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Identification of two small RNAs within the first 1.5-kb of the herpes simplex virus type 1–encoded latency-associated transcript

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The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) is abundantly expressed in latently infected neurons. In the rabbit or mouse ocular models of infection, expression of the first 1.5 kb of LAT coding sequences is sufficient for and necessary for wild-type levels of spontaneous reactivation from latency. The antiapoptosis functions of LAT, which maps to the same 1.5 kb of LAT, are important for the latency-reactivation cycle because replacement of LAT with other antiapoptosis genes (the baculovirus IAP gene or the bovine herpesvirus type 1 latency-related gene) restores wild-type levels of reactivation to a LAT null mutant. A recent study identified a micro-RNA within LAT that can inhibit apoptosis (Gupta *et al*, *Nature* 442: 82–85). In this study, the authors analyzed the first 1.5 kb of LAT for additional small RNAs that may have regulatory functions. Two LAT-specific small RNAs were detected in productively infected human neuroblastoma cells within the first 1.5 kb of LAT, in a region that is important for inhibiting apoptosis. Although these small RNAs possess extensive secondary structure and a stem-loop structure, bands migrating near 23 bases were not detected suggesting these small RNAs are not true micro-RNAs. Both of the small LAT-specific RNAs have the potential to base pair with the ICP4 mRNA. These two small LAT RNAs may play a role in the latency-reactivation cycle by reducing apoptosis and/or by reducing ICP4 RNA expression. *Journal of NeuroVirology* (2008) **14,** 41–52.

Keywords: apoptosis; HSV-1; ICP4; latency associated transcript (LAT); small regulatory RNAs

Introduction

Approximately 90% of adults in the United States are infected with herpes simplex virus type 1 (HSV-1) (Nahmias and Roizman, 1973; Whitley, 1997). Recurrent ocular HSV-1 is the leading cause of infectious corneal blindness in industrialized nations (Nesburn, 1983). HSV-1–induced encephalitis (HSE) is a severe form of focal necrotizing encephalitis that affects at least 2000 individuals each year in the United States (Gesser and Koo, 1997; Lohr *et al*, 1990; Whitley, 1991, 1997). Acute infection is typically initiated in the mucocutaneous epithelium, which results in HSV-1 establishing latency in sensory neurons located in trigeminal ganglia (TG) or sacral dorsal root ganglia (Jones, 1998; Wagner and Bloom, 1997). Despite a vigorous immune response during acute infection, latency is established. HSV-1 can periodically reactivate from latency, resulting in virus shedding and recurrent disease.

A single region within the viral long repeats is abundantly transcribed in latently infected neurons,

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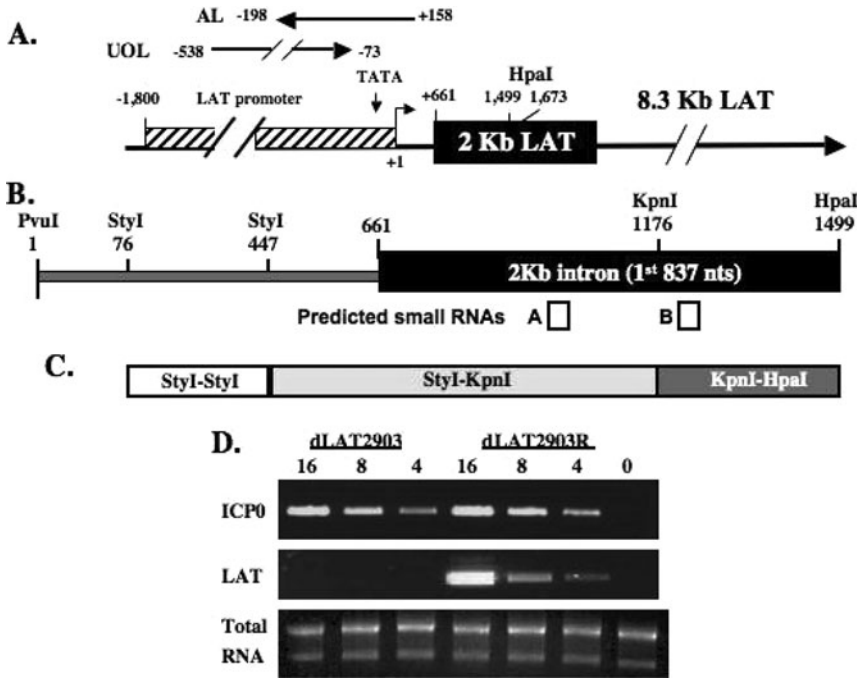

Figure 1 Schematic of LAT gene, location of putative small RNAs in LAT, and expression of viral genes during productive infection. **Panel A:** Diagram of the 8.3kb LAT region within the HSV-1 genome. The map shows the location of known genes that overlap the first 1.5 kb of LAT coding sequences: AL, UOL, and 2Kb stable intron. **Panel B:** Schematic of the first 1.5-kb of LAT, relevant restriction enzyme sites used in this study, and location of the first 837 nts of the stable 2 kb LAT. The predicted LAT small RNA A and B within the 1.5 kb region that were identified by computer analysis are denoted by the small rectangles. The parameters for identifying the small RNA structures were described in the materials and methods. **Panel C:** A plasmid containing the 1.5-kb LAT fragment was digested with StyI, KpnI, and HpaI to yield the restriction enzyme fragments depicted. These fragments were separated by 5% polyacrylamide gel electrophoresis, purified, and radiolabeled (see materials and methods). **Panel D:** Human neuroblastoma cells (SK-N-SH) were infected for the designated time (h) using a multiplicity of 5 PFU per cell. Total RNA was prepared as described in the materials and methods. Random primers were used for priming cDNA synthesis. The LAT primers were ttggcggtaaccccgattgtttatctcagg and tcgttccgtcgccgggatgtttcgttcgt, which yields a 200 bp product. The ICP0 primers were acagacccccaacacctaca and gcgtatgagtcagtgggga, which yields a 150 bp product. All primers are listed in a 5′-3′ direction.

