THE ROLE OF HBZ IN HTLV-1 BIOLOGY

DISSERTATION

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ABSTRACT

Human T-cell leukemia virus (HTLV)-1 and (HTLV-2) are closely related human retroviruses in genomic organization and at the nucleotide level. In tissue culture, HTLV-1 and HTLV-2 both have the capacity to transform primary T-cells. However, HTLV-1 has been identified as the causative agent for two human diseases, adult T-cell leukemia/lymphoma (ATLL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), whereas HTLV-2 is much less pathogenic and does not exhibit a direct link to neoplasia. Chapter 1 reviews on important aspects of HTLV-1 pathobiology and highlights insightful comparative studies between HTLV-1 and HTLV-2.

In the data Chapters 2-4 of this dissertation, we sought to broaden our knowledge of HTLV-1 at the molecular level using in vitro culture assays and two HTLV-1 animal model systems focusing on the contribution of Hbz in virus biology. By utilizing these systems we will gain a better understanding in the contribution of Hbz to virus infectivity, how it contributes to infected cell growth, and its involvement in the development of leukemogenesis. The antisense strand of the HTLV-1 genome encodes HBZ, a novel nuclear b-ZIP protein, which in over-expression assays down-regulates Tax oncoprotein-
induced viral transcription. In Chapter 2, we investigated the contribution of HBZ to HTLV-1-mediated immortalization of primary T-lymphocytes in vitro and HTLV-1 infection in a rabbit animal model. HTLV-1 HBZ mutant viruses were generated and evaluated for viral gene expression, protein production, and immortalization capacity. Biological properties of HBZ mutant viruses in vitro were indistinguishable from wild-type HTLV-1 providing the first direct evidence that HBZ is dispensable for viral replication and cellular immortalization in culture. Rabbits inoculated with irradiated cells expressing HTLV-1 HBZ mutant viruses became persistently infected. However, these rabbits displayed a decreased antibody response to viral gene products and reduced proviral copies in PBMCs as compared to wild-type HTLV-1 infected animals. This study demonstrated that retroviruses utilize negative strand-encoded proteins in the establishment of chronic viral infections.

In most adult T-cell leukemia/lymphoma (ATLL) cells, Tax oncoprotein expression is typically low or undetectable, whereas Hbz gene expression is maintained suggesting that Hbz expression may support infected cell survival and ultimately, leukemogenesis. Emerging data indicates that HBZ protein functions by interacting with CREB and Jun family members altering transcription factor binding and transactivation of both viral and cellular promoters. Herein, lentiviral vectors that express Hbz-specific short hairpin (sh)RNA effectively decreased both Hbz mRNA and HBZ protein expression in transduced HTLV-1-transformed SLB-1 T-cells. Hbz knockdown correlated with a significant decrease in T-cell proliferation in culture. Both SLB-1 and SLB-1-Hbz knockdown cells engrafted into inoculated NOD/SCIDγchain-/- mice to form solid tumors that also infiltrated multiple tissues. However, tumor formation and organ
infiltration was significantly decreased in animals challenged with SLB-1-\textit{Hbz} knockdown cells. Taken together our data indicate that \textit{Hbz} expression enhances the proliferative capacity of HTLV-1 infected T-cells playing a critical role in cell survival and ultimately HTLV-1 tumorigenesis in the infected host.

In Chapter 4 we utilized tissue culture experiments to expand on the findings from Chapter 3. Prior studies indicated that the \textit{Hbz} mRNA supported cell growth, but the experiments failed to address the contribution of the HBZ protein. The goal of Chapter 4 was to determine if both the \textit{Hbz} mRNA and HBZ protein contribute to the enhancement of cell proliferation in culture. Our data indicate for the first time that the \textit{Hbz} mRNA and HBZ protein independently enhance cell proliferation in overexpression studies and in the context of an infectious replicating virus. Taken together, the findings in this chapter support the hypothesis that, a unique HTLV-1 nonstructural gene \textit{Hbz}, has pleiotropic functions in two distinct molecular forms that specifically manipulate the cellular microenvironment to facilitate malignant cell growth.

Overall, generally the experiments in this dissertation utilize methods that manipulate a viral genetic element in the context of full-length proviral clone to analyze the function and mechanism of action in a system that closely mimics \textit{in vivo} HTLV-1 infection. The work performed in this dissertation utilizing animal model systems were the first in the field to evaluate the contribution of \textit{Hbz} in the context of a replicating virus on HTLV-1 infection, cell proliferation, and tumor development \textit{in vivo}. Our studies provide important insight into the molecular pathogenesis of HTLV-1 and ultimately the contribution of the novel antisense gene \textit{Hbz} in leukemogenesis.
Dedicated to:
My parents, Christopher and Terry
My grandparents, Jim and Eileen
and my wife, Megan
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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) are complex retroviruses that have been studied intensely for nearly 30 years because of their association with neoplasia, neuropathology, and/or their primary T lymphocyte transforming capacity. HTLV-1 and HTLV-2 are highly homologous at the nucleotide sequence level, but the clinical manifestations differ significantly. HTLV-1 is the causative agent of adult T-cell leukemia lymphoma (ATLL), an aggressive CD4+ T-cell malignancy, and immune-mediated disorders including HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). In contrast, HTLV-2 is much less pathogenic which as been associated with only a single case of atypical hairy-cell leukemia and a few cases of neurological disease reported in infected carriers. Both HTLV-1 and HTLV-2 efficiently transform T-lymphocytes in cell culture and persist in infected individuals or experimental animals. The study of HTLV, particularly the properties of the viral Tax oncoprotein, has allowed investigators to dissect many cellular processes, several of which are likely key contributors to the pathobiology of the virus. Furthermore, studies utilizing genetically manipulated infectious molecular clones of HTLV-1 and HTLV-2 in cell culture and a rabbit model of infection have revealed a
critical supporting role of other viral gene products in virus biology and provided fundamental insights into the mechanisms of pathogenic outcomes associated with the infection of HTLV-1 or HTLV-2.

1.2 Human T-cell leukemia virus discovery and epidemiology

The first pathogenic human retrovirus to be identified and later causally linked to neoplastic malignancy was HTLV-1. T-cell lines established from patients with leukemia and cutaneous T-cell lymphoma were shown to produce viral particles as well as viral antigens that are reactive against patients sera with adult T-cell leukemia/lymphoma. A vast number of molecular and epidemiological studies have provided evidence that HTLV-1 is the etiological agent of ATLL and the chronic slowly progressive neurological disorder HTLV-1 associated myelopathy/tropical spastic parapareisis (HAM/TSP).

HTLV-2 was discovered a few years after the discovery of HTLV-1 in a patient with a rare variant with hairy cell leukemia and in another patient with a CD8+ T-leukemia and a coexisting hairy B-cell leukemia. HTLV-2 appears to play a rare role in neurological and lymphoproliferative disorders, but a causal link of HTLV-2 viral infection to neoplasia has yet to be established.

HTLV-1 and HTLV-2 share a similar genome and nucleotide sequence organization (Figure 1.1). Despite these similarities of the human T-cell leukemia viruses (HTLVs) their evolutionary relationships are hypothesized to have originated independently from distinct lineages from the simian T-cell leukemia virus type 1 and 2. Two more recent HTLV isolates (HTLV-3 and HTLV-4) have been identified in
inhabitants from south Cameroon whose sera exhibited HTLV indeterminate serologies. Phylogenetically, HTLV-3 is related to type 3 simian T-cell leukemia virus (STLV), whereas HTLV-4 belongs to a distinct group phylogenetically unrelated to the currently known STLVs and HTLVs. A causal link to disease following infection with HTLV-3 and HTLV-4 is yet to be established.

The geographic distribution of HTLV-1 and HTLV-2 are distinct to regions of the world. It is estimated that 10-20 million people worldwide are infected with HTLV-1, where it is endemic in locations within Japan, Africa, South America, and the Caribbean basin. It is predicted that after a long latency of 20-50 years only 3.0-5.0% of HTLV-1 infected individuals will develop ATLL and 0.25-3.0% will develop HAM/TSP. The geographic origin of HTLV-2 is less defined and is somewhat concentrated in intravenous drug users (IVDUs) and their sexual contacts in Europe, Southeast Asia, and the Americas including the United States.

The small percentages of infected individuals that develop HTLV-1-associated diseases have a chronic, but lifelong infection. HTLV-1 is a cell associated virus and for efficient transmission, infected cells must be passed from the infected individual by cell-cell contact. When an infected cell contacts an uninfected cell, a microtubule-organizing center (MTOC) is polarized at the cell–cell junction, and a virological synapse forms at the interface. Thereafter, the HTLV-1 Gag (explained in later later section) complex and viral genomic RNAs accumulate at the synapse and egress into the uninfected cell.

Infection by HTLV-1 free virions is low, thus the primary route of infection is by cell-to-cell contact from breast feeding via breast milk, sexual contact, blood transfusions, and by IVDUs needle sharing.
Vertical transmission from mother-to-child depends on duration of breast feeding, ingestion of infected milk-borne lymphocytes, and maternal antibodies against HTLV. Bidirectional sexual transmission is possible yet there is higher likelihood that a woman would be infected due to sexual contact with an HTLV positive male compared to the converse scenario. These transmission patterns are also affected by other factors such as genital ulcers, high viral load, and anti-HTLV antibodies within the individuals. The most efficient route of HTLV transmission that leads to rapid disease development is blood transfusion as long as it has cellular blood components. It is estimated that the seroconversion rate in this case is up to 50%.

Finally, despite a wide variety of human and nonhuman cells that can be infected by HTLV \textit{in vitro} \textsuperscript{35,36} the \textit{in vivo} tropism or cellular targets are preferentially CD4+ T cells for HTLV-1, CD8+ and to some extent CD4+T cells for HTLV-2. A more detailed discussion of HTLV-1 and HTLV-2 cellular tropism will be expanded in a later section. \textsuperscript{37-40}

\textbf{1.3 HTLV classification, genome organization, and life cycle}

HTLVs are retroviruses which are members of the Deltaretrovirus group that includes bovine leukemia virus (BLV) and STLV-1, -2, and -3.\textsuperscript{41} HTLVs have a C-type morphology via electron microscopy where the virions assemble at the plasma membrane. The virions possess a central, symmetrically placed, spherical inner core that contains two copies of ~ 9kb linear, positive sense, single stranded RNA genome as a ribonucleoprotein complex. The viron includes tRNA from the host cell that serves as a
primer for reverse transcription. Structural and enzymatic components include nucleocapsid, capsid, matrix, reverse transcriptase, integrase and protease respectively. The HTLV-1 virion is coated by the outer envelope surface and transmembrane subunits, composed of viral and cellular proteins as well as lipids. The RNA genome organization as well as that of the DNA intermediate (provirus) of all HTLVs is very similar. The RNA genome is capped at the 5’ end and contains a poly(A) chain of around 200 nucleotides at the 3’ end. The proviral genome contains a long terminal repeat (LTR) at each end which encompasses sequence blocks that are essential for integration viral replication and regulation of gene expression. Each LTR is composed of a unique 3’ (U3) region present only at the 3’ end of the viral RNA genome, a repeated (R) region present at both ends of the viral RNA genome, and a unique 5’ (U5) region present only at the 5’ end of the viral RNA genome. The majority of the HTLV genome between the LTRs encodes Gag, Pol and Env in addition to four open reading frames (ORFs) in HTLV-1 and five ORFs in HTLV-2 present in what was initially referred to as the X region. The replication life cycle of the HTLVs are similar to that of other retroviruses. Briefly, the viral glycoprotein 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 TM component of Env. This is followed by the release of the uncoated viral core into the cytoplasm of the host cell. Then, due to a unique enzyme produced by all retroviruses
(reverse transcriptase), including HTLVs, the RNA genome is reverse transcribed to generate a double stranded linear DNA intermediate that is transported to the nucleus where it gets stably integrated into the host genome. Integration requires the enzymatic activity of the viral protein, integrase. At this point, the integrated HTLV genome is referred to as a provirus and becomes part of the host genome. Afterwards, the cellular RNA polymerase II mediates transcription from the viral promoter present in the U3 region of the 5’ LTR, resulting in multiple viral transcripts that are spliced (with the exception of the full length genomic gag/pol mRNA), processed, exported to the cytoplasm, and translated into viral proteins. The assembly of the virion and packaging of genomic RNA follows. HTLV utilizes a sequence called the Psi element (ψ) for encapsidation.\(^\text{43}\) Since ψ is spliced out in all other transcripts, only unspliced genomic RNA is packaged into the virion that buds at the plasma 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.\(^\text{44,45}\)

1.4 HTLV-1 pathogenesis

HTLV-1 infection has been linked to two major diseases: ATLL and HAM/TSP. HTLV-1 has also been implicated as an etiologic agent for polymyositis, polyarthritis, uveitis, infectious dermatitis, and virulent strongyloidiasis.\(^\text{3,46-50}\)
1.4.1 ATLL

