes as presented in this study suggest the possibility of antisense regulation of LAT by AL or vice versa. This may be important in the latency-reactivation cycle. We found an apparent discordance between transcription of AL and LAT in tissue culture. AL transcription is greatest prior to the time that LAT expression becomes readily apparent. Transcription of AL decreases after 6 h p.i., the time that LAT expression increases dramatically. This is consistent with the possibility of some form of antisense regulation between AL and LAT. It has been reported elsewhere that dexamethasone-induced reactivation of bovine herpesvirus 1 results in transient reduction of LAT expression (27). This decrease in LAT may be a key factor in reactivation. It is possible that a reactivation stimulus either directly or indirectly up regulates AL transcription and that this in turn down regulates LAT, which then leads to reactivation.

We (21, 24) and others (41) have suggested elsewhere that LAT, or the region of the HSV-1 genome near the start of the primary LAT transcript, may be involved in viral virulence. Zhu et al. (41) described a 0.7-kb RNA that overlaps the LAT promoter, is transcribed in the LAT direction, and terminates just prior to the start of LAT. They also showed that a mutant with a deletion in this 0.7-kb region has altered virulence in mice. Interestingly, this mutant also deletes a portion of the AL gene. The altered virulence of this mutant may therefore be due to partial deletion of the 0.7-kb LAT sense RNA or partial deletion of the AL gene. We have also reported alterations in viral virulence in three mutants with deletions within the 5′ end of the primary 8.3-kb LAT transcript (21, 24). dLAT1.5 has a deletion of LAT nucleotides 76 to 1499 (corresponding to AL nucleotides 82 to −1342). dLAT371 has a deletion of LAT nucleotides 76 to 447 (corresponding to AL nucleotides 82 to −289). Both of these mutants express the remainder of LAT. In contrast, LAT2.9A, which contains the same deletion as dLAT371, also does not express LAT nucleotides 1500 to 8324 (the end of LAT). These deletions do not alter the structure of the genomic region encoding the 0.7-kb LAT sense RNA. However, they may impact expression of the 0.7-kb RNA, since expression of this RNA was abolished in an HSV-1 strain 17syn+ mutant similar to dLAT371 (41). Thus, it remains possible that the altered virulence phenotypes of all four of these mutants (one directly affecting the 0.7-kb transcript and AL and three directly affecting LAT and AL and probably indirectly affecting the 0.7-kb RNA) may be due to AL.

LAT-null mutants that also delete AL or significantly disrupt the AL ORF do not appear to have altered virulence (3, 4, 14, 20, 30). It is therefore possible that LAT and AL have opposing and balanced functions with respect to viral virulence. Blocking expression of both genes simultaneously would then have no obvious virulence phenotype. In contrast, three mutants with deletions that remove the 5′ end of AL (AL-null mutants) but do not disrupt the LAT promoter (and hence allow the mutants to continue to express the region of LAT outside of the deletion) do have altered virulence (21, 24). It is possible that this is the result of altering the balance between an AL function and a LAT function. In addition, the virulence phenotypes of these three mutants differ from each other and also differ between rabbits and mice. Since each of these mutants expresses different LAT transcripts, it is also possible that there is an intricate balance between one or more AL functions and functions due to different regions of LAT.

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