and this transcript is referred to as the latency-associated transcript (LAT) (Croen *et al*, 1987; Deatly *et al*, 1987, 1988; Krause *et al*, 1988; Mitchell *et al*, 1990; Rock *et al*, 1987; Stevens *et al*, 1987; Wagner *et al*, 1988a, 1988b). Mice, rabbits, or humans latently infected with HSV-1 express LAT. The primary LAT transcript is approximately 8.3 kb (Deatly *et al*, 1988; Rock *et al*, 1987; Zwaagstra *et al*, 1990) (see Figure 1A for a schematic of the LAT gene). Splicing of the 8.3 kb transcript yields a stable 2-kb LAT and an unstable 6.3-kb LAT. The 2-kb LAT can also be further spliced in infected neurons (Mador *et al*, 1995). The majority of the 2-kb LAT is not capped or polyadenylated, and appears to be a circularized stable intron (Farrell *et al*, 1991; Krummenacher *et al*, 1997). Numerous studies have demonstrated that LAT significantly enhances the latency-reactivation cycle in small animal models (reviewed in Jones, 1998, 2003; Wagner and Bloom, 1997). For example, the HSV-1 McKrae strain is frequently shed in tears of infected rabbits because of spontaneous reactivation (Perng *et al*, 1994, 1996a, 1996b, 1996c, 1999). Unlike the wild-type (wt) McKrae, a LAT deletion mutant (dLAT2903) does not induce high levels of spontaneous reactivation in rabbits (Perng *et al*, 1994, 1996c) or high levels of induced reactivation in mice (Perng *et al*, 2001). dLAT2903 contains a deletion from −161 to +1667 relative to the start of the primary 8.3-kb LAT, and thus does not express detectable levels of LAT (Perng *et al*, 1994, 1996c). The spontaneous reactivation phenotype of dLAT2903 in rabbits, and the explant-induced reactivation phenotype of dLAT2903 in mice are restored to wt levels when the first 1.5 kb of LAT-coding sequences (LAT nucleotides 1 to 1499) driven by the LAT promoter is inserted into an ectopic location in the virus (Jin *et al*, 2003; Perng *et al*, 1996c). These studies indicate that the first 1.5 kb of LAT, which contains only the first 837 nucleotides of the stable 2-kb LAT, is sufficient to support high wt levels of reactivation in the rabbit and mouse. LAT may also enhance establishment of latency in mice (Sawtell and Thompson, 1992; Thompson and Sawtell, 1997) and rabbits (Perng *et al*, 2000), which would increase the pool of latently infected neurons, and consequently may increase the frequency of reactivation from latency.

LAT interferes with apoptosis in transiently transfected cells (Ahmed *et al*, 2002; Inman *et al*, 2001; Jin *et al*, 2003; Peng *et al*, 2000, 2003). Plasmids

expressing LAT products inhibit caspase 8– and caspase 9–induced apoptosis (Henderson *et al*, 2002; Jin *et al*, 2003), the major apoptosis pathways in mammals. LAT also reduces apoptosis of productively infected tissue culture cells (Jin *et al*, 2004), and promotes neuronal survival in trigemal ganglia (TG) of infected rabbits (Perng *et al*, 2000) and mice (Ahmed *et al*, 2002; Branco and Fraser, 2005). Inhibiting apoptosis appears to be the most important latency related function of LAT because two antiapoptosis genes, the bovine herpesvirus 1 (BHV-1) LAT homologue (Mott *et al*, 2003; Perng *et al*, 2002) and the baculovirus IAP gene (Jin *et al*, 2005), restore wt levels of spontaneous reactivation to a LAT null mutant. LAT may not encode a protein (Drolet *et al*, 1998), suggesting non–protein-coding RNAs regulate the latency-reactivation cycle.

Considerable evidence has demonstrated that small noncoding RNAs can regulate gene expression (Dykxhoorn *et al*, 2003; Hanon, 2002), promote neuronal differentiation (Kuwabera *et al*, 2004), or inhibit apoptosis (Xu *et al*, 2004). There are numerous types of small noncoding RNA: short interfering (si) RNA (Finnegan and Matzke, 2003), small temporal RNA (Banerjee and Slack, 2002), heterochromatic siRNA (Reinhart and Bartel, 2002), tiny noncoding RNAs (Fraser, 2003), and micro-RNAs (miRNAs) (Bartel, 2004), for example. miRNAs are non–protein-coding RNA molecules that are synthesized in the nucleus as 70- to 90-nucleotide precursors, and then processed into 21- to 23-nucleotide single-stranded RNA by the Dicer nuclease in the cytoplasm (Bartel, 2004). Dicer also processes short interfering RNA (siRNA). Following the discovery of five miRNAs encoded within the Epstein-Barr virus (EBV) genome (Pfeffer *et al*, 2004), miRNAs have been identified in several herpesviruses as well as other DNA viruses. For example, Kaposis sarcoma-associated herpes virus (KSHV) (Cai *et al*, 2005; Pfeffer *et al*, 2005; Samols *et al*, 2005), mouse gammaherpesvirus 68 (MGHV68) (Pfeffer *et al*, 2005), human cytomegalovirus (HCMV) (Dunn *et al*, 2005; Grey *et al*, 2005; Pfeffer *et al*, 2005), HSV-1 (Gupta *et al*, 2006; Pfeffer *et al*, 2004), Marek's disease virus (Burnside *et al*, 2006), and simian virus 40 (SV40) (Sullivan *et al*, 2005). The miRNAs encoded by EBV, KSHV, and MGHV68 were detected in latently infected cell lines. In the KSHV genome, 11 miRNA precursors produce 17 mature miRNAs that are clustered within the K12 gene, which is expressed during latency. A recent study predicted that HSV-1 encodes eight miRNAs, three of which are located in the 8.3-kb LAT region (Pfeffer *et al*, 2004). Another study reported that a miRNA is encoded from a site that is approximately 450 bases upstream from the start site of LAT (Cui *et al*, 2006). A subset of the Marek's disease virus miRNAs map to the LAT regions as well. An EBV RNA that is abundantly expressed in latently infected B cells (EBER), but does not encode a protein, interferes with interferon (IFN)-α–induced apoptosis in Burkitts lymphoma by binding to the double-stranded RNA-activated protein kinase (Nanbo *et al*, 2002). Thus, expression of small regulatory RNAs during herpesvirus latency is a common theme.