In 1977, clustered populations of adult T-cell leukemia/lymphoma (ATLL) patients in areas of Japan, lead researches to suggest that a transmissible agent was involved causing the disease.\textsuperscript{51} Later it was found that HTLV-1 was the causative agent for ATLL. ATLL is clinically characterized in five different stages: aggressive lymphoproliferative disease: 1) asymptomatic, 2) pre-leukemic, 3) chronic/smoldering, 4) lymphoma, and 5) acute. The majority of infected people are asymptomatic, yet these individuals are still able to transmit the virus. Infected T-cells typically have highly lobulated or classical “flower-shaped” nuclei with a CD2+, CD3+, CD4+, CD8-, CD25+, and HLA-DR+ cell surface phenotype.\textsuperscript{51} Approximately one-half of the pre-leukemic patients undergo spontaneous regression, whereas some progress to the next phase, smoldering ATLL which is characterized with skin lesions and marrow involvement, or chronic ATLL with elevated numbers of circulating leukemic cells. In the final stage of disease progression, a proportion of patients will develop acute ATLL which is clinically characterized by hypercalcemia, skin lesions, elevated levels of LDH, lymphadenopathy, lymphomatous meningitis, lytic bone lesions, spleen or liver involvement, and immunodeficiency.\textsuperscript{52} The median survival time for acute ATLL patients is 6-10 months even with intense chemotherapy.\textsuperscript{53} HTLV integration seems to be random within infected individuals\textsuperscript{54}, where the leukemic cells are mono or oligoclonal in origin.\textsuperscript{55,56} The clonality of infected cells indicates that a selection of secondary events in a cell in which viral replication is mainly a consequence of mitotic division of infected cell rather than the typical reverse transcriptase-mediated expansion and reinfection.
1.4.2 HAM/TSP

A small fraction of infected people (0.2-5%) develop HAM/TSP, which is a chronic progressive demyelinating disease that predominantly affects the spinal cord and is more prevalent in females than in males.\textsuperscript{26,58} In sharp contrast to ATLL, the onset of HAM/TSP is much faster and can progress in as little as 6 months after transfusion.\textsuperscript{59, 60} Initial symptoms of HAM/TSP include weakness and stiffness of the lower limbs. As the disease progresses, more symptoms such as constipation, impotence and hyperreflexia may present. Pathologic analyses have revealed perivascular and parenchymal infiltration of mononuclear lymphoid cells that correlate with myelin and axonal degeneration, ultimately resulting in severe degeneration of white matter. Several lines of evidence indicate that virus-host immune system interaction plays a critical role in the development of HAM/TSP. It has been shown that high proviral load accompanied with increased viral gene expression leads to the processing and presentation of HTLV-1-specific peptides, especially those of Tax. Subsequently, this results in the activation and expansion of antigen-specific CD4+ and CD8+ T-cells. The localized infiltration of HTLV-1-specific CD8+ cytotoxic T lymphocytes (CTL) in the central nervous system implicates those cells in the progression of disease development.

1.5 HTLV-2 pathogenesis

Contrary to HTLV-1, the disease association for HTLV-2 is less clear and currently lacks solid epidemiological evidence linking infection to disease. HTLV-2 was isolated from patients with a rare variant of hairy cell leukemia, CD8+ T-cell leukemia, and large granular lymphocytic leukemia.\textsuperscript{9} Theses findings suggest some association
between HTLV-2 and T-cell lymphoproliferative 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 a few reports that associate HTLV-2 infection with chronic neurodegenerative diseases.\textsuperscript{7} Interestingly, HTLV-2 infection has been reported in a patient with a chronic progressive neurological disease that is clinically identical to HAM/TSP.\textsuperscript{8} HTLV-2 is also associated with increased incidence of pneumonia and bronchitis, inflammatory conditions such as arthritis, and perhaps with increased mortality.\textsuperscript{62, 63}

1.6 HTLV structural and enzymatic proteins

HTLVs utilize ribosomal frameshifting and alternative splicing as mechanisms to produce structural, regulatory, and accessory proteins from the approximately 9 kb coding region. HTLVs encode for the structural and enzymatic proteins: Gag, Pol, Pro, and Env. The structural proteins are encoded by \textit{gag} and \textit{env} genes, and the \textit{pol} gene provides most of the enzymatic activity. Generally, HTLV-1 and HTLV-2’s structural proteins are similar to other retroviruses; yet they do not appear to ensure proper and efficient cell free transmission for reasons that are currently unknown.\textsuperscript{64}

1.6.1 Gag

The main function of Gag (group specific antigen) is to promote the assembly and release of virus particles, even in the absence of any other viral protein or packaged RNA. HTLV Gag is made as a polypeptide precursor. A critical processing step leads to the proteolytic cleavage and generation of the matrix (MA, p19), capsid (CA, p24), and
nucleocapsid (NC, p15) proteins. Targeting of MA and its precursor p55 to the inner surface of the plasma membrane is made possible by myristylation at the N-terminal end of the protein. Like other retroviruses, HTLV MA has an N-terminal cluster of basic amino acids that have been shown to play multiple roles in virus replication such as infectivity, particle release, precursor cleavage and ultimately cell-to-cell transmission. The other two Gag products (CA and NC) of HTLV have not been studied in detail but their contribution to the overall replication of the virus is likely similar to other retroviruses including Moloney MLV and HIV-1.

1.6.2 Pro

A frameshift occurs during the synthesis of Gag which is needed to produce protease (Pro) whose reading frame overlaps the 3’ end of gag and the 5’ part of pol. The function of Pro is to process the precursor of the Gag and Pol polypeptide to produce smaller functional units. Thus, Pro is essential for the maturation of HTLV viral particles.

1.6.3 Pol

A ribosomal frameshift also occurs during the translation of Gag to result in the synthesis of Gag-Pol. The C-terminal region of Gag-Pol precursor is cleaved by Pro to release integrase (IN). The integrase protein is needed for the stable integration of the provirus into the host cell genome. The remaining part of Pol contains the reverse transcriptase activity (RT) at the N-terminus and the RNase H activity at the C-terminus.
These activities cooperate following HTLV infection to generate the double-stranded DNA intermediate from the RNA genome termed the provirus.\textsuperscript{71}

1.6.4 Env

The incompletely spliced mRNA in HTLV encodes for the Envelope (Env) protein. This protein is post-translationally modified, which results in a 61-69 kDa glycoprotein.\textsuperscript{72,73} The Envelope precursor is cleaved by a cellular protease to form the surface (SU, gp46) and transmembrane (TM, gp21) subunits. These Env proteins are organized as oligomers, but at earlier stages Env is assembled in the endoplasmic reticulum (ER) as a dimmer.\textsuperscript{74} The SU glycoprotein harbors the receptor binding determinants which are composed of three domains. The TM on the other hand possesses a fusion peptide at the N-terminus and is directly responsible for the initiation of fusion pores. Mutational analysis of TM also indicates that it functions in post-fusion events that are required for infection.\textsuperscript{68}

1.7 HTLV cellular tropism

HTLV-1 and HTLV-2 exhibit different \textit{in vivo} T-cell tropisms and this has been hypothesized to be important for their distinct leukemogenic capacity.\textsuperscript{75,76} Investigation of HTLV cell tropism in asymptomatic patients and those with neurological disease indicated that HTLV-1 has a preferential tropism for CD4\textsuperscript{+} T-cells with CD8\textsuperscript{+} T-cells being an additional viral reservoir in HAM/TSP patients. In contrast, HTLV-2 \textit{in vivo} tropism is less clear but seems to favor CD8\textsuperscript{+} T-lymphocytes. One study revealed that proviral sequences were detected predominantly in CD8\textsuperscript{+} T-lymphocytes from HTLV-2-
infected individuals, whereas others have detected HTLV-2 in both CD4+ and CD8+ T-cell subsets, with a greater proviral burden in CD8+ T-cells. The distinct in vivo T-cell tropism of HTLV-1 and HTLV-2 has been recapitulated in vitro using immortalization/transformation assays where irradiated HTLV producer cells were cocultured with freshly isolated human PBMCs. Results from these studies showed that the majority of cells transformed by HTLV-1 in vitro were CD4+ T-lymphocytes, whereas HTLV-2 preferentially transformed CD8+ T-cells. Studies using HTLV recombinant infectious clones indicated that Tax and overlapping Rex (discussed later) did not confer the distinct HTLV-1 and HTLV-2 transformation tropism in vitro. This suggested that other viral genes or sequences were responsible for the differential ability to transform CD4+ or CD8+ T-cells. Follow-up recombinant studies revealed that the env gene was the major viral determinant of the distinct in vitro cellular transformation tropism of HTLV-1 and HTLV-2. The tropism was hypothesized to be a post entry phenomenon since at the time HTLV-1 and HTLV-2 were considered to utilize the same cellular receptor.

Using various assay systems several cell surface molecules have been shown to be important for HTLV entry into cells including the glucose transporter 1 (GLUT1), heparin sulfate proteoglycans (HSPGs), and neuropilin-1 (NRP1) suggesting that the HTLV receptor may be multi component. Recently, a careful examination of the cell surface of activated primary T-cells revealed that CD4+ T-cells expressed significantly higher levels of HSPGs than CD8+ T-cells, whereas CD8+ T-cells expressed GLUT1 at dramatically higher levels than CD4+ T-cells. Jones et al showed that HTLV-2 Env binding and viral entry were significantly higher on CD8+ T-cells
while HTLV-1 Env binding and viral entry were higher on CD4+ T-cells. Moreover, they reported that the over expression of GLUT1 in CD4+ T-cells enhanced HTLV-2 entry, while expression of HSPGs on CD8+ T-cells increased HTLV-1 entry. These studies demonstrate that HTLV-1 and HTLV-2 differ in their T-cell entry requirements and together with the viral recombinant studies suggest that the distinct differences in the in vitro cellular transformation tropism and in vivo pathobiology of these viruses result from different interactions between their related Env proteins and molecules on CD4+ and CD8+ T-cells involved in entry.

Mentioned earlier, cell-free HTLV-1 virions are poorly infectious in vitro for their primary target cells, CD4+ T-cells. In a recent study, HTLV-1 was found to efficiently infect myeloid and plasmacytoid dendritic cells (DCs). Moreover, DCs exposed to HTLV-1, both before and after being productively infected, can rapidly, efficiently and reproducibly transfer virus to autologous primary CD4+ T-cells. The DC-mediated transfer of HTLV-1 involves heparin sulfate proteoglycans and neuropilin-1 and results in long-term productive infection of the CD4+ T-cells. This study, along with observations of HTLV-1-infected DCs in the peripheral blood of infected individuals, indicates that DCs have a central role in HTLV-1 transmission, and persistence in vivo.

1.8 Cellular transformation and pathogenesis

HTLV-1 and HTLV-2 display distinct clinical manifestations, but a distinct feature of both these viruses is their ability to infect and transform primary human T-lymphocytes in cell culture. Although the molecular basis for cellular transformation is not completely understood, data generated from multiple experimental systems clearly
identifies the viral transactivator Tax as the critical determinant. Initial experiments revealed that Tax alone will morphologically transform rodent fibroblasts, induce tumors in transgenic mice, and immortalize primary human T-cells. More recently, studies using infectious molecular clones showed directly that Tax is essential for HTLV-1 and HTLV-2-mediated cellular transformation of primary human T-cells. A key advantage of using a molecular clone approach, compared to over-expression studies, is that the transforming capacity is evaluated in the context of all viral genes using the virus’s natural target cells, primary human T-lymphocytes. The precise mechanism by which Tax initiates the malignant process is unclear but is proposed to involve several points of cellular dysregulation ultimately culminating in the accumulation of genetic mutations and uncontrolled lymphocyte growth. Although there are many similarities between HTLV-1 and HTLV-2 Tax (Tax-1 and Tax-2, respectively), a number of distinct phenotypic differences have been documented in certain cell culture model systems. These differences have been hypothesized to hold the key as to why HTLV-1 and not HTLV-2 is associated with disease. Although Tax is clearly critical for the transforming capacity of the virus, data is emerging that other viral genes have important roles in the biology of the virus and ultimately its oncogenic potential. Specific Tax activities implicated in the transformation process and the supporting contribution of other viral gene products will be detailed in the next sections.

In addition to transactivating the viral promoter, Tax modulates the transcription or activity of numerous cellular genes involved in cell growth and survival, cell cycle control, genetic instability, and DNA damage/repair. The ability to modulate the expression or activity of a variety of viral and cellular gene products is proposed to be the
key mechanism by which Tax induces cellular transformation.

1.9 HTLV regulatory proteins Rex and Tax

HTLV encodes two positive regulatory proteins, Tax and Rex, from the same completely-spliced mRNA in separate but overlapping reading frames.

