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The Half-Life of the HSV-1 1.5 kb LAT Intron is similar to the half-Life of the 2.0 kb LAT Intron

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Abstract

Herpes Simplex Virus type 1 (HSV-1) establishes a latent infection in the sensory neurons of the peripheral nervous system of humans. Although about 80 genes are expressed during the lytic cycle of the virus infection, essentially only one gene is expressed during the latent cycle. This gene is known as the latency associated transcript (LAT) and it appears to play a role in the latency cycle through an anti-apoptotic function in the 5' end of the gene and miRNA encoded along the length of the transcript which down regulate some of the viral immediate early (IE) gene products. The LAT gene is about 8.3 kb long and consists of two exons separated by an unusual intron. The intron between the exons consists of two nested introns. This arrangement of introns has been called a twintron. Furthermore, the larger (2 kb) intron has been shown to be very stable. In this study we measure the stability of the shorter 1.5 kb nested intron and find its half-life is similar to the longer intron. This was achieved by deleting the 0.5 kb overlapping intron from a plasmid construct designed to express the LAT transcript from a tet-inducible promoter, and measuring the half-life of the 1.5 kb intron in tissue culture cells. This finding supports the hypothesis that it is the common branch-point region of these nested introns that is responsible for their stability.

Keywords

LAT; stable intron; half-life; HSV; latency

Introduction

Herpes Simplex virus-1 (HSV-1) is a large DNA virus that expresses over 70 genes during its lytic infection. It has the ability to establish latent infections in neurons of the sensory ganglia, particularly the trigeminal ganglia (Stevens, 1989; Wagner and Bloom, 1997). Latency-associated transcripts (LATs) are the only detectable transcripts produced during latent infection (Deatly *et al*, 1987; Spivack and Fraser, 1987; Stevens *et al*, 1987). It has been reported that the LATs play a role in 1) establishing latency (Margolis *et al*, 2007), 2) maintaining latency (Garber *et al*, 1997; Mador *et al*, 1998), 3) reactivation of the virus from latency, and 4) protection of neurons from apoptosis (Inman *et al*, 2001; Perng *et al*, 2000). More recently, LATs have been shown to give rise to microRNAs (Umbach *et al*, 2008) and to protect cells from granzyme-b induced apoptosis and CD-8 T cell killing (Jiang *et al*, 2011). Some of these claims have been disputed and the true functions of LATs regarding latency remain somewhat speculative.

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The LATs are a family of transcripts comprised of an 8.3 kb primary transcript, mLAT, found in low levels, and 3 introns (2.0 kb LAT found at high levels during lytic and latent infection; 1.5 kb LAT found at lower levels and only during latency; and 0.5 kb LAT not detected but predicted) that are processed from mLAT (Figure 1) (Farrell *et al*, 1991). The spliced 6.3 kb exon product cannot easily be detected, although the 2.0 and 1.5 kb introns can easily be seen using Northern blot analysis (Spivack and Fraser, 1987; Zabolotny *et al*, 1997). Translation of a protein from the spliced LAT exon mRNA remains to be convincingly shown (Henderson *et al*, 2009), nevertheless it is clear that the exon 1 region of the LAT is required for protection from apoptosis (Ahmed *et al*, 2002; Inman *et al*, 2001; Perng *et al*, 2000). Functions for the 1.5 kb LAT intron are yet to be determined, but the 2 kb LAT appears to play a role in up-regulation of certain heat shock proteins (Atanasiu *et al*, 2006).

The 1.5 kb LAT intron is entirely encoded within the 2.0 kb intron and thus has been referred to as a twintron (Borah *et al*, 2009; Copertino and Hallick, 1991; Scamborova *et al*, 2004). Both introns are found in the cell nucleus during latency. The LAT introns can be described as a complex twintron as three overlapping introns (2.0 kb, 1.5 kb and 0.5 kb), can be produced from the LAT transcript. Interestingly, formation of the 1.5 kb and 2.0 kb introns is mutually exclusive, as the 1.5 kb intron is produced after removal of a 0.5 kb LAT intron from within the 2.0 kb coding region of the primary 8.3 kb transcript. Therefore 2.0 kb and 1.5 kb introns cannot be produced from the same transcript (see Fig 1).

