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Detection and Quantitation of HTLV-1 and HTLV-2 mRNA Species by Real-time RT-PCR

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Summary

HTLV-1 and HTLV-2 are highly related delta-retroviruses that infect and transform T-lymphocytes, but have distinct pathogenic properties. HTLV replication and survival requires the expression of multiple gene products from an unspliced and a series of highly related alternatively spliced mRNA species. To date, the comparative levels of all known HTLV-1 and HTLV-2 viral mRNAs in different transformed cell lines and at different stages of virus infection have not been assessed. In this study, we compiled a series of oligonucleotide primer pairs and probes to quantify both HTLV-1 and HTLV-2 mRNA species using real-time RT-PCR. The optimized reaction for detection of each mRNA had amplification efficiency greater than 90% with a linear range spanning 25 to 2.5×10^7 copies. The R^2 s of all standard curves were greater than 0.97. Quantitation of HTLV mRNAs between different cell lines showed variability (gag/pol $\geq$ tax/rex $>$ env $\geq$ accessory proteins), but the overall levels of each mRNA relative to each other within a cell line were similar. These results provide a method to quantify all specific mRNAs from both HTLV-1 and HTLV-2, which can be used to evaluate further viral gene expression and correlate transcript levels to key stages of the virus life cycle and ultimately, pathogenesis.

Keywords

HTLV-1; HTLV-2; leukemia; mRNA; real-time RT-PCR

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) are highly related complex retroviruses that transform T-lymphocytes in cell culture and persist in infected individuals (Feuer and Green, 2005). However, the clinical manifestations of these two viruses differ significantly. HTLV-1 preferentially targets and transforms CD4⁺ T-cells and is the

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etiologic agent of adult T-cell leukemia (ATL) (Yoshida et al., 1982) and HTLV associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al., 1986). HTLV-2 is much less pathogenic, but has been associated with a few cases of atypical hairy cell leukemia and neurological disease (Hjelle et al., 1992; Kalyanaraman et al., 1982). Elucidation of the mechanisms by which infection with these two viruses results in distinct outcomes will provide fundamental insights into the initiation of multistep leukemogenesis.

Cytoplasmic expression of unspliced and a complex array of mono-or bicistronic alternatively spliced HTLV mRNAs results in production of the virion-containing, regulatory, and accessory gene products (Fig. 1). Both HTLV-1 and HTLV-2 express the structural and enzymatic proteins Gag, Pol and Pro from the unspliced full length mRNA (Lee et al., 1984; Nam et al., 1988). This unspliced mRNA also serves as genomic RNA to be packaged into progeny virions. Env is expressed from a singly spliced mRNA (Paine et al., 1994) and plays a major role in receptor recognition and target cell infection. Env is also the determinant for differences in transformation tropism exhibited between HTLV-1 and HTLV-2 (Xie and Green, 2005). HTLV-1 and HTLV-2 express a doubly or completely-spliced mRNA that produces the positive regulatory proteins Tax from the pX open reading frame (ORF) IV and Rex from the partially overlapping ORF III. Tax increases the rate of transcription from the viral long terminal repeat (LTR) (Cann et al., 1985; Felber et al., 1985; Inoue et al., 1987) and modulates the transcription or activity of numerous cellular genes involved in cell growth and differentiation, cell cycle control, and DNA repair (Leung and Nabel, 1988; Mulloy et al., 1998; Ressler et al., 1997; Schmitt et al., 1998; Siekevitz et al., 1987). Compelling evidence indicates that the pleiotropic effects of Tax on cellular processes are required for the transforming or oncogenic capacity of HTLV (Endo et al., 2002; Grossman et al., 1995; Robek and Ratner, 1999; Ross et al., 2000; Ross et al., 1996; Wycuff and Marriott, 2005). Rex acts post-transcriptionally by preferentially binding, stabilizing, and selectively exporting intron-containing viral mRNAs from the nucleus to the cytoplasm (Younis and Green, 2005). The function of amino terminal truncated forms of Rex (p21rex in HTLV-1 and p22/p20rex in HTLV-2) expressed from singly spliced mRNAs have yet to be clearly defined, but some evidence suggests that they may interfere with full-length Rex localization and function (Ciminale et al., 1997; Kubota et al., 1996; Shuh et al., 1999).

The accessory proteins of HTLV are encoded by several pX ORFs between env and the 3' LTR. In HTLV-1, p12 and p27 are encoded by ORF I and p13 and p30 by ORF II. p12 is potentially expressed from both single and doubly spliced mRNAs, localizes to the ER and cis-Golgi apparatus, and activates cells by regulating calcium signaling (Ding et al., 2001; Ding et al., 2003). p12 enhances LFA-1 T-cell adhesion in a calcium-dependent manner (Kim et al., 2006) and associates with cellular proteins including the 16kDa subunit of the vacuolar ATPase, IL-2 receptor β and γ chains, and MHC class I heavy chain (Johnson et al., 2000; Koralnik et al., 1995; Nicot et al., 2001). Together, these activities likely play key roles in viral infection, spread and escape from the immune system and are consistent with the requirement for p12 in the efficient infection of quiescent T-lymphocytes in culture by HTLV-1 (Albrecht et al., 2000) and persistence of the virus in inoculated rabbits (Collins et al., 1998). Furthermore, p12 appears to be preferentially expressed over p27. Although there is evidence that p27 is expressed *in vivo* (Pique et al., 2000) and *in vitro* (Ciminale et al., 1992), the precise function of p27 in HTLV-1 replication remains unclear.

p30, expressed from a doubly-spliced mRNA, is a multifunctional regulator that differentially modulates viral and/or cellular gene expression at the transcriptional level through association with cellular proteins including p300/CBP and TIP60 (Awasthi et al., 2005; Zhang et al., 2001; Zhang et al., 2000) and post-transcriptionally via binding and retaining tax/rex mRNA in the nucleus (Nicot et al., 2004; Younis et al., 2006; Younis et al., 2004). Although p30 is dispensable *in vitro* for replication and cellular transformation (Derse et al., 1997; Robek et

al., 1998), it is required to promote virus survival and persistence in infected animals (Silverman et al., 2004). p13, expressed from a singly-spliced mRNA, localizes to the mitochondria, has a suppressive effect on cell growth *in vitro* and plays an essential biological role during the early phase of infection *in vivo* (Hiraragi et al., 2006; Silic-Benussi et al., 2004).