1.9.1 ORF-III Rex-1 and Rex-2

The 189 amino acid Rex-1 and 170 amino acid Rex-2 proteins encoded by ORF III of the pX region share 60% homology at the amino acid level. When analyzed by SDS-PAGE, Rex-1 has an apparent size of 27 kDa, and Rex-2 is detected as two major bands of 24 and 26 kDa. The two isoforms of Rex-2, p24\textsuperscript{rex} and p26\textsuperscript{rex}, have the same amino acid backbone but differ by post-translational modifications; specifically serine phosphorylations resulting in a conformational change.\textsuperscript{84-86} HTLV-1 and HTLV-2 also produce truncated forms of Rex from alternatively spliced mRNAs. These proteins, named p21\textsuperscript{rex-1} and p22/20\textsuperscript{rex-2}, lack N-terminal sequences of Rex responsible for nuclear import and RNA binding, and interfere with Rex localization and function.\textsuperscript{87-89}

HTLV Rex is a trans-acting regulatory protein. It is required for efficient cytoplasmic accumulation of the mRNA templates for the viral structural and enzymatic proteins.\textsuperscript{90-92} Rex facilitates export of unspliced and singly spliced viral mRNA from the nucleus into the cytoplasm. In the absence of Rex, these viral mRNAs remain sequestered in the nucleus until they are either spliced or degraded.\textsuperscript{93} Rex-mediated RNA export requires the direct interaction of Rex with a cis-acting sequence termed the Rex response element (RxRE).\textsuperscript{94-97} RxRE-1 is located in the U3/R region of the 3’ LTR, and
it is composed of 205 nucleotides and forms a stem-loop secondary structure. Substitution and deletion mutations revealed that the proper RxRE secondary structure is essential for Rex function.\textsuperscript{95,97} Although the precise mechanism by which Rex mediates nucleus to cytoplasm export of viral mRNA is not completely understood, it is known that HTLV-1 Rex may use mechanisms similar to HIV Rev which involves the CRM1/exportin 1 pathway.\textsuperscript{95,98} Ye \textit{et al.} utilized an infectious molecular clone to investigate the contribution of Rex in HTLV-1 immortalization of primary T-cells \textit{in vitro} and viral survival in an infectious rabbit animal model.\textsuperscript{99} It was reported that the Rex function to modulate viral gene expression and virion production is not required for \textit{in vitro} immortalization of primary human T-lymphocytes by HTLV-1. However, this Rex deficient virus was significantly hampered in its ability to spread and persist in inoculated rabbits.

The accumulation of viral structural proteins is dependent on Rex, yet 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 protein is being made, 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 incompletely spliced mRNA in the cytoplasm, leading to the production of structural and enzymatic gene products and assembly of virus particles. Therefore, Rex is considered to be a positive regulator that controls the switch between early, latent and late, productive infection. Recently, Rex and p30 accessory protein (p30 discussed in more detail later) were found to form ternary ribonucleoprotein complexes onto specific viral mRNA.\textsuperscript{100} Rex was able to counteract p30-mediated suppression of
viral expression and restore cytoplasmic tax/rex mRNA and Tax protein expression demonstrating a complex regulatory mechanism of antagonizing post-transcriptional regulators.\textsuperscript{100}

\textbf{1.9.2 ORF-IV Tax-1 and Tax-2}

HTLV Tax is a pleiotropic phosphoprotein that regulates several aspects of viral replication as well as HTLV-mediated transformation of T-lymphocytes. HTLV-1 Tax protein is predominantly nuclear but has been shown to shuttle between the nucleus and cytoplasm.\textsuperscript{101} Tax-2, on the other hand, was reported to be located predominantly in the cytoplasm of the HTLV-2 immortalized or transformed infected T-cells.\textsuperscript{102} Tax-1 is 40 kDa and 353 a.a. in size, while Tax-2a is 37 kDa and 331 a.a. in size.\textsuperscript{70}

\textbf{1.10 Tax and viral transcription}

Tax (Transcriptional Activator of pX region) is one of the first viral proteins expressed early after viral infection and is a transactivator of viral gene expression. Tax transcriptionally activities the HTLV promoter through the three 21 bp repeat sequences termed the Tax response element (TRE) found in the U3 region of the LTR.\textsuperscript{103,104} The TRE contains DNA sequences identical to part of the cyclic adenosine monophosphate (cAMP)-responsive element (CRE). The CRE, which is contained in many cellular gene promoters, is responsive to cAMP and binds members of the CRE binding protein/activating transcription factor (CREB/ATF-1) family of transcription factors in a Tax-dependent manner.\textsuperscript{82,105-109} \textit{In vitro}, Tax contacts GC-rich DNA which flanks the TRE-1 sequence and recruits the cellular coactivator (CREB) to the transcription
complex. The Tax/CREB dimmer interacts with the CRE-like sequence of the viral promoter to activate viral transcription. Tax directly interacts with CREB-binding protein (CBP), and p300, to form a Tax/CREB/p300/CBP complex. The recruitment of another host cell factor PCAF, which directly interacts with Tax, is essential for transcription initiation. Tax also modulates the activity of other cellular transcription factors including serum response factor (SRF) and AP-1 which activate a plethora of early response genes that regulate proliferation. Tax-1 and Tax-2 mutants have been identified that fail to activate the CREB/ATF pathway and are defective for transactivation of the viral promoter. Over expression of these and other Tax mutants in various assay systems have been invaluable for dissecting cell signaling pathways and for determining the interplay between Tax and cellular transformation. However, Tax functional analysis in the context of an infectious virus presented a unique challenge since a knockout of Tax, or more specifically the inability of Tax to activate the CREB/ATF pathway disrupts overall viral gene expression and replication thus resulting in essentially a dead virus. Ross et al. circumvented this problem by generating a unique HTLV-2 provirus, which replicates by a Tax-independent mechanism due to replacement of the TRE with the cytomegalovirus immediate-early promoter enhancer. The advantage of this novel virus is that viral gene expression and replication is not significantly disrupted by mutations in Tax. Initial Tax knockout studies revealed that Tax was required for T-lymphocyte transformation providing the first direct evidence in the context of a virus that Tax was the critical viral transforming protein. Subsequent studies revealed that CREB/ATF activation by Tax was required to promote sustained cell growth and IL-2 independent cellular transformation.
1.11 Tax and NFkB

In addition to transactivating the viral promoter Tax modulates the transcription or activity of numerous cellular genes involved in cell growth and survival, cell cycle control, and DNA damage/repair.\textsuperscript{82} One of the major cell growth and survival pathways that Tax targets involves the activation of NFkB.\textsuperscript{115-122} Tax directly interacts with IKK$\gamma$ which ultimately induces the phosphorylation and degradation of IKB$\beta$. This results in the nuclear expression of NFkB which leads to the expression of many gene promoters including IL-2, IL-2 receptor $\alpha$, IL-3 and GM-CSF.\textsuperscript{123,124,125,126,127} Mutational analysis of Tax-1 and Tax-2 has identified specific mutants and domains important for activation of NFkB signaling.\textsuperscript{112,128} Coculture studies utilizing HTLV-1 and HTLV-2 infectious molecular clones indicated that immortalization of T-lymphocytes in cell culture is dependent on Tax activation of NFkB.\textsuperscript{83,114,129} The role for NFkB activation by Tax in the HTLV-1 malignant process is also supported by \textit{in vivo} observations. Additionally, NFkB and NFkB target genes are found to be activated in ATLL, ATLL transplanted NOD-SCID$\gamma$ knockout mice, and tumors arising in Tax transgenic mice.\textsuperscript{130} Approaches to block NFkB using drugs or peptide inhibitors have resulted in tumor cell regression in various animal models.\textsuperscript{130} There does however remain a disconnect between Tax activation of NFkB and ATLL, since many leukemic cells no longer express Tax, but show constitutive NFkB activation. Thus, it is clear that Tax activation of NFkB provides a critical proliferative or survival signal early in the cellular transformation process, but not the maintenance of the leukemic state.
1.12 Tax and cell cycle control

Perturbation of the cell cycle is a common feature in the transformation of cells by viral oncoproteins. Tax has been shown to modify the cell cycle by directly binding to cellular factors that are the gate keepers of cell cycle control. A major mitogenic activity of Tax is its ability to override cell cycle control and stimulate G1-to-S-phase transition in HTLV-1 infected and Tax expressing cells.\textsuperscript{131,132} In mammalian cells progression through the cell cycle is tightly controlled through timely activation and degradation of proteins called cyclins and cyclin-dependent kinases (Cdks). Cyclins and Cdks form complexes that function to phosphorylate target proteins within specific regulatory cascades. Phosphorylation specificity of residues and targets results in the activation or inactivation of proteins that will collectively dictate passage through the cell cycle. The G1-to-S-phase transition is stimulated by Tax in three ways: 1) transcriptional up regulation of cyclin D2; 2) direct binding and activation of the kinase holoenzyme; and 3) repression of Cdk inhibitors. Tax induces an increase in IL-2R signaling which increases the transcriptional expression of the G1 cyclin D2 by directly activating its promoter.\textsuperscript{133-135} Tax modifies the cell cycle by directly binding cyclin dependent kinases (CDK)-4 and (CDK)-6 and by repressing inhibitors such as the INK4A-D and KIP1.\textsuperscript{82,111,136} The resulting activation of the cyclin D/CDK4/6 kinase holoenzyme results in the hyper-phosphorylation of the Rb tumor suppressor.\textsuperscript{137} Rb has been shown to be the major tumor suppressor that regulates G1-to-S phase transition. It functions by binding and inhibiting the transcription factor E2F1 which regulates genes involved in S phase progression and/or apoptosis.\textsuperscript{132,135,138} Normal cell cycle progression into S phase is
marked by the phosphorylation of Rb by the cyclin D holoenzyme leading to its proteasomal degradation. In addition to activating the holoenzyme, Tax may also bind Rb directly inducing its degradation and subsequent release of the transcription factor E2F1 promoting G1-S transition.\textsuperscript{138,139}

1.13 Tax (PDZ) peptide binding motif (PBM)

Recently a PDZ binding motif (PBM) has been identified in the C-terminal fragment of Tax-1; this motif attracted additional interest because of its absence in Tax-2. The PDZ domain was named after the first identified PDZ-containing proteins, post-synaptic density protein (PSD-95), \textit{Drosophila} discs large protein (DLG) and epithelial tight junction protein (Zonula Occludens-1). It is one of the protein-protein interaction modules commonly used in eukaryotic cells to recruit and organize proteins to sites of cellular signaling. Tax-1, via its PBM, has been shown to interact with the human homolog of \textit{Drosophila melanogaster} discs large tumor suppressor protein, hDLG1.\textsuperscript{140} Tax-1 competes with the binding domain of hDLG and APC tumor suppressor protein and rescues cells from cell cycle arrest induced by hDLG. A chimeric Tax-2 encoding the last 53 amino acids of Tax-1 (Tax221), which contains the PBM, demonstrated an increased transforming potential in rat fibroblast cells.\textsuperscript{141} It was demonstrated further that deletion of the PBM from Tax-1 abrogates hDLG binding and results in reduced transformation activity in rat fibroblasts and in an IL-2 dependent mouse cell line.\textsuperscript{142,143} The contribution of the Tax-1 PBM to HTLV-induced proliferation and immortalization of primary T-cells \textit{in vitro} and viral survival in an infectious rabbit animal model was recently investigated.\textsuperscript{144} Using both virus gene knockout and knockin approaches the
Tax-1 PBM was found to significantly increase both HTLV-1- and HTLV-2-induced primary T-cell proliferation. Viral infection and persistence were severely attenuated in rabbits inoculated with an HTLV-1 virus containing a deletion in the four amino acid PBM motif. Together, these studies support the conclusion that the PBM of Tax-1 and its interacting partners, the cellular PDZ domain containing proteins (eg. hDLG1), are important in cellular transformation. Thus, the absence of the PDZ domain in Tax-2 may be a major determinant of the differences in pathogenicity between HTLV-1 and HTLV-2.

1.14 Tax in DNA damage and genetic instability

The majority of cancer cells contain a large number of chromosomal abnormalities including deletions, translocations, and aneuploidy. Structurally damaged DNA is quite often found in immortalized cells in culture and in HTLV transformed T-lymphocytes isolated from patients. As discussed earlier, Tax has the ability to manipulate multiple cellular proteins, signaling pathways, and critical checkpoints. These activities can have detrimental effects on chromosomal integrity, but 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. DNA mistakes are normally identified and corrected by multiple cellular checkpoints and mechanisms. Tax has been shown to not only abrogate DNA damage-induced checkpoints that normally monitor chromosomal integrity, but it also represses the expression of several overlapping DNA repair pathways including the expression of DNA β-polymerase which is used for nucleotide excision repair (NER),
base excision repair (BER)\textsuperscript{150-152}, mismatch repair (MMR), and recombination repair.\textsuperscript{153} It is not hard to imagine that the suppression of these pathways by Tax would destroy the integrity of the cell and create an environment that would allow the incidence of DNA damage to increase.