Little is known of the mechanism of LAT twintron splicing regulation. However it is recognized that correct splicing plays an important role in the establishment and maintenance of latency (Kang *et al*, 2003). In drosophila, prospero is a transcription factor, important for neuronal differentiation and development of drosophila CNS (Doe *et al*, 1991; Vaessin *et al*, 1991); the prospero gene contains a twintron, and by utilizing alternative splicing, two isoforms of the prospero protein are produced, namely pros-S, and pros-L. Moreover, the splicing is stage dependent as the pros-L predominates during the first half of Drosophila embryogenesis while pros-S is more abundant at later stages (Scamborova *et al*, 2004). By extrapolation to the LAT introns, perhaps the 1.5 kb and the 2.0 kb LAT intron production is infection stage dependent, and the mRNAs, that are the product of splicing of the primary LAT 8.3 kb transcript, perform unique functions in differentiated neuronal cells during the latent infection phase of infection. Interestingly the 2.0 kb intron is accumulated during acute infection and throughout latency, but the 1.5 kb intron only accumulates after a period of latency in neurons (Spivack and Fraser, 1988a). It is also interesting that although the 2.0 kb intron is accumulated in tissue culture infections, the 1.5 kb LAT is never accumulated in tissue culture infections suggesting that it requires a neuron infected with a latent HSV in order to accumulate 1.5 kb intron. The twintron appears to be unique to HSV-1. No 1.5 kb intron is detected in trigeminal ganglia neurons acutely infected with HSV-2 (Mitchell *et al*, 1990).

Introns are normally degraded rapidly after splicing (Moore *et al*, 1993), however the LAT introns are unusually stable. This stability is most likely due to a guanosine branch point upstream from a stem-loop structure, forming an unusually stable lariat at the 3' end (Krummenacher *et al*, 1997; Zabolotny *et al*, 1997). Previously published data determined that the half-life of the 2.0 kb intron is approximately 24hrs in SY5Y and COS-1 cells (Thomas *et al*, 2002). In this study we compare the half-life of the 1.5 kb and 2.0 kb LAT introns using a tetracycline-repressible system in Vero cells and Northern blot quantitation and show that they are similar.

Materials and Methods

Plasmids

pTet-tTak and pTet-Splice plasmids were obtained from Gibco-BRL. Construction of the plasmid pTet-LAT in pTet-Splice (Gibco-BRL) was as previously described (Thomas *et al*, 2002). This plasmid expresses the stable 2.0 kb LAT intron. The plasmid has a minimal hCMV immediate-early promoter regulated by Tn10 (a tetracycline-resistant operon). The pTet-tTak plasmid encodes a fusion protein of a tetracycline DNA binding domain and the transactivation domain of HSV VP16. In the absence of tetracycline, the pTet-tTak encoded activator binds to the Tet operator sequence in the LAT plasmid and transcription occurs. In the presence of tetracycline, the activator cannot bind to DNA, allowing only low-level basal transcription.

Cell culture and Transfection

Vero cells (African Green Monkey kidney epithelial cells) were maintained in T-175 flask in Gibco's Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% serum, and antibiotics Penicillin and Streptomycin at 37 degrees in a humidified incubator with 5% CO₂. For transfection 6.5×10⁵ cells were seeded in 60mm culture dishes to grow overnight to a confluence of >90%. 5 µg of control plasmids pcDNA3 and pGFP, and 2.5 µg of plasmids pTet-LAT and pTet 1.5 kb were diluted in Lipofectamine 2000 and Opti-MEM mixture based on manufacturers recommendations. The cells were incubated in the plasmids/lipofectamine 2000 mixture in Opti-MEM for 5 hours, then DMEM with 10% serum and pen/strep was added, and samples were kept in a 37 degrees 5% CO₂ humidified incubator for 24 hours. After the incubation, 3µg/ml tetracycline in DMEM was added to the test samples, while in control samples, no tet, pGFP, and pcDNA3 only the media was replaced with fresh one.

Whole RNA collection and RNA extraction

TRIzol was used to harvest total cellular RNA from the samples at 0, 1, 3, 6, 9, 12 and 24 hr post-tetracycline treatment, while controls always tet, no tet, pGFP and pcDNA3 were harvested at the end with 24 hr time-point. For RNA purification, chloroform and isopropanol were used to get rid of proteins, lipids and cellular debris, then RNA was precipitated using 70% ethanol and was resuspended in 0.1% v/v diethylpyrocarbonate (DEPC) treated water.

