KINETIC ANALYSIS OF HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 GENE EXPRESSION

DISSERTATION

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School of The Ohio State University

By

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ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) are closely related human retroviruses that transform T lymphocytes in cell culture and persist in infected individuals. HTLV-1 infection is clearly associated with leukemia/lymphoma and neurological disease, whereas HTLV-2 disease association is less compelling. 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 levels of viral gene expression throughout the process of infection, cellular transformation, and pathogenesis have not been experimentally assessed. We posit that having a concise viral gene expression profile will provide important insight into the function of specific viral genes and their role in the biology and pathogenesis of HTLV-1.

We first compiled and 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 followed by hybridization increased the specificity and sensitivity of the assay. The splice site-specific primers used in our experiments allowed differentiation of the closely related alternatively spliced mRNAs.
Specifically designed Taqman probes hybridized only to the segment to be amplified and emit signal upon amplification, which results in increased detection specificity. The assay offered a wide dynamic range as seen by our ability to quantify specific cDNA plasmid samples accurately at concentrations from 25 copies to $2.5 \times 10^7$ copies per reaction. Quantitation of HTLV mRNAs between different cell lines showed variability ($gag/pol \geq tax/rex > env \geq$ accessory genes), but the overall levels of each mRNA relative to each other within a cell line were similar. Our analysis also provided important information on some splice acceptor site utilization.

In Chapter 3 we utilized our newly developed real-time RT-PCR assay to determine the kinetics of viral gene expression in three systems: 293T cells transiently transfected with HTLV-1 proviral plasmid, human PBMCs infected with HTLV-1 in vitro, and PBMCs harvested from HTLV-1-inoculated rabbits. The HTLV-1 gene expression profiles in transiently transfected and infected cells were similar; over time, all transcripts increased and then maintained stable levels. The $gag/pol$, $tax/rex$, and $env$ mRNAs were detected first and at the highest levels, whereas expression of the accessory genes, including the anti-sense $hbz$, was significantly lower than $tax/rex$. In infected rabbits, $tax/rex$ and $gag/pol$ mRNA levels peaked early after inoculation and progressively decreased, which correlated inversely with the proviral load and host antibody response against viral proteins. Interestingly, $hbz$ mRNA was detectable at one week post-infection and increased and stabilized. This study provides the first
evidence linking *hbz* expression to proviral load and the survival of the virus infected cell in the host.

Lastly, in Chapter 4 we further explored the role that the HTLV-1 post-transcriptional regulator, Rex, played in viral gene expression. It was known that Rex utilized specific host machinery to actively export *gag/pol* unspliced and *env* incompletely-spliced viral mRNA from the nucleus to the cytoplasm. However, all HTLV-1 sense transcripts contain the Rex response element (RxRE) in their 3’ untranslated region. The contribution of Rex to the export and expression of completely spliced HTLV-1 mRNAs, particularly those encoding the accessory proteins, had been difficult to assess due to their low abundance in cells. Using our quantitative realtime RT-PCR assay we confirmed the effect of Rex on the export and expression level of unspliced *gag/pol* mRNA, singly-spliced *env* mRNA, and doubly-spliced *tax/rex* mRNA. Furthermore, we showed that Rex slightly decreased the overall expression level of singly and doubly spliced mRNAs encoding Tax, HBZ, and the accessory proteins, but had no significant effect on the export of these mRNAs. Rex RNA-pull down experiments demonstrated that Rex associated not only with the *gag/pol* and *env* mRNA, but also associated with all other singly and doubly spliced viral mRNAs encoding regulatory and accessory proteins. In contrast, the *hbz* antisense mRNA, which does not contain the RxRE, was not bound by Rex. Our results are the first to quantitatively measure the effects of Rex on the expression level and export of
HTLV-1 accessory protein mRNAs and to show that Rex associates with all the RxRE containing positive sense HTLV-1 mRNA transcripts. The Rex association with HTLV-1 transcripts provides the possibility of post-transcriptional regulations by Rex other than export.
Dedicated to my parents
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>vii</td>
</tr>
<tr>
<td>Vita</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xv</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Literature review</td>
<td>1</td>
</tr>
<tr>
<td>1.1. HTLV discovery and epidemiology</td>
<td>1</td>
</tr>
<tr>
<td>1.2. HTLV pathogenesis and disease association</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1. ATLL</td>
<td>3</td>
</tr>
<tr>
<td>1.2.2. HAM/TSP</td>
<td>5</td>
</tr>
<tr>
<td>1.2.3. Other HTLV-1 associated diseases</td>
<td>6</td>
</tr>
<tr>
<td>1.2.4. HTLV-2 pathogenesis</td>
<td>6</td>
</tr>
<tr>
<td>1.3. HTLV replication life cycle</td>
<td>7</td>
</tr>
<tr>
<td>1.4. HTLV genome structure</td>
<td>8</td>
</tr>
<tr>
<td>1.5. HTLV genes</td>
<td>11</td>
</tr>
<tr>
<td>1.5.1. HTLV structural and enzymatic proteins</td>
<td>12</td>
</tr>
<tr>
<td>1.5.2. Tax</td>
<td>13</td>
</tr>
<tr>
<td>1.5.3. Rex</td>
<td>17</td>
</tr>
<tr>
<td>1.5.4. ORF-II p30</td>
<td>22</td>
</tr>
<tr>
<td>1.5.5. ORF-II p13</td>
<td>24</td>
</tr>
<tr>
<td>1.5.6. ORF-I p12 and p27</td>
<td>26</td>
</tr>
<tr>
<td>1.5.7. HBZ</td>
<td>27</td>
</tr>
<tr>
<td>1.5.8. HTLV-2 accessory proteins</td>
<td>29</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>1.6</td>
<td>Regulation of HTLV gene expression by cellular factors and machinery</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Transcription activation</td>
</tr>
<tr>
<td>1.6.2</td>
<td>Post-transcriptional modification of HTLV transcripts</td>
</tr>
<tr>
<td>1.7</td>
<td>HTLV experiment systems</td>
</tr>
<tr>
<td>1.7.1</td>
<td>Cell culture</td>
</tr>
<tr>
<td>1.7.2</td>
<td>Rabbit model of HTLV infection</td>
</tr>
<tr>
<td>1.7.3</td>
<td>Real-time RT-PCR</td>
</tr>
<tr>
<td>2</td>
<td>Detection and quantitation of HTLV-1 and HTLV-2 mRNA species by real-time RT-PCR</td>
</tr>
<tr>
<td>2.1</td>
<td>Abstract</td>
</tr>
<tr>
<td>2.2</td>
<td>Introduction</td>
</tr>
<tr>
<td>2.3</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>2.4</td>
<td>Results</td>
</tr>
<tr>
<td>2.5</td>
<td>Discussion</td>
</tr>
<tr>
<td>3</td>
<td>Kinetic analysis of HTLV-1 gene expression in cell culture and infected animals</td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
</tr>
<tr>
<td>3.3</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
</tr>
<tr>
<td>4</td>
<td>HTLV-1 regulatory and accessory gene transcripts are Rex associated but not dependent on Rex for export</td>
</tr>
<tr>
<td>4.1</td>
<td>Abstract</td>
</tr>
<tr>
<td>4.2</td>
<td>Introduction</td>
</tr>
<tr>
<td>4.3</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>4.4</td>
<td>Results</td>
</tr>
<tr>
<td>4.5</td>
<td>Discussion</td>
</tr>
<tr>
<td>5</td>
<td>Synopsis and future studies</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>5.2</td>
<td>Assay development</td>
</tr>
<tr>
<td>5.3</td>
<td>Gene expression kinetics of HTLV-1</td>
</tr>
<tr>
<td>5.4</td>
<td>The role of Rex in post-transcriptional regulation of HTLV-1 mRNAs</td>
</tr>
<tr>
<td>References</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Primers and probes used for HTLV-1 mRNA species</td>
<td>69</td>
</tr>
<tr>
<td>2.2 Primers and probes used for HTLV-2 mRNA species</td>
<td>69</td>
</tr>
<tr>
<td>2.3 Ct value of hGAPDH gene and proviral load quantitation result in all cell lines used</td>
<td>69</td>
</tr>
<tr>
<td>3.1 Kinetic analysis of HTLV-1 transcript expression in transfected cells</td>
<td>97</td>
</tr>
<tr>
<td>4.1 Quantification of HTLV-1 mRNA level change in wtHTLV-1 or HTLV-1Rex-transfected 293T cells</td>
<td>127</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Genome organization of HTLV-1 and HTLV-2 and their gene products.</td>
<td>40</td>
</tr>
<tr>
<td>1.2</td>
<td>Key highlights of HTLV-1 regulatory and accessory protein functions within the context of a cell</td>
<td>42</td>
</tr>
<tr>
<td>2.1</td>
<td>Genome organization of HTLV-1 and HTLV-2 and their unspliced, singly spliced and doubly spliced mRNAs</td>
<td>70</td>
</tr>
<tr>
<td>2.2</td>
<td>Specific detection of HTLV gag/pol mRNA in cell lines</td>
<td>72</td>
</tr>
<tr>
<td>2.3</td>
<td>Quantitation of HTLV-1 mRNAs by real-time RT-PCR</td>
<td>73</td>
</tr>
<tr>
<td>2.4</td>
<td>Quantitation of HTLV-2 mRNAs by real-time RT-PCR</td>
<td>74</td>
</tr>
<tr>
<td>3.1</td>
<td>Provirus genome of HTLV-1 and its unspliced, singly spliced, and doubly spliced mRNAs</td>
<td>98</td>
</tr>
<tr>
<td>3.2</td>
<td>Time course of HTLV-1 protein expression following transient transfection</td>
<td>99</td>
</tr>
<tr>
<td>3.3</td>
<td>HTLV-1 T-lymphocyte immortalization assay</td>
<td>100</td>
</tr>
<tr>
<td>3.4</td>
<td>Assessment of HTLV-1 infection in rabbits</td>
<td>102</td>
</tr>
<tr>
<td>3.5</td>
<td>Kinetics and profile of HTLV-1 mRNA expression in infected rabbits</td>
<td>103</td>
</tr>
<tr>
<td>3.6</td>
<td>Relationship over time between HTLV-1 proviral load and tax/rex and hbz mRNA levels and in infected rabbits</td>
<td>104</td>
</tr>
</tbody>
</table>
4.1 Rex and p19 Gag protein expression in transiently transfected 293T cells...... 128

4.2 Nuclear/cytoplasmic fractionation and assessment of Rex function on viral mRNA export................................................................. 129

4.3 The Rex association with HTLV-1 transcripts in vivo ......................... 131
1.1 Human T-cell Leukemia Virus discovery and epidemiology

HTLV-1 was first isolated in 1980 from two T-lymphoblastoid cell lines, HUT 102 and CTCL-3, which were established from a patient with cutaneous T-cell lymphoma\textsuperscript{1,2}. A year later, HTLV-1 was isolated from another cell line, MT-1, which was derived from a patient with adult T-cell leukemia (ATL). Further serological, immunologic, and genetic studies have identified HTLV-1 as a pathogen for ATL\textsuperscript{2-5} and a variety of immune-mediated disorders including the chronic neurological disease termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)\textsuperscript{6,7}.

HTLV-2 was first identified in MoT, a T-cell line established from a patient with of hairy cell leukemia. Serum from the patient contained antibodies to the major internal core protein (p24) of HTLV-1, but with significantly different immunological cross-reactivity from all the HTLV-1 isolates. Therefore, this virus was designated as HTLV-2\textsuperscript{8,9}. HTLV-2 is much less pathogenic in comparison to HTLV-1, and its
etiological role in disease induction remains unclear. In addition to several cases of atypical hairy-cell leukemia, HTLV-2 has been implicated in a few cases of neurological disease similar to HAM/TSP 10-12.

Approximately 10-20 million individuals world-wide are estimated to be infected with HTLV-1 13, which is endemic in Japan, the Caribbean basin, central and west Africa, southeastern United States, Melanesia, parts of south America, and in certain populations in the middle east and India 14-21. After a long latency of 20 or more years 22,23, it is predicted that only 2-5% of HTLV-1 infected individuals will develop ATL 24, and 0.25-3% will develop HAM/TSP 7,25,26. HTLV-2 is endemic in intravenous drug users (IDU) and their sexual contacts in the United States, Europe, south America, and southeast Asia 27-30. Interestingly, certain Amerindian populations with distinct cultural, ethnical, and geographical distributions are also infected with HTLV-2 31-35.

The low percentages of infected people that develop HTLV-associated diseases indicate that HTLV induces a chronic, lifelong, but typically asymptomatic infection. Because HTLV cell-free virions are poorly infectious, the main route of transmission occurs via cell-cell contact from breast feeding 36,37, sexual contact 38, blood transfusion 39, and by needle sharing among IDUs. Mother-to-child transmission depends on ingestion of infected milk-borne lymphocytes, duration of breast feeding, mother’s proviral load and maternal antibody titers against HTLV 40. Although sexual transmission could be bidirectional, studies have demonstrated higher transmission
efficiency in the direction of male to female verses female to male \(^{38}\). The most efficient route of HTLV transmission is blood transfusion as long as it has cellular blood components as opposed to plasma. It is estimated that the sero-conversion rate in this form of transmission ranges from 40\% to 60\%\(^{41}\). These findings clearly show that natural transmission requires live HTLV-infected cells.

1.2 HTLV pathogenesis and disease association

HTLV-1 was the first retrovirus linked to human disease. HTLV-1 infection is associated with ATL \(^{1,2}\), HAM/TSP \(^{25,42}\), uveitis and infective dermatitis \(^{43}\). HTLV-2 infection is rarely associated with T-cell lymphoproliferative disorders such as Hairy Cell Leukemia (HCL) \(^8\), CD8+ T-cell leukemia \(^{44}\), Mycosis Fungoides \(^{45}\), and large granular lymphocytic leukemia \(^{46}\). The diseases associated with HTLV especially leukemia are multistep processes that require the accumulation of genetic modifications over a long period of time. For decades, scientists have been trying to understand the pathogenesis and find ways to treat HTLV associated disease.

1.2.1 Adult T-cell Leukemia

Adult T-cell Leukemia (ATL) seems to be associated with HTLV-1 transmission in early life. Only a small proportion of HTLV-1-infected individuals, especially those who acquire the virus as a result of breast feeding, develop ATL after a long latent
period \(^{24,47}\). Possible factors for this association are the primary infection before maturation of the immune system and the prolonged incubation period between infection and disease. It has been speculated that the development of ATL involves two stages, the initial polyclonal infection stage due to HTLV-1 replication and spread, and the monoclonal expansion stage of the infected T-lymphocytes \(^{48,49}\).

The clinical manifestations of ATL include malaise, fever, dyspnea, lymph node enlargement, hepatosplenomegaly, jaundice, and drowsiness. Another unique clinical characteristic of ATL is the massive infiltration of leukemic cells in the skin, liver, spleen, gastrointestinal tract and lung. Constitutive overexpression of various cytokines and chemokines likely provide the basis for the lymphocytic infiltration. In addition, abnormal laboratory findings including marked leukocytosis, hypercalcemia, high serum levels of lactate dehydrogenase (LDH) and a soluble form of interleukin-2 receptor (IL-2R) \(\alpha\)-chain, and the appearance of characteristic leukemic cells with deeply convoluted or lobulated nuclei (flower cells). These morphologically abnormal T-cells are the hallmark of HTLV-1 infection and have a mature phenotype (CD2+CD3+CD4+CD8-CD25+ and HLA-DR+) \(^{50}\). Immune suppression and opportunistic infections by bacteria, fungi, protozoa and viruses have also been reported in ATL patients, contributing to their poor prognosis \(^{51,52}\).

Based on tumor lesions in various organs and clinical course, ATL is classified into four subtypes: acute, chronic, smoldering and lymphoma. The acute subtype has the
highest incidence and shows the clinical characteristics listed above. The chronic and smoldering subtypes are less aggressive and may represent transitional states to the development of acute ATL. Patients with chronic ATL generally have an elevated number of circulating ATL cells and display mild symptoms, whereas patients with smoldering ATL present with skin lesions, lymph node enlargement and marrow involvement. The predominant feature of lymphoma type ATL is lymph node enlargement but not leukemic manifestations\textsuperscript{53,54}.

1.2.2 HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Five years after HTLV-1 identification, two different groups reported that patients with a chronic inflammatory neurological disease, known as tropical spastic paraparesis (TSP), were sero-positive for HTLV-1\textsuperscript{7,25}. To date, HAM/TSP has been described in all areas of the world known to be endemic for HTLV-1. Clinically, HAM/TSP is characterized by muscle weakness, spasticity in the lower extremities, urinary/fecal incontinence and mild peripheral sensory loss\textsuperscript{55}. Frequent clonal proliferation of HTLV-1-infected T cells, a phenomenon in ATL patients, was observed in the peripheral blood or in the cerebrospinal fluid of HAM/TSP patients\textsuperscript{56}. It has been demonstrated that high HTLV-1 proviral load and host genetic factors are two important contributing factors to the development of HAM/TSP\textsuperscript{55,57,58}.
1.2.3 Other HTLV-1 associated diseases

HTLV-1 has been associated with several other diseases, although some of them were established based on limited data and the definitive role of HTLV-1 remains to be elucidated. Patients with uveitis from Kyushu, Japan were reported to have high anti-HTLV-1 antibody titer and high proviral loads in the peripheral blood. There are also some reports of high sero-prevalence of HTLV-1 in patients with Sjögren’s syndrome. Moreover, HTLV-1 infection has been associated with some other diseases, including T lymphocyte alveolitis, arthropathy, dermatitis, polymyositis, and lymphadenitis.

1.2.4 HTLV-2 pathogenesis

Unlike HTLV-1, the disease association for HTLV-2 is less clear and lacks solid epidemiological evidence. However, the isolation of HTLV-2 from patients with a rare variant of HCL, CD8+ T-cell leukemia, Mycosis Fungoides, and large granular lymphocytic leukemia suggests some association between HTLV-2 and T-cell lympho-proliferative disorders. Consistent with the in vitro tropism of HTLV-2, several of the HTLV-2 associated leukemia cases show CD8+ T-cell lineage. Furthermore, there are reports that associate HTLV-2 with spastic ataxia and chronic neurodegenerative diseases. Interestingly, HTLV-2 infection has been reported in a patient with a chronic progressive neurological disease that is clinically identical to
HAM/TSP. HTLV-2 is also associated with increased incidence of pneumonia and bronchitis, inflammatory conditions such as arthritis. Finally, it has been reported that upregulated HTLV-1/HTLV-2 virus expression and disease manifestations occur during coinfection with Human immunodeficiency virus (HIV), sometimes in association with normal CD4 counts.

1.3 HTLV replication life cycle

The overall replication life cycle of HTLV is similar to that of other retroviruses. Briefly, the viral glycoprotein surface unit (SU) component of Env recognizes and binds to a cell surface receptor(s) leading to the viral and host membrane fusion that is facilitated by the transmembrane (TM) component of Env. Upon entry into the host cell, the virus uncoating process takes place and releases the viral core into the cytoplasm of the infected cell. The viral RNA genome is reverse transcribed by the viral encoded and virion incorporated reverse transcriptase (RT) to generate a double stranded linear DNA intermediate. This viral DNA intermediate is then transported to the nucleus where it gets stably integrated into the host genome by integrase (IN), another virion containing enzyme. The integrated HTLV DNA genome is also referred to as a provirus. Following integration, the provirus utilizes the cellular RNA polymerase II together with specific viral proteins and cellular transcription machinery to initiate transcription from the viral promoter present in the U3 region of the 5’ long
terminal repeat (LTR). Transcribed viral mRNA can be directly exported into the cytoplasm to be translated into essential viral structural and enzymatic proteins such as Gag, Pol and Pro, or serve as genomic RNA to be packaged into new virions. On the other hand, recognition of the multiple splice donor and acceptor sites within the unspliced mRNA leads to a group of alternatively spliced viral mRNAs that are exported and translated into the Env protein, regulatory proteins and accessory proteins. The assembly of the virion and packaging of genomic RNA follows. Packaged virions bud at the cell membrane. The released virions are immature and the activity of protease is needed for the proteolytic cleavage of structural proteins and maturation of the virions to generate an infectious particle. It is important to note that in general efficient HTLV infection requires cell to cell contact.