Chromosomal numerical abnormalities (aneuploidy and/or polyploidy) and structurally damaged DNA are common features of cancer cells. Genetic instability, in HTLV-1 infected and ATLL cells have been identified.\textsuperscript{154,113} Currently it is unclear if aneuploidy is the cause or consequence of cellular transformation.\textsuperscript{155} Aneuploidy can arise from multipolar mitosis, which typically happens when mistaken centrosome replication generates greater than two spindle poles in a single cell. Several studies have shown that HTLV-1 Tax induces multipolar mitosis by targeting and disrupting the function of two key cellular proteins TAX1BP2 and RANBP1. The interaction of Tax with these proteins increases abnormal duplication of centrioles and fragments spindle poles enhancing multipolar segregation.\textsuperscript{156,157}

The mitotic spindle assembly checkpoint (MSC) ensures that the correct number of chromosomes align properly before the transition to anaphase. Studies have reported that perturbation of the MSC correlates with the development of aneuploidy.\textsuperscript{158} The MSC is regulated by multiple proteins including the family of mitotic arrest defective proteins (MAD)-1, -2, and -3. Interestingly, Tax binds and inactivates MAD1 and in Tax expressing cells aberrant cytokinesis results in multinuclei formation.\textsuperscript{159,160} MAD1 is needed to deliver MAD2 to the kinetochores, where they function as a heterodimer to regulate proper microtubule attachment and correct chromosomal segregation during mitosis.\textsuperscript{161} Interestingly in the presence of Tax, both MAD1 and MAD2 were
mislocalized from the nucleus to the cytoplasm. Thus, the sequestering of MAD1 by Tax inhibits proper localization of MAD2 which results in defective MSC and is consistent with aneuploidy and ATLL progression. In more recent findings, Tax also supports the unscheduled degradation of securin and cyclin B1 by binding to and activating the anaphase promoting complex (APC). With this interaction, Tax is thought to induce early mitotic exit which contributes to abnormal chromosome segregation and subsequent aneuploidy.

1.15 HTLV-1 accessory proteins

Nearly a decade after the discovery of HTLV and the Tax and Rex positive transregulatory proteins, additional alternatively spliced viral mRNAs containing novel ORFs were identified and characterized. Based on protein sizes expressed from cDNA expression plasmids, HTLV-1 accessory proteins encoded by ORF I and II were named p12/p27 and p30/p13, respectively (Figure 1.2). Although the mRNAs encoding these proteins are well documented in HTLV-infected individuals, to date, detection of these proteins in infected cells has remained elusive. However, cytotoxic T-lymphocytes (CTLs) and serum from HTLV-1-infected individuals or experimentally infected rabbits have been demonstrated to recognize peptides against these proteins providing indirect evidence of expression in vivo.

1.15.1 HTLV-1 ORF I p12/p27

p12 appears to be a modulator of T-lymphocyte proliferation and immune function. Although it localizes to endomembranes, particularly the endoplasmic
reticulum and Golgi, it has been shown to interact with IL-2 receptor \( \alpha \) and \( \gamma_c \) chains and MHC class I heavy chains disrupting their surface expression. It interacts with the 16kDa subunit of the vacuolar ATPase, a complex important for the function of lysosomes and endosomes and implicated in transformation pathways. p12 also interacts with calnexin and calreticulin, important ER regulators of calcium release, NFAT transcriptional activation, and the regulation of T-cell proliferation. Initial studies utilizing an infectious molecular clone indicated that abrogation of p12 message or protein had no effect on viral replication and immortalization of primary T-lymphocytes.\(^{164}\) Subsequently, studies revealed the essential role of p12 in the establishment of persistent \textit{in vivo} viral infection using the rabbit model of infection.\(^{165}\) Studies by Albrecht \textit{et al}, demonstrated that p12 is required for optimal viral infectivity in quiescent but non activated primary cells, which suggests a role for p12 in T-cell activation.\(^{166}\) More recently, a study using a p12 deficient virus indicated that p12 promoted cell-to-cell spread by inducing LFA-1 clustering on T-cells via calcium-dependent signaling.\(^{167}\) Together these findings suggest that p12 is a multifunctional protein that facilitates viral infection, host cell proliferation and survival, and helps infected cells escape from host immune surveillance.

The 152 a.a. p27 is generated from a doubly-spliced mRNA and shares the first 20 a.a. with Rex and the last 99 a.a. with p12. The exact role of p27 in HTLV-1 replication is not known; however, a study established that CTLs against p27 protein are generated during HTLV-I infection providing evidence for the \textit{in vivo} chronic production of p27.\(^{168}\)
1.15.2 HTVL-1 ORF II p30/13

p30 is expressed from a doubly-spliced mRNA, localizes to the nucleus/nucleolus and physically interacts with CREB binding protein (CBP)/p300, TIP60, and Rex. In vitro studies have demonstrated that at low concentrations, p30 differentially regulates cellular and viral promoters through an interaction with CBP/p300. At high concentration, p30 functions as a repressor of viral gene transcription by competing with Tax for CBP/p300. Similarly, p30 may also repress cellular gene transcription from CREB-responsive promoters by sequestering the limited amount cellular CBP/p300. p30 via its interaction with TIP60 interacts with Myc-containing transcription complexes and enhances Myc-dependent cellular transformation of human fibroblasts. p30 has also been shown to repress viral replication at the post-transcriptional level by binding to and retaining tax/rex mRNA in the nucleus. By suppressing Tax protein expression p30 attenuates HTLV-1 transcription. More recently it was reported that p30 and the positive posttranscriptional regulator, Rex-1, form ribonucleoprotein complexes specifically on tax/rex mRNA. Rex counteracts p30-mediated suppression of viral expression and restores cytoplasmic tax/rex mRNA and Tax protein expression. These results explain the selective nuclear retention of tax/rex mRNA but not other viral mRNAs by p30. Together, this suggests that p30 may govern the switch between viral latency and replication. Similar posttranscriptional regulation has been reported in HTLV-2, mediated by the p28 ORF II protein. Interestingly, p28 does not appear to share the transcriptional properties of p30. A recent report showed that HTLV-1 p30 expression results in activation of the G2-M cell cycle checkpoint in Jurkat T-cells. This suggests p30 is involved in events that would promote early viral spread
and T-cell survival.\textsuperscript{171} Although p30 is dispensable for HTLV-1-mediated cellular transformation in culture inoculation of rabbits with a p30 deficient virus revealed that p30 expression is required early on in infection to sustain high viral loads in rabbits and promote persistence.\textsuperscript{172} Thus, it is becoming clear that p30 is a multifunctional protein that may assist the virus at many levels contributing to virus survival and pathogenesis.

p13 is expressed from a singly-spliced mRNA. Its open reading frame corresponds to the 87 carboxy terminal amino acids of p30.\textsuperscript{163} Unlike p30, p13 localizes to the mitochondria, alters its morphology by disrupting the inner membrane potential and ion flux, and binds farnesyl pyrophosphate synthesize, an enzyme involved in posttranslational farnesylation of Ras. These properties suggest that p13 is involved in cell signaling and apoptosis. p13 was found to negatively influence cell proliferation at high density in cell culture and to interfere with tumor growth in a nude mouse transplant model.\textsuperscript{173} In the context of an infectious molecular clone p13 is dispensable for HTLV-1 infection and immortalization of PBMCs in culture\textsuperscript{164}, whereas rabbits inoculated with a p13 deficient virus failed to induce a significant immune response and establish a persistent infection.\textsuperscript{174}

\textbf{1.15.3 HBZ antisense protein}

It has only been recently that retrovirus natural antisense transcripts have received significant attention. The HTLV-1 basic leucine zipper factor (\textit{Hbz}) gene is encoded from the minus strand of the proviral genome; the mRNA is synthesized from a promoter located in the 3’ LTR.\textsuperscript{175,176} In addition to HTLV-1, antisense viral transcripts have been identified in other retroviruses including HIV-1, feline immunodeficiency virus (FIV),
simian T-cell leukemia virus-1 (STLV-1), and recently in HTLV-3. Proteins from these transcripts have been postulated to play key roles in the infection cycle and/or pathophysiology of the virus. Interestingly, HTLV-2 does not code for a minus strand \textit{Hbz} transcript and is not linked to neoplasia suggesting \textit{Hbz} may contribute to the development of leukemogenesis. HTLV-3 codes for a truncated form of the HBZ protein, although it remains unknown if this virus induces cancer. Current findings allow one to speculate that HTLV strains that induce T-cell neoplasia encode genes in their negative strand that may play a role in oncogenesis. Multiple \textit{Hbz} mRNA transcripts have been identified in HTLV-1 positive and ATLL cell lines that differ in N-terminal amino acid sequences (Figure 1.3). The HBZ protein contains an N-terminal transcriptional activation domain, nuclear localization signal, basic region, modulatory domain, and a leucine zipper motif in its C-terminus (Figure 1.2). In Chapter 3, which corroborates the previous findings of Murata \textit{et al}, we show that the \textit{Hbz} spliced major transcript is expressed at high levels in HTLV-1 infected and ATLL cells and that the spliced isoform of HBZ is the only protein detectable by molecular approaches. Recent research in the field suggests that the \textit{Hbz} gene may function in two molecular forms; mRNA and protein. Exogenously overexpressed HBZ protein interacts with CREB, and CREB-2 which down-regulates Tax-mediated HTLV-1 transcription and interacts with and disrupts the DNA binding activity of JunB and c-Jun (AP-1 components). In addition, HBZ promotes c-Jun degradation through a proteosome-dependent degradation pathway. HBZ also interacts with JunD to activate the transcription of JunD-dependent promoters. The Jun family of transcription factors regulates gene expression of a number of cellular genes via interactions with the AP-1 sites present in the promoter
region. HBZ increases the expression of human Telomerase reverse transcriptase (hTERT) due to the presence of five putative AP-1 binding sites within the hTERT promoter.\textsuperscript{111,183,186} Therefore, it has been hypothesized that HBZ may play an important role in HTLV-1 biology and the development of leukemia by counteracting the effects of Tax-mediated transcription and/or attenuating or activating cellular gene expression.

In Chapter 2 of this dissertation utilizing a HBZ knockout infectious molecular clone we found that the HBZ protein is dispensable for immortalization/transformation of primary T-lymphocytes in cell culture. Furthermore, rabbits infected with this HBZ protein knockout virus became persistently infected.\textsuperscript{187} However, these rabbits displayed a decreased antibody response to viral gene products and reduced proviral load in peripheral blood mononuclear cells (PBMCs) as compared to wild type HTLV-1 infected animals. This provides important evidence that HBZ is required for the establishment of chronic viral infections \textit{in vivo}.\textsuperscript{187}

Interestingly, in leukemic cells isolated from ATLL patients the 5’-LTR of the provirus is often deleted or hypermethylated resulting in loss or suppression of the viral genes encoded on the plus strand of HTLV-1. However, the 3’LTR, which contains the Hbz promoter, is hypomethylated and unaffected by epigenetic changes. Additionally, the Hbz nucleotide sequence and splice acceptor site of the spliced major transcript is well conserved in HTLV-1 positive and ATLL cells. Hbz mRNA is expressed in all ATLL cells characterized to date and suppression of Hbz gene transcription by short interfering RNA inhibits proliferation of these cells.\textsuperscript{188} In addition, it was shown by mutational analysis that secondary structure of Hbz mRNA rather than HBZ protein promoted proliferation of a human T-cell line.\textsuperscript{188} In support of this conclusion, DNA microarray
analysis demonstrated that \textit{Hbz} mRNA up-regulated the cellular transcription factor \textit{E2F1} and subsequently many cellular E2F1-responsive genes providing further evidence that \textit{Hbz} could be critical for the regulation of cell proliferation and development of ATLL. Additional findings from the same study using transgenic mice with \textit{Hbz} under the control of the mouse CD4 promoter/enhancer revealed that \textit{Hbz} promoted the proliferation CD4+ T-lymphocytes \textit{in vivo}. These findings are consistent with the conclusion that \textit{Hbz} is not only involved in the progression of oncogenesis, but also plays a critical role in HTLV-1-associated chronic inflammatory diseases.\textsuperscript{175,188} In Chapters 3 and 4 of this dissertation we support the findings that the \textit{Hbz} gene is contributing to HTLV-1 leukemic process by showing that \textit{Hbz} supports proliferation of infected T-cells and is contributes to tumor formation in immunocompromised mice.

1.16 HTLV-2 accessory proteins

The accessory proteins in HTLV-2 are produced from pX ORF-I (p10), ORF-II (p28), ORF-III (p22/p20), and ORF-V (p11). To date limited detailed or comprehensive studies have been done to identify the function of these proteins. However, the fact that the pX region from which they are expressed is well conserved among deltaretroviruses indicates an important functional contribution of these proteins to virus biology. A study showed direct evidence on the significance of HTLV-2 accessory proteins in which the clone was modified to eliminate the expression of accessory genes in the pX region. This virus was able to replicate and infect human PBMCs \textit{in vitro}, but it was attenuated in terms of infectivity and persistence in an \textit{in vivo} rabbit model.\textsuperscript{189} Since all accessory genes were deleted simultaneously in that study, the individual contribution of each gene
was not elucidated. Below is a brief description of the proteins generated from transient transfection of these cDNAs of ORF-I, ORF-II, and ORF-V.