Gel electrophoresis and RNA transfer to membrane

10 µg of each RNA time point sample, and 3µg RNA Millennium™ Marker were denatured with deionized glyoxal mixture (Russell, 2001); denatured RNA samples were mixed with RNA loading buffer and loaded on gel prepared with 1.5% agarose in 1X BPTE buffer (Russell, 2001) along with ethidium bromide. The gel was ran at 100V for 2 hours, which resulted in separation of RNA bands based on size. After the electrophoresis, under UV illumination a picture was taken to locate 1.5 kb, 2.0 kb and 18s RNA bands on gel. The gel was then rinsed with 0.1% DEPC water, then was gently washed sequentially with 0.05N Sodium hydroxide, and 20X SSC buffer (Russell, 2001). For northern hybridization the RNA on gel was transferred to GeneScreen Plus® Hybridization Transfer Membrane using vacuum transfer at 50psi for 2 hours. The membrane was then reverse glyoxylated by shaking in boiling 20mM RNase-free Tris-HCL for 5 min. The membrane was UV cross-linked using Stratalinker (Stratagene).

Northern Hybridization

BstEII-BstEII fragment was cut from PstI-MluI cassette to be used as a probe (Figure 1). 25ng of the BstE II-BstE II probe was denatured and mixed with RadPrime DNA Labeling System along with 50 μ Ci alpha-32 P labeled dCTP and incubated overnight at 55 degrees. Next morning, the membrane was washed sequentially with 1XSSC and 0.1% SDS, then with 0.5X SSC and 0.1% SDS. After drying, the membrane was sealed in Amersham Biosciences phosphor storage cassette for 24 hours. After the exposure, blot was viewed using Typhoon phosphorimager, the data was analyzed by measuring volume of different bands using the Image quant Software version 1.2. To offset the dilution of LAT signal due to cellular replication, the individual volume reports were normalized by multiplying with $e(t)\ln 2/t_D$ (where t is the time of tetracycline repression, and t_D is the doubling time of the cells) as described by Thames et al (Thames *et al*, 2000). The normalized sample volumes were measured by using “Always tet” as a standard, and then converted into a fraction of the 0hr sample. For drawing graphs Microsoft Excel software was used. LAT expression of 1.5 kb and 2.0 kb was averaged based on the triplicate experiments, and was plotted versus time. Using Regression analysis, the half-life of the 1.5 kb and the 2.0 kb LAT introns was calculated. For statistical analysis, Student's t-test was used to measure if the two introns have similar half-lives.

Results

Isolation and cloning of the 1.5-kb LAT intron RNA

We have eliminated the 0.5 kb intron sequence (coordinates 119737–120295) from the 2.0 kb intron, and constructed a plasmid with only the ability to make the 1.5-kb LAT intron (119461–120480). Briefly, cDNA was synthesized from RNA isolated from mouse trigeminal ganglia latently infected with HSV-1 strain 17⁺ using reverse transcriptase (Promega) and Oligo-dT (Promega) according to the manufacturer. The cDNA obtained was used as a template for a PCR reaction. Primers PF (TCCATCCAATCTGTGGACGAAG) and PR2 (TGTTGGGCAGGCTCTGGTGTGA) were used to amplify a 1.03 fragment corresponding to the 2.0 kb LAT intron and an overlapping 0.53kb fragment corresponding to the 1.5 kb intron. The 0.53 kb product of the PCR reaction was separated by gel electrophoresis and cloned into vector pGem-T. Recombinants were screened for the presence of a 168bp Hpa-Hpa product, and confirmed by sequencing.

The 131bp *Hin*II-*Hpa*I fragment (119614–120302) from clone pGem-T 131, the *Hpa*I-*Hpa*I (120302–120470) and *Hpa*I-*Hind*III (120470–121597) fragments were ligated into pGEMPst/Mlu plasmid, which contains the LAT Pst-Mlu sequence (digested with *Hind*III and *Hpa*I) to yield the plasmid pGEM1.5LAT. This insert, containing the 1.5-kb intron, was further subcloned in pcDNA3 (Invitrogen) to yield pcDNA1.5LAT, and was further cloned into the pTet-Splice (Gibco-BRL) plasmid for use in intron half- life measurement experiments.