In HTLV-2, the accessory proteins include p10 encoded by ORF I, p28 by ORF II, and p11 by ORF V (Ciminale et al., 1995). HTLV-2 p28, with the potential to be expressed from two distinct singly-spliced mRNAs (both of these mRNAs also have the potential to produce p22/p20rex), is at least in part functionally homologous to HTLV-1 p30. It functions to repress viral replication post-transcriptionally by retaining tax/rex mRNA in the nucleus (Younis et al., 2004). p10 and p11 are expressed from the same doubly spliced mRNA in separate but overlapping reading frames. Although less is known about the role these two proteins play in the biology of HTLV-2, p10, like HTLV-1 p12, binds to the free chain of MHC class I but not to the IL-2R β and γ chains (Johnson et al., 2000) and p11 binds to MHC class I heavy chain (Johnson et al., 2001).

HTLV-1 expresses a minus-strand singly-spliced mRNA that is transcribed by a functional promoter present in the antisense strand of the proviral genome (Cavanagh et al., 2006; Larocca et al., 1989). This transcript encodes HBZ (HTLV-1 b-ZIP factor) and has not been detected in HTLV-2. Exogenously over-expressed HBZ down-regulates Tax-induced HTLV-1 transcription and interacts with and disrupts the DNA binding activity of ATF-4, JunB and c-Jun (Basbous et al., 2003; Gaudray et al., 2002; Matsumoto et al., 2005). Compared to wild type HTLV-1, HBZ mutant viruses, while retaining the ability to infect and immortalize T-cells in culture, when introduced into rabbits elicited diminished antibody response to viral gene products and generated reduced numbers of proviral DNA copies in PBMCs (Arnold et al., 2006). In addition, suppression of HBZ gene transcription by short interfering RNA appears to inhibit the proliferation of cells derived from ATL patients and, conversely, over-expression of HBZ mRNA promoted the proliferation of a human T-cell line (Satou et al., 2006). Together these data suggest that the HBZ gene may have a bimodal function in two different molecular forms.

Previously, real-time RT-PCR has been used to quantify some pX mRNAs of HTLV-1 (Princler et al., 2003). The goal of this paper was to compile and develop a panel of oligonucleotide primer pairs and probes to be used in Taqman real-time RT-PCR to reproducibly quantify and compare all expressed HTLV-1 and HTLV-2 mRNAs. Real-time RT-PCR monitors the fluorescence emitted during each PCR cycle as an indication of amplicon production during the reaction, as opposed to endpoint detection used in other quantitative RT-PCR methods. It allows quantitation of the initial amount of template most specifically, accurately, and with high sensitivity over a wide dynamic range. In the present study, this procedure was used to quantify HTLV-1 and HTLV-2 mRNAs in established transformed T-cell lines, stable provirus transfected producer B-cell lines, and newly immortalized primary T-lymphocytes. Since some of the HTLV accessory proteins have not been detected in infected cells by western blot, the HTLV mRNA expression profile will provide useful information in assessing the function of specific viral genes in the process of infection and various stages of cellular transformation and ultimately pathogenesis.

2. Material and methods

2.1. Cell lines

Established transformed human T-cell lines SLB-1 (HTLV-1) and MoT (HTLV-2), stable provirus transfected B-cell lines 729ACHneo (HTLV-1) and 729pH6neo (HTLV-2), and the human 729 B-cell line were maintained in Iscove's medium supplemented with 10% fetal

bovine serum (FBS), 2mM glutamine, penicillin (100 U/mL), and streptomycin (100µg/mL). Immortalized primary human T-lymphocyte cell lines PBL-ACH (HTLV-1) and PBL-pH6 (HTLV-2) were grown in RPMI medium supplemented with 20% FBS, antibiotics, and 10U/mL interleukin-2 (IL-2).