A miRNA encoded within LAT-coding sequences was recently reported to inhibit apoptosis (Gupta *et al*, 2006). Most of the sequences that encompass the LAT miRNA are contained within the StyI-StyI region located within LAT-coding sequences 76 to 447 (Figure 1B). Although this miRNA appears to have functional significance, deletion of the LAT StyI-StyI region does not always eliminate the antiapoptosis function of LAT (Jin *et al*, 2003; Peng *et al*, 2004), or impair reactivation from latency in a rabbit (Perng *et al*, 1996a) or mouse (Maggioncalda *et al*, 1994) model of infection. Conversely, there appears to be a strain-specific role for these sequences in rabbits because deletion of the StyI-StyI fragment from HSV-1 strain McKrae, but not strain 17syn+, has no effect on *in vivo* reactivation from latency in a rabbit ocular model of infection (Hill *et al*, 1996; Loutsch *et al*, 1999). In addition, if only the first 1.5 kb of LAT-coding sequences is expressed in the McKrae strain, then deletion of the Sty-StyI fragment reduces spontaneous reactivation from latency in the rabbit ocular model of infection (Perng *et al*, 2001), suggesting that in the context of the entire LAT gene, one or more downstream regions somehow compensate for the loss of the StyI-StyI-region. Based on these observations, we hypothesized that LAT encodes several additional factors, likely including additional noncoding RNAs, that play roles in how LAT regulates the latency-reactivation cycle. To test this prediction, we searched for and report here the identification of two additional small RNAs within the first 1.5 kb of LAT that are expressed in productively infected human neuroblastoma cells. Both small RNAs have the potential to base pair with HSV-1 ICP4 mRNA suggesting the possibility of repressing expression of ICP4 RNA.

Results

The 1.5-kb LAT region contains at least two small RNAs that have structural features similar to miRNAs

Identification of miRNA has been primarily achieved by cloning small RNAs from a size-fractionated cDNA library (Huttenhofer and Vogel, 2006; Lagos-Quintana *et al*, 2001; Lee and Ambros, 2001; Pfeffer *et al*, 2005). This method is limited if large amounts of RNA are difficult to obtain, and if the transcript is expressed at low levels. HSV-1 establishes latency in TG sensory neurons, and only about 30% of the neurons are latently infected (Thompson and Sawtell, 1997). The first 1.5 kb of LAT-coding sequences inhibit apoptosis (Henderson *et al*, 2002; Inman *et al*, 2001; Jin *et al*, 2003), and confers wt levels of reactivation in rabbit and mouse ocular models of

infection when recombined into a LAT null mutant (Perng *et al*, 1994, 1996c). Although the first 1.5 kb of LAT contains one miRNA (Gupta *et al*, 2006), it is unlikely that this miRNA performs all of the functions ascribed to the 1.5 kb LAT. Consequently, we reexamined the 1.5-kb LAT region using computer-driven predictions to identify additional putative LAT small regulatory RNAs, for example miRNAs. miRNAs are generated when RNaseIII-like enzymes cleave precursors that contain certain "stem-loop" structures (Denli *et al*, 2004; Lee *et al*, 2002, 2003; Yi *et al*, 2003). Several software programs have been developed to predict miRNAs based on conserved sequences and structural features within stem-loops of other miRNAs. For example, the MiRScan program predicted nematode miRNAs (Lim *et al*, 2003) and the miRseeker program predicted *Drosophila* miRNAs (Lai *et al,* 2003). Although a previous study identified eight miRNAs within the HSV-1 genome using a probability approach (support vetor mapping convergence) (Pfeffer *et al*, 2005), no miRNAs were predicted within the first 1.5 kb of the LAT-coding sequences. This approach may have failed to identify potential miRNAs within the 1.5-kb LAT region because there is no sequence similarity to currently identified viral miRNAs, the region has high GC content, or other classes of small regulatory RNAs are encoded by this region. Consequently, we used the MFOLD program to search directly for stem-loop small RNA structures in the first 1.5 kb of LAT sequence. Two stem-loop structures that somewhat resemble pre-miRNAs were identified. These two small RNAs were designated LAT small RNA A and B (for location of these putative small RNAs; see Figure 1B).