1.4 HTLV genome structure

The HTLV provirus genome is flanked by 5’ and 3’ LTRs (Fig. 1.1). HTLV LTR is composed of a unique 3’ (U3) region, a repeated (R) region and a unique 5’ (U5) region. The total size of the HTLV-1 LTR is 755 nucleotides with U3, R, and U5 regions 352, 227 and 176 nucleotides, respectively. The total size of the HTLV-2 LTR is 763 nucleotides with U3, R, and U5 regions 314, 249 and 200 nucleotides, respectively. The U3 region contains sequences that control transcription of the provirus.
Particularly, three imperfect 21-nucleotide repeats, commonly referred to as Tax-responsive element 1 (TRE-1), are critical to Tax-mediated transcriptional activation\textsuperscript{71-73}. These TRE-1 sites are homologous to cellular cyclic AMP response element (CRE) binding protein (CREB) binding sites. Between the second (middle) and the third (promoter proximal) TRE-1, there is an additional Tax-responsive element TRE-2 or Ets responsive region-1 (ERR-1), which can also mediate trans-activation via the binding of many other cellular proteins such as Ets family proteins and c-Myb. \textit{In vivo} foot-printing on TRE-2 indicated that this region is bound by proteins in the presence or absence of Tax\textsuperscript{74-79}. Although Tax does not appear to bind directly to the viral promoter, it is proposed that Tax can stabilize the cellular protein-TRE complexes. On the other hand, the cooperative binding of Tax and some cellular proteins to the viral promoter may position Tax to interact with basal transcription factors CREB binding protein (CBP), transcription factor IIA (TFIIA), and TFIID\textsuperscript{80-83, 84}.

The viral LTR also contains some sequences that are important for viral replication. The U3 contains sequences responsible for termination and polyadenylation of mRNAs. The transcription start site is located at the U3/R junction. The attachment (\textit{att}) sites required for proviral integration are found in the 5’ terminal U3 and 3’ terminal U5 sequences. Downstream of the U5 region and upstream of the \textit{gag} gene is the primer binding site (pbs), which is a 18nt sequence that can hybridize with host tRNA necessary for priming viral reverse transcription.
A specific cis-acting sequence termed the Rex-responsive element (RxRE), which is the binding site of HTLV Rex protein, was first localized to the 3’ LTR of the virus, and subsequently was shown to be present, at least in part, in the 5’LTR as well. RxRE lies in the U3 and R regions of the 3’ LTR and forms a stable and complex secondary structure, consisting of four stem loops and a long stretch of stem structures. An inhibitory sequence, termed cis-acting repressive sequences (CRS) was also previously identified spanning the U5 region of the LTR and more recently identified overlapping the 3’ RxRE of the unspliced and incompletely spliced mRNAs of HTLV-1. Thus, Rex is proposed to bind specifically to RxRE and overcome the inhibitory effect of CRS in retaining the unspliced and incompletely spliced mRNA in the nucleus to export them into the cytoplasm.

Although the 5’-LTR and the 3’-LTR are identical in sequence, CpG methylation has been implicated in viral gene regulation in vivo. Selective hyper-methylation of the 5’-LTR correlates with latency of viral gene expression. Comparison of the LTRs of HTLV-1 and HTLV-2 reveals that the critical elements for viral gene expression including the TRE repeats, polyA signal and TATA box are well conserved.

The bulk of the HTLV genome between the LTRs is the coding region for viral genes. HTLV expresses multiple gene products from unspliced mRNA and a complex array of spliced mRNAs (Fig.1.1). HTLV-1 expresses at least eight positive sense transcripts and one negative sense transcript. As is the case for all replication
competent retroviruses, the genomic unspliced mRNA encodes group associated gene (Gag), polymerase (Pol), and protease (Pro) \(^{94,95}\). Four singly spliced mRNA species encode Env, p12, p21rex, and p13 and three doubly spliced mRNAs encode Tax, Rex, p27, and p30 \(^{96}\). An unspliced and two singly-spliced antisense transcripts encode HBZ. Similarly, the HTLV-2 genome expresses at least seven mRNAs all of the positive sense polarity. In addition to unspliced species which encode Gag, Pol, and Pro, three singly spliced mRNAs encode Env, p28, p22/p20Rex. The doubly spliced mRNAs encode Tax, Rex, p10, p11 and a putative as yet uncharacterized viral protein \(^{97}\).

### 1.5 HTLV genes

Expression of viral genes is regulated throughout the process of infection, immortalization/transformation and ultimately HTLV pathogenesis. Previous research showed that the structural, enzymatic and regulatory gene products play different roles in virus replication and pathogenesis. Recent studies have focused on trying to understand the function of the accessory proteins. They were not detected in HTLV infected cells and were initially considered to be dispensable for viral replication \(^{98,99}\). Studies carried out using mutant viral clones demonstrated that several HTLV-1 determinants, including HBZ \(^{100}\), ORFs X-I protein (p12) \(^{101,102}\), X-II proteins (p13, p30) \(^{101,103,104}\) as well as HTLV-2 p28 \(^{105}\) are dispensable for viral replication and
immortalization in vitro, but enhanced viral infectivity and persistence in inoculated rabbits. Those studies indicate a significant role of the accessory proteins in the life cycle of HTLV in the infected individual. Indirect evidence of the expression of the viral accessory proteins in vivo was from the studies of the immune response to the viral infection, including serum antibodies as well as cytotoxic T cells (CTL) response. In the remainder of this chapter, we will review the extensive work that has been done to study the function and expression of those viral proteins regulated by various viral and cellular factors at transcriptional and post-transcriptional level.

1.5.1 HTLV structural and enzymatic proteins

The full-length HTLV RNA is utilized both as virion genomic RNA and for the synthesis of Gag, Pol and Pro. A single-spliced sub-genomic mRNA encodes Env.

There are three mature Gag products, p19 matrix (MA), p24 capsid (CA), and p15 nucleocapsid (NC), generated from the p55 precursor by protease-mediated cleavage. NH₂-terminus myristoylation of p19 targets the p55 Gag precursor to the inner surface of the cell membrane. In addition to assembling into virions, some Gag protein including p19 can be secreted out of infected cells. The amount of secreted p19 usually correlates with the amount of virion produced from the cells, thus p19 production is commonly used as a measure of virion production.

The viral protease is produced from the same gag precursor mRNA via ribosomal
frame shifting. It is responsible for processing the mature Gag products as well as generating the mature form of itself by auto-catalyzed self-cleavage \(^4\). Another ribosomal frame shifting event is responsible for the translation of the pol gene, which encodes viral RT, IN and RNaseH. These activities cooperate after HTLV infection to generate the double-stranded DNA intermediate from the RNA genome.

Env is a glycoprotein of approximately 61-69 kDa depending on different cell lines studied \(^8\). Like those of other retroviruses, HTLV Env is synthesized in the endoplasmic reticulum (ER) as a precursor protein, glycosylated in the Golgi and cleaved into two mature products, SU (gp46) and TM (gp21). The interaction(s) between Env and cellular receptors mediate the first step of viral entry. Envelope is a major viral determinant of the distinct \textit{in vitro} cellular transformation tropism of HTLV-1 and HTLV-2 \(^\text{111}\).

\textbf{1.5.2 Tax}

Tax is one of the first viral proteins expressed early after viral infection and is a trans-activator of viral gene expression. Tax is encoded by a doubly spliced viral mRNA from open reading frame (ORF) IV. HTLV-1 Tax (Tax-1) is 353 amino acids, 40 kDa and HTLV-2 Tax (Tax-2) is 331 amino acids, 37 kDa in size \(^3\). Structure and function analysis has revealed at least four functional domains. The N-terminal amino acid residues 2 to 58 contain an activation domain and also a nuclear localization signal.
Residues 23 to 49 contain the zinc-binding domain (zinc finger) that very likely plays a critical role in protein-protein interactions. The central amino acids 161 to 211 constitute the structural domain, and the carboxyl-terminal 289 to 322 residues contain an activation domain. HTLV-1 Tax protein is predominantly nuclear but has been shown to shuttle between the nucleus and cytoplasm. Tax-2, on the other hand, was reported to be located predominantly in the cytoplasm of HTLV-2 immortalized or transformed T-cells.

Tax associates with the LTR at the TRE, which contains DNA sequences identical to part of CRE, which is contained in many cellular gene promoters. TRE is responsive to cyclic adenosine monophosphate (cAMP) and binds members of the CRE binding protein/activating transcription factor (CREB/ATF-1) family of transcription factors in a Tax-dependent manner. The exact detail of how Tax activates transcription through CREB/ATF is not completely understood. However the current model proposed is that the N-terminus of Tax directly binds CREB molecules docked at CRE sites in the viral TRE. Binding of Tax to CREB enhances CREB-CREB homo-dimerization and enhances DNA association. Enhanced CREB-dimerization at the LTR could result in Tax-Tax homo-dimerization, which facilitates recruitment of p300/CBP and p300/CBP-associated factor (PCAF). The interaction of Tax with p300/CBP and PCAF leads to acetylation of histones and remodeling of the restrictive chromatin structure. The direct interaction of Tax with CBP allows the
binding of the co-activator in the absence of CREB phosphorylation, permitting specific activation of the viral promoter \(^{122,123}\). In addition to functioning as a bridge between transcription factors and the basal transcription machinery, CBP/p300 is also a histone acetyltransferases (HAT) \(^{124,125}\). PCAF activity has been shown strongly associated with HAT activity \(^{126}\). Targeted histone acetylation is thought to neutralize the positive charge of the histone tails and relax the interaction between histones and negatively charged DNA. Thus, target gene promoters become accessible.

Despite the fact that histones acetylation was observed at specific sites within the proviral genome, histone deacetylases (HDAC) were shown to be present at the viral promoter. Inhibition of HDAC activity resulted in an increase in histone acetylation on the HTLV-1 promoter and a concomitant increase in viral RNA \(^{127}\). Over expression of HDAC1 represses Tax transactivation\(^{128}\).

In addition to trans-activation of viral gene expression, Tax is crucial for initiating malignant transformation leading to the development of ATL. The exact mechanism by which Tax induces cellular transformation is not fully understood. Tax-mediated modulation of multiple cellular genes and/or proteins involved in T-cell activation, cell cycle regulation, DNA damage/repair and apoptosis may play a crucial role in this process. Since transformation is a multistep process, it is reasonable to speculate that several of the distinct Tax functions may play important roles at different stages during transformation.
Through CREB, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and serum response factor (SRF) pathways, Tax trans-activates cellular promoters including those of cytokines (IL-13, IL-15), cytokine receptors (IL-2Rα) and co-stimulatory surface receptors (OX40/OX40L), leading to up-regulated protein expression and activated signaling cascades including Janus Kinase and Signal Transducers and Activator of Transcription (JAK/STAT), phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase (JNK)\textsuperscript{129-131}.

A major mitogenic activity of Tax is reflected in its regulation of cell cycle checkpoints\textsuperscript{132}. More specifically, Tax has been shown to induce G1-S progression via its functional crosstalk with p16\textsuperscript{INKa} as well as p18\textsuperscript{INK4c} \textsuperscript{134-136}. In addition, Tax up-regulates the level or activity of several cyclins such as cyclin D \textsuperscript{137,138}. Tax activates cyclin-dependent kinases (CDK) such as CDK4 and CDK6 \textsuperscript{139,140}, leading to phosphorylation of retinoblastoma protein (pRb) tumor suppressor family proteins\textsuperscript{141-143}.

Other features of HTLV transformed cells are genomic instability, aneuploidy, and impaired DNA repair and apoptosis, all of which are affected or regulated by Tax \textsuperscript{144-148}. Although there is no direct evidence that Tax directly induces DNA damage, the current accepted hypothesis is that Tax inhibits the ability of the cell to repair DNA damage introduced from exogenous sources. Through its interaction or modulation of DNA polymerase β and proliferating cell nuclear antigen (PCNA), Tax has the potential to
influence base excision repair \(^{149,150}\). On the other hand, Tax disrupts nucleotide excision repair of DNA \(^{151}\) which also correlates with the ability of Tax to induce PCNA \(^{152}\). Finally, ample evidence point at an intimate link between Tax and apoptosis despite the conflicting reports on whether Tax actually induces or protects from apoptosis \(^{153-159}\). Tax has evolved various strategies to negate cellular tumor suppressor p53, Rb and their abilities to dictate apoptosis in primary cells \(^{160,161}\).

### 1.5.3 Rex

The 189 amino acid HTLV-1 Rex (Rex-1) and 170 amino acid HTLV-2 Rex (Rex-2) proteins encoded by ORF III of the pX region share 60% homology at the amino acid level. When analyzed on SDS-PAGE, Rex-1 is 27 kDa, and Rex-2 is detected as two major bands of 24 and 26 kDa. The two isoforms of Rex-2, p24Rex and p26Rex, have the same amino acid backbone but differ by post-translational modification; specifically serine phosphorylations resulting in a conformational change \(^{162-164}\). Phospholated Rex protein localizes to nucleus, nucleoli, and nucleolar speckles in HTLV transiently transfected cells as well as in infected cells \(^{97,165-167}\). HTLV-1 and HTLV-2 also produce truncated forms of Rex from alternatively spliced mRNAs. These proteins, named p21Rex (HTLV-1) and p22/p20Rex (HTLV-2), lack N-terminal sequences of Rex responsible for nuclear export and RNA binding, and interfere with Rex localization and function \(^{165,168,169}\).
Structure-function analyses revealed biochemical and functional properties of discrete Rex domains. Both Rex-1 and Rex-2 have a RNA binding domain (RBD), nuclear localization signal (NLS), and an activation domain (AD) encompassing a nuclear exporting signal (NES) which is flanked by two multimerization domains (MD). In addition, a unique C-terminal domain has been described for Rex-2 that is a target for serine phosphorylation and may also contribute to efficient nucleo-cytoplastic shuttling. The RBD binds specifically to RxRE in viral mRNAs. The AD/NES interacts with chromosome region maintenance interacting protein 1 (CRM1)/exportin 1. These RNA-protein-protein interactions are very important for Rex function.

The Rex protein is required for the expression of the structural and enzymatic proteins that are translated from the unspliced gag/pol mRNA and singly spliced env mRNA. Studies using stably infected cells or transfection of plasmids have demonstrated that HTLV-1 Rex increase the amounts of unspliced viral RNA by reducing the rates of splicing and degradation of unspliced RNA in the nucleus. Rex also facilitates the nuclear-cytoplasmic transport of the unspliced and incompletely spliced viral mRNAs, resulting in increased structural and enzymatic protein production. Since the accumulation of viral structural proteins is dependent on Rex, and Rex itself is generated from completely spliced mRNA, the virus has a biphasic life cycle: an early Rex-independent phase and a late Rex-dependent phase. Early during
infection, when insufficient Rex is produced, most of the viral mRNAs are doubly spliced, due to default splicing by the host cellular machinery. Accumulation of sufficient levels of Rex results in the expression of unspliced and incompletely spliced mRNA in the cytoplasm, leading to the production of structural and enzymatic gene products and assembly of virus particles.

The mechanism of Rex function and the Rex transport cycle has been reviewed by Younis and Green. The proposed model of Rex transport cycle involves: (a) binding of Rex to the RxRE present in viral mRNA, resulting in the protection of this mRNA from splicing and/or degradation; (b) Rex multimerization; (c) formation of an RNA/Rex/CRM1/Ran-GTP complex; (d) interaction of the complexes with nuclear pore complex (NPC) and exit from the nucleus; (e) hydrolysis of Ran-GTP to Ran-GDP, resulting in dissociation of the complex and subsequent release of the cargo mRNA; and (f) return of Rex to nucleus to start another cycle. A second model suggests that Rex actively inhibits splicing of mRNA by stripping it of splicing factors. Once the mRNA is free of splicing factors the cellular machinery would recognize it as a processed mRNA and export it efficiently to the cytoplasm. Although there is some evidence for both models, the first is supported by a greater amount of published work.

RxRE of HTLV-1 is a stem loop structure of 205 nucleotides that is found in the 3’ LTR of viral mRNAs. Mutational analyses indicate that the actual Rex binding sub-domains in the ~200 nucleotide RxRE are relatively short. A stretch of 43
nucleotides constitutes the high affinity Rex-1 binding motif\textsuperscript{173,186} and is sufficient to mediate Rex function\textsuperscript{186,187}. It is likely that the free energy from a longer secondary structure ensures proper folding into a stable secondary structure with a high affinity binding site. Also, the ability of the RxRE to fold into stem loop structure brings the polyadenylation signal (AAUAAA) into close proximity to the GU rich polyadenylation site\textsuperscript{170,188}, giving the full RxRE a novel role in ensuring efficient polyadenylation of viral mRNA. Substitution and deletion studies have demonstrated that proper folding of RxRE is required to provide a docking site for Rex, and hence, is essential for its \textit{in vivo} function\textsuperscript{86,189}.

Unlike HIV-1, which contains Rev Responsive Element (RRE) only in its unspliced and incompletely spliced \textit{env} mRNA, all HTLV transcripts, except for the antisense transcript which encodes HBZ, contain the RxRE. However, it is still unclear how Rex preferentially regulates the unspliced and incompletely spliced versus the fully spliced mRNAs. In fact, in the context of the entire provirus, the increased levels of unspliced mRNAs in the presence of Rex are accompanied by a decrease in the steady state level of the doubly spliced \textit{tax/rex} message. Despite previous work on the Rex/RxRE association, no data has actually shown if Rex binds to any HTLV-1 mRNA transcribed \textit{in vivo} from provirus. The function of Rex on the export of completely spliced HTLV-1 mRNAs, especially accessory protein mRNA, has been poorly investigated due to their low abundance in provirus context. Work in Chapter 4 will
study the Rex association with viral mRNAs transcribed from provirus by Rex RNA-pull down (PD), and the Rex function on export/expression of all HTLV-1 mRNAs by real-time RT-PCR.

Other than Rex, certain cellular mRNA-binding proteins also bind to the RxRE. One of the more significant factors is heterogeneous ribonucleoprotein particle A1 (hnRNP A1), which competes with Rex-1 for binding to RxRE, resulting in impairment of Rex function in cells over-expressing hnRNP A1190 191.

A less investigated model for Rex function is that Rex may also increase the translational efficiency of Rex bound mRNA 192, which is consistent with HIV-1 Rev data 193,194. Additional support for this hypothesis comes from studies that show an association between Rex-1 and translational initiation factor 5A (eIF-5A) 195,196. Despite some evidence for this mechanistic effect of Rex, the primary function of Rex remains the nucleo-cytoplasmic export of unspliced and partially spliced viral mRNA. Therefore, Rex is considered to be a positive regulator that controls the switch between early, latent and late, productive infection. Since Rex has been shown to be essential for HTLV gene expression, the full understanding of its function and regulation remains a critical objective in HTLV research that could yield new strategies for therapeutic intervention.
1.5.4 ORF-II p30

HTLV-1 accessory protein p30 is a 241 amino acid nuclear/nucleolar protein that is encoded by ORF II from a doubly spliced transcripts \(^{197}\). Although p30 has been shown to be directly or indirectly associated with viral RNA, unlike Rex, it is a non-shuttling protein. Studies of p30 expression from its complete cDNA, including all coding and non-coding regions, revealed a strict dependence on the presence of Rex \(^{198}\).

p30 contains serine- and threonine-rich regions that share limited homology with the DNA binding domain of the transcription factor Oct-1 \(^{199}\), and a bipartite NLS \(^{198}\). p30 repressed the cellular CRE-driven reporter gene activity in a dose-dependent manner, with or without Tax expression. Small amounts of p30 enhanced HTLV-1 LTR-driven reporter gene activity, even in the presence of Tax, while higher concentrations of p30 repressed LTR-driven reporter gene activity \(^{200}\). p30 was demonstrated to co-localize with p300 in cell nuclei and directly binds to p300/CBP in cells. DNA binding assays confirmed the interference of p30 with the assembly of CREB-Tax-p300/CBP multi-protein complexes on 21-bp repeat oligo-nucleotides \textit{in vitro} \(^{201}\). Therefore, at the transcriptional level, p30 may differentially influence HTLV-1 replication or cellular gene expression, thus reducing immune recognition of infected cells. p30 also has been shown to enhance the transforming potential of Myc and activate the human cyclin D2 promoter, which are associated with accelerated G1-S
phase transition and multinucleation. These activities requires Tat interacting protein (TIP60) HAT activity and correlated with the stabilization of p30-Myc-TIP60 chromatin remodeling complex. Thus p30 may function as a retroviral modulator of Myc-TIP60-transforming interactions that may contribute to HTLV-1 leukemogenesis 202.