1.16.1 ORF-I p10

The HTLV-2 p10 protein is expressed from a doubly spliced bicistronic mRNA that also encodes ORF-V p11. p10 is an 83 a.a. protein that consists of the first 21 a.a. of Rex-2 linked to ORF-I-encoded sequences. Generally the p10 protein is hydrophobic and shows some homology to HTLV-1 p12. p10 is similar to p12, in that it associates with MHC-I but does not seem to bind to IL2-Rβ or γ or 16K.\textsuperscript{190,191} Despite the homology between p12 and p10, one could speculate that these two proteins may be functionally distinct\textsuperscript{190}. To support this concept, p12 localizes to the ER and cis-Golgi, whereas p10 accumulates in the periphery of nucleoli and in nuclear speckles. This distinct cellular localization of p10 could be attributed to the first 21 arginine-rich amino acids that are derived from the Rex-2 ORF and serve as nuclear/nucleolar localization signal.\textsuperscript{192}

1.16.2 ORF-II p28

The HTLV-2 accessory protein p28 can be translated from two singly spliced, bicistronic mRNAs. This protein is 216 a.a. in size with a predicted molecular weight of 23.9 kDa, thus suggesting that p28 is post-translationally modified. In comparison, the first 49 N-terminal amino acids of p28 reveal 77.5% identity with the C-terminal portion of HTLV-1 p30. p28 has been found to localize predominantly to the nucleus. 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.\textsuperscript{192} The p28 protein acts as a negative regulator of both Tax and Rex by binding to and retaining $tax/\text{rex}$ mRNA in the nucleus, thereby inhibiting virion production.\textsuperscript{193} Reduction of viral replication in a cell carrying the provirus may allow escape from immune recognition in an infected individual. In a study by Younis \textit{et al}, they found that p28 is recruited to the viral promoter in a Tax-dependent manner and travels with the transcription elongation machinery until its target mRNA is synthesized. The results of experiments artificially directing these proteins to the promoter indicated that p28, unlike HTLV-1 p30, displays no transcriptional activity. Furthermore, the tethering experiments using p28 further supports the function of it as a negative regulator.

Lastly, p28 has the potential to be expressed from two distinct singly-spliced mRNAs. These mRNAs use different splice acceptors positions and have the potential to encode both p28 and the truncated Rex products (p22/p20Rex). Ciminale \textit{et al}, were able to generate and sequence cDNAs corresponding to HTLV-2 accessory gene products using RT-PCR on RNA isolated from HTLV-2-chronically infected cell line (MoT).\textsuperscript{192} The p22 and p20 truncated Rex products that seem to have cytoplasmic localization, but their function has not been elucidated.

1.16.3 ORF-V p11

The protein p11 is produced using the AUG of Tax/Env that is linked to ORF-V sequences. On an SDS-PAGE the p11 protein produced seems to be larger than its predicted molecular weight of 8.4 kDa. A small portion of 10 a.a. in p11 shows high homology to part of the musculoaponeurotic fibrosarcoma (MAF) nuclear transforming
protein. However, a functional significance of this homology has not been tested yet.\textsuperscript{192} Like p10, p11 associates with MHC-I but does not bind to 16K, or IL2Rβ or γ.\textsuperscript{190} Finally, p11 localizes to the nucleus and, to a lesser extent, to the cytoplasm.\textsuperscript{192}

1.17 HTLV Experimental Systems

1.17.1 Cell culture

Since the discovery of HTLV almost 30 years ago, experimental assay systems for the study of the virus have been complicated by its poor replication in culture, consistent animal models, and frequency and time course of the disease as compared to the avian and murine retroviruses, as well as HIV-1. 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{194-197} However, only primary T-lymphocytes are susceptible to immortalization/transformation and this has historically represented an accepted experimental system for exploring the early events associated with malignancy. HTLV is a highly cell-associated virus; cell-free infection is very inefficient and efficient infection of cells requires co-cultivation of target cells, primary PBMCs, with irradiated HTLV-producer cells. 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 cocultivation. Transformation is defined as continuous growth in the absence of exogenous IL-2; the establishment of hearty IL-2-independent transformed T-cell lines usually requires months in culture.
Initial HTLV studies were restricted to examination of infected patient material, over-expression of individual viral genes using reporter assays in cell lines, or characterization of infected cell lines using viral isolates obtained directly from patients. Although these types of studies have and continue to be very informative, the understanding of HTLV biology and pathogenesis has benefited further from the isolation and manipulation of proviral clones capable of generating infectious virus, and the development and refinement of methodologies for characterization of these clones in primary human T-lymphocytes and relevant animal models.\textsuperscript{198-200}

\subsection*{1.17.2 HTLV animal models}

A variety of animal models of HTLV infection and transformation have provided important insight into the viral and host determinants of the malignant process. As with most animal models of infection and disease, the HTLV animal models each have unique advantages and disadvantages.\textsuperscript{130} HTLV consistently infects only rabbits, some non-human primates, and to a lesser extent rats. HTLV does not efficiently infect murine cells, thus limiting the mouse as an infection model.\textsuperscript{201-206} However, tumor transplant models in genetically engineered severe combined immunodeficiency (SCID) mice have yielded important information on the proliferative and tumorigenic potential of ATL cells as well as assessing tumor outgrowth for potential therapeutic drug invention.\textsuperscript{207,208} In addition, transgenic mouse models have provided important insight of the role of the viral Tax and Tax-mediated dysregulation of cellular processes in lymphocyte transformation and leukemogenesis.\textsuperscript{209} The squirrel monkey has been infected successfully with HTLV-1 and offers an attractive nonhuman primate model of HTLV-1 for vaccine
testing. Rats offer a model of the neurologic disease associated with the viral infection and have been a useful species to test the role of cell-mediated immunity to the infection.

Among the HTLV infection models, the rabbit has been used the most extensively because of the ease and consistency of HTLV transmission. However, in the majority of studies, the rabbit infection has only paralleled the asymptomatic infection in humans. Early studies utilizing the rabbit model of HTLV infection have provided important information regarding transmission of the virus, bodily fluids likely to contain the virus (blood, semen, breast milk), and effective methods to prevent the transmission. The rabbit model has also been used for the evaluation of immune response against the infection and in attempts to generate a vaccine. Early studies used the model to define the sequential development of antibodies to HTLV-1 in infected rabbits and to detect proviral DNA in infected tissue. More recently the rabbit model has been successfully used to evaluate infectious molecular clones of HTLV-1 and HTLV-2. Essentially, molecular cloned proviral DNA is transfected into human PBMCs or established cell lines to generate virus producer cells. Lethally irradiated producer cell lines are inoculated into rabbits where viral replication, immune response, and persistence are monitored over time.

1.18 Conclusions

HTLV-1 and HTLV-2 have the capacity to efficiently immortalize and transform T lymphocytes in vitro and persist in infected individuals or experimental animals. HTLV-1 infection leads to ATLL and HAM/TSP, whereas HTLV-2 infection is not
associated with leukemogenesis. This Chapter focused on important aspects of HTLV-1 pathobiology and where appropriate highlighted insightful comparative studies between HTLV-1 and HTLV-2. Multiple assay systems provide clear evidence that the viral Tax is the key player in HTLV-mediated oncogenesis. It has also become clear from studies utilizing infectious molecular clones in primary human T-cells in vitro and relevant animal model systems that other viral proteins play a supporting role. These types of studies will be instrumental to dissect the virus/host interactions associated with HTLV infection and survival, proliferation of infected cells, and the development of disease. Ultimately, these model systems can be used to understand the mechanism of viral pathogenesis and to develop potential therapeutic intervention strategies against HTLV-1.
Figure 1.1: Genome Organization of HTLV-1 and HTLV-2. HTLV-1 (A) and HTLV-2 (B) proviral genome in kilobases containing the long terminal repeats (LTR), mRNAs, and open reading frames (ORFs) are shown. ORFs are indicated by boxes: Structural and enzymatic proteins (black); regulatory protein ORFs (grey), accessory protein ORFs (white); HTLV-1 antisense ORF (shaded).
Figure 1.2: Structural and functional domains of HTLV-1 regulatory and accessory proteins. Highlighted within each protein are the identified domains required for protein function, host cofactor interactions, and cellular localization.
Figure 1.3: Illustration of Hbz transcripts and open reading frames. At the top is a cartoon illustration of the HTLV-1 genome and open reading frames (ORFs). Detailed below is Hbz including the unspliced mRNA transcript and ORF, the major (SP1) and minor (SP2) spliced transcripts and ORFs. Solid black line denotes mRNA and the asterisk in SP1 and SP2 denotes multiple transcription start sites. Only one transcription start site for the unspliced transcript has been identified. Splice donor (SD) and spliced acceptor (SA) are identified nucleotide based on negative strand proviral numbering. The protein translational start codons are highlighted.
CHAPTER 2

ENHANCEMENT OF INFECTIVITY AND PERSISTENCE IN VIVO BY HBZ, A NATURAL ANTISENSE CODED PROTEIN OF HTLV-1

2.1 Abstract

Natural antisense viral transcripts have been recognized in retroviruses including HTLV-1, HIV-1, and FIV and have been postulated to encode proteins important for the infection cycle and/or pathogenesis of the virus. The antisense strand of the HTLV-1 genome encodes HBZ, a novel nuclear b-ZIP protein, which in over-expression assays down-regulates Tax oncoprotein-induced viral transcription. Herein, we investigated the contribution of HBZ to HTLV-1-mediated immortalization of primary T lymphocytes in vitro and HTLV-1 infection in a rabbit animal model. HTLV-1 HBZ mutant viruses were generated and evaluated for viral gene expression, protein production, and immortalization capacity. Biological properties of HBZ mutant viruses in vitro were indistinguishable from wild type HTLV-1 providing the first direct evidence that HBZ is dispensable for viral replication and cellular immortalization. Rabbits inoculated with irradiated cells expressing HTLV-1 HBZ mutant viruses became persistently infected. However, these rabbits displayed a decreased antibody response to viral gene products and reduced proviral copies in PBMCs as compared to wild type HTLV-1 infected animals. Our findings indicated that HBZ was not required for in vitro cellular
immortalization, but enhanced infectivity and persistence in inoculated rabbits. This study demonstrates that retroviruses utilize negative strand-encoded proteins in the establishment of chronic viral infections.

2.2 Introduction

HTLV-1 infection causes adult T-cell leukemia/lymphoma and is associated with a variety of lymphocyte mediated diseases. Although infected subjects develop a vigorous and sustained immune response against the virus, infection is typically life long. The positive sense RNA genome of HTLV-1 encodes the typical retroviral structural and enzymatic genes gag, pol, and env, the positive regulatory gene products, Tax and Rex, and several accessory gene products, p12I, p27I, p13II, and p30II, which are important for viral infectivity and persistence in vivo. Tax, the transcriptional activator, is an important modulator of both viral and cellular gene expression and its expression is essential for HTLV-mediated cellular transformation of T lymphocytes. Tax transactivates not only the viral gene promoter, but many host cellular promoters through CREB, NFκB, and SRF pathways. The stimulation of such pathways by Tax leads to unregulated protein expression and heightened activation in signaling cascades including JAK/STAT, PI3Kinase, and JNK. It has also been shown that Tax can bind to cyclin-dependent kinase holoenzymes, inactivate tumor suppressors such as p53 and DLG, and has the ability to silence cellular checkpoints. These pleiotropic effects of Tax on the cell have suggested that there may be differences between the initiation and maintenance of transformation.
The majority of retroviral gene products are encoded by the sense strand of the genome. However, natural antisense viral transcripts have been recognized in retroviruses including HTLV-1, human immunodeficiency virus, and feline immunodeficiency virus. The novel HTLV-1 protein HBZ (HTLV-1 b-ZIP factor) is encoded by a minus-strand mRNA that is transcribed by a functional promoter present in the antisense strand of the HTLV-1 proviral genome. Since exogenously over-expressed HBZ down-regulates Tax-induced HTLV-1 transcription and interacts with and disrupts the DNA binding activity of JunB and c-Jun (AP-1 components), it has been hypothesized that HBZ may play an important role in HTLV-1 biology by counteracting the effects of Tax at the transcriptional level and attenuating the activation of AP-1.

In this study, we evaluated the functional role of HBZ in the context of an infectious molecular clone and determined its contribution to viral-induced immortalization in vitro and viral replication kinetics and persistence in vivo. Our findings indicated that the reported repressive effects of HTLV-1 HBZ on Tax and AP-1 were not sufficient to disrupt the capacity of the virus to immortalize primary T lymphocytes in vitro, but rather enhanced infectivity and modified virus persistence in vivo.

2.3 Materials and Methods

Cells

293T cells and 729 B cells were maintained in Dulbecco's modified Eagle and Iscoves medium, respectively. The media were supplemented to contain 10% fetal
bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Human peripheral blood mononuclear cells (hPBMCs) were isolated using Ficoll Hypaque (Amersham, Piscataway, NJ) and cultured in RPMI 1640 medium supplemented with 20% FCS, 2 mM glutamine and antibiotics in the presence or absence of 10U/mL recombinant human IL-2 (Roche Applied Biosciences, Indianapolis, IN). The protocol to obtain human blood was approved by The Ohio State University human subjects internal review board.