The 1.5-kb LAT fragment expresses two small RNAs during productive infection

To test whether the first 1.5 kb of LAT-coding sequence encodes small RNAs, restriction fragments derived from the first 1.5 kb of LAT were used to hybridize to the small RNA fraction prepared from SK-N-SH cells that were infected for 16 h with a LAT null mutant (dLAT2903) or the marker rescued LAT(+) virus (dLAT2903R). As expected, dLAT2903 did not express LAT; however, dLAT2903 and dLAT2903R expressed abundant levels of the ICP0 transcript (Figure 1D). The first 1.5 kb of the LAT fragment was digested into three fragments: (1) a StyI-StyI fragment (371 bp), (2) a StyI-KpnI fragment (729 bp), and (3) a KpnI-HpaI fragment (324 bp) (see Figure 1B and C). The restriction enzyme fragments were separated on a polyacrylamide gel, the respective fragment purified, and then radiolabeled. These individual radioactive fragments were used as probes to hybridize with a small RNA fraction. When the StyI-StyI fragment was used as a probe, specific hybridization to the small RNA fraction was not detected in infected or uninfected cells (Figure 2A). Although the StyI-StyI fragment contains part of the LAT miRNA that was recently described (Gupta *et al*, 2006), part of this miRNA is also located downstream of the StyI site at LAT nucleotide (nt) 447. This could explain why the StyI-StyI probe did not detect this miRNA. The StyI-KpnI probe hybridized to several specific

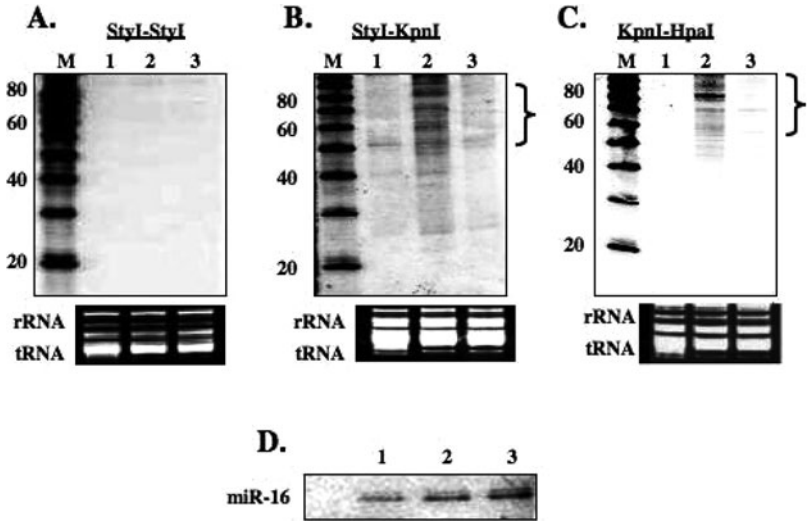

Figure 2 Identification of small RNAs encoded within the 1.5 kb LAT. Human neuroblastoma cells (SK-N-SH) were infected for 16 h using a multiplicity of 5 PFU per cell. Total small RNA was prepared as described in the materials and methods. Small RNA was subjected to Northern blot analysis using the designated LAT probes. Detection of small RNAs by Northern blot analysis using the LAT probe StyI-StyI (Panel A), *Sty*I-*Kpn*I (Panel B), or *Kpn*I-*Hpa*I (Panel D. Lane M: 100 bp ladder, bottom band is always 20 nt; Lane 1: Mock infected; Lane 2: cells were infected with the rescued virus dLAT2903R (LAT^+); Lane 3: infected with dLAT2903 (LAT^-). LAT specific small RNA are denoted by the brackets. Equal loading of the gel was monitored by ethidium bromide staining of tRNA and rRNA bands (bottom of each panel). **Panel D:** As a positive control, northern blots were hybridized to a probe specific for the mature human miR-16 probe. Lane 1: Mock infected; Lane 2: cells were infected with the rescued virus dLAT2903R (LAT^+); Lane 3: infected with dLAT2903 (LAT^-).

bands migrating between 50 and 80 nt in cells infected with dLAT2903R (Figure 2B, lane 2, denoted by the brackets). As expected, these bands were not detected in mock-infected cells or cells infected with dLAT2903 (Figure 2B, lanes 1 and 3, respectively). The KpnI-HpaI probe also hybridized to bands migrating between 50- and 80-nt band when SK-N-SH cells were infected with dLAT2903R (Figure 2C, lane 2, denoted by the bracket), but not with dLAT2903 infected cells or mock infected cells (Figure 2C, lanes 1 and 3, respectively).

As a control, the human miRNA, miR-16, was analyzed following infection. The mature miRNA-16 was readily detected under the same conditions regardless of the virus used to infect SK-N-SH cells (Figure 2D). In summary, the results of the Northern blot studies identified small RNAs within the first 1.5 kb of LAT that mapped to the location of the predicted LAT small RNA A and B within the 1.5-kb LAT fragment.

Cloning small RNA encoded by the LAT gene

Following cloning of the small RNA fraction prepared from infected cells, potential LAT+ clones were identified using Southern blotting hybridization with a probe generated from the LAT region. Those clones exhibiting a positive signal were analyzed by sequencing the inserts using a primer that flanked the 5′ terminus of the T7 primer site. Using NCBI BLASTnt, two clones were identified that corresponded to sequences from the first 1.5 kb of LAT (see Figure 3A for the location of these cloned small RNAs). The sequences of these two clones were compared to the predicted LAT small RNA sequences described above, using the online version of ClustalW (EMBL-EBI tools,