2.2. Plasmids

A corresponding proviral or cDNA plasmid clone was obtained or generated for each mRNA species to be detected and quantified. This plasmid DNA was used to determine primer and probe specificity and to optimize real-time PCR reaction conditions by generating a standard curve. Ten-fold serial dilutions of individual plasmids were made ranging from 2.5×10^0 to 2.5×10^7 copies. Standard dilutions were stored at -20°C in single use aliquots. SE356 (Ye et al., 2003), and JA662 (Arnold et al., 2006) were used to quantify HTLV-1 tax/rex, and HBZ mRNA, respectively. pMS9-7.8, pMT2-2.3, pMT2-5.4B, pMS9-1.8, and pMS9-11.1, specific for HTLV-1 p21rex, p12 (using splice acceptor site at nt6383), p12 (using splice acceptor site at nt6478), p27/p12 and p30 transcripts, respectively, were a generous gift from D. Derse (NIH, Frederick, MD) and previously described (Princlar et al., 2003). ML765, ML627, and ML766, used to quantify env transcripts are specific for HTLV-1 exon 1/exon 2 (terminal exon for env) junctions utilizing splice acceptor sites at nt 4501, nt 4641, and nt 4658, respectively and ML628, ML764, and ML760, specific for HTLV-1 p13, HBZ minor spliced transcript, and gag/pol unspliced transcript, respectively, were generated by PCR amplification followed by insertion into pCRScript (Stratagene, La Jolla, CA): ML765, partial exon 1 (nt 103–119) fused to exon 2 (nt 4501–4831) and partial intron 2 (nt 4832–4919); ML627, partial exon 1 (nt 100–119) fused to exon 2 (nt 4641–4831) and partial intron 2 (nt 4832–4919); ML766, partial exon 1 (nt 103–119) fused to exon 2 (nt 4658–4831) and partial intron 2 (nt 4832–4919); ML628, partial exon 1 (nt 102–119) fused to partial exon 3 (nt 6875–7170); ML764 (numbering is based on ACH antisense strand proviral sequence beginning with the last nt of U5 of the 3' LTR), exon 1 (nt 212–225) fused to partial exon 2 (nt 1765–1826); ML760, nt 920–1049. IY531, and IY595 (Younis et al., 2004) were used to quantify HTLV-2 tax/rex and env mRNAs, respectively. ML637, ML638, ML674 and ML761, specific for HTLV-2 p28, p22/p20rex-1, p28, p22/p20rex-2, p10/p11 and gag/pol transcripts, respectively were generated by PCR amplification of fragments across the appropriate splice junction followed by insertion into pCRScript (Stratagene, La Jolla, CA): ML637, partial exon 1 (nt 117–135) fused to partial terminal exon (nt 6630–6984); ML638, partial exon 1 (nt 117–135) fused to partial exon 3 (nt 6900–6984); ML674, partial exon 1 (nt 123–135) fused to exon 2 (nt 4730–4869) fused to partial terminal exon (nt 6493–6508); ML675, partial exon 1 (nt 123–135) fused to exon 2 (nt 4730–4869) fused to partial terminal exon (nt 6630–6645); ML761, nt 1887–1986. pBluescript-hGAPDH was a gift from K. Boris-Lawrie (Ohio State University, Columbus, OH).

2.3. RNA extraction, mRNA purification and cDNA synthesis

All cells for RNA extraction were in the log phase of growth at the time of harvesting. Cells were counted, harvested and washed with PBS prior to RNA extraction. Total RNA was extracted from 10^7 cells using the RNeasy kit and DNase treated columns followed by Poly A + mRNA isolation using the Oligotex kit (Qiagen Inc., Valencia, CA). First strand cDNAs were prepared with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA) using random hexamers as described by the manufacturer.

2.4. PCR primer pairs and specific probes

All primer pairs and probes for detection of HTLV-1 and HTLV-2 mRNAs are summarized in Table 1 (HTLV-1) and Table 2 (HTLV-2). Newly designed and generated primers used to specifically detect HTLV-1 transcripts include: [ENV4501-S] $5'-^{106}\text{CGTCCGCCGTCTAG}^{119\wedge 4501}\text{CCCT}^{4504}\text{-3'}$, [ENV4641-S]

5'-¹⁰⁶CGTCCGCCGTCTAG¹¹⁹Δ⁴⁶⁴¹CTTCC⁴⁶⁴⁵-3', [ENV4658-S]
 5'-¹⁰⁸TCCGCCGTCTAG¹¹⁹Δ⁴⁶⁵⁸CCGC⁴⁶⁶¹-3', [ENV-AS]
 5'-⁴⁹⁰¹ATTGTGAGAGTACAGCAGC⁴⁹¹⁹-3', [P13-S]
 5'-¹⁰⁷GTCCGCCGTCTAG¹¹⁹Δ⁶⁸⁷⁵CAGGT⁶⁸⁷⁹-3', [TR-AS]
 5'-⁷⁰⁵⁷CCGAACATAGTCCCCCAGAGA⁷⁰³⁷-3', [HBZMAP1]
 5'-¹⁹⁰⁵CTTCTAAGGATAGCAAACCGTCAAG¹⁸⁸¹-3', [HBZMAP2]
 5'-³⁵³ATGGCGGCCTCAG³⁶⁵Δ¹⁷⁶⁵GGCT¹⁷⁶⁸-3' [HBZminor-S]
 5'-²¹²CCGGCTGAGTCTAG²²⁵Δ¹⁷⁶⁵GGCTG¹⁷⁶⁹-3' [HBZ-AS]
 5'-¹⁸³⁰GCCCCGTCCACCAATTCT¹⁸¹³-3' (HBZ primer numbering is based on ACH antisense strand proviral sequence beginning with last nt of U5 of the 3' LTR). Primers #19 (5'-¹⁰³⁶GAGGGAGGAGCAAAGGTACTG¹⁰¹⁶-3'), #20 (5'-⁹³⁸AGCCCCCAGTTCATGCAGACC⁹⁵⁸-3') used to detect HTLV-1 gag/pol and H1JA2 (5'-⁶⁸⁸⁵AGGAGCGCCGTGAGCGCAAGT⁶⁸⁶⁵-3') used to detect HBZ antisense transcript have been described previously (Kusuhara et al., 1999, Arnold, 2006 #3637). Primers X2TR1-2 (5'-⁴⁸¹⁹ACCAACACCATGG⁴⁸³¹Δ⁶⁹⁵⁰CCCA⁶⁹⁵³-3'), P21Rex-S (5'-¹⁰⁷GTCCGCCGTCTAG¹¹⁹Δ⁶⁹⁵⁰CCCA⁶⁹⁵³-3'), P12-6383-S (5'-¹⁰⁷GTCCGCCGTCTAG¹¹⁹Δ⁶³⁸³CAAC⁶³⁸⁶-3'), P12-6478-S (5'-¹⁰⁷GTCCGCCGTCTAG¹¹⁹Δ⁶⁴⁷⁸CACT⁶⁴⁸¹-3'), P12-AS (5'-⁶⁵⁵²GGAGAAAGCAGGAAGAGC⁶⁵³⁵-3') P27S (5'-⁴⁸¹⁹ACCAACACCATGG⁴⁸³¹Δ⁶³⁸³CAACT⁶³⁸⁷-3'), X2P30-S (5'-⁴⁸¹⁹ACCAACACCATGG⁴⁸³¹Δ⁶⁴⁷⁸CACTA⁶⁴⁸²-3'), TR1-AS (5'-⁷¹⁷⁰GAGTCGAGGATAAGGAAC⁷¹⁵²-3'), have been described previously (Princlar et al., 2003). Primers designed and generated to quantify HTLV-2 mRNA species include: [GP2-S] 5'-¹⁹⁰⁴GCCTACCCAAGCGCTACTT¹⁹²²-3', [GP2-AS] 5'-¹⁹⁷⁰CCCGGGCAGGAGTGTCT¹⁹⁵⁴-3', [ENV2-AS] 5'-⁴⁸⁸⁸AGTAGGAAGAAAACATTACCCATGGT⁴⁸⁶³-3', [TR2-S] 5'-¹²³TGCTCCTCCCAAG¹³⁵Δ⁴⁷³⁰GAAGC⁴⁷³⁴-3', [TR2-AS] 5'-⁶⁹¹⁵AATCCTGGGAAATGGG⁶⁹⁰⁰Δ⁴⁸⁶⁹CCAT⁴⁸⁶⁶-3', [P28-1-S] 5'-¹²⁴GCTCCTCCCAAG¹³⁵Δ⁶⁶³⁰GCGCT⁶⁶³⁴-3', [P28-2-S] 5'-¹²³TGCTCCTCCCAAG¹³⁵Δ⁶⁹⁰⁰CCCAT⁶⁹⁰⁴-3', [P28-AS] 5'-⁶⁹⁸⁴GGACACCAATCGGCCTGTAC⁶⁹⁶⁵-3', [P10-AS] 5'-⁶⁵⁰⁶GGGAAAAGAAGGTC⁶⁴⁹³Δ⁴⁸⁶⁹CCATG⁴⁸⁶⁵-3', [?-AS] 5'-⁶⁶⁴³GCAGAAAGGAGCGC⁶⁶³⁰Δ⁴⁸⁶⁹CCAT⁴⁸⁶⁶-3'. Primers [hGAPDH-S] 5'-CATCAATGACCCCTTCATTGAC-3' and [hGAPDH-AS] 5'-CGCCCCACTTGATTTTGGGA-3' were used to quantify hGAPDH.