**Plasmids**

The plasmid containing the wtHTLV-1 infectious proviral clone, pACHneo, was used in this study.\(^99,199\) HTLV-1HBZΔLZ was generated by changing the sequence \(^{6760}\) ACTCCA \(^{6765}\) to GCTAGC, which introduced a diagnostic *NheI* restriction enzyme site that truncates or terminates the HBZ reading frame at amino acid 175 of the original published sequence \(^{28}\) (amino acid 172 of the *Hbz* major transcript) by deleting the majority of the C-terminal leucine zipper (LZ) region. This mutation did not alter any other known HTLV-1 ORFs. HTLV-1ΔHBZ was generated by introducing a G to A point mutation (nt 7258) that resulted in termination of the HBZ reading frame at amino acid 11\(^{176}\) (amino acid 8 of the *Hbz* major transcript). This single base mutation is thirty nucleotides 5’ to the p13\(^{II}\) ATG on the sense strand and resulted in an Arg to Gln amino acid change in the accessory protein p30\(^{II}\). This p30\(^{II}\) mutant displays a wild-type phenotype in both the transcriptional and post transcriptional assays previously used to define p30\(^{II}\) function *in vitro* \(^{170,234}\) (data not shown). HBZ and HBZΔLZ cDNA expression vectors (pME vector based) were generated from the ACH proviral clone
sequences (based on the *Hbz* major transcript\textsuperscript{181}). The LTR-1-Luc and TK-*Renilla* were described previously.\textsuperscript{170}

**Transfection, luciferase assay, and detection of viral p19 matrix antigen**

To measure Tax function, 1.5 x 10\textsuperscript{5} 293T cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA). The amount of DNA was kept constant and was composed of 0.1 µg of LTR-1-Luc reporter and 10 ng TK-*Renilla* along with 2 µg of an empty plasmid or proviral clones. Cell supernatants (48 h) were used for p19 enzyme-linked immunosorbent assay (ELISA) (Zeptometrix Corporation, Buffalo, N.Y.). Cell pellets were lysed and Tax activity was measured in light units as described previously.\textsuperscript{99} All experiments were performed independently three times in triplicate and the results were normalized for transfection efficiency using *Renilla* luciferase. Stable 729 transfectants containing proviral clones were isolated as described\textsuperscript{99} and cell clones were screened by p19 Gag expression in the cell supernatant by ELISA.

**DNA preparation and standard PCR**

DNA was isolated from 729 producer cell lines, immortalized human PBMCs, or infected rabbit PBMCs using PURGENE® DNA purification system (Gentra, Minneapolis, MN.). DNA (0.5 µg) was subjected to 35 cycle polymerase chain reaction (PCR) (95 for 5’, 95\textdegree{}C 1’, 56\textdegree{}C 1’, 72\textdegree{}C 1’, followed by 72\textdegree{}C 10’ and held at 4 \textdegree{}C). The HTLV-1-specific primer pair \textsuperscript{7145}GTCAAGCACAGCTTCCTCCTC\textsuperscript{7168} and \textsuperscript{7386}GGGGCACCAGTCGCCTTGTACACA\textsuperscript{7363} was used to amplify a 241 base pair fragment for sequencing to confirm the ΔHBZ mutation. The HTLV-1-specific primer
pair H1JA1 \(6659\)\text{TTATTGCAACCACATCGCCTCCAGCCTCCC}\(^{6688}\) and H1JA2 \(6885\)\text{AGGAGCGCCGTGAGCGCAAGT}\(^{6865}\) was used to amplify a 226 base pair fragment that was subjected to \textit{NheI} diagnostic digestion and sequenced to confirm the HBZ\(\Delta\)LZ mutation.

**RT-PCR and quantitative Taqman real-time RT-PCR**

RNA was extracted using the RNeasy kit (Quiagen, Valencia CA) from SLB-1, 729.ACHneo, and 729 uninfected control cells. Total RNA was subjected to three consecutive DNase treatments followed by OLIGOTEX polyA+ mRNA isolation (Quiagen, Valencia, CA). PolyA+ mRNA (25 ng) was subjected to reverse transcriptase (RT)-PCR using the first strand synthesis kit and the primer HMS\(^{6659}\)\text{TTATTGCAACCACATCGCCTCCAGCCTCCC}\(^{6688}\) (1uM) to generate cDNA from the minus strand \(Hbz\) transcript RNA. Duplicate reactions were performed in the presence and absence of RT to control for DNA background/contamination. The cDNAs then were subjected to standard PCR using the HTLV-1-specific primer pair H1JA1 and H1JA2. Human GAPDH was amplified using primers previously described.\(^{92}\) PCR amplified fragments were separated on a 1% agarose gel and visualized by ethidium bromide staining. Forty cycles of real-time Taqman® PCR were conducted to quantitate proviral copy number per cell in infected rabbit PBMC. Rabbit PBMC DNA was amplified in duplicate using the primers TaxS\(^{7335}\)\text{CGGATACCCCAGTCTACGTGTTT}\(^{7356}\) and TaxAS\(^{7495}\)\text{CTGAGCCGATAACGCATGTTCA}\(^{7476}\) and probe (5'-FAM-\text{ATCACCTGGGACCCCATCGATGGA}\(^{7476}\)-TAMARA-3') and final values were
averaged. The 25-µl reactions contained 500 ng rabbit PBMC DNA, 100 ng (25 ng/mL) of each primer and probe concentration of 100pmol/ul. Copy number was determined based on a standard curve generated from duplicate samples of log_{10} dilutions of a plasmid containing the Tax sequences. The copy number per cell value for a sample was generated based on the estimation that 1 µg of PBMC DNA is equivalent to 67,300 cells.

**Western blot**

Western blot was performed as described using rabbit anti pHBZ polyclonal antisera (1:500) and goat anti rabbit-conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ).

**Short-term coculture microtiter proliferation and long-term immortalization assays**

Short-term microtiter proliferation assays were performed as described previously. Briefly, 729 HTLV producer cells (200) were irradiated with 100 Gy (10,000 rads) and cocultured with 10^4 pre-stimulated PBMCs in the presence of human (h) IL-2 in 96-well round bottom plates. Wells were enumerated for growth and split 1:4 at weekly intervals. For long-term immortalization assays, 10^6 irradiated 729 producer cells were cocultivated with 2 × 10^6 freshly isolated PBMCs with 10U/ml hIL-2 in 24-well culture plates. The presence of HTLV expression was confirmed at weekly intervals by detection of p19 Gag protein in the culture supernatant using an ELISA. Viable cells also were counted by trypan blue exclusion. Cells inoculated with wtHTLV-1 and HTLV-1 HBZ mutant viruses that continued to produce p19 Gag antigen and
proliferate 12 weeks post-coculture in the presence of exogenous hIL-2 were identified as immortalized.

**Rabbit inoculation procedure**

Twelve-week-old specific pathogen-free female New Zealand White rabbits (Hazelton, Kalamazoo, MI) were inoculated via the lateral ear vein with approximately $1 \times 10^7$ gamma-irradiated 75 Gy (7,500 rads) 729 viral producer cells or uninfected control cells. At weeks 0, 2, 4, 6, and 8 after inoculation, 10 mL of blood were drawn from the central ear artery of each animal. This study protocol was approved by the University Laboratory Animal Resources (ULAR) of the Ohio State University. Serum reactivity to specific viral antigenic determinants was detected using a commercial HTLV-1 western blot assay (GeneLabs Diagnostics, Singapore). Serum (dilution of 1:400) showing reactivity to Gag (p24 or p19) and Env (gp21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity (data not shown). A commercial ELISA kit (Vironostika HTLV-1 MicroELISA system, BioMerieux Inc., Durham, NC) was used to quantitate HTLV-1 serum antibody. Plasma was diluted 1:12,000 to obtain values in the linear range of the assay and data were expressed as absorbance values.
2.4 Results

The $Hbz$ transcript was detected in HTLV-1 transformed and stably transfected cell lines.

It was reported previously that the $Hbz$ transcript was present in HTLV-1 infected SLB-1 cells, but in a relatively low quantity as compared to $tax/rex$ mRNA. In addition, the HBZ protein was detected in the HTLV-1 infected cell line C8166, but could not be detected in MT-2 cells. Since our studies make use of the HTLV-1 molecular proviral clone ACH, initial experiments were designed to verify the expression of the $Hbz$ mRNA transcript in the HTLV-1 producer cell line 729.ACHneo (referred to as 729.HTLV-1). This cell line produces HTLV-1 that has the capacity to infect and transform human PBMCs. Poly A+ RNA isolated from SLB-1, 729.HTLV-1, and uninfected 729 cells was subjected to standard RT-PCR in the presence and absence of reverse transcriptase. $Hbz$ mRNA was clearly detected in SLB-1 and 729.HTLV-1, but absent in 729 uninfected cells (Figure 2.1A). In addition, reactions in which the reverse transcriptase was omitted indicated that no amplified product resulting from DNA contamination was detected. Therefore, the ACH molecular clone produced a minus strand transcript reported to encode HBZ.

Construction and characterization of HTLV-1 HBZ mutant proviral clones.

Although informative, studies to date investigating the function or activity of HBZ have been performed in over expression systems outside the context of the entire provirus. In order to determine the role of HBZ in HTLV-1-mediated cellular
immortalization *in vitro* and viral persistence *in vivo*, we generated two mutant proviral clones. HTLV-1HBZΔLZ has a deletion in the C-terminal leucine zipper that is a key functional domain for b-ZIP proteins and is required for the association of HBZ with CREB-2, JunB and c-Jun.\(^{176,183,233}\) HTLV-1ΔHBZ has a severely truncated HBZ within the first 10 amino acids (Figure 2.1B). We first determined whether HBZ mutant proviruses had altered Tax-mediated LTR gene expression. Cotransfection of either HBZ wild type or HBZ mutant HTLV-1 proviral clones, as a source of Tax, and the LTR-1-Luc reporter revealed no significant difference in LTR-directed gene expression (Figure 2.2A). Moreover, cells transfected with either HBZ mutant proviral clone produced levels of p19 Gag in the culture supernatant similar to wild type HTLV-1 (Figure 2.2B). We next determined the effect of exogenously expressed HBZ on Tax-mediated transcription. Our results indicated that HBZ expressed from a cDNA vector significantly repressed Tax-mediated transcription in dose-dependent manner (Figure 2.3A). Importantly, the HBZΔLZ cDNA expression vector failed to repress Tax-mediated transcription (Figure 2.3A). In direct correlation, the addition of HBZ, but not HBZΔLZ, resulted in a dose-dependent reduction of p19 Gag in the supernatant of transfected cells (Figure 2.3B). Western blot analysis confirmed that the amount of HBZ or HBZΔLZ protein expressed correlated directly with the amount of plasmid DNA transfected, whereas a control cellular protein, β-actin remained unchanged (Figure 2.3C). Taken together these data support previous published work that the leucine zipper is a key functional domain required for protein interaction and transcriptional repression\(^{176,183,233}\) and also indicates that the repression is not the result of a significant RNA interference (RNAi) effect on Tax/Rex or Gag/Pol. These results are also consistent with the
conclusion that either HBZ is not expressed from the proviral clone following transient transfection or that the levels of HBZ expressed from the proviral clone are below the threshold concentration required for a detectable repression of viral transcription.

**HBZ repressed viral gene expression in stable viral producer cell clones.**

To determine the capacity of HBZ mutant proviral clones to synthesize viral proteins, direct viral replication, and induce cellular immortalization, stable 729 cell transfectants expressing the proviral clones were isolated and characterized. Each stable transfectant contained complete copies of the provirus with the expected mutations (data not shown). Using western blot analyses, HBZ was detected in the HTLV-1 transformed cell line SLB-1 and the virus producer cell line 729.HTLV-1. A smaller form of HBZ was detected in 729.HTLV-1HBZΔLZ consistent with the truncation and as expected HBZ was not detected in 729.HTLV-1ΔHBZ or in the negative control 729 (Figure 2.4A). To monitor the production of another viral protein in these mutant stable transfectants, the concentration of p19 Gag in the culture supernatant of several independently isolated cell clones was quantified by ELISA. p19 Gag expression can be variable from independent stable cell clones attributable to chromosomal location of proviral sequences and overall proviral copy number. However, we consistently observed a significant increase in p19 Gag production in cell clones expressing HBZ mutant proviral clones (Figure 2.4B), which is consistent with the conclusion that in stable cell lines the loss of HBZ function results in increased viral gene expression.
HBZ-deficient mutants promoted HTLV-1-induced proliferation and immortalization of PBMCs.

We next assessed the capacity of the HBZ mutant viruses to immortalize human PBMCs in coculture assays. Freshly isolated human PBMCs cocultured with lethally irradiated 729.HTLV-1, 729.HTLV-1ΔHBZ, or 729.HTLV-1HBZΔLZ in the presence of 10 U/ml of human IL-2 showed very similar progressive growth patterns consistent with the HTLV-1 immortalization process (Figure 2.5A). We also detected continuous accumulation of p19 Gag in the culture supernatant indicating viral replication and virion production (Figure 2.5B). Immortalized PBMCs harbored the expected HTLV-1 sequences suggesting that viral transmission was responsible for the immortalization of PBMCs (Figure 2.5C and data not shown). In an effort to obtain a more quantitative measure of the ability of these viruses to infect and immortalize PBMCs, a fixed number of PBMCs were cocultured with different dilutions of virus-producing cells in a 96-well plate assay. Since this assay is very stringent, slowly growing or non-dividing cells are eliminated very quickly and the percentage of surviving wells is an accurate measure of the immortalization efficiency of viruses. A Kaplan-Meier plot of HTLV-1-induced T-cell proliferation indicated that the percentage of wells containing proliferating lymphocytes was similar between HTLV-1 and HTLV-1 HBZ mutants (Figure 2.5D). Taken together, our results are consistent with the conclusion that HBZ is not required for efficient infectivity or HTLV-1-mediated immortalization of primary human T-lymphocytes in vitro.
HTLV-1 HBZ enhanced infectivity and persistence in inoculated rabbits.