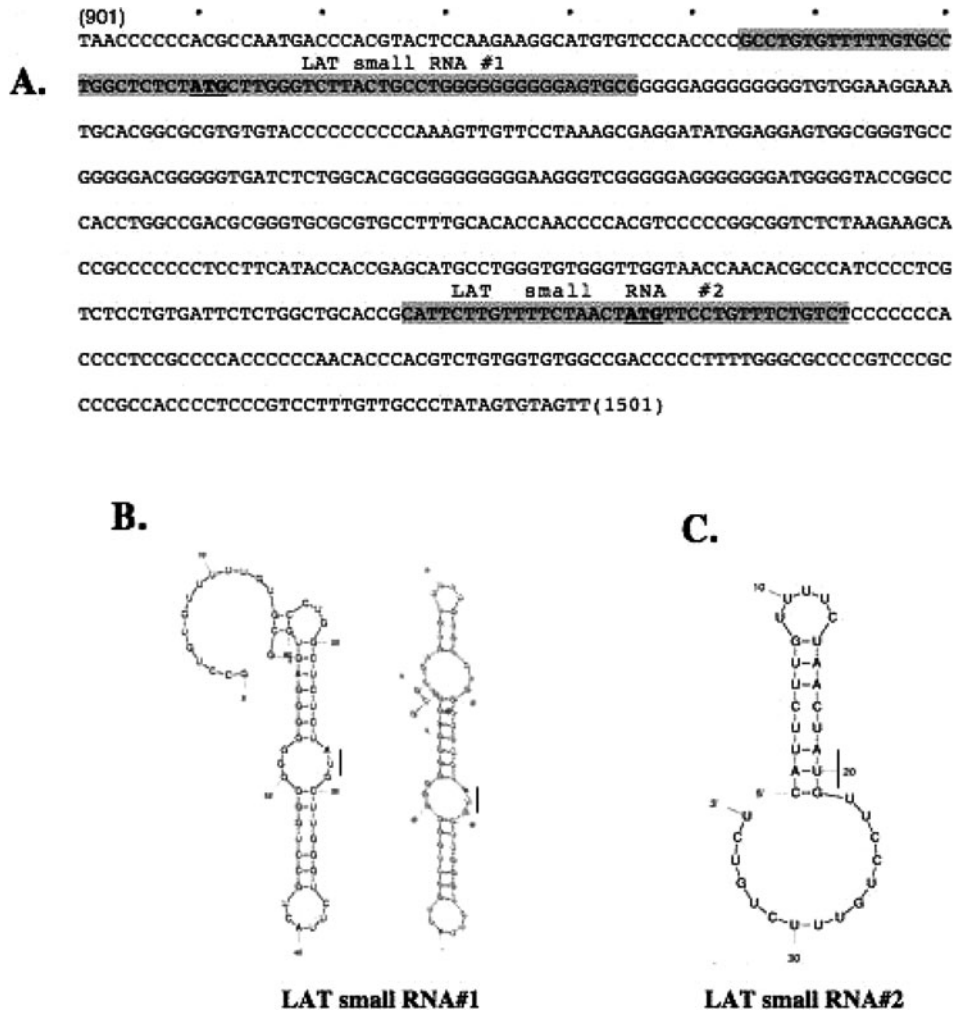

Figure 3 Location of LAT small RNAs. **Panel A:** Nucleotide sequence of LAT (+901-1501) that contain the cloned small RNAs. The grey shaded sequences denote the positions of LAT small RNA#1 and LAT small RNA #2. The bold and underlined ATG's are initiating methionines that are at the 5′ terminus of putative LAT ORFs, and were described previously (Drolet *et al*, 1999). **Panels B and C.** The predicted structure of the respective cloned LAT small RNAs was predicted using MFOLD. LAT small RNA #1 has two stable structures that were predicted by MFOLD. The vertical lines denote the ATGs present in the LAT small RNAs.

Table 1 HSV-1 potential targets that may base pair with LAT small RNA #1

Target gene	*Alignments*	*dG kcal/mol*	*Position*	*Gene Accession Number*	*Function*
ICP4	target 5' C C GGCG GGAGUCGGGCACG UCGU UCUCGGUCCGUGU miRNA 3'U AUC	−30.9	555–574	X06461	transcriptional activator
	target 5' G C GGGCGUGGGGGGC GGG CGCG UUCGUAUCUCUCG UCC GUGU miRNA 3' G	−34.9	6310–6331		
ICP0	target 5' GGGCGUGGAGGGU GGGCACG UUCGUAUCUCUCG UCCGUGU miRNA 3' G	−34.0	2967–2986	X04614	transcriptional activator
	target 5' AGG U GGG GGGGGGUCGGGCGC UUC UCUCUCGGUCCGUG miRNA 3' GUA U	−31.1	3913–39233		
Vmw65	target 5' C GCUGCACC GGCA GCCAGGCGCA UCGU CGGUCCGUGU miRNA 3'U AUCUCU	−29.1	1015–1037	X03141	activation of IE genes
UL8	target 5′ CGCCA GCGC GGAGCUGGGCGCG UUCG UCUCGGUCCGUGU miRNA 3′ UAUC	−31.4	4260–4281	M19120	DNA replication

Potential hybridization that is predicted to occur between the HSV-1 target gene and LAT small RNA. The structures (duplexes) exhibit hybridization obtained from RNA hybrid software: the top strand represents the target and the bottom strand is the small LAT RNA. The nucleotides above each duplex represent unpaired nucleotides that from unpaired gaps or loops.

http://www.ebi.ac.uk/clustalw/) and BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The first cloned LAT small RNA (designated LAT small RNA 1) was identical to the predicted LAT small RNA A, except that it was 10 nt shorter at the 3′ end and 6 nt longer at the 5′ end. LAT small RNA 1 has the potential to be folded into two stable structures (Figure 3B).

In contrast, the sequence of the second cloned LAT small RNA (LAT small RNA 2) did not correspond to LAT small RNA B, which was identified in the KpnI-HpaI fragment (Figures 1B and 3A). However, LAT small RNA 2 corresponded to a location within the KpnI-HpaI fragment (see Figure 3A), and LAT small RNA 2 contains extensive secondary structure with stem-loop structures (Figure 3C). The predicted structures of the two cloned LAT small RNAs lacked certain features of a microRNA stem-loop, and may instead represent novel small regulatory RNAs.