Measurement of Intron Half-Life

In order to measure the half-life of the 1.5 kb LAT intron, and to compare it to the 2.0 kb LAT intron, a tetracycline repressible system was employed (Gibco-BRL).

Vero cells were grown overnight in eleven 60 mm tissue culture dishes to a confluence of approximately 90%. Nine dishes of cells were co-transfected with plasmids 1.5 kb LAT, 2.0 kb LAT and pTet-tTak as described in the methods. The remaining two dishes of cells were transfected with pEGFP or pcDNA3 plasmid. After 24 hours of co-transfection, the transcription of both 1.5 kb and 2.0 kb intron RNAs was repressed by addition of tetracycline to the media of eight dishes (except the “no-tet” control). To measure the half-life, the total

cellular RNA was harvested at 0, 1, 3, 6, 9, 12, and 24 hours post-repression. We collected total cytoplasmic and nuclear RNA because we have previously shown that 2kb LAT intron is distributed between both compartments during tissue culture infection. The controls No-tet (Tetracycline was not added, not repressed), always tet (Tetracycline was always present in the media, always repressed), GFP (to measure transfection efficiency), and pcDNA3 (Empty vector used as negative control) were harvested along with the 24 hour time-point at the end. After separating the denatured RNA samples by agarose gel electrophoresis, the RNAs were transferred from the gel to a membrane using vacuum transfer as described in the methods. For Northern hybridization, the membrane was probed with 32P labeled BstEII-BstEII (120095–121053) fragment DNA, phosphorimaged and bands analyzed by imagequant software (as described in the Materials and Methods). Three independent experiments were performed. Figure 2 shows a typical image of a northern blot. Figures 3 and 4 show the averaged LAT expression vs time plot for the half-lives of the two introns. Using regression analysis the half-life of the 1.5 kb and 2.0 kb LAT was measured to be 14.8 hours (standard deviation= 5.5) and 13.5 hours (standard deviation=4.3). For statistical analysis, a two tailed t-test was used; at $p=0.05$, the difference between the half-lives of the two introns is insignificant, hence we conclude that they have a similar half-life.

Discussion

The 2.0 kb LAT intron is known to accumulate in host cells during the late lytic or latent infection cycle of HSV-1 (Dobson *et al*, 1989; Rodahl and Haarr, 1997; Spivack and Fraser, 1988b; Stevens *et al*, 1987; Wu *et al*, 1996). It has been reported to be unusually stable with a half life of several hours (Thomas *et al*, 2002). The reason behind its stability has been attributed to its non-linear lariat intron structure (Wu *et al*, 1998), and guanosine branch point upstream from a stem-loop structure, leading to inefficient debranching (Krummenacher *et al*, 1997; Zabolotny *et al*, 1997). After the removal of a 0.5 kb intron, which overlaps the 2.0 kb intron (Fig1), from the 8.3 kb primary LAT (mLAT), a 1.5 kb LAT intron is formed. Although this intron has been previously detected by Northern hybridization of latent peripheral nervous system tissue RNA (Spivack *et al*, 1991), its half-life has not yet been reported.

In this study we measured the half-life of the 1.5 kb LAT intron, using a tet (inducible plasmid - tissue culture system, and Northern hybridization of the resulting RNA. The half-life was found to be of the order of several hours - a half-life much longer than any normal intron. Comparison of the half-lives of the 1.5 kb and 2.0 kb introns by co-transfection of cells with both 1.5 kb and the 2.0 kb LAT expressing tet inducible plasmids, revealed that the average half-life of the 1.5 kb and the 2.0 kb LAT was 14.8 and 13.5 hours respectively.

Previously Thomas *et al.*, measured the half life of the 2.0 kb LAT intron, and reported it to be around 24 hours (Thomas *et al*, 2002). The averaged half-life we measured is different by a factor of 2. Given that the half-life is approximately $\times 10^4$ fold more stable than the average cell intron, we think that a factor of 2 is not significant difference. We are not sure what factors account for the difference, however the half -lives of the 2 introns are similar suggesting that the unusual intron stability is not affected by the loss of the 0.5 kb from the 1.5 kb intron lariat region.