Probes to detect amplified PCR products include [TMP-1] 5' FAM-⁶⁴⁸⁹TTCGCCTTCTCAGCCCCCTTGTCT⁶⁵¹¹-TAMRA3', [TMP-2] 5' FAM-⁷¹⁰⁵ATCACCTGGGACCCCATC⁷¹²²-TAMRA3' previously described (Princlar et al., 2003) and newly designed [TMP-3] 5' FAM-⁹⁹⁰CTGCCAAAGACCTCCAAGACCTCC¹⁰¹³-TAMRA3', [TMP-4] 5' FAM-⁴⁸⁷⁴CCCTCATCCTCGGTGATTACAGCCC⁴⁸⁹⁸-TAMRA3', [TMP-5] 5' FAM-TGGCAAATTCCATGGCACCGTC-TAMRA3', [TMP-6] 5' FAM-⁴⁸⁰⁰AAGCTGCATGCCCAAGACCAGACGCC⁴⁸²⁵-TAMRA3', [TMP-7] 5' FAM-⁶⁹³⁹ACCCCGTCTACGTGTTTGGCTATTG⁷³⁶³-TAMRA3', [TMP-8] 5' FAM-¹⁹²⁵CACAGGAGCCGACCTTACGGTTATACCC¹⁹⁵²-TAMRA3', [TMP-10] 5' FAM-⁷⁰⁴⁷ACTGTGTACAAGGCGACTGGTGCCC⁷⁰⁷¹-TAMRA3', [TMP-13] 5' FAM-¹⁷⁸²CCTGTGCCATGCCCGGAGGA¹⁸⁰¹-TAMRA3'.

2.5. Real-time PCR

The instrumentation and general principles of the Prism 7000 sequence detector system (Perkin Elmer/Applied Biosystems, Foster City, CA), have been described in detail in the operator's manual. PCR amplification was carried out in 96 well plates with optical caps. The final

reaction volume was 25 μ l consisting of 12.5 μ l Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA), 200nM specific probe (FAM and TAMRA labeled), 600nM of each specific primer, and 2.5 μ l of cDNA template. For each run, standard cDNA, sample cDNA and no template control were all assayed in duplicate. The reaction conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60 °C.

2.6. Genomic DNA extraction and proviral load measurement

Cells in log phase of growth were harvested, washed once with PBS, and enumerated. Genomic DNA was isolated from 5 $\times$ 10⁶ cells using the Puregene™ DNA isolation kit (Gentra, Minneapolis, MN) and HTLV proviral load was amplified by real-time PCR using primer/probes for respective gag/pol (Table 2A (HTLV-1) and Table 2B (HTLV-2)).