The LAT small RNAs in the first 1.5 kb of LAT-coding sequences are predicted to target the HSV-1 ICP4 gene

Small regulatory RNAs, including miRNA, can inhibit gene expression by degrading target mRNA or suppressing translation (Doench, 2004; Hutvagner and Zamore, 2002). Because LAT has been proposed to regulate viral gene expression (Garber *et al*, 1997), we used computational RNA hybridization to predict potential HSV-1 genes that would hybridize to LAT small RNA 1 or 2. Several potential target HSV-1 genes were identified and their binding sites are listed in Tables 1 (LAT small RNA 1) and 2 (LAT small RNA 2). For LAT small RNA 1, the candidate target genes were two intermediate-early (IE) genes (ICP4 and ICP0), a late gene (Vmw65), and an early gene (UL8). ICP4 also appeared to be a candidate target for LAT small RNA 2, as were VP22 and glycoprotein H. Because both LAT small RNA 1 and 2 were predicted to target ICP4 with a high degree of sequence complementarity, it is interesting to speculate that they may down-regulate ICP4 mRNA levels.

Discussion

In this study, we cloned and identified two small LAT RNAs that were expressed in productively infected human neuroblastoma cells from the first 1.5 kb of the LAT-coding sequences. Combined with the LAT miRNA that was recently described (Gupta *et al*, 2006), this makes a total of three small RNAs encoded from within the first 1.5 kb of LAT. We hypothesize that additional small RNAs may be present within this critical LAT region for the following

Table 2 HSV-1 potential targets that may base pair with LAT small RNA #2

Target gene	*Alignments*	*dG kcal/mol*	*Position*	*Gene accession number*	*Function*
ICP4	target 5' G CC G GG G 3' GGGCGGGG GCGGGAGCG GG AG GAGCGGGGG UCUGUCUU UGUCCUUGU UC UC UUUGUUCUU miRNA 3' A AA U AC 5'	−38.2	6318–6353	X06461	transcription
glycoprotein H	target 5' A C UG C C GG A 3' GGGCGG GAUGGGA G GGU GAAGAUGAG UG UCUGUC UUGUCCU U UCA CUUUUGUUC AC miRNA 3' U UG A AU UU 5'	−37.1	564–534	M14884	virus entry and fusion
VP22	target 5' G G C CU U 3' GGGC AGAAGC GGAGCG GG UGGGG CGAGG UCUG UCUUUG CCUUGU UC AUCUU GUUCU miRNA 3' U A A UU UAC 5'	−31.1	888–855	AY602769	virus tegument; virus assembly

Potential hybridization that is predicted to occur between the HSV-1 target gene and LAT small RNA. The structures (duplexes) exhibit hybridization obtained from RNA hybrid software: the top strand represents the target and the bottom strand is the small LAT RNA. The nucleotides above each deplex represent unpaired nucleotides that form unpaired gaps or loops.

reasons. First, the structure of the small RNAs identified in this study was nearly double stranded, which we believe hampered the addition of adapters to the small RNA. Secondly, the GC-rich nature of these sequences also reduced the efficiency of the cloning strategy that was used. In spite of the limitations of cloning small RNAs from the 1.5 kb of LAT, this study supports the concept that LAT encodes multiple small RNAs that may play a role in the latency-reactivation cycle. For example, computational RNA hybridization suggested that both of the LAT small RNAs bind to the HSV-1 ICP4 mRNA. This finding was consistent with three previous studies that concluded LAT represses ICP4 RNA expression (Chen *et al*, 1997; Garber *et al*, 1997; Mador *et al*, 1998).

An HSV-1 recombinant virus that expresses just the first 1.5 kb of the LAT-coding sequences (LAT3.3A) restores wt levels of spontaneous reactivation to a LAT null mutant (dLAT2903) (Perng *et al*, 1994, 1996c). This region is also capable of blocking apoptosis (Ahmed *et al*, 2002; Henderson *et al*, 2002; Inman *et al*, 2001; Jin *et al*, 2003; Peng *et al*, 2003; Perng *et al*, 2000). In addition, a LAT mutant that expresses all of LAT except for the first 1.7 kb of LAT has a reactivation phenotype indistinguishable from that of a LAT null mutant (Perng *et al*, 2001). Thus, this region is both required and sufficient for producing the high wt reactivation phenotype and for blocking apoptosis. Because the two small LAT RNAs described in this report map to this same first 1.5 kb of LAT, there is a correlation between LAT's ability to block apoptosis and to enhance the reactivation phenotype and the ability of LAT to express small RNA 1 and 2. It will be interesting to determine if one or both of these LAT small RNAs can inhibit apoptosis or reduce ICP4 RNA levels. Because LAT levels are reduced during reactivation from latency (Rock *et al*, 1987), it is probable that the levels of the LAT small RNAs are also reduced suggesting that their absence may disrupt the maintenance of latency. Non–protein-coding RNAs that regulate certain aspects of the latency-reactivation cycle may have a selective advantage relative to a protein that regulates latency because a latently infected neuron that does not express high levels of a protein would escape immune surveillance.