The identical half-live implies that the 1.5 kb LAT retains the structural features of the 2.0 kb LAT intron that make it unusually stable. Previously we hypothesized that the stability of the 2.0 kb intron is due to its unusual branch point and 3' hairpin structure (Krummenacher *et al*, 1997; Zabolotny *et al*, 1997). This region is not altered on removal of the 0.5 kb intron. This 0.5kb intron does have a consensus mammalian splice branch point and is not stable – never accumulating on Northern blots of infected cells.

Wu et al., suggested that the 1.5 kb LAT intron tends to accumulate more efficiently in latent stage of infection because it is slightly more stable than the 2.0 kb LAT, as the shorter lariats are more stable (Wu *et al*, 1998). Although the measured difference between the half-lives of the two introns is statistically insignificant, in each individual experiment we found that the half-life of the 1.5 kb LAT was slightly higher than the 2.0 kb LAT.

The 1.5 kb LAT is accumulated during latent infection stage (Wagner *et al*, 1988), in contrast with the 2.0 kb LAT which is found to accumulate to high levels both in late lytic and latent stages of infection (Dobson *et al*, 1989; Spivack and Fraser, 1988b; Stevens *et al*, 1987). Researchers working on euglena have suggested that splicing of the internal sequence is possible before, or along with the splicing of the external sequence, but never after the external sequence is spliced (Copertino *et al*, 1992). The 1.5 kb LAT is formed after removal of the 0.5 kb fragment from the 2.0 kb, this means that formation of 1.5 kb and the 2.0 kb cannot occur simultaneously from a single transcript. The 1.5 kb predominates in latent infection, and our observation that the half life of the 1.5 kb is similar to the 2.0 kb LAT intron, implies that the 1.5 kb is important for the virus latent cycle. Construction of a virus expressing only the 1.5 kb LAT intron will help resolve these issues.

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Figure 1. The LAT locus of the HSV-1 genome and Twintron processing

(A) The linear HSV-1 genome with the Unique Long (U_L) and Unique Short (U_S) regions flanked by the Terminal Repeat Long (TR_L), Internal Repeat Long (IR_L), Internal Repeat Short (IR_S), and the Terminal Repeat Short (TR_S) regions.

(B) The LAT locus maps to the repeat regions of the genome with the ICP0 and γ -34.5 genes overlapping the LAT primary 8.3 kb RNA. The L/ST's also overlap the LAT gene to some extent.

(C) The 8.3 kb primary LAT transcript is spliced to make a 2.0 kb intron that is unusually stable, and a 6 kb mRNA that is short lived and for which no protein has been conclusively identified.

(D) Alternatively the 8.3 kb primary transcript can be spliced to remove a 0.5 kb intron that is unstable and a 7.8 kb RNA that can be further spliced to reveal a 1.5 kb intron and the 6.3 kb RNA produced from the 2.0 kb splicing pathway. This arrangement of nested introns is called a Twintron.

Figure 2. Northern Blot of the 2.0 kb and 1.5 kb LAT introns

The half-life of the 1.5 kb and 2.0 kb LAT introns were measured with a tetracycline-repressible transient-transfection system. Vero cells were transfected with 2.5 μ g of pTet LAT and pTet 1.5LAT, and 5 μ g pTet tTak and incubated for 24 hours. The controls No-tet (not repressed), always tet (always repressed), GFP (to measure transfection efficiency), and pcDNA3 (negative control) were also incubated for 24 hours. Transcription was repressed with 3 μ g/ml tetracycline in the eight test-samples, and RNA was isolated at the times (in hours) indicated post-repression. 10 μ g of total RNA was analyzed by Northern hybridization using the BstE II-BstE II LAT specific probe.

Figure 3.

The 1.5 kb. half-life was quantitated using ImageQuant software version 1.2. and a Typhoon phosphorimager. Spot volumes were made equivalent to the “always tet” control, and three independent experimental blots analyzed with the phosphorimager and Immagequant software. Using the Microsoft Excel software, graphs for relative LAT signal vs time were plotted. Regression analysis was used to measure the half-life of both the introns.

Figure 4.

The 2.0 kb half-life was quantitated using ImageQuant software version 1.2. and a Typhoon phosphorimager. Spot volumes were made equivalent to the “always tet” control, and three independent experimental blots analyzed with the phosphorimager and Imagequant software. Using the Microsoft Excel software, graphs for relative LAT signal vs time were plotted. Regression analysis was used to measure the half-life of both the introns.