At the post transcriptional level, p30 has been shown to down-regulate tax/rex mRNA expression by retaining this mRNA in the nucleus 203,204. Because Tax and Rex are positive regulators of viral gene expression, their inhibition by p30 reduces virion production. Thus p30 inhibits virus expression at post-transcriptional level by reducing Tax and Rex expression.

HTLV-1 Rex and p30 are both RNA binding proteins regulating viral gene expression at post-transcriptional level. Rex interacts with RxRE and stimulates nuclear export of incompletely spliced viral RNAs thereby increasing production of virions. On the other hand, p30 is involved in the nuclear retention of the tax/rex mRNA leading to inhibition of virus expression. How these two proteins, with apparent opposite functions, integrate in the viral replication and transcription is not completely understood. Rex and p30 interaction was recently reported and this interaction is speculated to govern the switch between virus latency and replication 205. It was demonstrated that Rex and p30 form ribo-nucleoprotein ternary complexes specifically onto tax/rex mRNA. While p30 does not affect Rex-mediated nuclear
export of RNA, Rex partially counteracts p30-mediated viral suppression by rescuing the export of *tax/rex* mRNA\textsuperscript{205}.

Intracellular localizations of Tax, Rex and p30 and suggested that the transcriptional and post-transcriptional regulation of HTLV-1 gene expression depends on the concentration of select regulatory complexes at specific area of the nucleus. The nucleoli are the route of entry of Rex in the nucleus and the site where the Rex and CRM-1 assembled complexes are sequestered by the negative regulator p30. Assembly of Rex/CRM-1 complexes with their target RNA would occur after redistribution of these complexes to the nucleoplasm and their concentration at the boundary of the Tax nuclear bodies where Tax-mediated activation of gene expression occurs\textsuperscript{206}.

Initial studies suggested that ORF II was dispensable for Tax, Rex, or Env expression, as well as viral replication and immortalization of primary lymphocytes *in vitro*\textsuperscript{99,207,208}. However, pX ORF II is indeed necessary for maintenance of high viral loads *in vivo*\textsuperscript{209}.

**1.5.5 ORF-II p13**

Another accessory protein, p13 is also encoded by pX ORF II of HTLV-1. An internal methionine codon in p30 can be used to produce p13, which contains the C-terminal 87 amino acids of p30. Alternatively, p13 can be produced from a singly
spliced message by splicing of the first exon directly to the splice acceptor at position 6875\textsuperscript{96,197,210}. Compared to the transcriptional and post-transcriptional function of p30, less is known about the function of p13. Initial studies demonstrated p13 localization to the nucleus\textsuperscript{210}, but other reports show mitochondrial localization of the protein\textsuperscript{211,212}. A 10-amino-acid mitochondrial targeting sequence (MTS) in the N terminus of p13 is responsible for this localization\textsuperscript{211}.

The role of p13 in the induction of apoptosis is based on the observation that p13 disrupts the mitochondrial inner membrane potential and eventually alters mitochondrial morphology\textsuperscript{213}. p13 inserts into the inner mitochondrial membrane and alters mitochondrial conductance to Ca\textsuperscript{2+} and K\textsuperscript{+}, leading to swelling and collapse of inner mitochondria membrane potential\textsuperscript{213}. In the mitochondria, p13 interacts with farnesyl pyrophosphate synthase that is involved in the synthesis of a substrate required for the prenylation of Ras\textsuperscript{214}. Furthermore, p13 seems to sensitize cells to apoptosis induced by pro-apoptotic agents such as ceramide\textsuperscript{104}. The role of p13 in apoptosis and its interaction with the Ras signaling pathway ultimately results in reduction of tumor incidence and growth rate in tumorigenicity assays, indicating p13 as a negative regulator of cell growth. Although p13 is dispensable for viral replication and immortalization \textit{in vitro}, it is required for enhanced viral infectivity and persistence \textit{in vivo}\textsuperscript{215}.  

25
1.5.6 ORF-I p12 and p27

The 152 amino acids p27 is generated by a doubly-spliced mRNA from pX ORF I. The exact role of p27 in HTLV-1 replication is unknown; however, cytotoxic T lymphocytes (CTLs) against p27-specific peptides are generated during HTLV-1 infection, providing critical, although indirect, evidence for the \textit{in vivo} production of p27 \textsuperscript{96}.

Another HTLV-1 pX ORF I protein p12 is generated from both the doubly-spliced mRNA using an internal start codon downstream of p27 start codon, and singly spliced mRNAs with splicing acceptor sites at position 6383 or 6478. CTLs and serum from HTLV-1 infected individuals and experimentally infected rabbits have been demonstrated to recognize ORF I p12 derived peptide \textsuperscript{106,107}, which provides indirect evidence of the translation of p12 \textit{in vivo}. p12 is a 99 amino acid long, highly hydrophobic protein with two putative trans-membrane domains, four proline-rich (PXXP) Src homology 3 (SH3)-binding motifs, a leucine zipper motif, and a calcineurin-binding motif \textsuperscript{210,216-218}. p12 localizes to cellular endomembranes, particular ER and Golgi apparatus where it interacts with two ER resident proteins that regulate calcium signaling, calreticulin and calnexin \textsuperscript{219}. It also has been shown that p12 plays a role in T-cell activation by inducing nuclear factor of activated T cells (NFAT) gene expression in a calcium-dependent manner \textsuperscript{220,221} and stimulating IL-2 production \textsuperscript{222}. p12 interacts with the immature forms of IL-2R \(\beta\) and \(\gamma\) chains, leading
to their reduced expression on the cell surface \(^{223}\). p12 binds to the IL-2R \(\beta\) chain that is involved in the recruitment of the Janus-associated kinases Jak1 and Jak3, resulting in transcriptional activity of signal transducers and activators of transcription 5 (STAT5), thus providing a proliferative advantage to T-cells \(^{224}\). Collectively, the expression of p12 may decrease the threshold of T-cell activation through NFAT transcriptional activation and IL-2 production. p12 also increases the responsiveness to IL-2 by STAT5 activation. Working together, p12 favors T-cell entry into the S phase even in conditions of sub-optimal antigen stimulation \(^{98,224}\). Moreover, p12 binds to and directs the degradation of immature forms of the major histocompatibility complex class I (MHC-I) in transiently transfected and transduced cells but not immortalized T-lymphocytes. This observation suggests that p12 may interfere with antigen presentation and help infected cells escape from the immune surveillance early after infection \(^{98,225,226}\). Collectively, these observations of p12 suggest its potential role in enhancing infectivity, minimize immune recognition and facilitating viral replication \(^{102,220}\). While p12 is not necessary for HTLV-1 replication \textit{in vitro} \(^{99,101,208}\), p12 contributes to viral infectivity \textit{in vivo} \(^{102}\).

1.5.7 HBZ

In 1989 an mRNA with coding capacity in the minus strand of HTLV-1 was suggested to exist \(^{227}\). An unspliced mRNA was later characterized to be transcribed by a functional
promoter in the 3’ portion of the genome and two alternatively spliced variants (SP1 and SP2) initiated from a promoter in the 3’ LTR. These transcripts encode highly related HBZ isoforms (varying only in the first 8 amino acids). Translation of HBZ was determined to be mostly initiated from the first exon located in the 3’ LTR (SP1 mRNA) and polyadenylation of \( \text{hbz} \) mRNA was identified to be 1450 nucleotides downstream of the HBZ stop codon.

HBZ contains an N-terminal transcriptional activation domain and a C-terminal leucine zipper motif. Three distinct nuclear localization signals of HBZ target the protein to the nucleus, where it accumulated in nuclear speckles. HBZ interacts with CREB-2 and inhibit its binding to the HTLV-1 promoter, resulting in inhibition of Tax-dependent viral transcription. HBZ also interacts with members of the Jun family, JunB, c-Jun and JunD to modulate their transcriptional activity.

Besides simply inhibiting Tax, recent findings suggested that \( \text{hbz} \) mRNA supports proliferation of ATL cells. Suppression of \( \text{hbz} \) gene transcription by short interfering RNA (siRNA) significantly decreased proliferation of ATL cells. In contrast to \( \text{tax} \) mRNA, \( \text{hbz} \) mRNA is expressed in all fresh ATL cells tested from a large panel of cell clones. Lentiviral vectors that express \( \text{hbz} \)-specific short hairpin RNA effectively decreased both \( \text{hbz} \) mRNA and protein expression in transduced HTLV-1-transformed SLB-1 T-cells. \( \text{hbz} \) knockdown correlated with a significant decrease in T cell proliferation in culture and tumor formation in animals challenged with SLB-1-\( \text{hbz} \) knockdown cells.
In conclusion, the hbz gene might have a bimodal function, both at the mRNA and protein levels, which could represent an uncharacterized strategy to regulate viral replication and proliferation of infected T cells \(^{235}\). HBZ is dispensable for HTLV-1 infectivity and cellular immortalization of primary T lymphocytes in cell culture but required for viral persistence \textit{in vivo} \(^{100}\).

1.5.8 HTLV-2 accessory proteins

As with HTLV-1, the proximal pX region of HTLV-2 between \textit{env} and the last exon of \textit{tax/rex} is not required for \textit{in vitro} viral replication and cellular transformation, but plays an important role in maintaining high viral load and viral persistence \textit{in vivo} \(^{236,237}\). The proteins produced from HTLV-2 pX region are p10 (ORF-I), p28 (ORF-II), p22/p20 (ORF-III), and ORF-V (p11).

p10 and p11 are two HTLV-2 accessory proteins expressed from the same doubly spliced mRNA. These proteins are generally hydrophobic and show some homology to HTLV-1 p12. A study found that the MHC class I heavy chain is a common cellular target of HTLV-2 p10, p11 and HTLV-1 p12, proposing the potential role of p10 and p11 in interference of HTLV-2 viral antigen presentation \(^{225}\).

HTLV-2 p28 can be translated from two singly spliced, bi-cistronic mRNAs, with splice acceptor site at 6630 or 6900 based on plasmid pH6neo sequence. The protein is 216 amino acids in size with a predicted molecular mass of 23.9 kDa, indicating that
post-translational modification of this protein. Similar to p30, HTLV-2 p28 localizes to the nucleus and specifically inhibits the cytoplasmic export of *tax/rex* mRNA in the context of the provirus. Therefore, it functions as a post-transcriptional repressor of virion production and potentially promotes viral persistence *in vivo*\(^ {204}\). The same transcripts that encode p28 also have the potential to encode p22/p20, which are truncated Rex isoforms. There is no report about the function of p22/p20 to date. Although p28 could potentially be translated on *tax/rex* mRNA, it has been reported that when the AUGs for Tax and Rex are functional, there is a barely detectable amount of p28 that is produced from this mRNA\(^ {97}\).

### 1.6 Regulation of HTLV gene expression by cellular factors and machinery

#### 1.6.1 Transcription activation

Expression of all the viral genes discussed above depends first on transcription from viral LTRs of the integrated provirus. There have been extensive studies evaluating gene expression from the sense strand promoters found in both the 5’ and 3’ LTRs. 5’LTR and 3’LTR carry two identical U3 regions with two identical promoters. The 5’ promoter directs synthesis of the genomic RNA, whereas the 3’ promoter, if active, synthesizes RNAs that extend into the adjacent host cell genome\(^ {238}\). Studies with other retroviruses suggest that the downstream promoter is silent and that RNA polymerases initiating at the upstream promoter proceed through the 3’ LTR\(^ {239}\). A
recent report studying transcriptional control of *hbz* gene showed that promoters of both the spliced form of *hbz* gene and the unspliced form of *hbz* gene are TATA-less and contained initiators and downstream promoter elements 240. In addition to Tax trans-activation of viral promoter that we have discussed above, a lot of cellular transcription factors contribute to the up or down regulation of viral transcription. Nearly equal distribution of activator (Tax, CREB, ATF-1, ATF-2, c-Fos, and c- Jun) and regulatory proteins (CBP, p300, TAFII250, and polymerase II) were found to be bound at both the upstream and downstream promoters in HTLV-1 infected cell lines and ATL cells 238. The downstream promoter was transcriptional active, suggesting that the two promoters are functionally equivalent. Asymmetrical binding of HDAC-1,-2, and -3 was detected at both promoters to repress Tax trans-activation, which suggested that HTLV-1 transcriptional regulation at both the 5’ and 3’ LTRs is mediated, in part, through mutually exclusive binding of Tax and HDACs at the proviral promoters 238.

Among all the transcription factors bound to HTLV-1 LTR, AP-1 and CREB/ATF factors are capable of physically competing with each other for binding to and regulating transcriptional activation from the LTR 241,242. AP-1 is a family of bZIP transcription factors (C-Jun, JunB, JunD, c-Fos, DosB, Fra-1 and Fra-2), which function as dimers involved in the regulation of several myeloid-specific genes 243. Over-expression of the viral oncogene v-Jun, c-Jun alone, or c-Fos in combination with
c-Jun has previously been shown to up-regulate basal activation of the HTLV-1 LTR \(^{242,244}\). AP-1 mediated activation of the HTLV-1 LTR resulted from direct interactions between AP-1 and TRE-1 repeat II \(^{244}\). Additional studies demonstrated that Tax can also up-regulate AP-1 expression and DNA binding activity suggesting that Tax and AP-1 may function synergistically to trans-activate HTLV-1 viral gene expression \(^{245,246}\). HBZ has been shown to interact with c-Jun and repress c-Jun-mediated transcription by abrogating its DNA-binding activity \(^{247}\).

Sp1 is another cellular factor that binds to TRE and promotes a modest up regulation of HTLV-1 LTR activation. Sp factors belong to the zinc-finger transcription factor family known to play key roles not only in the regulation of constitutive gene expression, but also regulate cell type-specific and differentiation stage-specific gene expression \(^{248,249}\). Binding sites for factors of the Sp family (Sp1 and Sp3) have been identified to the TRE-1 repeat III and TRE-2 within the U3 region \(^{250,251}\) and the U5 region of the HTLV-1 LTR \(^{252}\). Sp1 and Sp3 binding to HTLV-1 LTR has been shown to participate in regulation of HTLV-1 viral gene expression \(^{253}\).

### 1.6.2 Post-transcriptional modification of HTLV transcripts

Following transcription, viral transcripts undergo a number of processing steps, including splicing and polyadenylation. The process of splicing involves recognition of signals at the extreme ends of the intron. The 5’ splice site (5’ss) is recognized through base pairing interactions with U1 snRNP, and the 3’ss via interactions of U2 snRNP with
the branch point (bpt) and U2AF with the polypyrimidine tract (ppt). The strength of the various interactions ultimately determines the efficiency of intron excision. However, the efficiency of the splice site can also be influenced by the action of members of the hnRNP and SR protein families binding to adjacent sequences. The requirement to generate multiple viral proteins from the single primary viral transcript required that HTLV evolve mechanisms to regulate the conversion of this transcript into multiple mRNAs. This processing must happen in a balanced fashion to generate the necessary levels of both structural/enzymatic and regulatory viral proteins required for appropriate regulation of HTLV expression and assembly of replication competent viral particles. Is there a specific pattern of HTLV gene expression? How is this pattern regulated over the process of infection and cellular transformation? In Chapters 2 and 3 we determined HTLV gene expression pattern as well as kinetics in various experimental systems to dissect the regulation of HTLV gene expression and potential undiscovered functions of viral proteins in viral replication and cellular transformation.

In addition to regulating the RNA splicing, HTLV also has evolved mechanisms to modulate recognition of its polyadenylation signals. Cleavage and polyadenylation occur at the 3’ end of the majority of transcripts and are important for downstream mRNA processing events such as export, translation and stability. In HTLV, the AAUAAA polyadenylation signal and the downstream GU-rich element is located both at the R region in 5’ and 3’ ends of the viral transcripts. While the use of the promoter proximal
polyadenylation signal would be detrimental to viral gene expression, HTLV has evolved mechanisms to alter the relative efficiencies of each of the signals. The RNA secondary structure formed by the 5’ R-U5 sequences blocks access to the polyadenylation machinery. On the other hand, the stem loop structure that formed at the 3’ LTR RxRE brings the polyadenylation signal into close proximity to the GU-rich polyadenylation site, ensuring efficient polyadenylation of viral mRNA.\textsuperscript{187}

Once processing is complete, the viral RNAs must be transported to the cytoplasm for subsequent translation. Recognition of fully processed mRNAs for export may occur by assembly of the exon junction complex (EJC) during splicing or by direct binding of factors to specific sequence elements within the RNA. Nuclear retention of unspliced and incompletely spliced HTLV mRNAs has been attributed to either partial spliceosome assembly or specific nuclear retention signal in the RNA. However, HTLV has evolved strategies (utilizing the viral Rex) to overcome nuclear retention of unspliced RNAs which facilitates the export of unspliced viral mRNA by recruiting CRM-1, as has discussed earlier in this chapter.

Translation initiation is the rate-limiting step in protein synthesis and the primary target for translational control. The majority of eukaryotic mRNAs use a cap-dependent mode of translation initiation in which the multi-component eIF4F complex binds the 5’-7mmpG cap of mRNA and recruits the small ribosomal subunit.\textsuperscript{254,255} Retroviral mRNAs are capped and polyadenylated, conferring them
cap-dependent translation initiation. However, the highly structured replication motifs between the 5’cap and the distal translation start codon of retroviral mRNAs have been directly demonstrated to inhibit efficient cap-dependent translation initiation at the downstream open reading frame. Two RNA structural motifs have been characterized to facilitate efficient translation initiation despite a complex 5’ UTR: internal ribosome entry site (IRES) and 5’ proximal post-transcriptional control element (PCE). While HTLV-1 5’UTR sequence does not support IRES initiation, it has been shown to contain a PCE to facilitate efficient translation of HTLV-1 gag mRNA through interaction with host RNA helicase A (RHA), resulting in more viral structural and enzymatic protein production.

Collectively, HTLV expresses multiple viral genes in the process of replication, infection and cellular transformation under the regulation of various viral and cellular factors. Characterization of the viral gene expression profile throughout the process of infection, immortalization/transformation, and ultimately HTLV pathogenesis is likely to provide key functional information on viral gene products and their potential contribution to these processes. To date, such an analysis has not been thoroughly evaluated due to a challenging assay system and the difficulty in detecting viral mRNAs present in low abundance with small changes over time. The ultimate fate of infected T cells in vivo likely depends on their ability to balance proliferation, cell cycle control and anti-apoptotic signals mediated by viral and cellular proteins, versus the ability to
evade the host immune response. Therefore, how the virus responds to environmental
signals and regulates viral and cellular gene expression is critical to its long-term
survival and persistence in the infected individuals.

1.7 HTLV experimental systems

1.7.1 Cell culture

Since the discovery of HTLV almost 30 years ago, experimental systems for the
study of the virus have been complicated by its poor replication in culture. Cell free
infection of HTLV is very inefficient and infection of cultured cells requires
co-cultivation of target cells with lethally irradiated virus producer cells. In cell
culture, HTLV has the capacity to infect a number of cell types including B-cells,
T-cells, endothelial cells, glial cells and monocytes of both human and nonhuman
origin\textsuperscript{259-262}. However, only primary T-lymphocytes are susceptible to
immortalization/transformation by both HTLV-1 and HTLV-2. Immortalization is
defined as continuous growth of T-lymphocytes in the presence of exogenous IL-2
typically evident in culture microscopically as refractile cell clusters within 7-10 weeks
of co-cultivation. Transformation is defined as continuous cell growth in the absence
of exogenous IL-2 and the establishment of IL-2 independent transformed T-cell line
usually requires months in culture. In our study detailed in Chapter 3, co-cultivation
assays were used to study kinetics of HTLV-1 gene expression profile following \textit{in vitro}
HTLV-1 infection of human PBMCs.