3. Results

3.1. HTLV-1 and HTLV-2 mRNAs

The mRNA species expressed by HTLV-1 and HTLV-2 and the corresponding proteins they putatively encode are depicted in Figure 1A and 1B, respectively. All but one of the HTLV-1 mRNA species are encoded by the positive sense strand of the proviral genome including the unspliced/genome RNA (Fig 1A). These mRNAs share common initiation and polyadenylation sites. All spliced mRNAs share the non-coding exon 1, which starts at the transcription initiation site at the beginning of R and ends at the major splice donor site at position 119. The terminal exon for env mRNA and exon 2 of the doubly spliced mRNAs are defined at the 5' end by alternative splice acceptor sites at positions 4501, 4641 (major use), or 4658; the splice donor site at position 4831 defines the 3' boundary of exon 2 of the doubly spliced mRNAs. Four alternative splice acceptor sites at positions 6383, 6478, 6875, and 6950 are utilized to generate the 3' exon of singly and doubly spliced mRNAs. All HTLV-1 mRNA nucleotide positions above can be converted to ACH provirus (Kimata et al., 1994) numbering by adding 351. Primers across splice junctions and specific probes were designed and utilized to detect and quantify closely related mRNA species. Because of the potential use for alternative splice acceptor sites (nt 4501, nt 4641 and nt 4658), quantitation of all env mRNA transcripts required three different splice site specific primer pairs. Similarly, utilization of alternative splice acceptor sites (nt 6383 or nt 6478) require two different splice site specific primer pairs to quantify singly spliced transcripts that have the potential to encode p12. Specific primers for the doubly spliced gene transcripts including tax/rex, p27/p12 and p30 were designed over the junction of exon 2 and their unique exon 3 junctions and thus each transcript could be quantified using a distinct single primer pair. The final spliced HBZ mRNA represents several closely related minus-strand singly-spliced mRNAs that are transcribed by a functional promoter present in the antisense strand of the 3' proviral genome. A recent report showed that there are multiple initiation sites for this mRNA in the 3' R U5 region (Cavanagh et al., 2006). Based on the ACH antisense strand proviral sequence (numbering begins with last nucleotide of U5 of the 3' LTR), there is a major transcript splice donor at position 365 and a minor transcript splice donor at position 225 both of which utilize the splice acceptor at position 1765. We designed and utilized two distinct primer pair sets to individually quantify both HBZ transcripts.

The splicing pattern for HTLV-2 is similar to HTLV-1 with the exception that HTLV-2 does not express an antisense (HBZ) transcript and alternative splice acceptor sites have not been reported for exon 2 (Fig. 1B). The spliced mRNAs all share a common non-coding first exon that initiates at the beginning of R and ends at the major splice donor site at position 135. The terminal exon for the singly spliced env mRNA and exon 2 of the doubly spliced mRNAs are defined at the 5' end by the splice acceptor site at position 4730; the splice donor site at position 4869 defines the 3' boundary of exon 2 of the doubly spliced mRNAs. Four alternative splice

acceptor sites at positions 6493, 6513, 6630, and 6900 are utilized to generate the 3' exons of the singly and doubly spliced mRNAs. All HTLV-2 mRNA nucleotide positions above can be converted to pH6neo provirus numbering by adding 314. It should be noted that two distinct singly spliced mRNAs using splice acceptors at positions 6630 or 6900 have the potential to encode both p28 and the truncated Rex products (p22/p20Rex). In addition, proteins translated from mRNAs in which the terminal exon uses splice acceptors at positions 6513 or 6630 (labeled ?) have yet to be defined.

3.2 Assay development and standardization

We employed site-specific primer pairs and Taqman probes to specifically quantify individual cDNAs in a mixture of highly related cDNA species. For each HTLV-1 and HTLV-2 mRNA a cDNA plasmid clone was obtained or generated and used to determine primer specificity and optimize real-time RT-PCR reaction conditions. Ten-fold serial dilutions of cDNA plasmid standards were made, ranging from 2.5 to 2.5×10^7 copies/reaction. The Prism 7000 sequence detector system records the fluorescence emitted at each annealing/extension step and plots it against the cycle number, generating amplification plots (data not shown). The threshold was set manually along the linear range of each plot; the cycle at which the amplification curve crosses the threshold is called the cycle threshold (Ct). A plot of the Ct versus the $\log_{10}$ value of the plasmid copy number results in a standard curve. Plasmid standards, oligonucleotide primer pairs, and probes used to specifically detect HTLV-1 mRNAs and HTLV-2 mRNAs are summarized in Table 1 and Table 2, respectively. The optimized standard curves for all primer pairs used in this study were linear over the 10^7 range with slopes ranging from -3.3 to -3.7 , indicating that the amplification efficiencies were greater than 90% (data not shown). The R^2 correlation coefficients of all the standard curves were greater than 0.97.

3.3. Quantitation of HTLV-1 and HTLV-2 mRNAs in cell lines

The cell lines used in this study included the uninfected human B-cell line 729, stably transfected viral producer B-cell lines 729ACHneo (HTLV-1) and 729pH6neo (HTLV-2), transformed T-cell lines SLB-1 (HTLV-1) and MoT (HTLV-2), and the newly immortalized human T-lymphocyte lines PBL-ACH (HTLV-1) and PBL-Ph6 (HTLV-2). It is well known that HTLV-expressing cell lines in culture are highly variable for proviral copy number and, in many cases, cell lines can contain partially deleted proviruses. These cell lines are no exception and proviral copy numbers ranged from approximately 1 to 6 per cell as determined by real-time PCR (Table 3). Poly A+ mRNA was isolated from 10^6 cells from each cell line that was characterized. We first determined whether the poly A+ mRNA samples were free of DNA contamination by standard reverse transcriptase PCR using gag/pol specific primers. As expected, in the presence of reverse transcriptase a gag/pol product was amplified in all HTLV-1 and HTLV-2 cell lines, whereas no product was detected in the absence of reverse transcriptase, confirming no viral DNA contamination (Fig. 2). Equal amounts of poly A+ mRNA from each cell line were converted to cDNA and amplified by real-time PCR. We initially performed real-time PCR on the cDNA for GAPDH to determine if it would be an acceptable normalization standard throughout our studies. Our results indicated that the Ct's for GAPDH amplification were similar and highly reproducible among the seven cell lines, ranging from 18.01 to 19.17 and thus validating its use as an appropriate standard for comparison of HTLV mRNA levels between cell lines (Table 3). Copy numbers of all HTLV mRNAs were calculated from standard curve plots using cDNA plasmids and then normalized to 10^6 copies of GAPDH cDNA.