To evaluate the role of HBZ in vivo, we compared the abilities of 729, 729.HTLV-1, 729.HTLV-1ΔHBZ, or 729.HTLV-1HBZΔLZ cell lines to transmit virus to rabbits, which is an established model of infection and persistence. Rabbits were inoculated with lethally irradiated cell lines (cell inoculums were equilibrated based on their p19 Gag production) and blood was drawn at biweekly intervals. Antibody response to viral antigens was detected by western blot in all rabbits inoculated with cells expressing either wild type HTLV-1 or HBZ mutant viruses, and the antibody titers in the majority of the rabbits increased over the time course of the study (data not shown). Moreover, quantitative comparison of antibody responses between each rabbit was performed using a HTLV-specific ELISA (Figure 2.6A). Statistical analysis of titers at both four and eight weeks post-inoculation revealed a significantly lower antibody response to HTLV-1 antigens in the 729.HTLV-1HBZΔLZ and 729.HTLV-1ΔHBZ-inoculated rabbits as compared to the wild-type control group. Consistent with our antibody data, HTLV-1 DNA sequences were detected in all wild type HTLV-1 and HBZ mutant virus-infected rabbits (Figure 2.6B). However, quantitative real-time Taqman PCR revealed that as early as two weeks post inoculation, and later at eight weeks post inoculation, proviral loads in rabbits infected with either HBZ mutant virus were lower than rabbits inoculated with wild type HTLV-1 (Figure 2.6B). Proviral loads in both wild type and HBZ mutant virus-infected rabbits correlated with the observed antibody responses (Figure 2.6A). Diagnostic DNA PCR analyses and or nucleotide sequencing performed on PBMCs from rabbits eight weeks post-infection indicated that the infected cells contained the expected viral sequences (Figure 2.6C and data not shown).
together, our results indicated that HBZ, while dispensable for HTLV-1 infection, attenuated parameters of virus replication such as antibody response to viral antigens and proviral loads, which were decreased compared to wild type HTLV-1 infected rabbits. This attenuation is apparent within two weeks post inoculation suggesting that HBZ function is required early for efficient replication and survival in the host. In addition, since ΔHBZ and HBZΔLZ mutants displayed a similar phenotype in vivo, we concluded that the leucine zipper domain was critical for HBZ functional activity.

2.5 Discussion

The role of the novel HTLV-1 negative strand gene product, HBZ, in viral replication and/or pathogenesis remains to be defined. Exogenously over expressed HBZ has been shown to interact with several cellular transcriptional factors such as the cAMP response element binding protein (CREB)-2, JunD, JunB and c-Jun and is a negative regulator of Tax-mediated HTLV-1 transcription. In the present study, site directed mutations were introduced in an infectious molecular clone of HTLV-1 to severely truncate HBZ or delete the carboxy terminal leucine zipper domain, while maintaining the ability to express other viral gene products. We examined the expression of HBZ and determined its biological significance for the immortalization of primary human T lymphocytes in vitro and viral persistence in vivo. We showed that the HBZ antisense viral transcript (Figure 2.1A) and protein (Figure 2.4A) were expressed from the ACH HTLV-1 molecular proviral clone. Consistent with a previous report, quantitative real-time RT-PCR revealed that, although variable with each individual cell line, the level of HBZ mRNA transcription was approximately 20 to 50-fold lower than
the abundant tax/rex mRNA (data not shown). We observed that in the context of a proviral clone, the repressive effects of HBZ on Tax transcription were not apparent following transient transfection (Figure 2.2A), but confirmed that over expression of HBZ from a cDNA plasmid down-regulated Tax-mediated viral transcription (Figure 2.3A). We further demonstrated that the repressive effects of HBZ on Tax-mediated transcription were detectable in cell lines stably harboring the proviral clone (Figure 2.4B). Therefore, we speculate that Hbz gene expression is temporally regulated and not expressed following transient proviral DNA plasmid delivery or that a threshold level of HBZ is required for the repressive activity.

Data from our short-term proliferation and immortalization assays indicated that the reported repressive effects of the HTLV-1 HBZ on Tax and AP-1\textsuperscript{183,233} were not sufficient to disrupt the capacity of the virus to infect, induce proliferation, and/or immortalize primary T lymphocytes \textit{in vitro} (Figure 2.5A and 2.5D). Therefore, similar to the HTLV-1 pX open reading frame (ORF) I and II encoded accessory proteins\textsuperscript{164,238}, HBZ appears to be dispensable for efficient viral infectivity, replication and primary T-lymphocyte immortalization capacity \textit{in vitro}.

Based on the efficient infectivity and immortalization of cells \textit{in vitro} and our findings that 729-HTLV-1ΔHBZ and 729-HTLV-1HBZΔLZ inoculated rabbits became infected with HTLV-1, we hypothesized that the biological function of HBZ and its role in HTLV-1 biology is not likely during the early phase of viral infection. However, future experiments designed to quantitatively assess viral infectivity of rabbits at 1-2 days post inoculation will ultimately be required to definitively rule out an early block in infection \textit{in vivo}. It is clear that soon after inoculation, some parameters of viral
replication are attenuated in HTLV-1 HBZ mutant virus-infected animals. We begin to observe a reduction in proviral load as early as two weeks post inoculation as compared to the wild type virus (Figure 2.6B). By eight weeks HBZ mutant inoculated rabbits show a significant reduction in antibody response to viral gene products and a 15 to 20 fold drop in proviral load as compared to the wild type virus (Figure 2.6A and 2.6B). Combined with the data that HTLV-1 HBZ repressed Tax-mediated transcription and attenuated AP-1 activity, we speculated that HBZ might function in concert with other viral gene products to tightly regulate viral replication and/or influence the infected lymphocyte to ultimately promote infected cell survival, viral spread, and assist in establishment of persistent infection. Interestingly, HBZ’s repressive function and potential contribution to HTLV-1 replication or leukemogenesis may be somewhat analogous to what has been reported for the HTLV-1 p30\textsuperscript{II} accessory protein\textsuperscript{239}. p30\textsuperscript{II} selectively increases key regulatory genes, influences T-cell signaling, apoptosis, and the cell cycle, but overall represses cellular gene expression. Many of these affects appear to counteract the HTLV-1 oncoprotein Tax. It remains a possibility that HBZ and p30\textsuperscript{II} work synergistically to ultimately modulate viral and cellular gene expression during different stages of the infection to promote virus survival.

Since HBZ is expressed from an antisense transcript it might be predicted to result in a short interfering RNA (siRNA) or dsRNA effect ultimately resulting in a nucleic acid-based adaptive immunity. Recent evidence indicates that that HIV-1 encodes viral siRNA precursors in its genome and provokes an RNA silencing response\textsuperscript{240}. However, a domain of the HIV-1 Tat protein has been identified which suppresses the production of siRNAs and the cell’s RNA silencing defense\textsuperscript{240}. Thus far, there is no evidence to suggest
that the *Hbz* antisense transcript induces RNA interference (RNAi). On the contrary, data presented in Figure 2.4A and 2.4B indicates that the HBZΔLZ expression vector, which makes the same RNA transcript as the HBZ vector with the exception of four nucleotide changes, fails to repress Tax activity and p19 Gag protein expression. It still however remains a possibility that the *Hbz* antisense transcript may have an RNAi effect on the ORF I or II encoded proteins, but this is currently difficult to test since these proteins are not expressed at detectable levels from the provirus. It is exciting to speculate that HTLV-1, like HIV-1 may also have a suppressor of RNA silencing, but further studies are required to determine if the virus encodes such an activity.

In summary, our work provides the first demonstration that retroviruses utilize novel negative strand gene products to enhance virus replication *in vivo*. Further studies are warranted to explore the effects of HTLV-1 HBZ on repression of viral transcription and its potential role in innate, cytotoxic T cell, and NK cell immune surveillance. More broadly, our data elucidate future directions for studies to understand the role of negative strand-encoded proteins in retroviral mediated disease and offer new targets for therapy to disrupt virus replication in infected subjects.

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**Figures**

**Figure 2.1:** Detection of the *Hbz* RNA transcript. (A) *Hbz* transcript was detected in Poly A+ RNA isolated from SLB-1 and 729.HTLV-1 using standard RT-PCR. First strand synthesis with a specific oligo designed to copy only HTLV-1 antisense RNA containing the HBZ coding sequence was performed in the presence and absence of reverse transcriptase. The 226 bp PCR product was separated on a 2% agarose gel and visualized by ethidium bromide staining. (B) Schematic representation of the complete HTLV-1 proviral genome is shown. LTRs are depicted with their U3, R, U5 regions. The location of the viral open reading frames and the opposite strand HBZ are indicated. The reported HBZ coding sequence has been expanded showing the transactivation domain, basic region, and leucine-zipper region and the two HBZ truncation mutants generated for this study (*HBZΔLZ* and *ΔHBZ*).
Figure 2.2: Characterization of proviral clones *in vitro*. 293 T cells (1.5x10^5) were cotransfected with 2 µg of wtHTLV-1, HTLV-1ΔHBZ, HTLV-1HBZΔLZ proviral clones or negative control DNA along with 0.1 µg of LTR-1-Luc and 0.01 µg of TK-Renilla. All transfections were performed in triplicate and normalized to TK-Renilla to control for transfection efficiency. Cell lysates or supernatants were harvested 48 h post-transfection. (A) Measure of Tax activity presented as relative luciferase units. (B) Measure of p19 Gag in the cellular supernatants.
Figure 2.3: Exogenously expressed HBZ results in dose-dependent repression of Tax-mediated transcription and p19 Gag production. 293T cells (1.5x10^5) were cotransfected with 1 µg of wtHTLV-1 proviral clone or negative control DNA, 0.1 µg of LTR-1-Luc and 0.01 µg of TK-Renilla, and varying concentrations (0.1-0.4 µg) of HBZ or HBZΔLZ expression vectors as indicated. (A) Tax function was measured as firefly luciferase activity (RLU, relative light units) from LTR-Luc normalized to Renilla luciferase activity. (B) Culture supernatant was collected from cells in panel A and assayed for p19 Gag production by ELISA. (C) Western blot analysis to confirm increasing
concentrations of HBZ and HBZΔLZ used in panels A and B. β-actin levels were assessed as a loading control. (*) represents statistically significant dose-dependent reduction of Tax transactivation activity or p19 Gag production. Statistical significance was determined by ANOVA followed by Tukey’s test.
Figure 2.4: HBZ and p19 Gag protein expression in stably transfected cell lines. (A) HBZ protein was detected in stable provirus expressing cell lines by western blot using a rabbit polyclonal antibody against HBZ. HBZ was not detected in 729 control and 729.HTLV-1ΔHBZ as expected. HBZ polypeptide of the expected molecular weight was detected in SLB-1, 729.HTLV-1, and 729.HTLV-1HBZΔLZ. (B) HTLV-1 p19 Gag was quantified by ELISA from three independently isolated stable 729 transfectants expressing wild type HTLV-1, HTLV-1ΔHBZ, or HTLV-1HBZΔLZ. HBZ mutant virus producers expressed statistically greater amounts of p19 Gag than wild type virus producers consistent with a repressive role of HBZ on viral transcription. p19 Gag production of the two mutant virus producers (HTLV-1ΔHBZ and HTLV-1HBZΔLZ) were not statistical significant. Statistical significance was determined by ANOVA followed by Tukey’s test.
Figure 2.5: HTLV-1 T-lymphocyte proliferation and immortalization assays. PBMC (2x10^6) donor cells were cultured with (1x10^6) irradiated producer cells as indicated in 24 well plates. (A) Representative growth curve is presented showing cell viability at weekly intervals. The mean and standard deviation of each time point was determined from three random independent samples. (B) HTLV-1 gene expression was quantified by
detection of Gag protein in the culture supernatant using ELISA. (C) The HTLV-1 genome fragment containing the HBZ coding region was amplified by PCR from DNA of immortalized PBMCs as indicated (HTLV-1HBZΔLZ DNA was cut by Nhel). (D) Prestimulated PBMCs (10^4) were cocultured with 2000 irradiated 729 stable producer cells in 96 well plates. The Kaplan-Meier plot shows the percentages of proliferating wells as a function of time (wks). Results indicated that the percentage of wells containing proliferating lymphocytes was similar between wtHTLV-1 and HBZ mutant viruses.
**Figure 2.6:** Assessment of HTLV-1 infection in rabbits. (A) Antibody response against HTLV-1 from each rabbit was measured by anti-HTLV-1 ELISA assay, using both HTLV-1 Gag and envelope proteins as antigens. Each dot represents the absorbance value of a single inoculated rabbit at 0, 2, 4, 6, and 8 wks post inoculation within each group. Inoculum as indicated below includes 729.HTLV-1 (n=5), 729.HTLV-1ΔHBZ (n=6), 729.HTLV-1ΔHBZΔLZ (n=6), or 729/media (n=2). The horizontal line represents the average of the rabbit group at each weekly time point and the dotted line represents
three times the standard deviation of uninfected control values. (B) Genomic DNA was isolated from rabbit PBMCs and subjected to standard PCR using HTLV-1-specific primers (670/671). No amplified PCR fragment (-); Amplified PCR fragment (+). Genomic DNA was isolated from rabbit PBMCs (wk 2 and 8 post inoculation) and subjected to real time Taqman PCR. Numbers in parentheses at wk 2 and 8 denote copy number per cell in rabbit PBMC. Copy numbers in rabbits inoculated with the mutant proviruses at wk 8 were significantly different than wild type as determined by ANOVA followed by Tukey’s test (p<0.00032). (C) The HTLV-1 genome fragment containing the HBZ coding region was amplified by PCR from DNA of PBMCs from a representative of at least one rabbit of each group (wk 8 post inoculation). The expected HBZ mutations in rabbit PBMCs were confirmed using the diagnostic restriction enzyme NheI for HTLV-1HBZΔLZ and further confirmed by nucleotide sequencing (data not shown).
CHAPTER 3