1.7.2 Rabbit model of HTLV-1 infection

Animal models of HTLV-1 and HTLV-2 infection and transformation have provided critical knowledge of HTLV infection, transmission and immune response to the viral infection. HTLV-1 consistently infects rabbits, some non-human primates, and to a lesser extent, rats. Rabbits are used extensively as a model of HTLV infection because of the easy and consistent transmission of the virus in this species. Rabbits infected with HTLV-1 do not typically develop disease, however, the rabbit model has been used to verify routes of HTLV-1 transmission and immune response against HTLV-1 infection. Inoculation of rabbits with HTLV-1-infected cell lines derived from ATL or HAM/TSP patients demonstrate heterogenous biological response to HTLV-1 infection. Infectious molecular clones of HTLV-1 were first developed in the mid-1990s. These molecular clones were used to immortalize human PBMCs to create the ACH cell line, which was then used to infect rabbits. It was demonstrated that the lethally irradiated ACH cell line successfully establishes infection in the PBMCs of rabbits. Subsequently, ACH clones with mutations within the ORF encoding Rex, p12, p13, p30 and HBZ, were inoculated into rabbits to demonstrate the necessity of these proteins for establishment of infection and maintenance of proviral loads.
The rabbit model of HTLV-1 infection was utilized in Chapter 3 to study HTLV-1 gene expression kinetic profile \textit{in vivo}.

\subsection{1.7.3 Real-time RT-PCR}

Real-time RT PCR quantifies the initial amount of the template most specifically, reproducibly, and is extremely sensitive. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle as opposed to the endpoint detection so it is accurate and does not need post amplification analysis. This helps to increase throughput and reduce the chances of carry-over contamination. Real-time RT PCR also offers a much wider dynamic range of up to $10^7$-fold, compared to 1000-fold in conventional RT-PCR. A wide dynamic range means that a wide range of concentration ratios of target and control can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantification.

Alternative splicing of RNAs is an important way of virus to regulate gene expression. The combined amplification/hybridization approach of real-time RT-PCR assays is ideally suited for the detection and quantification of known splice variants, as they can be distinguished reliably by a combination of exon-specific primers and an appropriate probe.

Real-time RT-PCR significantly simplifies and accelerates the process of producing
reproducible quantification of mRNAs. With the use of appropriate standard curves, absolute copy numbers of mRNA can be easily calculated. Kinetic RT-PCR assays are readily standardized, making the comparison of results from different laboratories easier and more reliable. Therefore, real-time RT-PCR must be the method of choice for any experiment requiring sensitive, specific and reproducible quantification of mRNA.
Figure 1.1 Genome organization of HTLV-1 and HTLV-2 and their gene products. The provirus is flanked by 5’ and 3’ LTRs and consists of \textit{gag}, \textit{pro}, \textit{pol}, \textit{env} genes and genes of the pX region, encoding regulatory proteins Tax, Rex, and other accessory proteins. (A) HTLV-1 expresses at least eight positive sense transcripts and one negative sense transcript as shown. The numbers indicated by open or closed arrows represent two splice donor sites (nt119 and nt4831) and seven splice acceptor sites (nt4501, 4641, 4658, 6383, 6478, 6875 and 6950). The genomic unspliced mRNA encodes the Gag, Pol, and Pro proteins. Four singly spliced mRNAs encode Env, p21rex, p12 and p13. The three doubly spliced mRNAs encode Tax, Rex, p27 and p30. The singly spliced antisense transcript initiates at multiple sites in the 3’LTR and encodes Hbz. Exon 2 of env and p12, as well as all the doubly spliced mRNAs, has the potential to utilize multiple splice acceptor sites, which are depicted by gray lines. (B) HTLV-2 has similar genome structure with HTLV-1 and expresses at least seven mRNAs all of the positive sense polarity. Again, numbers indicated by open or closed arrows represent two splice donor sites (nt135 and 4869) and five splice acceptor sites (nt4730, 6493, 6630 and 6900). In addition to the unspliced mRNA species which have the same coding potential as HTLV-1, HTLV-2 has three singly spliced mRNAs encode Env, p28 and p20/p22Rex. The three doubly spliced mRNAs encode Tax, Rex, p10, p11 and an uncharacterized viral protein. 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 lines designate exons and dotted lines introns.
Figure 1.1
Figure 1.2  Key highlights of HTLV-1 regulatory and accessory protein functions within the context of a cell. Clockwise from the upper left side of the figure:
1. p13. p13 inserts into the inner mitochondrial membrane and alters mitochondrial conductance to Ca\(^{2+}\) and K\(^{+}\), leading to swelling and collapse of inner mitochondria membrane potential \(^{213}\). In the mitochondria, p13 interacts with farnesyl pyrophosphate synthase (FPPS) that is involved in the synthesis of a substrate required for the post-translational farnesylation of Ras \(^{214}\).
2. p12. p12 expression increases basal intracellular calcium in T cells, leading to increased NFAT-mediated transcription \(^{220,221}\) and LFA-1 clustering \(^{275}\). p12 binds to newly synthesized MHC-I-heavy chain (Hc) before its association with \(\beta\)-2-microglobulin and most of the MHC class I bound to p12 is routed from the Golgi to the cytosol and targeted to the proteasome for degradation, resulting in less surface expression of MHC-presenting peptide \(^{225,226}\). p12 protein also binds the IL-2R, which is involved in Jak/STAT pathway, leading to an increase in STAT5 transcriptional activity \(^{223,224}\).
3. Rex. Rex preferentially binds to, stabilizes, and exports intron-containing viral mRNAs from the nucleus to the cytoplasm \(^{177,178,181}\). Rex also inhibits splicing and its association with viral mRNA after export likely facilitates polysome loading and increased translation efficiency \(^{194}\).
4. p30. p30 associates with p300/CBP and TIP60 to modulate viral and cellular gene transcription \(^{201,202}\). p30 also binds and retains tax/rex mRNA in the nucleus downregulating viral replication \(^{204,276}\).
5. HBZ. HBZ interacts with CREB-2 and abrogates its binding to the HTLV-1 promoter inhibiting Tax-dependent viral transcription \(^{228}\). HBZ also interacts with members of the Jun family, JunB, c-Jun and JunD to modulate their transcriptional activity \(^{231,232}\). Hbz mRNA up regulates the transcription of E2F1 and many E2F1 targeted genes, leading to cell proliferation \(^{233}\).
6. Tax. Tax trans-activates viral transcription by binding to CREB and recruiting P300/CBP \(^{277}\). Tax activates NF-\(\kappa\)B and Akt signaling pathways, to promote cell survival and proliferation \(^{278,279}\). Tax activates CDK4 and CDK6, leading to the hyper-phosphorylation or proteasomal degradation of the retinoblastoma (RB) tumor suppressor protein. The phosphorylation/degradation frees the E2F1 transcription factor, accelerating G1 to S cell cycle transition \(^{139,280,281}\).
CHAPTER 2

DETECTION AND QUANTITATION OF HTLV-1 AND HTLV-2 mRNA SPECIES BY REAL-TIME RT-PCR

2.1 Abstract

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 $2.5 \times 10^7$ 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 further evaluate viral gene expression and correlate transcript levels to key stages of the virus life cycle and ultimately, pathogenesis.

2.2. 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. However, the clinical manifestations of these two viruses differ significantly. HTLV-1 preferentially targets and transforms CD4+ T-cells and is the etiologic agent of adult T-cell leukemia (ATL) and HTLV associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-2 is much less pathogenic, but has been associated with a few cases of atypical hairy cell leukemia and neurological disease. 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.2.1). Both HTLV-1 and HTLV-2 express the structural and enzymatic proteins Gag, Pol and Pro from the unspliced full length
mRNA\textsuperscript{94,95}. This unspliced mRNA also serves as genomic RNA to be packaged into progeny virions. Env is expressed from a singly spliced mRNA\textsuperscript{283} and plays a major role in receptor recognition and target cell infection. Env also is the determinant for differences in transformation tropism exhibited between HTLV-1 and HTLV-2\textsuperscript{111}. 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)\textsuperscript{71,178,277} and modulates the transcription or activity of numerous cellular genes involved in cell growth and differentiation, cell cycle control, and DNA repair\textsuperscript{150,284-287}. Compelling evidence indicates that the pleiotropic effects of Tax on cellular processes are required for the transforming or oncogenic capacity of HTLV\textsuperscript{148,288-292}. Rex acts post-transcriptionally by preferentially binding, stabilizing, and selectively exporting intron-containing viral mRNAs from the nucleus to the cytoplasm\textsuperscript{89}. 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\textsuperscript{165,168,293}.

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\textsuperscript{219,222}. p12 enhances LFA-1 T-cell adhesion in a calcium-dependent manner\textsuperscript{275} 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\textsuperscript{224,225,294}. 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\textsuperscript{101} and persistence of the virus in inoculated rabbits\textsuperscript{295}. Furthermore, p12 appears to be preferentially expressed over p27. Although there is evidence that p27 is expressed \textit{in vivo}\textsuperscript{107} and \textit{in vitro}\textsuperscript{96}, 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\textsuperscript{200-202} and post-transcriptionally via binding and retaining tax/rex mRNA in the nucleus\textsuperscript{203,204,296}. Although p30 is dispensable \textit{in vitro} for replication and cellular transformation\textsuperscript{99,208}, it is required to promote virus survival and persistence in infected animals\textsuperscript{103}. p13, expressed from a singly-spliced mRNA, localizes to the mitochondria, has a suppressive effect on cell growth \textit{in vitro} and plays an essential biological role during the early phase of infection \textit{in vivo}\textsuperscript{215,297}.
In HTLV-2, the accessory proteins include p10 encoded by ORF I, p28 by ORF II, and p11 by ORF V \(^{97}\). 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/p20\(\text{rex}\)), is at least in part functionally homologous to HTLV-1 p30. It functions to repress viral replication post-transcriptionally by retaining tax/\(\text{rex}\) mRNA in the nucleus \(^{204}\). 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 \(\beta\) and \(\gamma\) chains \(^{225}\) and p11 binds to MHC class I heavy chain \(^{226}\).

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 \(^{227,229}\). This transcript encodes HBZ 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 \(^{228,231,247}\). 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 \(^{100}\). 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 \textit{hbz} mRNA promoted the proliferation of a human T-cell line \textsuperscript{233}. Together these data suggest that the \textit{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 \textsuperscript{298}. 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.
2.3. Material and methods

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).

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 2.5x10^1 to 2.5x10^7 copies. Standard dilutions were stored at -20°C in single use aliquots. SE356^{299}, and JA662^{100} 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 298. 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 204 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).

**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.

**PCR primer pairs and specific probes**

All primer pairs and probes for detection of HTLV-1 and HTLV-2 mRNAs are summarized in Table 2.1 (HTLV-1) and Table 2.2 (HTLV-2). Newly designed and generated primers used to specifically detect HTLV-1 transcripts include: [ENV4501-S] $5'\quad^{106}\text{CGTCCGCCGTCTAG}^{119}_{A,4501}\text{CCCT}^{4504}_{-3'}$, [ENV4641-S]
5'-106 CGTCGCCGCTCTAGCTTCC4645-3', [ENV4658-S]
5'-108 TCCCGCGCTCTAGCCGC4661-3', [ENV-AS]
5'-4001 ATTGTGAGAGTACAGCAGC4919-3', [P13-S]
5'-107 GTCCGCCCCCTCTAGCAGGT6879-3', [TR-AS]
5'-7057 CCGAACATAGTCCCCCAGAGA7037-3', [HBZMAP1]
5'-1905 CTTCTAAGGATAGCAAACCGTCAAG1881-3', [HBZMAP2]
5'-355 ATGGCGGCCTCAG365GGCT1768-3' [HBZminor-S]
5'-212 CCGGCTGAGTCTAGGGCTG1769-3' [HBZ-AS]
5'-1830 GCCCGTACCACAAATTCTCT1813-3' (HBZ primer numbering is based on ACH antisense strand proviral sequence beginning with last nt of U5 of the 3’ LTR). Primers #19 (5'-1036GAGGGAGGAGCAAGTACTG1016-3'), #20 (5'-938 AGCCCCCAGTTCATGCAGAC958-3’) used to detect HTLV-1 gag/pol and H1JA2 (5'-6885 AGGAGCGCCGTGAGCGCAAGT6865-3’) used to detect HBZ antisense transcript have been described previously #3637. Primers X2TR1-2 (5'- 4819 ACCAACACCATGG4831,6950 CCCA6953-3’), P21Rex-S
(5'-107 GTCCGCCGCTCTAG119,6950 CCCA6953-3’), P12-6383-S
(5'-107 GTCCGCCGCTCTAG119,6383 CAAC6386-3’), P12-6478-S
(5'-107 GTCCGCCGCTCTAG119,6478 CACT6481-3’), P12-AS
(5’-6552 GGAGAAAGCAGGAAGGC6535-3’), P27S
(5’-4819 ACCAACACCATGG4831,6383 CAACT6387-3’), X2P30-S
Primers designed and generated to quantify HTLV-2 mRNA species include: [GP2-S] 
5'-1904GCCTACCCAAGCGCTACTT1922-3', [GP2-AS]
5'-1970CCCGGGCACGAGTGTCT1954-3', [ENV2-AS]
5'-4888AGTAGGAAAGAAACATTACCACATGGT4863-3', [TR2-S]
5'-123TGCTCTCCCAAG135GAAGC4734-3', [TR2-AS]
5'-6915AATCCTGGGAAATGGG6900CCAT4866-3', [P28-1-S]
5'-124GCTCCTCCCAAG135GCGCT6634-3', [P28-2-S]
5'-123TGCTCTCCCAAG135CCCAT6904-3', [P28-AS]
5'-6984GGACACCAATCGGCCTGTAC6965-3', [P10-AS]
5'-6643GGGAAAAGAAGGTC6493CCAT4865-3', [?-AS]
5'-6643GCAGAAAGGAGCGC6630CCAT4866-3'. Primers [hGAPDH-S]
5'-CATCAATGACCCCTTCATTGAC-3' and [hGAPDH-AS]
5'-CGCCCCACTTGATTATTTGGA-3' were used to quantify hGAPDH.

Probes to detect amplified PCR products include [TMP-1] 
5'FAM-6489TTCGCCTTCTCAGCCCCTTGTCT6511-TAMRA3’, [TMP-2] 
5'FAM-7105ATCACCTGGGACCCCATC7122-TAMRA3’ previously described 298 and newly designed [TMP-3] 
5'FAM-900CTGCCAAAGACCTCAAGACCTCC1013-TAMRA3’, [TMP-4]
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 25μl consisting of 12.5μl 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 μl 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.

5’FAM-4874CCCTCATCCTCGGTGATTACAGCCC4898-TAMRA3’, [TMP-5]
5’FAM-TGGCAAATTCCATGGCACCGTC-TAMRA3’, [TMP-6]
5’FAM-4800AAGCTGCATGCCCAAGACCAGACCGCC4825-TAMRA3’, [TMP-7]
5’FAM-6939ACCCGTCCTACGTGTTTGCTATTG7363-TAMRA3’, [TMP-8]
5’FAM-1925CACAGGAGCGACCTACGGTTATACCC1952-TAMRA3’, [TMP-10]
5’FAM-7047ACTGTGTACAAGGCAGCTGTTGCC7071-TAMRA3’, [TMP-13]
5’FAM-1782CCTGTGCCATGCCGGAGA1801-TAMRA3’.
**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 5x10^6 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)).

**2.4. Results**

**2.4.1. 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 is encoded by the positive sense strand of the proviral genome including the unspliced/genome RNA (Fig 2.1.A). 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 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. 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. 2.1.B). 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.

2.4.2 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 25 to 2.5 x10⁷ 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 log₁₀ 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 2.1 and Table 2.2, respectively. The optimized standard curves for all primer pairs used in this study were linear over the 10⁷ range with slopes ranging from -3.3 to -3.7, indicating that the amplification efficiencies were greater than 90% (data not shown). The R² correlation coefficients of all the standard curves were greater than 0.97.

2.4.3. 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 2.3). Poly A+ mRNA was isolated from $10^7$ 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. 2.2). 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 Cts 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 2.3). 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.
2.4.3.1 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 (Fig. 2.3). 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 ≥ 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 x 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 > p13 > p27/p12 > p30); the 
antisense transcript that encodes HBZ was approximately 10-fold lower than tax/rex 
mRNA (4 x 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 hbz 
major transcript was at least 100-fold more abundant than the hbz minor transcript.

2.4.3.2 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 (Fig.2.4). In the newly immortalized PBL-pH6 cell line, the four most 
abundant mRNAs included gag/pol, tax/rex, env, and p28, p22/p20rex-1 (gag/pol > 
tax/rex > p28, p20/p22rex-1 > 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/p20 -2 and the doubly-spliced mRNA whose potential protein product(s) has yet to be characterized (p? > p28, p22/p20-2 > 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 298.

2.5. Discussion

Alternative splicing of mRNAs is important for cell-specific gene regulation 300. 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 x 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. 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 2.3) 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, 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. 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.

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. 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-fold to 1000-fold less than the p28, p22/p20rex-1 mRNA transcript.

In conclusion, we present an accurate and reproducible methodology to specifically detect 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.
Acknowledgements

We thank Tim Vojt for preparation of the figures and Kate Hayes for editorial comments. This work was supported by grants from the National Institutes of Health (CA100730 and CA077556).
Table 2.1 Primers and probes used for HTLV-1 mRNA species

<table>
<thead>
<tr>
<th>HTLV-1 mRNA</th>
<th>cDNA plasmid</th>
<th>HTLV Sequence in Plasmid Standards</th>
<th>5’ primer</th>
<th>3’ primer</th>
<th>Taqman probe</th>
</tr>
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<tr>
<td>gag/pol</td>
<td>ML760</td>
<td>920-1049</td>
<td>#20</td>
<td>#19</td>
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<td>env-4501</td>
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Table 2.2 Primers and probes used for HTLV-2 mRNA species

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<th>HTLV Sequence in Plasmid Standards</th>
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<th>3’ primer</th>
<th>Taqman probe</th>
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<td>TMP-6</td>
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<td>TMP-6</td>
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Table 2.3. Ct value of hGAPDH gene and proviral load quantitation result in all cell lines used.

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<th>SLB-1</th>
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<th>PBL-ACH</th>
<th>729Uninf</th>
<th>MoT</th>
<th>729pH6neo</th>
<th>PBL-pH6</th>
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<td>Ct value</td>
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<td>18.80±0.16</td>
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<td>Pro-load</td>
<td>1.26±0.30</td>
<td>5.12±0.55</td>
<td>0.9±0.08</td>
<td>0</td>
<td>1.55±0.70</td>
<td>5.10±0.29</td>
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**Figure 2.1:** 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/p22rex-1), or 6900 (p28, p22/p22rex-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 2.1
Figure 2.2: 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 2.3:** 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 2.4: 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 729pHneo 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.
CHAPTER 3

KINETIC ANALYSIS OF HUMAN T-CELL LEUKEMIA VIRUS TYPE I GENE EXPRESSION IN CELL CULTURE AND INFECTED ANIMALS

3.1 Abstract

HTLV-1 infection causes adult T-cell leukemia and is associated with a variety of lymphocyte-mediated disorders. It has been hypothesized that a highly regulated pattern of HTLV-1 gene expression is critical for virus survival and disease pathogenesis. In this study, real-time RT-PCR was used to determine the kinetics of viral gene expression in cells transiently transfected with an HTLV-1 proviral plasmid, newly infected human PBMCs, and PBMCs from newly infected rabbits. The HTLV-1 gene expression profiles in transiently transfected and infected cells were similar; over time, all transcripts increased and then maintained stable levels. \textit{gag/pol}, \textit{tax/rex}, and \textit{env} mRNA were detected first and at the highest levels, whereas expression of the accessory genes, including the anti-sense \textit{hbz}, were significantly lower than \textit{tax/rex} (ranging from 1-4 logs depending on the specific mRNA). In infected rabbits, \textit{tax/rex} and \textit{gag/pol} mRNA levels peaked early after inoculation and progressively decreased,
which correlated inversely with the proviral load and host antibody response against viral proteins. Interestingly, \textit{hbz} mRNA was detectable at one week post-infection and increased and stabilized. Expression of all other HTLV-1 genes in infected rabbit PBMCs were at or below our limit of detection. This analysis provides insight into viral gene expression under various \textit{in vitro} and \textit{in vivo} experimental conditions. Our \textit{in vivo} data indicate that under immune pressure there is an inverse correlation between \textit{tax/rex} and \textit{hbz} mRNA expression over time, which provides the first evidence linking \textit{hbz} expression to proviral load and the survival of the virus infected cell in the host.