3.3.1 HTLV-1 mRNA levels—The total mRNA copy number was approximately 8-fold higher in the established transformed T-cell line SLB-1 and the newly immortalized primary T-lymphocytes PBL-ACH as compared to the stable provirus transfected producer B-cells, 729ACHneo. In all three cell lines, the four most abundant mRNAs included gag/pol, tax/rex,

env, and p21rex. In SLB-1, env mRNA was approximately 10-fold lower than gag/pol, tax/rex, and p21rex mRNA; tax/rex mRNA was present at approximately 50- to 1000-fold higher levels than the mRNAs that encode the accessory proteins p27, p12, p30 and p13 ($p12 \geq p13 > p27/p12 > p30$); the antisense transcript that encodes HBZ was approximately 100-fold lower than tax/rex mRNA, but at levels closer to the high end of the accessory protein transcripts for p12 and p13. Since the immortalized IL-2 dependent cell line PBL-ACH is newly established and has been in culture for a limited amount of time, we consider it to be the most representative of early infection. In this cell line, gag/pol mRNA was the most abundant (5.7×10^6 copies per 10^6 copies of GAPDH). Tax/rex mRNA, which encodes the transforming protein Tax, was approximately 30-fold less in the PBL-ACH cell line than in the established transformed T-cell line. It is possible that this lower level of expression relative to SLB-1 cells may correlate with selection time in culture and IL-2 dependence. Tax/rex mRNA was approximately 2-fold and 3.5-fold higher than env and p21rex mRNA, respectively. The mRNAs that encode the accessory proteins p27, p12, p30 and p13 ranged from 7- to 20-fold lower than tax/rex mRNA ($p12 \geq p13 > p27/p12 > p30$); the antisense transcript that encodes HBZ was approximately 10-fold lower than tax/rex mRNA (4×10^4 copies per 10^6 copies of GAPDH). In the stable provirus transfected producer B cell line 729ACHneo, the accessory gene transcripts (p12, p27/p12, p30, and p13) were relatively lower (10- to 1000-fold) than tax/rex, env, p21rex, or HBZ. It should be noted that in all three cell lines the env transcript using the splice acceptor site at position 4641 was the most abundant of the three potential env transcripts; the p12 transcript using the splice acceptor site at position 6383 was at least 10-fold more abundant than the p12 transcript using splice acceptor site at position 6478; and, the HBZmajor transcript was at least 100-fold more abundant than the HBZminor transcript.

3.3.2 HTLV-2 mRNA levels—The total mRNA copy number was slightly higher (approximately 2- to 4-fold) in the established transformed T-cell line MoT and the newly immortalized primary T-lymphocyte line PBL-pH6 than in the stable provirus transfected producer B cell line 729pH6neo. In the newly immortalized PBL-pH6 cell line, the four most abundant mRNAs included gag/pol, tax/rex, env, and p28, p22/p20rex-1 ($\text{gag/pol} \geq \text{tax/rex} \geq \text{p28, p20/p22rex-1} > \text{env}$). HTLV-2 env mRNA was approximately 6-fold lower than gag/pol, tax/rex, and p28, p22/p20rex-1, which were similar in magnitude; tax/rex mRNA was present at approximately a 65- to 1400-fold higher level than the mRNAs that encode p10/p11, p28, p22/p20rex-2 and the doubly-spliced mRNA whose potential protein product(s) has yet to be characterized ($p? > \text{p28, p22/p20-2} > \text{p10/p11}$). Interestingly, in MoT p28, p22/p20rex-1 mRNA was the most abundant, which was 2-to 5-fold higher than gag/pol and tax/rex mRNAs, respectively. This observation is similar to what has been reported for the HTLV-1 transformed T-cell line MT2 (p21rex-encoding mRNA was the most abundant) and could be attributed to an increased number of deleted proviruses in this cell line (Princlar et al., 2003).

4. Discussion

Alternative splicing of mRNAs is important for cell-specific gene regulation (Lopez, 1998). Both HTLV-1 and HTLV-2 produce a set of closely related mRNAs by alternative splicing. The levels of all specific viral mRNAs in different HTLV-1 and HTLV-2 transformed cell lines and at different stages of virus infection have not been assessed and compared to date. The viral gene expression profile will provide useful information in assessing the function of specific viral genes in the process of viral infection and cellular transformation. In this study, we compiled and/or generated a series of oligonucleotide primer pairs and probes to quantify both HTLV-1 and HTLV-2 mRNA species using real-time RT-PCR. The combination of amplification/hybridization increases the specificity and sensitivity of the assay. The splice site-specific primers used in our experiments allow differentiation of the closely related alternatively spliced mRNAs. Specifically designed Taqman probes hybridize only to the segment to be amplified and emit signal upon amplification, which results in increased

detection specificity. The assay offers a wide dynamic range as seen by our ability to quantify specific cDNA plasmid samples accurately at concentrations from 25 copies to 2.5×10^7 copies per reaction. The 96-well plate format allows multiple samples to be analyzed independently in the same run so the reaction output is high for each experiment. The use of an internal control standard (GAPDH for these experiments) allows the normalization of gene expression in different cell lines and the ability to compare relative levels of transcript within and between cell types.