We were unable to consistently detect mature miRNA bands (approximately 22 nt RNA species) using probes that contain LAT small RNA 1 or 2. In contrast, the human miR-16 was readily detected in the same human cell line (both infected and uninfected) (Figure 2D), suggesting that procedures used in this study were sensitive enough for detecting mature miRNAs. A recent study has indicated that intronic miRNA precursors can bypass Drosha processing, but are still functional (Ruby *et al*, 2007). Because both LAT small RNAs described in this study are present in the LAT stable 2-kb intron, they may be examples of unprocessed miRNAs. All miRNAs possess a unique property because they exist as a double-stranded structure known as a stem loop (reviewed by Bartel, 2004; Finnegan and Matzke, 2003). Although LAT small RNA 1 can fold back on itself, it lacks the typical loop structure. LAT small RNA 2 has a large 3′ unpaired end that is predicted by MFOLD to have high free energy when folded. Thus, LAT small RNA 2 does not fit the typical miRNA structure. LAT small RNA 2 also contained large asymmetric structures. Finally, LAT small RNA 1 or 2 was not detected using standard miRNA computer programs, and they

do not appear to contain consensus Drosha cleavage sites close to the precursors. Consequently, we suggest that LAT small RNA 1 or 2 are not true miRNAs, and may be tiny noncoding RNAs perhaps similar to those identified in *Caenorhabditis elegans*. (Ohler, 2004). These tiny noncoding RNAs can also regulate gene expression (Fraser, 2003).

Inhibiting apoptosis appears to be the most important function of LAT because two antiapoptosis genes, the bovine herpesvirus type 1 (BHV-1) LAT homologue (Mott *et al*, 2003; Perng *et al*, 2002) and the baculovirus IAP gene (Jin *et al*, 2005), restore wt levels of spontaneous reactivation to a LAT null mutant. We suggest that expression of LAT small RNA 1 and 2 may play a role in inhibiting apoptosis because small non–protein-coding RNAs can regulate cell death (Iseni *et al*, 2002; Schors *et al*, 2002; Xu *et al*, 2004). For example, expression of the human miRNA-21 inhibits caspase activation and consequently apoptosis (Chan *et al*, 2005). Furthermore, the miRNA-17-92 cluster regulates expression of the proapoptotic transcription factor, E2F (He *et al*, 2005; O'Donnell *et al*, 2005). The EBV EBER, which is a small noncoding RNA that is not a miRNA, can inhibit apoptosis and promote latency (Nanbo *et al*, 2002). A blast analysis of the human genome demonstrated that LAT small RNA 1 and 2 have the potential to base pair with specific human mRNAs, some of which regulate apoptosis (unpublished data). Because miRNAs have the potential to inhibit expression of several target genes (Lim *et al*, 2005), LAT-encoded small RNAs may target other genes besides ICP4. The LAT small RNAs may also interact with mitochondria and inhibit release of proapoptotic factors (cytochrome *c* and Smac/Diablo, for example) because GC-rich RNA molecules (approximately 100 nucleotides in length) can bind phospholipid membranes and alter their permeability (Khvorova *et al*, 1999). Both LAT small RNAs contain ATGs that are situated to be initiating codons of potential small open reading frames (ORFs) present in the first 1.5 kb of LAT (Drolet *et al*, 1998). Mutagenesis of these ATGs impairs the ability of the first 1.5 kb of LAT to inhibit apoptosis (Carpenter *et al*, 2007). Although we do not know if these mutations alter the expression of the small RNAs, mutagenesis of the ATG $\rightarrow$ TTG in LAT small RNA 1 and LAT small RNA 2 alters the secondary structure of each (data not shown). It is also possible that mutagenesis of the ATG $\rightarrow$ TTG influences the ability of the LAT small RNAs to interact with cellular factors. Studies are in progress to examine whether the LAT small RNAs can directly inhibit apoptosis, and/or ICP4 RNA synthesis.

Materials and methods

Prediction of small RNA with structures resembling miRNAs within LAT

Based on the characteristics of 35 known virus miRNAs and their precursors published in the miRBase sequence database (http://microrna.sanger.ac.uk/sequences/index.shtml) release 7.1, five distinctive parameters were chosen to identify potential miRNAs in the 1.5-kb LAT region (accession number X14112.1, genomic nt 118801 to 120300, corresponding to LAT nt 1 to 1499). The miRNA precursor forms 50- to 100-nt-long hairpin loops with at least a 23-bp stem arm. The minimum folding energy of the precursor sequence is -20 kcal/mol. No more than a 5-unpaired-base bulge is allowed; and the distance from the end of the miRNA to the base of the loop in the hairpin precursor is 2 to 9 bases. Lastly, potential precursors that include the mature miRNA sequence typically contain a U at the first position, and this feature was included in the search. The MFOLD computer program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) was used to identify the secondary structure of potential miRNA precursors in the 1.5 kb of LAT (Zuker, 2003).

Cells and viruses

Cells were plated at a density of 5×10^5 cells/100-mm plastic dish in Earl's modified Eagle's medium supplemented with 10% fetal bovine serum. All medium contained penicillin (10 U/ml) and streptomycin (100 μg/ml). SK-N-SH cells (human neuroblastoma) were obtained from the American Type Culture Collection (Rockville, MD). All parental and mutant viruses were plaque purified three times and passed one or two times prior to use. dLAT2903 (LAT mutant), dLAT2903R (LAT rescued virus), and the McKrae strain were described previously (Perng *et al*, 1994, 1996c, 2001). Rabbit skin cells were used for preparation of virus stocks.