\section*{3.2 Introduction}

Human T-cell leukemia virus type 1 (HTLV-1) is a complex oncogenic retrovirus that causes adult T-cell leukemia/lymphoma (ATLL) after a long clinically latent period (>30 years). HTLV-1 has the capacity to infect and transform primary human T-lymphocytes both in cell culture and infected individuals \textsuperscript{274,301}. However, the relationship of specific viral gene expression and infected cell survival, ultimately resulting in oncogenic transformation of T-lymphocytes, is not completely understood.

HTLV-1 utilizes both strands of its proviral genome to express multiple gene products from unspliced mRNAs and a complex array of alternative spliced mRNAs. In addition to the \textit{gag}, \textit{pol}, and \textit{env} genes that encode the structural and enzymatic proteins of all replication competent retroviruses, HTLV-1 encodes the regulatory \textit{tax}
and rex genes and open reading frame (ORF) I and II accessory genes from the positive genome strand. The negative sense strand of the genome encodes the HTLV-1 B-Zip (HBZ) accessory protein. Tax increases the rate of viral gene transcription from the promoter located in the viral long terminal repeat (LTR) \(^{71,178,277}\); furthermore, Tax modulates the transcription or activity of numerous cellular genes involved in cell growth and differentiation, cell cycle control, and DNA repair \(^{150,284,286,287,302}\). Compelling evidence implicates Tax as the key viral protein required for cellular transformation and oncogenesis \(^{282,288,292}\). The second regulatory protein, Rex, acts post transcriptionally by preferentially binding, stabilizing, and selectively exporting the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, essentially regulating production of the virion components \(^{89}\).

To date, the accessory proteins (p12, p30, and p13) have not been detected in HTLV infected or transformed cells, but functional activities have been revealed based on over-expression studies \(^{276}\). p12 activates cells by regulating calcium signaling \(^{219}\). p12 also enhances LFA-1 T-cell adhesion \(^{275}\) and associates with cellular proteins including the 16kDa subunit of the vacuolar ATPase, IL-2 receptor β and γ chains, and the MHC class I heavy chain \(^{224,225,294}\). p30 differentially modulates viral and/or cellular gene expression at the transcriptional level through association with p300/CBP and TIP60 and post transcriptionally via binding and retaining tax/rex mRNA in the nucleus \(^{201,202}\). p13 also has suppressive effects on cell growth in cell culture \(^{297}\).
Contrary to p12, p30, and p13, the antisense HBZ protein is detected in most HTLV-1-transformed cell lines, HTLV-1 proviral plasmid transfected cells, and ATL patient samples $^{100,229,233,303}$. HBZ protein can interact with CREB and Jun family members altering transcription factor binding and transactivation of both viral and cellular promoters $^{235}$. $hbz$ gene expression also has been linked to cellular proliferation $^{233,304}$. Interestingly, all of the accessory proteins are dispensable for viral replication and cellular transformation under standard cell culture conditions $^{98-100}$. However, in vivo studies using a rabbit model of infection have revealed that p12, p30, p13, and HBZ are important for the enhancement of infectivity and the establishment of persistent infection $^{100,102,103,215}$, indicating that the activities of these proteins likely play key roles in viral infection, spread, and escape from the immune system.

Characterization of the viral gene expression profile throughout the processes of infection and immortalization/transformation is likely to provide key functional information on viral gene products and their potential contribution to HTLV pathogenesis. To date, such extensive analysis has not been undertaken due to a challenging assay system and the difficulty in detecting viral mRNAs present in low abundance. The ultimate fate of HTLV-1 infected T-cells in vivo likely depends on their ability to balance proliferation, cell cycle control and anti-apoptotic signals that are mediated by viral and cellular proteins, versus the ability to evade the host immune response. Therefore, how the virus responds to environmental signals and regulates
viral and cellular gene expression are critical to its long-term survival and persistence in infected individuals.

Real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) is a highly sensitive method allowing quantitation of low concentration mRNA transcripts and discrimination of small changes in gene expression. We previously developed a series of oligonucleotide primer pairs and probes to quantitate all HTLV-1 mRNA species using Taqman real-time RT-PCR. In the present study, we utilize this approach to measure the kinetics of viral gene expression in cells transiently transfected with an HTLV-1 proviral plasmid, in human PBMCs newly infected with HTLV-1 in culture, and in PBMCs harvested from HTLV-1 inoculated rabbits. The HTLV-1 gene expression profile in transiently transfected and infected cells were similar with a general increase in all transcripts over time. gag/pol, tax/rex, and env mRNAs were expressed first and at the highest levels. In infected rabbits, tax/rex and gag/pol mRNA peaked early after inoculation and progressively decreased in contrast to increasing proviral load and the host antibody response against viral proteins. hbz mRNA level was low but detectable one week post-infection and increased over time. Although our in vitro analysis reveals that tax/rex and gag/pol mRNAs are expressed first followed by subsequent expression of all other transcripts (all increasing and then stabilizing over time), we detect no apparent regulatory control of specific transcripts. However, our in vivo analysis showed that tax/rex and gag/pol mRNAs were expressed
at the highest levels immediately after infection and then progressively declined over time, eventually stabilizing at low levels. Conversely, *hbz* was expressed at a low level early after infection, continued to increase before reaching a plateau, which was in direct correlation with proviral load levels in infected rabbit PBMCs. Therefore, our results revealed an inverse correlation between *tax/rex* and *hbz* mRNA expression over time providing the first evidence linking *hbz* expression to proviral load and the survival of the virus infected cell in an infected host.

3.3 Materials and Methods

**Cells and plasmids**

293T cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and antibiotics [2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL)]. 729 is a human B-lymphoblast cell line. 729ACHneo (referred to as 729HTLV-1) is a 729-derived stable ACHneo transfectant cell line that produces HTLV-1 with the capacity to infect and transform human peripheral blood T-lymphocytes. Both 729 and 729HTLV-1 cells were maintained in Iscove’s medium supplemented with 10% FBS and antibiotics. Human and rabbit peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque (Amersham, Piscataway, NJ) and Percoll (Amersham, Piscataway, NJ), respectively, and cultured in RPMI 1640 medium supplemented with 20% FBS, antibiotics, and 10
U/mL recombinant human interleukin-2 (IL-2; Roche Applied Biosciences, Indianapolis, IN).

The HTLV-1 proviral plasmid ACHneo has been described previously. Plasmids containing specific HTLV-1 cDNA gene sequences and human GAPDH cDNA gene sequences have been described and were used to generate standard curves for the Taqman real-time PCR assays. Plasmid ML789 is pCRScript-derived (Stratagene, La Jolla, CA) and contains a rabbit GAPDH DNA gene fragment that was amplified from rabbit PBMC genomic DNA.

**Transient transfection, p19 ELISA and Western blot**

293T cells (8 x 10⁵) were plated 24 hours prior to transfection with 8 µg of the ACHneo plasmid using the Lipofectamine method (Invitrogen, Carlsbad, CA). Cells and culture supernatants were harvested at 4, 12, 20, 28, 44 and 60 hrs post-transfection. p19 ELISA (Zeptometrix Corporation, Buffalo, NY) was performed to quantitate HTLV-1 Gag protein production in the culture supernatants. Cell pellets were lysed for Western blot analysis to detect HTLV-1 p24, Tax, and Rex protein expression. Western blots were performed as described. The following dilutions were used for individual antibodies against different viral proteins: HTLV-1 rabbit anti-Rex1 polyclonal antisera (1:1000); HTLV-1 mouse anti-Tax1 monoclonal antibody (NIH cat. No. 1318) (1:1000); HTLV-1 mouse anti-p24 (clone 46/3.24.4 ZeptoMetrix Corp,
Horseradish peroxidase-conjugated anti-IgG (mouse or rabbit as appropriate) was used as the secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and proteins were visualized using the electrochemiluminescence (ECL) Western blot analysis system (Amersham Biosciences, Piscataway, NJ).

**Long term immortalization assay**

Irradiated 729HTLV-1 producer cells (1 x 10⁶) were cocultured with 2 x 10⁶ freshly isolated PBMCs in the presence of IL-2 (10 U/ml) in 24-well culture plates. An immortalization growth curve was generated by enumerating viable cells per well (an average of three wells per time point) by trypan blue exclusion at weekly intervals. HTLV-1 expression was confirmed by detection of p19 Gag protein in culture supernatants using an ELISA at weekly intervals. Immortalized cells were defined as cells with continuous proliferation >8 wks post-coculture.

**Rabbit inoculation procedures**

Twelve-week-old specific pathogen-free New Zealand White rabbits (Hazelton, Kalamazoo, MI) were inoculated via the lateral ear vein with 10⁷ gamma-irradiated (7500 rad) 729HTLV-1 (nine rabbits) or uninfected 729 control cells (two rabbits). At 0, 1, 2, 4, 6, and 8 wks post-inoculation, 15 mL of blood was drawn from the central ear artery of each animal for testing. This protocol was approved by the University
Laboratory Animal Resources (ULAR) of the Ohio State University. Plasma serum reactivity to specific viral antigenic determinants was detected using a commercial HTLV-1 Western blot assay (GeneLabs Diagnostics, Singapore) with modification in which goat anti-human IgG conjugated with alkaline phosphatase was substituted with goat anti-rabbit IgG conjugated with alkaline phosphatase (Chemicon, Temecula, CA). Serum (dilution of 1:200) showing reactivity to Gag (p24 or p19) and Env (gp21 or gp46) antigens was classified as positive.

**DNA/RNA preparation and cDNA synthesis**

Genomic DNA was extracted from $10^7$ rabbit PBMCs using the Easy-DNA kit (Invitrogen Corp., Carlsbad, CA). Total RNA was extracted from transfected 293T, HTLV-1 immortalized human T-lymphocytes, or rabbit PBMCs using the RNeasy kit and subjected to DNase treated columns. Poly A+ mRNA was isolated from transfected cells or immortalized cell lines 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 and 200 ng (total mRNA) or 80 ng (poly A+ mRNA) of input RNA per reaction as described by the manufacturer.
Taqman real-time PCR and real-time RT-PCR

Taqman real-time PCR (Applied Biosystems, Foster City, CA) was performed to quantitate the proviral copy number per cell in infected rabbit PBMCs. 500 ng rabbit genomic DNA were put into each reaction as template for Taqman real-time PCR using HTLV-1 specific primers #19 and #20 and probe TMP-3 as previously described. Absolute copy number was determined by extrapolation against a standard curve generated from log_{10} dilutions of plasmid DNA containing known copies of the gag/pol sequence. Each reaction was performed in duplicate and averaged. The copy number-per-cell value for a sample was generated based on the estimation that one μg rabbit PBMC DNA is equivalent to 134,600 cells. The specific primers, probes, and protocol for Taqman real-time RT-PCR to quantitate all HTLV-1 mRNA and human GAPDH mRNA were previously described. Rabbit GAPDH mRNA was quantified using primers rGAPDH-S 5’-GATGCTGGTGCCGAGTGACGTGG-3’ and rGAPDH-AS 5’-GTGGTGCAAGGTGCTGCTGA-3’ and probe BY-1 5’FAM-ACCACCATGGAGAAGGGCGG-TAMRA3’. Total mRNA copy number was determined using a standard curve generated from log_{10} dilutions of a plasmid containing the corresponding cDNA sequence for each mRNA and normalized to one million copies of human or rabbit GAPDH mRNA.
3.4 Results

3.4.1 Kinetic analysis of HTLV-1 gene expression in transiently transfected 293T cells.

Multiple gene products from unspliced mRNAs and a complex array of alternative spliced mRNAs are expressed from HTLV-1 (Fig. 3.1). Although HTLV-1 gene expression appears to be highly regulated, a kinetic expression profile of all the viral genes has been difficult to assess experimentally. We previously generated and characterized a series of oligonucleotide primer pairs and probes to specifically quantitate all HTLV-1 mRNA species using Taqman real-time RT-PCR\(^{305}\). We initially determined the HTLV-1 gene expression profile at defined time points following the transient transfection of 293T cells with the HTLV-1 proviral plasmid, ACHneo. Cells were transfected and harvested at 0, 4, 12, 20, 28 and 44 hrs post-transfection, and poly A+ mRNA was isolated. We confirmed that the poly A+ mRNA samples from each time point were free of DNA contamination as determined by the failure of gag/pol specific primers to amplify a fragment by standard RT-PCR in the absence of RT (data not shown). cDNA was synthesized from poly A+ mRNA using RT and random hexamers and further subjected to real-time PCR with specific primers and probes to quantitate the cellular GAPDH mRNA (to be used as a normalization control) and each specific viral mRNA. As early as 4 hrs post-transfection, we were able to detect unspliced gag/pol/genome mRNA and tax/rex
doubly-spliced mRNA (Table 3.1). At 4 hrs, *tax/rex* mRNA was the most abundant transcript, approximately 2-3 fold higher than the full length *gag/pol* mRNA ($5.5 \times 10^2$ vs $1.93 \times 10^2$ per $10^6$ copies of GAPDH mRNA). However, over time (12 to 44 hrs), *tax/rex* and *gag/pol* genome mRNAs increased by several logs, with the full-length *gag/pol* mRNA consistently expressed at the highest concentration. Expression of *env* and the accessory genes were first detected at 12 hrs and increased slightly over the time course, however, their levels ranged from 1-4 logs lower than *tax/rex* depending on the specific mRNA. P30 gene expression remained below our detection limit throughout the time course (<25 copies). Failure to detect p30 expression is consistent with our previous study in which p30 mRNA expression levels in HTLV-1 transformed cell lines were 1-2 logs lower than other accessory gene expression levels.

To determine if viral protein production kinetics correlated with mRNA expression kinetics, Western blot was performed on transfected cell lysates to detect HTLV-1 p24Gag, Tax, and Rex proteins. Viral protein expression was detected in cell lysates initially at 12 hrs post-transfection. In direct correlation with mRNA expression, protein levels increased and then plateaued at later time points (Fig 3.2A). Quantitation of p19 Gag in the supernatants of transfected cells, which is a measure of new virion production, showed a similar pattern (Fig. 3.2B). Taken together, our results revealed that following transient transfection of cells, all HTLV-1 gene transcripts increased over time eventually reaching a plateau at 28-44 hrs. *Gag/pol, tax/rex,* and
env mRNAs were expressed first and at the highest level, whereas expression of the accessory genes including the anti-sense *hbz* was significantly lower than *tax/rex*.

### 3.4.2 Kinetic analysis of HTLV-1 gene expression in human PBMCs infected with HTLV-1 in cell culture.

We next characterized HTLV-1 gene expression in newly infected cells and throughout the immortalization process *in vitro*. Freshly isolated human PBMCs were cocultivated with lethally irradiated 729HTLV-1 producer cells in the presence of a low concentration of recombinant IL-2 (10 U/ml). Cell number and viability were monitored at weekly intervals to follow the immortalization process and the characteristic expansion of T-cells from the PBMC mixed cell population. A growth curve of a representative assay indicated a progressive loss of viable cells over time in cocultures containing irradiated uninfected 729 cells and PBMCs (Fig. 3.3A). In contrast, immortalization clearly was apparent in the PBMC/729HTLV-1 coculture in which the cell number increased early followed by a cell number decrease as the newly infected cells undergo a proliferative burst and subsequent crisis stage before recovering. After eight wks, surviving cells harbor provirus, continue to proliferate, and are considered to be immortalized by HTLV-1 274,308. We detected p19 Gag in the culture supernatants confirming HTLV-1 infection, replication, and virion production (Fig. 3.3B). PBMCs from random cocultured wells were harvested each week for poly A+
mRNA extraction. Poly A+ mRNA was subjected to real-time RT-PCR for quantitation of each specific HTLV-1 mRNA. HTLV-1 mRNA expression following infection of PBMCs was consistent with the cell growth and immortalization observations (Fig. 3.3C): HTLV-1 mRNA levels increased from wks 1-5, significantly decreased during the typical cell growth crisis stage (wks 6, 7 & 8), and slowly recovered to relatively high steady state levels as the surviving newly immortalized cells expanded. The general gene expression profile in newly infected PBMCs was similar to cells transiently transfected with proviral plasmid: full length gag/pol (genome) mRNA (approximately $10^6$ copies per $10^6$ copies of GAPDH mRNA) > doubly spliced tax/rex mRNA ($10^5$ copies) > singly spliced env mRNA ($10^4$ copies).

The transcripts encoding the accessory proteins (p21, p12, p30, and p13), including the anti-sense mRNA encoding HBZ, were significantly lower than tax/rex mRNA (ranging from 1-4 logs depending on the specific mRNA). Other than gag/pol and tax/rex transcripts being expressed first and consistently at the highest levels throughout the time course, there did not appear to be a highly regulated viral gene expression pattern following transfection or infection of cells in vitro.
3.4.3 Kinetic analysis of HTLV-1 gene expression in PBMCs from HTLV-1-inoculated rabbits.

To evaluate the HTLV-1 gene expression profile in vivo, we utilized our established rabbit model of infection and persistence. Twelve month-old New Zealand white rabbits were inoculated with $1 \times 10^7 \gamma$-irradiated 729HTLV-1 producer cells (nine rabbits) or uninfected 729 cells as a control (two rabbits). Rabbit blood was drawn at weeks 0, 1, 2, 4, 6, and 8 after inoculation and plasma and PBMCs were isolated for further analysis. To determine the serologic response of rabbits to the inocula, we measured anti-HTLV-1 antibody responses in rabbits by Western blot analysis. A representative seroconversion pattern from each of the inoculated groups is shown in Figure 3.4A (rabbit plasma was diluted 1:200). Seroconversion was detected in the 729HTLV-1-inoculated rabbits starting at week 2 and antibody titers rose over the time course of the experiment. As expected, we were unable to detect any antibody response in rabbits inoculated with control 729 cells. To further confirm infection status and quantitate the HTLV-1 proviral load in inoculated rabbits over time, DNA was extracted from isolated PBMCs and subjected to real-time PCR (Fig. 3.4B). Integrated HTLV-1 proviral DNA was detected as early as two wks post-inoculation, and although variable for individual rabbits, loads increased over time. By wk 8, the HTLV-1 proviral loads in the nine infected rabbits varied from 0.02 to 0.14 copies per cell with a mean proviral load value of 0.043 (43 infected cells per $10^3$ rabbit PBMCs).
To evaluate the kinetics of HTLV-1 gene expression in inoculated rabbits, total mRNA was extracted from rabbit PBMCs harvested at week 0, 1, 2, 4, 6 and 8 post-inoculation and subjected to real-time RT PCR. *tax/rex* mRNA was expressed very early after infection and at the highest levels of all viral mRNAs detected (3000 copies per $10^6$ GAPDH mRNA copies). After one-two wks, *tax/rex* mRNA progressively decreased and stabilized at relatively low levels (Fig. 3.5A). *gag/pol* mRNA expression mirrored that of *tax/rex* mRNA expression, but with an average magnitude of approximately four-fold lower at its peak (Fig. 3.5B). With the exception of *hbz* mRNA, all other HTLV-1 mRNAs in rabbit PBMCs including those encoding Env and the accessory proteins were below our limit of detection (25 copies, data not shown). *hbz* mRNA was detectable at low levels early after inoculation and slowly increased and stabilized (Fig. 3.5C). Interestingly, at eight wks post-infection the *hbz* mRNA was expressed at the highest concentration (an average of nine-fold higher than *tax/rex* mRNA).