Our experiments quantified HTLV mRNAs in established transformed T-cell lines, stable provirus transfected producer cell lines, and newly immortalized primary T-lymphocyte cell lines. These cell lines expressed variable levels of the different viral mRNA but in general, a similar mRNA expression pattern emerged. We found that the total viral mRNA expression was about 2- to 7-fold higher for HTLV-1 than HTLV-2. This result is consistent with studies that demonstrated that HTLV-1 has stronger intrinsic promoter and Tax-1 transactivation activity than HTLV-2 (Ye et al., 2003). When comparing total HTLV-1 mRNA levels among the different cell lines tested, SLB-1 and PBL-ACH cells were higher than 729ACHneo cells. A similar result was observed for HTLV-2. We quantified the DNA proviral load in these cell lines and our results indicated that the proviral load (Table 3) was not proportional to the different mRNA levels. The simplest explanation for this is that the differential expression is attributable to the cell type (T-cell > B-cell). T-cells are the natural target cell for both HTLV-1 and HTLV-2. Another possibility is that the transcriptional activity is greater in cell lines in which proviruses are established by natural infection versus stable transfection. Moreover, our results showed a reverse correlation between viral mRNA levels and the antisense transcript encoding HBZ (2- to 4-fold higher levels of HBZ mRNA in 729ACHneo vs SLB-1 or PBL-ACH and lower total viral mRNA levels), supporting the hypothesis that HBZ may play an important role in HTLV-1 biology by counteracting the effects of Tax at the level of transcription.

Among all the mRNA species of HTLV-1, consistently the most abundant was the unspliced mRNA encoding the Gag/Pro/Pol proteins. The second in abundance was the tax/rex mRNA followed by the env mRNA. High level expression of these mRNAs would be expected in actively replicating cells in culture that are devoid of any immune surveillance. Tax and Rex are positive regulators of gene expression and are required for efficient production of the structural and enzymatic proteins Gag, Pol, and Env. By comparison, the expression levels of all accessory protein mRNAs were low. SLB-1 and PBL-ACH cells contained similar levels while the accessory protein mRNAs in 729ACHneo were approximately 10-fold lower. HBZ mRNA levels were similar in SLB-1 and PBL-ACH, yet slightly higher in 729ACHneo. The assumption, supported for at least Tax, Rex, and p21Rex proteins (Shuh et al., 1999), is that relative viral mRNA levels are proportional to the levels of their corresponding proteins produced. Gag, Env, Tax, Rex, and truncated Rex are relatively easy to detect by western blot (data not shown) and their corresponding mRNA levels were high in the cell lines tested. HBZ has been detected by western blot in SLB-1 and 729ACHneo (Arnold et al., 2006). The accessory proteins (p30, p12, p27, and p13) have not been detected in infected cell lines by western blot and the quantitation results show their corresponding mRNA levels are relatively low (approximately 10- to 10,000-fold lower than tax/rex mRNA).

Our analysis provided important information on some splice acceptor site utilization. Quantitation of the env transcripts revealed that the splice acceptor site at position 4641 is utilized 2- to 5 fold more than nearby splice acceptor sites at position 4511 and 4658. In addition, for the p12 singly spliced transcripts we found that splice acceptor site at position 6383 is favored over splice acceptor site at position 6478 by approximately 16- to 35-fold. Lastly, the HBZ major transcript is at least 100-fold greater than the HBZ minor transcript

indicating preferred utilization of splice acceptor site at position 365 vs 225 (Cavanagh et al., 2006).

The total HTLV-2 mRNA copy number was slightly higher in MoT and PBL-pH6 than in 729pH6neo. The relative copy numbers for gag/pol, tax/rex, and p28, p22/p20rex-1 mRNAs were similar, whereas env was approximately 10-fold less and p28, p22/p20rex-2 mRNA approximately 100-fold less. The mRNA levels for p10/p11 and p? were extremely low. Like HTLV-1, Gag, Env, Tax, Rex, and truncated Rex of HTLV-2 can be detected consistently by western blot in infected cell lines (data not shown). p28 has functional analogy with HTLV-1 p30 and to date, p28 like p30, has not been detected in HTLV infected cells by western blot (Younis et al., 2004). Indeed, by real-time PCR, p30 mRNA levels were low, which is consistent with the failure to detect p30 protein. However, p28, p22/p20rex-1 mRNA levels were demonstrated to be comparable or greater than gag/pol and tax/rex mRNA levels. One possible explanation for the failure to detect p28 is that the translation of truncated forms of Rex is favored by both p28, p22/p20rex-1 and/or p28, p22/p20rex-2 mRNA transcripts. An alternate possibility is that p28 is expressed preferentially from the p28 p22/p20rex-2 mRNA transcript, which was approximately 100- to 1000-fold less than the p28, p22/p20rex-1 mRNA transcript.

In conclusion, an accurate and reproducible methodology is described to detect specifically and compare HTLV-1 and HTLV-2 mRNA species. Our goal is to use this approach to measure the temporal viral gene expression profile at defined time points following infection of primary human T-lymphocytes in culture as well as in inoculated rabbits. This technique will be particularly valuable for dissecting the phenotypes of specific viral gene mutations in culture and in the infected host. Furthermore, the results will provide important information about the possible contribution of specific viral proteins to viral infection as well as cellular immortalization/transformation and ultimately HTLV pathogenesis.

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Figure 1.

Genome organization of HTLV-1 and HTLV-2 and their unspliced, singly spliced and doubly spliced mRNAs. (A). HTLV-1 expresses at least eight positive sense transcripts and one negative sense transcript. The genomic unspliced mRNA encodes the Gag, Pol and Pro proteins. Four singly spliced mRNA species are the result of splicing of exon 1 (nt 1–119) to splice acceptors at positions 4501, 4641, or 4658 (Env), 6383 or 6478 (p12), 6950 (p21rex), and 6875 (p13). The three doubly spliced mRNAs include exon 1, exon 2 (4641–4831) and a third exon that starts at position 6950 (Tax/Rex), 6478 (p30), or 6383 (p27/p12). The singly spliced antisense transcript (HBZ) initiates at multiple sites in the 3'LTR and utilizes a splice donor site at position 225 (minor) or 365 (major) and a splice acceptor site at nt position 1765. (B). Similarly, the HTLV-2 genome expresses at least seven mRNAs all of the positive sense polarity. In addition to the unspliced species, three singly spliced mRNAs contain exon 1 (nt 1–135) linked to splice acceptor sites at 4730 (Env), 6630 (p28, p22/p20rex-1), or 6900 (p28, p22/p20rex-2). The major doubly spliced mRNA encodes Tax/Rex and contains exon 1 and exon 2 (nt 4730–4869) linked to a splice acceptor at position 6900. The other doubly spliced