Isolation of small RNA and Northern blot analysis to detect small RNAs

Small RNA was extracted from SK-N-SH cells using the mirVana miRNA Isolation kit (Ambion, TX) according to the manufacturer's instruction. Five to 10 μg of small RNA was loaded onto a 12% polyacrylamide gel prepared using the SequaGel Sequencing System Kit (National Diagnostics, GA). After electrophoresis at 450 V for approximately 2.5 h, the gel was stained with 4 ug/ml EtBr in $0.5 \times$ TBE, a photograph taken to visualize tRNA and 5S rRNA (78 and 120 nt). The levels of tRNA and rRNA were used to ensure that similar levels of total RNA were loaded on each lane. The RNA in the gel was transferred to a Nytran supercharge membrane (Bioscience, NH) using a Trans-Blot SD semidry electrophoresis transfer cell (BioRad, CA) at a constant current of 3.5 mA/cm^2 for 30 min. The RNA was then cross-linked to the membrane using UV light (Spectrolinker XL-1000; Spectronics), and the membrane was baked for 45 min at 80°C.

Radioactive labeling of DNA or RNA probes

Oligonucleotide probes: A total of 10 pmol of the human miR-16 oligonucleotide (5′cgccaatatttacgtgctgcta3′) was 5′-end labeled using [γ-^{32}P]ATP

(Amersham Bioscience, NJ) and T4 polynucleotide kinase (New England Biolabs, MA). Labeled oligonucleotides were separated from unincorporated nucleotides by purification on G-25 Sephadex columns (Roche, IN) according to the manufacturer's instruction.

DNA probes: For Southern blot, a 692-bp polymerase chain reaction (PCR) product that encompasses a portion of the 1.5-kb LAT fragment was amplified with using the primer pair 5′AGTCAGACTCTGTTACTTACCCGTCCG3′ (LAT nt 748 to 774) and 5′AACTACACTATAGGGCAA CAAAGGACGGGAGGG3′ (LAT nt 1409 to 1441), digested with the EaeI restriction enzyme, purified with PureLink Quick Gel Extraction Kit (Invitrogen, CA), and labeled with [γ-^{32}P]ATP (Amersham Bioscience, NJ).

A plasmid containing the first 1.5 kb of LAT was digested with various restriction enzymes to prepare the respective LAT probes (Figure 1). The DNA fragments were cut from the agarose gel and purified with a Gene Clean III kit (Qiagene, CA). Probes were labeled with [α-^{32}P]dCTP using the Radprimer DNA Labeling System (Invitrogen, CA) and used in Northern blot hybridizations. Blots were prehybridized in ULTRAhybTM-oligo buffer (Ambion, TX) for 2 h at 42°C. Denatured radiolabeled probes were added and incubated for 16 h at 42°C.

Cloning small RNAs from infected cells
Forty micrograms of small RNAs and 200 μg of total RNA were loaded in a 12% polyacrylamide gel prepared using SequaGel Sequencing System Kit (National Diagnostics, GA). The gel was electrophoresed at 300 V for 3 h in DEPC-treated 0.5 × TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH = 8). To visualize RNA, the gel was stained for 10 min in 5 μg/ml EtBr diluted in diethylpyrocarbonate treated 0.5 × TBE buffer. A gel slice containing RNAs from approximately 15 to 100 nt was cut and purified according to an Ambion protocol (http://www.ambion.com/techlib/append/supp/miRNA_gel_purif.pdf) with minor modifications. The concentration of RNA was measured and this RNA was used for cloning. Amplification of cDNAs was performed with Global MicroRNA Amplification Kit (SBI, CA) according to the manufacturer's protocol. PCR products were cloned in a plasmid vector pGEM-T and pGEM-T Easy Vector Systems (Promega, WI). Plasmids were then transformed into *Escherichia coli* strain DH5-α (Invitrogen, CA) and plated on agar plates containing 100 μg/ml ampicillin. After incubation over night at 37°C, clones were placed into 5 ml of liquid LB medium containing 100 μg/ml ampicillin and incubated for 8 h at 37°C with shaking at 220 rpm. Plasmid DNA was purified with GeneJET Plasmid Miniprep Kit (Fermentas, MD) and 0.2 μg of plasmid DNA for each sample was loaded onto a 0.8% agarose gel. The gel was electrophoresed at 80 V (Fisher Scientific FB300) for 4 h. DNA was visualized by staining the gel for 15 min in 0.5 μg/ml EtBr.

Plasmid DNA was transferred to a positively charged nylon membrane Hybond N plus 0.45 μm (Amersham Biosciences, NJ) by capillary transfer in 0.4 M NaOH for 18 h. To fix the DNA to the membrane, the membrane was UV cross-linked and baked for 1 h at 80°C. Southern blotting was performed to detect LAT+ clones. The membrane was prehybridized for 1 h with OligoULTRAHyb (Ambion, TX) and hybridized with the radiolabeled probe for 16 h at 42°C. Following hybridization, the membrane was washed two times with 2 × SSC at room temperature for 5 min and then two times with 0.1% sodium dodecyl sulfate (SDS) and 0.1 × SSC at 60°C for 15 min (1 × SSC is 150 mM Nacl, 15 mM Na citrate (pH 7.0)). The membrane was then exposed to x-ray film for 1 week at −80°C and developed. Plasmid pUC19 containing the first 1.5 kb of LAT was used as a positive control and pGEM-T and pUC19 empty vectors were used as negative controls. Inserts from clones that gave a positive hybridization signal were sequenced. BLASTnt in NCBI was performed with the obtained sequences to identify the source of the cloned RNAs.

Prediction of potential targets in HSV-1 genome
The RNA hybrid programmer from http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html was used to search for miRNA targets in the HSV-1 genome (gene accession number X14112.1). The target sites were predicted on the basis of two properties: free energy of RNA-RNA duplexes (−25 kcal/mol); and sequence complementarities at the 5′ end, in which six to eight nucleotides are critical for miRNAs to suppress their targets. These parameters also considered that base pairs at the 3′ end can compensate for imperfect 5′ binding to its target (Brennecke *et al*, 2005).

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