We next compared *tax/rex* and *hbz* gene expression directly to proviral load in an effort to understand the relationship between specific gene expression and viral spread and persistence. Typical of HTLV-1 infection, proviral load in PBMCs was low early after infection and increased over time until a set point unique to each infected individual was reached (Fig. 3.4B). We noted that the rabbits that expressed the highest levels of *tax/rex* mRNA at the early time points had lower proviral loads at later
weeks; this likely reflective of a more robust immune response and elimination of infected cells (compare Fig. 3.4B with Fig. 3.5A). Our data also indicated that tax/rex mRNA expression was highest early after infection and decreased, which correlated inversely with proviral load (Fig. 3.6A). In contrast, hbz mRNA expression started out low then slowly increased and stabilized in direct correlation with proviral load (Fig. 3.6B). The timing of expression of hbz is consistent with its reported cellular proliferative function and its maintenance in ATL cells in which the Tax oncoprotein is rarely expressed.

3.5 Discussion

Although compelling evidence indicates that the HTLV-1 Tax protein is critical for viral oncogenesis, the viral gene expression profile throughout the immortalization process and the relationship of individual gene expression to infected cell survival and cellular transformation have not been assessed. Alternative splicing of mRNA is important for specific gene regulation and HTLV-1 produces a set of closely related mRNAs by alternative splicing. We and others have hypothesized that a precise blueprint of the viral gene expression profile might provide useful information for dissecting the functional role of specific viral genes in the process of viral infection and cellular transformation. In this study, real-time RT-PCR was used to determine the kinetics of viral gene expression using three systems: 293T cells transiently transfected
with HTLV-1 proviral plasmid, human PBMCs infected with HTLV-1 in vitro, and PBMCs harvested from HTLV-1-inoculated rabbits. Our results provide a composite picture of HTLV-1 gene expression under multiple experimental systems and most notably provides the first kinetic mRNA expression analysis in a newly infected host. More importantly, we provide the first evidence linking \(hbz\) expression to proviral load and the survival of the virus infected cell in vivo.

Transient transfection of 293T cells with the HTLV-1 plasmid revealed \(tax/rex\) completely-spliced mRNA as early as four hrs post-transfection at approximately two-fold greater levels than unspliced \(gag/pol\)/genome mRNA. Subsequently (12-44 h), transcription of these two mRNAs increased dramatically then stabilized with the \(gag/pol\) mRNA expressed at the highest concentration. Doubly or completely spliced mRNA (encoding Tax and Rex) was expressed first followed by a significant increase in unspliced and incompletely-spliced transcripts. These results fit a model where, initially, Tax enhances viral transcription, but as Rex increases in concentration, it functions to inhibit splicing and facilitate export of the unspliced and singly-spliced mRNAs. Expression of \(env\) and the other accessory genes was detected at 12 hrs, increased slightly over time, and plateaued at levels significantly (1-4 logs) lower than \(tax/rex\) or \(gag/pol\). Thus, it is clear from our results that the incompletely-spliced and doubly-spliced transcripts are not generated at the same rate from full-length transcripts.

Kinetic analysis of HTLV-1 mRNA expression in newly infected human PBMCs
showed a specific gene expression pattern for HTLV-1 mRNA similar to the results from proviral plasmid transfected 293T cells where transcript levels were consistent with the cell growth and the immortalization process. All HTLV-1 mRNA levels increased between weeks one through five with gag/pol, tax/rex, and env expressed at the highest concentrations. However, during the typical cell growth crisis stage between weeks six through eight, all transcript levels declined significantly (mirroring cell growth), but slowly recovered to steady state and relatively high levels as the surviving newly immortalized cells expanded. The levels of pX transcripts (encoding Tax, Rex and accessory gene products) in newly immortalized human cells were consistent with previous studies of HTLV-1 gene expression in established transformed cell lines (tax/rex > p21rex ≥ p12, p27/p12, p13, p30) 298,305. However, our data provide the first kinetic expression analysis of the antisense hbz transcript that we observed to mirror the expression pattern and levels of the other accessory gene transcripts; gradual increase over time and stabilizing approximately two logs below tax/rex mRNA levels. Although functional over-expression studies revealed that p30 and HBZ can modulate viral gene expression by distinct mechanisms at the transcriptional level (p30 and HBZ) and the post transcriptional level (p30) 100,201-204, there was no obvious alteration in overall or specific gene expression that could be directly correlated with their mRNA expression pattern. One study showed that in the context of a proviral clone, the repressive effects of HBZ on Tax transcription were not
apparent following transient transfection \(^{100}\). It is possible that these proteins may not
achieve a threshold level or be expressed within the proper microenvironment to
function \textit{in vitro}, results consistent with both p30 and HBZ being dispensable in cell
culture but required for efficient infectivity and persistence in infected rabbits \(^{99,100,209}\).

Rabbits inoculated with HTLV-1 induce a significant antibody response to viral
antigens and become persistently infected. Proviral loads were variable among
individual rabbits, but in general increased over time before stabilizing or reaching a set
point. Our kinetic analysis of HTLV-1 mRNA expression in newly infected rabbits
indicated that \textit{tax/rex} mRNA was the most abundant mRNA detected and expression
inversely correlated with proviral loads. \textit{tax/rex} mRNA peaked very early and after
1-2 wks post-infection progressively decreased and stabilized at relatively low levels.
\textit{gag/pol} mRNA expression mirrored that of \textit{tax/rex} mRNA expression, but with an
average magnitude of approximately four-fold lower at its peak. DNA methylation of
retroviruses is one host defense mechanism for inactivating retrovirus gene expression
\(^{309}\) which may ultimately allow virus infected cells to escape from the host immune
system and establish a latent state. Consistent with our observed mRNA expression
pattern in infected rabbits over time, a previous study reported that the \textit{gag/pol} and \textit{env}
regions of the HTLV-1 proviruses in infected individuals at the time of seroconversion
are methylated and that methylation continues to progress into the 5’ LTR. \(^{310}\). Moreover,
DNA methylation of the HTLV-1 5’LTR has been shown to silence viral gene
transcription in leukemia cells\textsuperscript{92,93}. The finding that the 3’LTR is unmethylated in all ATL cases and carriers\textsuperscript{310} is consistent with the expression of \textit{hbz} in ATL cells and its constant and stable expression in HTLV-1 infected rabbits. Relative expression of \textit{tax/rex} mRNA was significantly lower \textit{in vivo} (approximately 1-to-2 logs) as compared to cell culture. This difference can be attributed to a lower ratio of infected-to-uninfected cells within PBMCs, but also likely reflects immune regulation and clearance of infected cells. Tax has the remarkable ability to promote the proliferation of infected cells. However, it is also a major target of CTL \textit{in vivo}\textsuperscript{311}. Interestingly, rabbits that display a higher \textit{tax/rex} mRNA level at earlier weeks tend to have a lower proviral load at later weeks, clearly suggesting negative pressure on \textit{tax/rex} mRNA-expressing cells.

With the exception of \textit{hbz} mRNA, all other HTLV-1 mRNAs in rabbit PBMCs including those encoding Env and the accessory proteins were below our limit of detection; this was consistent with the low percentage of infected cells \textit{in vivo} and the low level of expression of these specific mRNA in cultured or transfected cells. In contrast to \textit{tax/rex} and \textit{gag/pol} mRNA, \textit{hbz} mRNA expression appeared to start low and slowly increase and stabilize over time showing a direct correlation with proviral load, thus supporting the conclusion that \textit{hbz} mRNA expression remains relatively constant in the infected cell and increases with infected cell number. \textit{hbz} is expressed in most all ATL cells, whereas \textit{tax/rex} is low or rarely expressed\textsuperscript{229,233,303}. A snapshot of \textit{hbz} and
tax/rex mRNA expression levels in primary ATL cells revealed high \( hbz \) and low \( tax/rex \) mRNA expression levels \(^{312} \). Consistent with our results, these authors also showed that \( hbz \) mRNA expression level significantly correlated with infected cell number. Taken together, the data strongly implicate a key role for \( hbz \) expression in infected cell survival and disease. Indeed, suppression of \( hbz \) gene expression by siRNA inhibited the proliferation of HTLV-1 primary immortalized T-lymphocytes and transformed cell lines derived from ATL patients \(^{233,304} \). Moreover, the knockdown of \( hbz \) expression significantly decreased tumor formation and tissue infiltration in a mouse tumorigenicity transplant model \(^{304} \).

In summary, this study provides a quantitative analysis of HTLV-1 gene expression using various experimental systems. Our \textit{in vivo} data indicate that under immune pressure, there is an inverse correlation between \( tax/rex \) and \( hbz \) mRNA expression over time and provides the first evidence linking \( hbz \) expression to proviral load and cell survival. These results are consistent with the maintenance of expression of \( hbz \) in most ATL cells and support an important role for \( hbz \) in leukemogenesis.

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Table 3.1. Kinetic analysis of HTLV-1 transcript expression in transfected 293T cells

<table>
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<th>mRNA Transcript</th>
<th>0</th>
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<th>12</th>
<th>20</th>
<th>28</th>
<th>44</th>
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<td>1.93E+02</td>
<td>9.33E+04</td>
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<td>2.09E+05</td>
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<td>7.77E+04</td>
<td>1.26E+05</td>
<td>1.08E+05</td>
</tr>
<tr>
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<td>0.00E+00</td>
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<td>5.27E+03</td>
</tr>
<tr>
<td>p21rex</td>
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<td>0.00E+00</td>
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<td>1.08E+02</td>
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*293T cells were transfected with the HTLV-1 proviral clone ACHneo and RNA was isolated at indicated time points post-transfection. Numbers shown are the copy number average of two transfections and are normalized to one million copies of GAPDH. Detection limit cutoff is 25 copies.
Figure 3.1. Provirus genome of HTLV-1 and its unsliced, singly spliced, and doubly spliced mRNAs. At least eight positive sense transcripts and one negative sense transcript are expressed by HTLV-1. The genomic unsliced 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 major splice acceptors at positions 4641 (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 Hbz singly-spliced major anti-sense transcript initiates at multiple sites in the 3’LTR and utilizes a splice donor site at position 365 and a splice acceptor site at nt position 1765 (Hbz unsliced and minor spliced transcript not shown). 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 anti-sense transcript. Black lines designate exons and dotted lines designate introns. Major utilized splice donor (open triangles) and splice acceptor (closed triangles) sites are indicated above the unsliced mRNA.
Figure 3.2 Time course of HTLV-1 protein expression following transient transfection. 293T cells (8 x 10^5) were transfected with eight μg HTLV-1 ACHneo plasmid by lipofectamine in 100mm plates. Culture supernatants and cell lysates were harvested at 12, 20, 28, 44, and 60 hrs post-transfection. (A) Detection of HTLV-1 p24, Rex, and Tax protein expression in transfected cell lysates by Western blot analysis. β-actin levels were assessed as a loading control. (B) p19 Gag ELISA was used to quantify HTLV-1 viral particle production in culture supernatant. p19 was first detected in the supernatant at 12 hrs post-transfection. Error bars indicate standard deviation of two independent transfections performed in triplicate.
**Figure 3.3.** HTLV-1 T-lymphocyte immortalization assay. Freshly isolated human PBMCs (2 x 10^6) were cultured with 10^6 γ-irradiated 729HTLV-1 producer cells in 24-well plates. (A) Representative growth curve is presented to show cell viability as determined by trypan blue exclusion at weekly intervals. Numbers shown at each time point are the average of three random wells with error bars denoting standard deviation. (B) p19 Gag ELISA was used to quantify HTLV-1 virion production by detecting Gag protein in the culture supernatants. Numbers shown at each time point are the average of three random wells with error bars denoting standard deviation. First time point begins at three wks when there is no remaining signal in the supernatant from irradiated producer cells. (C) Real-time RT PCR was performed on cells from triplicate wells at weekly intervals (1-8) and at a final 14 wk time point to quantify the HTLV-1 transcript levels throughout the immortalization process. The data is presented graphically with error bars denoting standard deviation with the average numbers displayed below. Human GAPDH mRNA was used as the internal control and numbers presented are normalized to 1 x 10^6 copies of hGAPDH mRNA.
Figure 3.3
Figure 3.4. Assessment of HTLV-1 infection in rabbits. Twelve month-old New Zealand white rabbits were inoculated with $1 \times 10^7 \gamma$-irradiated 729HTLV-1 producer cells (nine total rabbits) or uninfected 729 cells as a control (two total rabbits). Following inoculation, 15 mls of blood were drawn from each rabbit at wks 0, 1, 2, 4, 6 and 8 for collection of sera and rabbit PBMCs. (A) Sera from inoculated rabbits were tested for reactivity to specific HTLV-1 proteins by Western blot. A representative rabbit from each group as indicated is shown with reactive viral proteins labeled on the right. (B) Genomic DNA was isolated from rabbit PBMCs and subjected to Taqman real-time PCR using HTLV-1 specific primers #19 and #20 and probe TMP-3; the standard curve was generated by serial 10-fold dilutions of plasmid DNA. The proviral load is plotted against weeks post-infection with the actual copy number per cell displayed below. The copy number per cell value for a sample was generated based on the estimation that one $\mu$g of PBMC DNA is equivalent to 134,600 cells.
Figure 3.5. Kinetics and profile of HTLV-1 mRNA expression in infected rabbits. RNA extracted from rabbit PBMCs (harvested from rabbits at wk 0, 1, 2, 4, 6 and 8 post-inoculation described in Fig. 4 legend) were subject to Real-Time RT PCR to quantitate *tax/rex* mRNA expression (A), *gag/pol* and full length genomic mRNA expression (B), and *Hbz* anti-sense mRNA expression (C). Numbers shown are values normalized to one million copies of rabbit GAPDH mRNA.
Figure 3.6. Relationship over time between HTLV-1 proviral load and tax/rex or Hbz mRNA levels in infected rabbits. We used log-transformation of both proviral load (data taken from Fig 4) and tax/rex or Hbz mRNA (data taken from Fig. 5) to stabilize the variance. Linear mixed modeling was employed to take account of correlated multiple observations in a time series from the same rabbit. (A) The data shows a negative correlation between log tax/rex mRNA expression and log proviral load in HTLV-1 infected rabbits (p = 0.0003). (B) The data shows a direct correlation between log Hbz mRNA and log proviral load in HTLV-1 infected rabbits (p = 0.0001). Each weekly time point is designated by a distinct symbol as indicated and each individual infected rabbit per weekly time point is denoted (1-9) inside the symbol.
Figure 3.6

A

\[ \log \text{te/ce mRNA copy number (normalized to 10^6 rGAPDH)} \]

\[ \log \text{proviral load per PBMC} \]

B

\[ \log H\alpha_2 \text{mRNA copy number (normalized to 10^6 rGAPDH)} \]

\[ \log \text{proviral load per PBMC} \]

- week 1
- week 2
- week 4
- week 6
- week 8

\[ p = 0.0003 \]

\[ p = 0.0001 \]
CHAPTER 4

HTLV-1 REGULATORY AND ACCESSORY GENE TRANSCRIPTS ARE REX ASSOCIATED BUT NOT DEPENDENT ON REX FOR EXPORT

4.1 Abstract

The HTLV-1 Rex protein is a post-transcriptional regulator that utilizes specific host machinery to actively export gag/pol unspliced and env incompletely-spliced viral mRNA from the nucleus to the cytoplasm. However, all HTLV-1 sense transcripts contain the Rex response element (RxRE) in their 3’ untranslated region. The contribution of Rex to the export and expression of completely spliced HTLV-1 mRNAs, particularly those encoding the accessory proteins, has been difficult to assess due to their low abundance in cells. The precise understanding of Rex function and regulation remains a critical goal in HTLV-1 research, which could yield new strategies for therapeutic intervention. Herein, we confirmed the effect of Rex on the export and expression level of unspliced gag/pol mRNA, singly-spliced env mRNA, and doubly-spliced tax/rex mRNA. Furthermore, we showed that Rex slightly decreased the overall expression level of singly and doubly spliced mRNAs encoding Tax, HBZ,
and the accessory proteins, but had no significant effect on the export of these mRNAs. Rex RNA-pull down (PD) experiments demonstrated that Rex associated not only with the gag/pol and env mRNA, but also associated with all other singly and doubly spliced viral mRNAs encoding regulatory and accessory proteins. In contrast, the hbz antisense mRNA, which does not contain the RxRE, was not bound by Rex. Our results are the first to quantitatively measure the effects of Rex on the expression level and export of HTLV-1 accessory protein mRNAs and to show that Rex associates with all the positive sense RxRE containing HTLV-1 mRNA transcripts.

4.2 Introduction

A critical step in the life cycle of complex retroviruses, including HTLV-1 and HTLV-2, is the ability of the viruses to adopt a mechanism by which the unspliced and the partially spliced mRNAs are exported out of the nucleus; the default pathway is to completely splice out introns prior to export. The Rex protein of HTLV-1, first defined in 1988, was found to be a post-transcriptional regulator that utilizes specific host machinery to actively export unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm. Translation of these transcripts into structural and enzymatic proteins is required for virion assembly and the formation of infectious particles. As a result of the positive regulation of the gag/pol and env mRNAs, Rex indirectly inhibits the expression of doubly-spliced mRNA which encodes itself and the
regulatory protein Tax. Since Rex has been shown to be essential for HTLV-1 gene expression, the full understanding of its function and regulation remains a critical objective in HTLV research that could yield new strategies for therapeutic intervention.

HTLV-1 Rex is a 27-kD nuclear phosphoprotein that localizes to nucleus/nucleolus in transiently transfected cells as well as HTLV-1 infected and transformed cell lines. Mutational analyses of Rex-1 have defined several domains critical for their functional properties. These include the arginine-rich N-terminal sequences that serve both as an RNA binding domain and as a nuclear localization signal, the central leucine-rich activation domain encompassing the nuclear export signal (NES), and the multimerization domain composed of two regions flanking the NES. The functional activity of Rex requires two specific cis-acting sequences in the viral LTR, the Rex Response Element (RxRE) and the cis-acting repressive sequence (CRS).

In HTLV-1, the RxRE, comprised of 205 nucleotides, is located in the U3/R region of the 3’ long terminal repeat (LTR) and at least partly in the 5’-LTR. The CRS is located independently in the 5’LTR and 3’LTR overlapping the 3’ RxRE. Substitution and deletion studies have demonstrated that the proper folding of the RxRE is required to provide a docking site for Rex, and hence, is essential for its in vivo function. The RxRE must be maintained in the RNA molecule in the sense orientation to function. Biochemical characterization of both HTLV-1 and HTLV-2 Rex activity using in vitro synthesized RxRE and purified Rex protein indicated that
Rex directly binds RxRE sequences in both gel mobility shift and immunoprecipitation 

Previous studies have identified cellular proteins including CRM1, eIF-5A and hRIP/RAB that interact with Rex via the activation domain (AD)/NES. The most functionally significant interaction is with CRM1, which bridges Rex to several cellular proteins at the nuclear pore complex (NPC). The proposed working model for Rex function is that the CRS retains the unspliced mRNA in the nucleus and prevents it from degradation until Rex binds the RxRE. The Rex/RxRE interaction overcomes the inhibitory effect of the CRS and facilitates CRM-1-dependent mRNA export to the cytoplasm. Export requires the multimerization of multiple molecules of Rex on the RxRE followed by formation of an RNA/Rex/CRM-1/Ran-GTP complex which facilitates interaction with the NPC and exit from the nucleus. The complex dissociates upon the hydrolysis of Ran-GTP to Ran-GDP and the cargo mRNA is released. Subsequently, Rex shuttles back to the nucleus to start another cycle, or possibly, like the HIV-1 Rev protein, continues its interaction with the mRNA in the cytoplasm to promote polysomal association and translation of the mRNA.