mRNAs contain exon 1 and 2 linked to a splice acceptor at 6493 (p11/p10) or 6630 (?, putative and uncharacterized). In both panels, nucleotide numbering starts at the beginning of the R region for the positive sense transcripts and the last nucleotide of U5 in the 3'LTR for the antisense transcript. Black or gray (multiple boundaries due to splice site utilization) lines designate exons and dotted lines introns. Open or closed triangles represent splice donor and splice acceptor sites, respectively.

Figure 2.

Specific detection of HTLV gag/pol mRNA in cell lines. Poly A⁺ mRNA (0.05 µg) isolated from HTLV-1 cell lines (SLB-1, PBL-ACH, and 729ACHneo), HTLV-2 cell lines (MoT, PBL-pH6, and 726pH6neo), and negative control (729), was subjected to an uncoupled 35 cycle RT-PCR using gag/pol specific primers in the presence (+) or absence (-) of reverse transcriptase (RT). The 99 bp PCR products for HTLV-1 (panel A) and HTLV-2 (panel B) gag/pol were separated on a 2% agarose gel and visualized by ethidium bromide staining. These results demonstrated that gag/pol mRNA was expressed in the appropriate cell lines and absent in the control. The failure to detect a signal in the absence of RT confirmed that the poly A⁺ mRNA was free of proviral or plasmid DNA contamination.

Figure 3.

Quantitation of HTLV-1 mRNAs by real-time RT-PCR. Real-time RT-PCR was performed on poly A+ mRNA isolated from HTLV-1 cell lines SLB-1, PBL-ACH, and 729ACHneo as described in the Materials and Methods. Primer pairs and probes to specifically detect viral mRNA species as indicated are summarized in Table 1. Data is presented in histogram form with standard deviations from triplicate experiments. Total copy number below was determined using plasmid DNA standards and normalized to 10^6 copies of GAPDH mRNA.

Figure 4.

Quantitation of HTLV-2 mRNAs by real-time RT-PCR. Real-time RT-PCR was performed on poly A+ mRNA isolated from HTLV-2 cell lines MoT, PBL-pH6, and 729pH6neo as described in the Materials and Methods. Primer pairs and probes to specifically detect viral mRNA species as indicated are summarized in Table 2. Data is presented in histogram form with standard deviations from triplicate experiments. Total copy number below was determined using plasmid DNA standards and normalized to 10^6 copies of GAPDH mRNA.

Table 1
Primers and probes used for HTLV V-1 mRNA species

HTLV-1 mRNA	cDNA plasmid	HTLV Sequence in Plasmid Standards	5' primer	3' primer	Taqman probe
gag/pol	ML760	920-1049	#20	#19	TMP-3
Env-4501	ML765	103-119^4501-4919	ENV4501-S	ENV-AS	TMP-4
Env-4641	ML627	100-119^4641-4919	ENV4641-S	ENV-AS	TMP-4
Env-4658	ML766	103-119^4658-4919	ENV4658-S	ENV-AS	TMP-4
Tax/Rex	SE356	4741-4831^6950-7954	X2TR1-2	TR1-AS	TMP-2
p21Rex	pMS9-7.8	58-119^6950-7286	P21REX-S	TR1-AS	TMP-2
p13	ML628	102-119^6875-7170	P13-S	TR-AS	TMP-10
p12-6383	pMT2-2.3	109-119^6383-6549	P12-6383-S	P12-AS	TMP-1
p12-6478	pMT2-5.4B	108-119^6478-6549	P12-6478-S	P12-AS	TMP-1
p27/p12	pMS9-1.8	4676-4831^6383-6549	P27S	P12-AS	TMP-1
P30	pMS9-11.1	4819-4831^6478-6549	X2P30	H1J42	TMP-1
HBZ-365major	JA662	353-365^1765-2373	HBZMAP1	HBZMAP2	TMP-13
HBZ-225minor	ML764	212-225^1765-1828	HBZminor-S	HBZ-AS	TMP-13

Table 2
Primers and probes used for HTLV V-2 mRNA species

HTLV-2 mRNA	cDNA plasmid	HTLV Sequence in Plasmid Standards	5' primer	3' primer	Taqman probe
gag/pol Env Tax/Rex p28,p22/p20rex-1 p28,p22/p20rex-2 p10/p11 p?	ML761	1887^1986	GP2-S	GP2-AS	TMP-8
	IY595	2-135^4730-8239	TR2-S	ENV2-AS	TMP-6
	IY531	2-135^4730-4869^6900-8239	TR2-S	TR2-AS	TMP-6
	ML637	117-135^6630-6984	P28-1-S	P28-AS	TMP-7
	ML638	117-135^6900-6984	P28-2-S	P28-AS	TMP-7
	ML674	123-135^4730-4869^6493-6508	TR2-S	P10-AS	TMP-6
	ML675	123-135^4730-4869^6630-6645	TR2-S	M-AS	TMP-6

Table 3
Ct value of hGAPDH gene and proviral load quantitation result in all cell lines used.

	SLB-1	729ACHneo	PBL-ACH	729Uninf	MoT	729pHneo	PBL-pH6
Ct value	18.59±0.39	18.01±0.03	19.17±0.06	18.80±0.16	19.14±0.15	18.47±0.11	18.56±0.28
Pro-load	1.26±0.30	5.12±0.55	0.9±0.08	0	1.55±0.70	5.10±0.29	0.76±0.2