Data suggests that both Rex and HIV-1 Rev may actively inhibit the splicing machinery prior to facilitating mRNA export. Rex has been shown to bind pre-mRNA splicing factors such as SF2/ASF and inhibit mRNA splicing in vitro. In fact, in the context of the entire provirus, the increased levels of unspliced and incompletely spliced mRNA produced in the presence of Rex are accompanied by a
decrease in the steady state level of the doubly spliced tax/rex message\textsuperscript{183,192}. Unlike HIV-1, all HTLV transcripts, except for the anti-sense transcript which encodes HBZ, contain RxRE. To date, the effect that Rex has on the export and expression of completely spliced HTLV-1 accessory gene mRNA has been poorly investigated due to the low abundance of these mRNAs. Initially considered to be dispensable, HTLV-1 accessory proteins have been recently shown to play an important role in viral replication and persistence in a rabbit model of infection\textsuperscript{100,102,103,215}. Studies to reveal the regulation of their expression become essential to understanding viral pathogenesis. In this study, we investigated the role that Rex plays in the regulation of the expression of all HTLV-1 mRNA transcripts. In addition to confirming that loss of Rex results in a decrease of unspliced gag/pol and incompletely-spliced env mRNAs with a concomitant 2-fold increase in tax/rex mRNA, we show that in the absence of Rex the accessory gene mRNAs also increased 2 to 4 fold. Nuclear/cytoplasmic fractionation was performed to determine the effect of Rex on export of accessory protein mRNAs. Consistent with previously reports, Rex stimulated the export of unspliced gag/pol and incompletely spliced env mRNA, but had no significant effect on the export of doubly spliced tax/rex mRNAs\textsuperscript{192}. However, we show that the completely spliced viral mRNAs encoding accessory proteins, including the antisense mRNA encoding HBZ are not dependent on Rex for export. Rex RNA-PD experiments demonstrated that Rex associated not only with the gag/pol and env mRNA, but also associated with all other
singly and doubly spliced viral mRNAs encoding regulatory and accessory proteins. In contrast, the \textit{hbz} antisense mRNA which does not contain the RxRE, was not bound by Rex. Our results are the first to quantitatively measure the effects of Rex on the expression level and export of HTLV-1 accessory protein mRNAs and to show that Rex associates with all the positive sense RxRE containing HTLV-1 mRNA transcripts.

4.3 Materials and Methods

Plasmids and Cell lines

The ACH HTLV-1 proviral plasmid (wtHTLV-1), Rex-defective proviral plasmid (HTLV-1Rex-), and HTLV-1 Rex cDNA expression plasmid SE356 been described previously \cite{307}. BC12 expression vector control and CMV-Luc reporter plasmid have been described previously \cite{324}. The S-tagged expression vector MK935StagRex-1 was constructed by inserting the HTLV-1 Rex open reading frame into the Sma I and BamHI sites of pTriEx4-Neo (Novagen, Madison, WI) in-frame with the amino-terminal S tag and His tag. 293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, penicillin (100U/mL), and streptomycin (100μg/mL).
Transient transfection, western blot and p19 ELISA

8×10^5 293T cells were plated in 100mm culture dishes, grown for 24 h, and then transfected with 8 μg of wtHTLV-1, HTLV-1Rex- proviral plasmid or the Rex expression plasmid SE356 using Lipofectamine (Invitrogen, Carlsbad, CA). 24 h post transfection, cell lysates were subjected to SDS-PAGE and western blot analysis as previously described. Rex was detected by HTLV-1 rabbit anti-Rex-1 polyclonal antisera (1:1000) and horseradish peroxidase-linked anti-IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin detected with anti-β-actin rabbit monoclonal antibody and goat anti rabbit-IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. Proteins were visualized using the electrochemiluminescence (ECL) Western blot analysis system (Santa Cruz Biotechnology, Santa Cruz, CA).

Culture supernatants were harvested at endpoint 48 h or time points 4h, 16h, 28h, 44h and 52 hour post transfection and subjected to p19 ELISA (Zeptometrix Corporation, Buffalo, NY) as a quantitative measure of HTLV-1 virion production.

RNA extraction and real-time RT-PCR

293T cells transfected with wtHTLV-1 or HTLV-1Rex- plasmids were harvested 48 h post transfection and subjected to RNA extraction using the RNeasy kit (Qiagen, Valencia, CA) with on column DNase treatment (Qiagen, Valencia, CA). To remove all
possible plasmid DNA contamination carried over from transfection extracted total RNA was further treated with DNase (Roche, Mannheim, Germany) for 30min at 37°C (repeated three times). RNA clean step from RNeasy kit (Qiagen, Valencia, CA) followed DNase treatment. Equivalent amounts of purified RNA was subjected to reverse transcriptase PCR (RT-PCR) using the SuperScript™ First-Strand Synthesis kit (Invitrogen Corp., Carlsbad, CA) with random hexamers. Reactions were performed in the presence and absence of RT as a further control for DNA contamination. RNA extraction and first strand cDNA synthesis from cytoplasmic and nuclear fractions and pre-IP and post-IP cell lysates followed the same procedure as described above.

Taqman real-time RT-PCR was performed to quantify levels of all HTLV-1 mRNA using splice junction specific primers and probes for each mRNA as described previously 305. Quantitation results were normalized to transfection efficiency and the cellular gene hGAPDH. The quantities of a specific viral mRNA species in HTLV-1Rex- plasmid transfected cells and the quantities in wtHTLV-1 plasmid transfected cells were compared to assess the impact of Rex on the viral mRNA expression level; the quantities of this specific viral mRNA species in the cytoplasm and the quantities in whole cell (from both the cytoplasm and the nuclear fraction) were compared to indicate the viral mRNA export. All experiments were repeated three times with triplicate transfections each time.
Nuclear and cytoplasm fractionation

293T cells transfected with HTLV-1 or HTLV-1Rex- proviral plasmids were harvested at 48 h post transfection and washed in 1mL of PBS. The cell pellet was re-suspended in 450 μl hypotonic buffer (10 mM HEPES, pH 7.9 @ 4°C, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) with 50μl 5% NP40 solution, vortexed at maximum speed for 30 sec and placed on ice for 10 min. The suspension was subjected to 3000 rpm spin for 3 min. The supernatant was transferred to a new eppendorff tube and re-spun to remove any of the residual nuclear fractions. The supernatant after a second spin was collected and stored as the cytoplasmic fraction. The nuclear pellet was washed twice with 500μl of hypotonic buffer. To assess the integrity of the nuclear/cytoplasmic fractionation, 60μg of total protein from the cytoplasmic fraction, nuclear fraction or the total cell lysates were subjected to SDS-PAGE followed by western blot. Western blot was performed as described using mouse anti-histone monoclonal antibody (1:1000, Abcam, Cambridge, MA) (nuclear), mouse anti-α-tubulin monoclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) (cytoplasmic), and rabbit anti-β-actin polyclonal antibody (1:1000, Abcam, Cambridge, MA) (nuclear and cytoplasmic).
Rex RNA-pull down (PD)

293T cells were transiently co-transfected with 5 μg of wtHTLV-1 proviral plasmid and 3μg of BC12 plasmid or 5μg of HTLV-1Rex- proviral plasmid and 3μg of S-tag-Rex expression plasmid. 293T cells transfected with 8μg of BC12 were used as control. Transfections were performed in triplicate to normalize for transfection efficiency. 48 h post-transfection, cells transfected with each plasmid were harvested, combined and then redivided into three fractions from which one fraction was used as pre-PD sample for RNA extraction and the other two fractions were subjected to Rex RNA-PD. Briefly, cells were washed in PBS and pelleted. The cells were then resuspended and lysed in 1X PLB (Promega, Madison, WI) containing protease inhibitors cocktail tablet (Roche, Mannheim, Germany) followed by incubation on ice for 30 min. Lysate was cleared by maximum speed centrifugation for 10 min. 500μg of total cellular lysate was incubated with 50μl of S-protein conjugated beads (Novagen, Madison, WI) overnight at 4°C with gentle agitation. Beads were washed 3 times in a low salt modified RIPA buffer (0.05M Tris PH8.0 HCl, 0.1% SDS, 1.0% Triton-X, 0.15M NaCl). S-tag Rex captured on beads was confirmed by western blot analysis with anti-Rex antibody. Rex bound RNA was extracted from beads using RNA lysis buffer as detailed above in the RNA Extraction and Real-time PCR section.

The primer pairs to detect all HTLV-1 viral transcripts were described previously and include #19 and #20 for the full length gag/pol mRNA, ENV4641-S and Env-AS
for env mRNA, ENV4641-S and TR exon 2/3 for tax/rex mRNA, P21REX-S and TR1-AS for p21rex mRNA, P13-S and P13-AS for p13 mRNA, P12-6383-S and P12-AS for p12 mRNA, P27S and P12-AS for p27/p12 mRNA, X2P30 and P30-AS for p30 mRNA, HBZMAP1 and HBZMAP2 for hbz mRNA. It is important to note that primer TR exon 2/3 (5’-CCTGGGAAGTGGGCCATG-3’) was designed to specifically detect tax/rex mRNAs transcribed from proviral plasmid and not from the cDNA S-tag Rex plasmid. PCR cycle number was adjusted to maximize detection (40-50 cycles) and was typically based on mRNA concentrations in cells.

4.4 Results

4.4.1 Deletion of Rex impairs viral replication

Our studies made use of two previously characterized proviral plasmid clones; wtHTLV-1 (ACH) and HTLV-1Rex-274. We first assessed Rex’s expression and function in transfected 293T cells transfected with these proviral clones. As expected, western blot analysis demonstrated that Rex was expressed in wtHTLV-1 transfected 293T cells, whereas it was absent in HTLV-1Rex- transfected cells (Fig.4.1A). Next, the effect that Rex had on p19 Gag production in transfected cells overtime was assessed by ELISA. Cells transfected with the wtHTLV-1 clone produced p19 Gag starting at 16 h post transfection which continued to increase to approximately 600pg/ml at 52 h (Fig.4.1B). In contrast, p19 Gag production in cells transfected with
the HTLV-1Rex- clone were below the limit of detection (<25 pg/ml) throughout the time course. However, this does not rule out low level of virion production or the capacity to transmit the virus by cell to cell contact as has been shown previously 274.

**4.4.2 Rex affects the steady-state levels of all HTLV-1 mRNAs**

To assess the effect of Rex on the expression level of individual viral mRNAs, wtHTLV-1 and HTLV-1Rex- plasmid were transfected into 293T cells and 48 h post transfection each viral mRNA species was quantified (Table 4.1). The full length mRNA encoding *gag/pol* and the incompletely spliced mRNA encoding *env* were about 50% and 70% in HTLV-1Rex- transfected cells as compared to wtHTLV-1 transfected cells, whereas the doubly-spliced mRNA encoding Tax and Rex regulatory proteins was approximately 2 fold greater. These results are consistent with one previous report and validates our quantitative methodology 192. We next determined the Rex effect on HTLV-1 accessory gene expression. HTLV-1 mRNA species encoding accessory proteins and antisense mRNA encoding HBZ protein were all shown to increase in the absence of Rex. P12, p27, p13, p30 and *hbz* mRNA increased approximately 2 fold, while p21rex increased 4 fold (Table 4.1). If normalized to *tax/rex* mRNA level, there was not significant difference in the relative ratio of these accessory protein mRNAs in HTLV-1Rex- than in wtHTLV-1 transfected cells, within tow fold difference. Thus, in HTLV-1Rex- transfected cells, the completely spliced viral mRNA level increase
slightly at the expense of the full length gag/pol mRNA and the incompletely spliced env mRNA. However, the total viral mRNA level does not change dramatically, with a slight increase about 2 fold in HTLV-1Rex- than in wtHTLV-1 transfected cells.

4.4.3 HTLV-1 completely spliced mRNA and anti-sense mRNA encoding HBZ is not dependent on Rex for export

Although Rex has been reported to be required for export of the full length and incompletely spliced mRNA, no studies have been done to evaluate whether Rex functions on the export of the completely spliced mRNA. To determine whether Rex affects the subcellular distribution of HTLV-1 mRNAs, we quantitated nuclear and cytoplasmic viral mRNAs in wtHTLV-1 and HTLV-1Rex- transfected 293T cells. The validity of our fractionation protocol was confirmed by western blot against α-tublin, histoneH1 and β-actin (Fig. 4.2A). As expected α-tublin was detected only in cytoplasmic and total cell fractions histone H1 was detected only in nuclear and total cell fractions, and β-actin, used as a loading control, was found evenly distributed in all fractions. RNAs prepared from these fractions were quantified by our previously developed real-time RT-PCR assay. Data for each specific viral mRNA is presented in Figure 4.2B as a ratio of cytoplasmic mRNA over total mRNA (nuclear +cytoplasmic). The effect of Rex on export is determined by comparing HTLV-1 transfected cells to HTLV-1Rex- transfected cells. Consistent with previous reports, we
showed that the export of full length (gag/pol) mRNA and incompletely spliced env mRNA were significantly reduced in the absence of Rex protein (p<0.05) whereas tax/rex mRNA export was not affected. Furthermore, our results indicate no significant effect of Rex on the export of completely spliced viral mRNA encoding accessory proteins p21Rex, p12, p27, p13, p30 and the spliced antisense transcript encoding HBZ (Fig. 4.2B).

### 4.4.4 All HTLV-1 sense transcripts are associated with Rex.

The Rex protein redistributes HTLV-1 accessory gene expression by prevent splicing of unspliced viral transcripts, but does not affect the export of these accessory gene transcripts. However, the fact is that all HTLV-1 sense transcripts contain RxRE elements in their 3’ ends. Why then is only the unspliced gag/pol mRNA and incompletely spliced env mRNA dependent on Rex for export? We next addressed whether the completely spliced viral mRNAs associate with Rex. To study the association of Rex with HTLV-1 RxRE containing transcripts, 293T cells were cotransfected with HTLV-1Rex- and an S-tag-Rex plasmid that expresses functional Rex. Transfected cells were harvested 48 h post transfection and subjected to S-tag protein bound beads to pull down the Rex-mRNA protein complexes. 293T cells transfected with empty vector BC12, or HTLV-1Rex- plasmid only were used as control. The Rex protein is pulled down from HTLV-1Rex- and Stag-Rex cotransfected 293T
cells, but not from BC12 or HTLV-1Rex-transfected cells (Fig. 4.3A). Total RNA was extracted from pre-PD and post-PD cell lysates followed by RT-PCR with specific primers to identify viral mRNA that was associated with Rex (Fig. 4.3B). Possible DNA contamination was controlled for by performing PCR in the absence of a reverse transcriptase (RT) step. All viral transcripts including gag/pol, env, tax/rex, p21rex, p12, p27, p13, p30 and hbz mRNA can be detected prior to IP from HTLV-1Rex-transfected or HTLV-1Rex-/Stag-Rex cotransfected cells, but not in BC12 transfected cells, confirming expression of these viral transcripts from provirus plasmid transfection. Following PD for S-tag-Rex gag/pol mRNA and env mRNA were amplified from HTLV-1Rex-/Stag-Rex co-transfected 293 T cells, but not HTLV-1Rex- or BC12 transfected 293T cells. These results are consistent with the known Rex function and indicate that gag/pol and env mRNA associate with Rex protein in vivo. A specific amplified product was not detected in PCR performed in the absence of RT-dependent cDNA synthesis, confirming no DNA contamination. Moreover, these results are consistent with the Rex function on export of these mRNA species. To specifically detect tax/rex mRNA transcribed from proviral plasmid and not tax/rex mRNA transcribed from an exon 2/3 cDNA Stag-Rex expression plasmid, primers were designed with sense primer across the exon1/2 splice junction and anti-sense primer across the exon2/3 splice junction. Our results indicate that tax/rex mRNA transcribed from provirus is also bound by Rex protein. Other transcripts that have the RxRE
element including p21rex, p13, p30, p12 and p27 were also present in the Rex/RNA complex. Interestingly the *hbz* major spliced transcript could not be detected from RNA isolated from Stag-Rex pull down cell lysates. This result demonstrated that all HTLV-1 transcripts containing the RxRE element are associated with the Rex protein *in vivo*, while *hbz* antisense transcript is not bound by Rex.

### 4.5 Discussion

HTLV-1 gene expression is regulated by the viral regulatory proteins at both the transcriptional and post-transcriptional levels. The post-transcriptional regulator Rex is produced from a doubly spliced mRNA that also expresses Tax. Export of unspliced *gag/pol* mRNA and the singly-spliced *env* mRNA are dependent on Rex, which recognizes and binds to RxRE sequences found in the 3’ untranslated region of their mRNAs. The effect of Rex on completely-spliced viral accessory mRNAs has been less studied due to the difficulty of evaluating low abundance mRNA species. Unlike HIV-1, of which the Rev response element (RRE) is located only in unspliced and incompletely spliced viral transcripts, the RxRE of HTLV is found in every sense transcript including the all the completely spliced mRNAs. Although the Rex and RxRE association has been studied extensively *in vitro* utilizing purified Rex protein and synthesized RxRE sequence, the association of Rex with RxRE in cells has not been definitively shown. In this study, we used transient transfection of HTLV-1 proviral
clones, in conjunction with our well developed real-time RT-PCR assay and Rex RNA-IP, to examine Rex function and binding in its natural context. We demonstrated that Rex slightly increases the abundance of the completely-spliced HTLV-1 accessory protein mRNAs, but does not affect their export. The RNA-PD results showed that although the completely-spliced HTLV-1 mRNAs are not dependent on Rex for export, all transcripts containing the RxRE were Rex associated. These results suggest a possible post-transcriptional function of Rex on the expression of the completely spliced mRNAs need to be further investigated.

In HTLV-1, the requirement to generate multiple proteins from the single primary viral transcript necessitated that HTLV-1 evolve mechanisms to regulate the conversion of this transcript into multiple alternatively spliced mRNA. This processing must happen in a balanced fashion to generate the necessary levels of both structural and regulatory proteins required for appropriate regulation of HTLV-1 expression and assembly of replication competent viral particles. When we quantifying each viral mRNA species in wtHTLV-1 or HTLV-1Rex- transfected 293T cells, the result showed that the absence of Rex increased the abundance of completely spliced viral mRNAs in the context of provirus transcription while decrease the full length and incompletely spliced mRNAs, indicating the absence of Rex is able to disturb the balanced gene expression and cause re-distribution of viral genes. Previously research investigated into the possible feedback control of Rex on pX mRNA splicing indicated that Rex does
not regulate alternative splicing of regulatory and accessory gene\textsuperscript{298}. Consistently, our result showed that there was not significant difference in the relative ratio of these accessory protein mRNAs in HTLV-1Rex- than in wtHTLV-1 transfected cells. Thus, we believe this re-distribution is caused by increasing level of completely spliced genes at the expense of unspliced viral genes. The total viral mRNA level does not change dramatically, with a slight increase about 2 fold in HTLV-1Rex- than in wtHTLV-1 transfected cells. The ultimate effect of Rex on total viral mRNA expression would be determined by RNA degradation rate and Tax trans-activation. Actually, it was discovered that there were lower levels of intra-nuclear degradation of the intron-containing retroviral transcripts in fibroblasts than those in T-cells\textsuperscript{325}. Previous research showed that in transfected fibroblast, the total amount of viral RNA was not affected by Rex production, while in human cord blood T-cells, the total amount of viral transcripts decreased slightly in the absence of Rex\textsuperscript{177}. Another study showed that in transfected JM4 T-cells, the total amount of HTLV-2 transcripts decreased slightly, approximately 4 fold, due to degradation of viral mRNA in the absence of Rex. Thus, Rex could either increase or decrease the total viral mRNA level depending on the balance of the cell type specific degradation rate of intro-containing retroviral transcripts, Rex stabilization or export of unspliced viral transcripts, and the Tax trans-activation of total viral gene expression.

We determined the function of Rex on export of viral mRNA by real-time RT-PCR
quantitation of nuclear and cytoplasm viral mRNAs. We showed that Rex facilitates the nuclear-cytoplasmic transport of unspliced and incompletely spliced viral mRNA, but has no significant effects on the export of completely spliced viral mRNAs including the antisense \( \text{hbz} \) mRNA. Our Rex/RNA-PD results showed that all HTLV-1 sense transcripts that contain the RxRE were Rex associated. The association of \( \text{gag/pol} \) and \( \text{env} \) mRNAs with the Rex protein is consistent with their dependence on Rex for export. However, although not dependent on Rex for export, the completely spliced viral mRNAs were also found to be Rex associated. Rex has been shown to associate with \( \text{tax/rex} \) mRNA\(^{326}\). Accessory protein p30 retains \( \text{tax/rex} \) mRNA in the nucleus, but does not affect Rex-mediated nuclear export of unspliced RNA. In contrast, Rex was able to counteract p30 mediated suppression of viral expression and restore cytoplasmic \( \text{tax/rex} \) mRNA and Tax protein expression\(^{326}\). Also, Rex has been shown to be dispensable for HTLV-1 immortalization of PBMCs \textit{in vitro} but required for efficient virus production and infection in the rabbit model\(^{274}\). Taken together, the postulation of Rex selective modulation of mRNA export would be: Rex-bound transcripts are not Rex dependent unless their export pathway is harmed by some other features. In unspliced mRNAs, the intron sequence and CRS retain those mRNA from export until Rex binds and utilizes CRM-1 pathway\(^{182,327,328}\). In doubly spliced mRNA encoding Tax and Rex, p30 suppresses viral expression by retaining this mRNA in the nucleus, but Rex was able to counteract p30 mediated suppression of viral
expression and restore cytoplasmic tax/rex mRNA and Tax protein expression \(^{326}\). In infected rabbits, HTLV-1 gene expression is down regulated by immune system and the microcellular environment \textit{in vivo} may require Rex to modulate viral gene expression for virus survival and persistence.

We also found that in the absence of functional Rex, the viral p19 Gag expression level in the supernatant was significantly reduced. However, low but detectable levels of p19 Gag were accumulated over time in the HTLV-1Rex- transfected cells. A previous study indicated that the Gag production is modulated approximately 116-fold by Rex \(^{274}\). The level of gag/pol and env cytoplasmic RNAs increased 7- to 9- fold in the presence of Rex \(^{192}\). Consistent with this result we also showed that the modulation at the protein level is much higher than at mRNA level. In HIV-1, in addition to the export of viral mRNA, Rev is also believed to enhance the translation of viral RNA. It has been shown to promote the loading of gag/pol and env mRNA on to polysomes \(^{194}\). Rev elevates the amount of gag mRNA in the cytoplasm by only 8-16 fold while the amount of Gag protein increases by over 800 fold \(^{194}\). In addition to increasing the cytoplasmic level of full length HIV-1 mRNA and dramatically increasing Gag protein expression, Rev has little or no effect on cytoplasmic accumulation of singly spliced HIV-1 mRNAs, vif, vpr and env/vpu. However, expression of those proteins was greatly reduced in the absence of Rev. Analysis of the cytoplasmic RNA revealed that in the absence of Rev or RRE, the cytoplasmic vif, vpr
and env/vpu mRNAs were not associated with polysomes. Viral RNA must undergo some remodeling following export to allow it to continue on the path of translation or packaging into assembling virions. Here, we postulate that HTLV-1 Rex functions in a similar manner to facilitate the translation of HTLV-1 mRNAs. In our result, every HTLV-1 sense transcripts containing RxRE was found to be Rex associated although not all of them are dependent on Rex for export. It is possible that those viral mRNAs use Rex to recruit certain cellular proteins to help with remodeling or polysome loading, thus increase the translation efficiency. Further studies will be required to fully understand the possible post-transcriptional functions of Rex on the expression of the completely spliced mRNAs.

In summary, the complex control of HTLV-1 mRNA expression would allow precise regulation of distinct viral mRNAs and proteins in particular stages of its life cycle and/or cellular environment. Our work provides valuable data in investigating the complexity in the Rex regulation of HTLV-1 gene expression. The Rex association with viral transcripts provides the possibility of post-transcriptional modifications by Rex other than export. Further studies may provide insight as to what cellular pathway are been used by Rex to regulate specific gene expression at viral infection, latency and cellular transformation. Targeting selected Rex regulatory functions at right stages of viral life cycle would contribute to therapeutics of ATL.
Table 4.1 Quantification of HTLV-1 mRNA level change in wtHTLV-1 or HTLV-1Rex-transfected 293T cells.

<table>
<thead>
<tr>
<th></th>
<th>gag/pol</th>
<th>env</th>
<th>tax/rex</th>
<th>Hbz</th>
<th>p21rex</th>
<th>p12</th>
<th>p13</th>
<th>p30</th>
<th>p27</th>
<th>total</th>
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<tbody>
<tr>
<td>HTLV-1Rex-</td>
<td>0.49</td>
<td>0.70</td>
<td>2.08</td>
<td>1.54</td>
<td>4.43</td>
<td>1.25</td>
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<td>1.60</td>
<td>2.04</td>
<td>2.16</td>
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<td>HTLV-1</td>
<td></td>
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pX mRNA relative fold change (normalized to tax/rex)  

mRNA was isolated from transfected 293T cells 48 hours post transfection and subjected to Taqman real-time RT-PCR. After normalizing to transfection efficiency, each mRNA species was compared between HTLV-1Rex- and wtHTLV-1 transfection (second row). The fold change of pX mRNA was normalized to tax/rex mRNA (third row).
**Figure 4.1** Rex and p19 Gag protein expression in transiently transfected 293T cells. (A) 293T cells were transfected with wtHTLV-1, HTLV-1Rex-, SE356 (tax/rex) or BC12 control plasmids. 48 h post transfection, Rex proteins were detected by western blot analysis using a rabbit anti Rex-1 polyclonal antibody (B) p19 Gag expression kinetics following transient transfection of 293T cells. Cells transfected with wtHTLV-1 resulted in increasing levels p19 Gag antigen over time. Limited viral Gag antigen was detected with HTLV-1Rex-, but accumulated to a detectable level (over background) by 52 h post transfection. Numbers shown are the average of three independent transfections.
Figure 4.2. Nuclear/cytoplasmic fractionation and assessment of Rex function on viral mRNA export. (A) 293T cells were transfected with 8μg of wtHTLV-1 or HTLV-1Rex- proviral clones, and 0.5μg of CMV-Luc to normalize for transfection efficiency. 48 h post transfection, 1 million cells were subjected to luciferase assay. Nuclear and cytoplasmic fractionation was performed on the remainder cells and the integrity of the fractionation was validated by western blot against α-tublin (cytoplasmic), histone H1 (nuclear) and β-actin (throughout the cell). 60 μg of protein according to Bradford assay from each fraction was loaded per lane. (B) RNA was extracted from nuclear and cytoplasmic fractions and quantified by real-time RT-PCR. Bars represent the percentage of each viral mRNA export in wtHTLV-1 transfected cells (white bar) and HTLV-1Rex- transfected cells (grey bar). * Statistically significant reduction of viral mRNA export in the absence of Rex. Statistical significance was determined by analysis of variance (ANOVA) test. Bars represent the average value of 4 independent experiments, each with duplicate transfections. Error bars denote standard deviations.
Figure 4.2
Figure 4.3. The Rex association with HTLV-1 transcripts *in vivo*. (A) 293T cells were co-transfected with HTLV-1Rex- and Stag-Rex plasmid. Transfections with HTLV-1Rex- only or BC12 plasmid were used as controls. 48 h post transfection, cells were harvested for immuno-precipitation with anti-Stag antibody as described in Materials and Methods. Pre-IP and Post-IP cell lysates were run on SDS-PAGE followed by western blot against the Rex protein. (B) Total RNA was extracted from pre-IP and post-IP cell lysates followed by PCR in the presence (+) or absence (-) of RT with specific primers to identify viral mRNAs that are associated with Rex. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.
5.1. Introduction

HTLV-1 and HTLV-2 are pathogenic human retroviruses that infect 10-25 million people worldwide. They both have the capacity to transform primary human T cells in cell culture and infected individuals. In vitro co-culture assays have identified Tax as the major protein determinant essential to the HTLV mediated T cell transformation process, whereas the accessory proteins p12, p13, and p30 are dispensable. However, the accessory proteins play an important role in viral infectivity and persistence in vivo. How each specific viral gene product contributes to the process of infection, cellular transformation, and pathogenesis is of great interest. We posit that having a concise viral gene expression profile will provide important insight into the function of specific viral genes and their role in the biology and pathogenesis of HTLV-1. In this dissertation, we sought to broaden our knowledge of HTLV gene expression kinetics in cell culture and in the HTLV-1 infected animal model by developing a real-time
RT-PCR methodology for all HTLV-1 genes. We also focused our attention on understanding the role of Rex in the regulation of HTLV-1 gene expression, with specific emphasis on whether Rex regulates the completely spliced viral mRNA encoded accessory protein. Chapter 1 focused on reviewing HTLV gene expression regulation by both viral and cellular factors. Chapter 2 detailed the development and optimization of a real-time RT-PCR approach to quantify all HTLV-1 and HTLV-2 mRNA species accurately and reproducibly. Chapter 3 determined HTLV-1 gene expression kinetics under various experimental systems in cell culture and in inoculated rabbits. Chapter 4 studied the effects of Rex on the expression level and export of completely spliced HTLV-1 mRNA. The studies in this dissertation combined with future perspectives have been and will be instrumental to understand the virus gene expression regulation associated with HTLV infection, proliferation/survival of infected cells, and ultimately pathogenesis.

5.2 Assay Development

Both HTLV-1 and HTLV-2 produce a set of closely related mRNAs by alternative splicing. We compiled and generated a series of primer pairs and probes to quantify both HTLV-1 and HTLV-2 mRNA species using real-time RT-PCR. The developed assay offers a wide dynamic range as seen by our ability to quantify specific cDNA samples accurately at concentrations from 25 copies to $2.5 \times 10^7$ copies per reaction.
HTLV-1 and HTLV-2 mRNAs in established transformed T-cell lines, stable provirus transfected producer cell lines, and newly immortalized primary T-lymphocyte cell lines were quantified using our newly developed assay. These cell lines expressed variable levels of the different viral mRNA transcripts but in general, a similar pattern emerged for HTLV-1 and HTLV-2 gene expression, respectively. Among all the mRNA species of HTLV-1, the most abundant was consistently the unspliced \textit{gag/pol} mRNA. The second in abundance was the \textit{tax/rex} mRNA followed by the \textit{env} mRNA. High levels of these mRNAs would be expected in actively replicating cells in culture that are devoid of any immune surveillance. Efficient production of the structural and enzymatic proteins Gag, Pol, and Env for the assembly of new virions requires that the regulatory gene Tax and Rex are expressed at high levels. In comparison, the expression levels of all accessory protein mRNAs were 1 to 4 logs lower than \textit{tax/rex} mRNA. The total viral mRNA expression was about 2-7 fold lower for HTLV-2 than HTLV-1, which is consistent with studies demonstrating that HTLV-1 has a stronger intrinsic promoter than HTLV-2 and Tax-1 displays a higher transactivation activity than Tax-2. The relative level for HTLV-2 \textit{gag/pol}, \textit{tax/rex} and \textit{p28,p22/p20rex-1} mRNAs were similar, whereas \textit{env} mRNA was approximately 10-fold less and \textit{p28,p22/p20rex-2} mRNA was approximately 100-fold less than \textit{tax/rex} mRNA. The mRNA level for \textit{p10/p11} protein was extremely low. A specific gene expression pattern of HTLV-1 and HTLV-2 suggests that viral genes are regulated, but raises the question of whether regulation is at
a certain stage or throughout the process of infection and transformation. Our analysis also provided quantitative information on some splice donor and acceptor site utilization, particularly for HTLV-1 \textit{env}, p12 and \textit{hbz} transcripts. In conclusion, we developed an accurate and reproducible methodology to detect specifically and compare HTLV-1 and HTLV-2 mRNA gene expression. This technique will be particularly valuable for measuring the kinetic gene expression profile following infection of primary human T-lymphocytes in culture as well as in inoculated rabbits. Furthermore it can be used to dissect the phenotypes of specific viral gene mutations in culture and in the infected host, for a better understanding of the function of a specific viral gene in the natural infection process. This technique could also be used to characterize viral gene expression profile in HTLV-1 asymptomatic carriers, HAM/TSP patients and ATL patients, to determine the relationship among viral gene expression, proviral load and disease outcome.

5.3 Gene Expression Kinetics of HTLV-1

To date, the levels of all specific viral mRNAs in different HTLV-1 and HTLV-2 transformed cell lines as well as at different stages of virus infection have not been assessed. We used real-time RT-PCR to determine the viral gene expression profile over time in 293T cells transiently transfected with HTLV-1 proviral plasmid, human PBMCs infected with HTLV-1 in culture, and PBMCs harvested from HTLV-1
inoculated rabbits. Results from in vitro experiments including both transient transfection and infectivity assays confirmed the specific pattern we have characterized in Chapter 2. However, the gene expression kinetics does not show an intricate regulation of specific viral mRNAs over time as has been hypothesized. We see all mRNA levels increase following transfection or infection and then plateau. Since the cellular environment in vitro differs significantly from those in vivo (e.g. immune surveillance and organism cellular micro-environment), we sought to determine HTLV-1 gene expression kinetics in infected rabbits, which is an established animal model of HTLV infection. Our results show the regulation of specific mRNA kinetics. Early after inoculation, tax/rex mRNA is expressed at high levels (wk1-2) followed by gradual decrease and stabilization at relatively low levels (wk4-8). The gag/pol full length mRNA mirrored tax/rex mRNA kinetics, but with an average magnitude of approximately four-fold lower at its peak. The tax/rex mRNA expression is in reverse correlation with proviral load in HTLV-1 infected rabbits. It was known that Tax has a remarkable potency to promote the proliferation of infected cells in vitro. However, it is also a major target of CTL response in vivo. The negative correlation between tax/rex mRNA level and the proviral load is consistent with the conclusion that Tax expression is severely inhibited in vivo by specific immune response. mRNA expression of all other viral genes, with the exception of hbz, was at or below our limit of detection. hbz was found to be expressed immediately after infection and increased
over time, which is in direct correlation with proviral load. This was a very exciting result since originally, Tax was considered to be the determinant for HTLV-1 infected cell proliferation. However, Tax expression is repressed in ATL cells but HBZ expression is maintained. Our results indicate that \textit{hbz} gene is important for proliferation of infected cells \textit{in vivo}.

HBZ has been reported to be dispensable for viral replication and cellular immortalization \textit{in vitro}, but required for enhanced infectivity and persistence \textit{in vivo}. Rabbits inoculated with HBZ mutant viruses have decreased antibody response and significantly lower proviral load in rabbit PBMCs \textsuperscript{100}. Repression of \textit{hbz} gene expression by siRNA inhibits the proliferation of cells derived form ATL patients, while over-expression of \textit{hbz} mRNA promoted the proliferation of human T-cell line \textsuperscript{233,234}. It remains to be determined if the \textit{in vivo} requirement of \textit{hbz} is dependent on its specific function to repress Tax-mediated trans-activation, or \textit{hbz} has direct or indirect roles in maintaining viral load and the promotion of infected cell proliferation. A more detailed understanding of the \textit{in vivo} role of \textit{hbz} might be gained by studying the gene expression kinetics in \textit{hbz} mutant virus infected rabbits in comparison to wild type HTLV-1 infected rabbits. Taking into account the data that HTLV-1 \textit{hbz} represses Tax-mediated transcription and attenuates AP-1 activity and our novel data that in HTLV-1 wild-type virus infected rabbits Tax expression level spiked and decreased overtime whereas \textit{hbz} expression level remained constant, we speculate that \textit{hbz} might
function \textit{in vivo} at the protein level to down-regulate viral transcription in order for infected cells to survive the immune surveillance pressure; and possibly at mRNA level to support infected cell proliferation.\textsuperscript{233}

Other than \textit{hbz}, which represses Tax-mediated transcription, previous work also has shown a counteractive effect of the HTLV-1 accessory protein, p30, against Tax. We speculate that \textit{hbz} and p30 may work synergistically to ultimately modulate viral and cellular gene expression during different stages of the infection to promote virus survival. Although we were unable to obtain p30 mRNA kinetics \textit{in vivo} because of its low level expression, the expression profile of other viral genes in p30 mutant virus inoculated rabbits would provide information about this synergistically modulation and potentially lead us to a better understanding of \textit{hbz} and p30 function in viral spread and infected cell survival.

Although we have a clearer picture about HTLV-1 gene expression in the immortalization process \textit{in vitro} and infection \textit{in vivo}, gene expression of HTLV-2 in the process of infection and cellular transformation has not been investigated. A better picture of HTLV-2 gene expression kinetics would not only help understand HTLV-2 gene regulation and transformation mechanism, but would also benefit HTLV-1 research by comparative study.
5. 4 The Role of Rex in Post-transcriptional Regulation of HTLV-1 mRNAs.

The HTLV Rex protein is a post-transcriptional regulator that utilizes specific host machinery to actively export unspliced \((gag/pol)\) and incompletely spliced \((env)\) viral mRNAs from the nucleus to the cytoplasm. However, the role of Rex on the export and expression of HTLV-1 accessory protein mRNA, has been poorly investigated due to their low abundance in cells. In Chapter 4, in addition to confirming the effect of Rex on the export and expression level of unspliced \(gag/pol\) mRNA, singly spliced \(env\) mRNA, and doubly spliced \(tax/rex\) mRNA, we showed that Rex slightly decreased the steady state expression level of singly and doubly spliced mRNAs encoding accessory proteins including the antisense mRNA encoding HBZ, but had no significant effects on the export of these mRNAs. Rex RNA-PD studies demonstrated that Rex associated not only with the \(gag/pol\) and \(env\) viral mRNA, but also was found bound to all other singly- and doubly-spliced viral mRNAs encoding regulatory and accessory proteins. In contrast, \(hbz\) antisense mRNA, which does not contain the RxRE, was not bound by Rex. This result is the first to show the quantitative effects of Rex on the expression level and export of HTLV-1 accessory protein mRNAs. This study is also the first to show that Rex associates with all positive sense HTLV-1 mRNA transcripts. It is important to note that they all contain the RxRE. We postulate that HTLV-1 Rex has an additional role in post-transcriptional modification of viral mRNAs other than at the step of export. In HIV-1, in addition to the export of viral mRNA, Rev is also believed
to enhance the translation of viral RNA. It has been shown to promote the loading of $gag/pol$ and $env$ mRNA onto polysomes $^{194}$. In our result, every HTLV-1 sense transcript containing RxRE was found to be Rex associated although not all of them are dependent on Rex for export. It is possible that those viral mRNAs use Rex to recruit certain cellular proteins to help with remodeling or loading these mRNAs onto polysomes, thus increasing their translation efficiency. To analyze the role of Rex in translation of HTLV-1 mRNAs, the association of the cytoplasmic HTLV-1 mRNA with ribosomes could be examined in the presence or absence of Rex protein expression. Our hypothesis is that HTLV-1 mRNA produced in the absence of Rex would be excluded from polysomes, while HTLV-1 mRNA produced in the presence of Rex would be associated with polysomes and produce viral protein. Other possible post-transcriptional function of Rex on the expression of the completely spliced mRNAs also needs to be further investigated.

HTLV pathogenesis is still a significant problem to be solved in HTLV basic research. Thorough investigations of HTLV gene expression and regulation will be significant in understanding the basic biology of this virus and associated malignancies. HTLV research has been focused on individual viral gene and resulted in great amount of data in revealing the role of specific viral proteins. To better understand the natural course of infection, the study of the HTLV-1 gene expression provides a more complete understanding of the regulation of this retrovirus.
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