HUMAN T-CELL LEUKEMIA VIRUS TYPE-1 ANTISENSE-ENCODED GENE, HBZ, PROMOTES T LYMPHOCYTE PROLIFERATION

3.1 Abstract

Human T-cell leukemia virus type 1 (HTLV-1) basic leucine zipper factor (HBZ) is dispensable for HTLV-1-mediated cellular transformation in cell culture, but is required for efficient viral infectivity and persistence in inoculated rabbits. In most adult T-cell leukemia (ATL) cells, Tax oncoprotein expression is typically low or undetectable, whereas Hbz gene expression is maintained suggesting that Hbz may support infected cell survival and ultimately, leukemogenesis. Emerging data indicates that HBZ protein functions by interacting with CREB and Jun family members altering transcription factor binding and transactivation of both viral and cellular promoters. Herein, lentiviral vectors that express Hbz-specific short hairpin (sh)RNA effectively decreased both Hbz mRNA and protein expression in transduced HTLV-1-transformed SLB-1 T-cells. Hbz knockdown correlated with a significant decrease in T-cell proliferation in culture. Both SLB-1 and SLB-1-Hbz knockdown cells engrafted into inoculated NOD/SCIDγchain-/- mice to form solid clustered tumor masses that infiltrated into surrounding tissues. However, tumor formation and tumor cell organ tissue progression was significantly decreased in animals challenged with SLB-1-Hbz knockdown cells. Taken together our
data indicate that $Hbz$ expression enhances the proliferative capacity of HTLV-1 infected T-cells playing a critical role in cell survival and ultimately HTLV-1 tumorigenesis in the infected host.

### 3.2 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection causes adult T-cell leukemia/lymphoma (ATLL) and is associated with a variety of lymphocyte-mediated disorders.\(^{241}\) Although HTLV-1 can immortalize/transform primary human T-lymphocytes efficiently in culture, the disease penetrance within infected individuals is only 2-5% and typically requires an extensive latency period. Following infection of the T-lymphocyte, ATLL is thought to arise from a multitude of factors that include both genetic and epigenetic changes in the cell that accumulate over time.\(^{111}\) Although many aspects of HTLV-1 biology have been elucidated, the detailed mechanisms behind ATLL development and occurrence remains ill-defined.

HTLV-1 is a highly cell-associated virus; thus, efficient infection requires direct cell-to-cell contact.\(^{76}\) In the infected individual, the increase in viral progeny occurs primarily by clonal expansion of the infected T-cells, which is dependent on the action of the positive regulatory ($\text{tax}$ and $\text{rex}$) and accessory genes ($\text{p13}$, $\text{p30}$, and $\text{p12}$) encoded on the plus strand of the genome.\(^{111}\) Although the precise mechanism remains unclear, the accessory gene products are dispensable in standard culture assays of virus-mediated cellular transformation, but are important for viral infectivity and persistence \textit{in vivo}.\(^{163,165,172,242}\) Tax is the viral transcriptional transactivator that also activates many cellular signaling pathways including nuclear factor kappa-B (NF-KB), cAMP response
element binding protein (CREB), activator protein-1 (AP-1), and serum responsive factor (SRF). In addition, Tax has been shown to inactivate p53, which results in inhibition of apoptosis. Tax can activate cyclin-dependent kinases, CDK-4 and CDK-6, through direct protein binding leading to the hyperphosphorylation and degradation of retinoblastoma (RB), which then frees the E2F1 transcription factor, thereby accelerating cell-cycle transition from G1 to S. Compelling experimental evidence suggests that these pleiotropic effects of Tax on cell biology are required for the transforming or oncogenic capacity of HTLV-1 and thus are critical in the early stage of the malignant process. However, there remains a disconnect between tax gene expression and ATLL. Although most leukemic cells isolated from patients maintain the 3’ portion of the HTLV-1 genome, Tax typically is not expressed, which has been attributed to a deletion of the 5’ LTR/promoter or to gene inactivation by mutations or DNA methylation. Thus, the loss of Tax expression actually may enable ATLL cells to evade host immune surveillance, since Tax elicits a strong CTL response in vivo and Tax-expressing cells would rapidly be targeted for elimination.

The HTLV-1 basic leucine zipper factor gene (Hbz) is located in the 3’ portion of the provirus and is maintained in ATLL cells. Unlike the other HTLV-1 genes, Hbz is encoded by the minus or antisense strand of the genome. Both spliced and unspliced Hbz transcripts with the potential to encode highly related protein isoforms have been detected in most ATLL cells. Exogenously over-expressed HBZ protein interacts with CREB-2 to down-regulate Tax-mediated HTLV-1 transcription and also interacts with and disrupts the DNA binding activity of JunB and c-Jun (AP-1 components). HBZ also interacts with JunD to activate the transcription of JunD-
dependent promoters. One recent mutational study provided additional evidence that the Hbz mRNA, not HBZ protein, had the capacity to induce T-cell proliferation by up-regulating cellular E2F1, which has lead to the novel hypothesis that Hbz exists in two molecular forms (RNA and protein) that may play an important role in HTLV-1 biology, promoting cell survival by counteracting the effects of Tax-mediated transcription and/or attenuating or activating cellular gene expression. Consistent with this hypothesis, previous studies from our lab utilizing an HBZ deficient infectious molecular clone indicated that the HBZ protein is dispensable for immortalization/transformation of primary T-lymphocytes in cell culture, but in a rabbit animal model HBZ protein knockout viruses were attenuated for viral infectivity, replication, and persistence.

In this study, we generated and utilized a panel of lentiviral vectors that express Hbz-specific short hairpin (sh)RNAs to determine the role of Hbz in T-cell proliferation in vitro and tumor growth in the NOD/SCIDγc-/- (NOG) transplant mouse model. We show that Hbz-specific shRNAs effectively decreased both Hbz mRNA and protein expression, which correlated with significantly decreased cellular proliferation of an established HTLV-1 T-cell line and newly HTLV-1 immortalized IL-2-dependent T-lymphocytes. Importantly, we show directly that global viral mRNA and protein levels in vector transduced cells were unaffected by Hbz-specific shRNA expression. In NOG mice, wild-type and Hbz-knockdown SLB-1 cells engrafted to form solid clustered tumor masses that infiltrated into surrounding organ tissues, but tumor formation and tumor cell organ tissue progression were reduced significantly in animals challenged with SLB-1 Hbz-knockdown cells. Taken together, our data are consistent with the conclusions that Hbz expression enhances the proliferative capacity of HTLV-1 infected cells and plays a
critical role in cell survival and ultimately, HTLV-1 tumorigenesis.

3.3 Materials and methods

Cells

293T cells were maintained in Dulbecco’s modified Eagle medium. SLB-1, 729ACH, MT-1, MT-2, C8166, and PBLACH (HTLV-1 positive cell lines) and HTLV-1 negative Jurkat T-cells were maintained in Iscove’s medium. All media were supplemented to contain 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). PBLACH, in vitro immortalized human peripheral blood T-lymphocytes, also were supplemented with recombinant human IL-2 at 10U/mL.

Plasmids

The pME-based HBZ cDNA expression vector (based on the Hbz major transcript) was described previously. The third generation (pRNAT-U6.2/Lenti) lentiviral vector designed to generate small hairpin (sh) RNA from the U6.2 promoter and GFP from the cytomegalovirus (CMV) promoter was purchased from the GenScript Corporation (Piscataway, NJ). The vector control with no insert was designated V1. The Hbz-specific and scrambled target sequences were cloned into the lentiviral vector using the unique restriction sites BamHI and XhoI. Vectors expressing Hbz transcript target sequences (see Figure 3.1 for location) were: V2, 5’ AGGACAAGGAGGAGGAGG 3’; V3, 5’ ACAGCATAGTGCTAGGAAA 3’, and; V4, 5’ CGGCCTCAGGGCTGTTTCG 3’. Sequence and numbering are based on
the positive sense nucleotide sequence of the ACH proviral clone. V2 and V3 target sequences were previously described, siRNA4 and siRNA31, respectively and are expected to target all Hbz transcripts, but also have the potential for off target affects on HTLV-1 sense strand mRNAs. The target sequence for V4 was designed specifically to span the splice junction and target the Hbz major transcript (Hbz-S1). V5 contains a scrambled nucleotide sequence designed to form a non-specific hairpin shRNA and not target Hbz, other HTLV-1 genes, or cellular genes.

**Transient transfection**

To measure the ability of each lentiviral vector plasmid to knock down HBZ protein expression transiently, $1.5 \times 10^5$ 293T cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA). The amount of DNA was kept constant and was composed of 0.2 µg of HBZ cDNA plasmid along with 1 µg lentiviral vector plasmid (V1-V5) or empty plasmid DNA as negative control. Three independent wells were transfected for each sample and time point. Cells were harvested at 24 and 48 h and lysed in 1X passive lysis buffer (Promega, Madison, WI). All experiments were performed independently three times in triplicate. Transfection efficiency was determined by normalization to GFP expression as measured by fluorescence microscopy.

**Western blot**

Western blot analysis was performed as described on transiently transfected 293T cells, HTLV-1 positive cell lines, and stable vector infected SLB-1 cell clones using rabbit anti-HBZ polyclonal antiserum (1:500) and goat anti rabbit-conjugated with
horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). GFP and β-actin were detected using (1:1000) anti-GFP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin rabbit monoclonal antibody, respectively, and goat-anti rabbit conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). HTLV-1 Tax was detected using (1:1000) mouse hybridoma monoclonal antibody and HRP-conjugated goat anti-mouse secondary antibody. Proteins were visualized using the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ).

Virus particle production and cell infection

Plasmid lentiviral vectors (V1-V5) were isolated from bacterial cultures and sent to the Ohio State University Comprehensive Cancer Center/Cincinnati Children’s Hospital Medical Center viral vector core or virus particle production. Lentiviruses were produced by transient cotransfection of lentiviral plasmid vectors and packaging plasmids pCDNA3.g/p.4xCTE, pRSV-rev, and M75-VSV-G. 293T cells were transfected using the calcium phosphate method (Sigma Aldrich) in DMEM/10% FBS in the presence of 25 mM chloroquine. At 12-18 h post-transfection, the media was changed and supernatants were collected at four 10-14 h intervals, 0.45 micron filtered, and frozen at -80°C. Supernatants then were concentrated by size exclusion using a 100 kDa filter (VivaScience) and infectious titers were determined by expression of GFP on HT1080 cells four days post-transduction. SLB-1 cells were infected at a multiplicity of infection (MOI) of two in supplemented Iscove’s media along with 8 ug/mL polybrene (Sigma). At 48 h post infection, viral inocula were removed and infected cells were selected by
growth in 1 mg/ml Geneticin (Gibco, Carlsbad, CA). Stable clones were isolated in bulk cultures four weeks following initial selection.

**RT-PCR and quantitative Taqman real-time RT-PCR**

Total RNA from $10^7$ HTLV-1 positive cell lines, stable lentivirus-infected SLB-1 cell lines (V1-V5), and uninfected Jurkat control cells was extracted using the RNeasy kit and DNase treated column (Quiagen, Valencia, CA.). Two ug of total RNA were subjected to reverse transcriptase (RT)-PCR using (1uM) oligo DT primer for first strand synthesis to generate cDNA libraries (Invitrogen). Forty cycles of real-time Taqman® PCR were conducted to quantitate mRNA transcripts from cDNA libraries as indicated. cDNA was amplified in duplicate using the primers and probes for *Hbz*, *tax/rex*, and *gag/pol* sequences as described. The final numbers were averaged and transcript quantity was determined based on a standard curve generated from duplicate samples of log$_{10}$ dilutions of a plasmid containing the *Hbz*, *tax/rex*, and *gag/pol* DNA sequences. The copy of each transcript was normalized to $10^6$ copies of hGAPDH. hGAPDH was amplified using primers previously described.

**Proliferation assay and cell viability**

Cell Titer 96® Aqueous One Solution Cell Proliferation Assays were performed on mock SLB-1 and stable lentivirus-infected SLB-1 cell lines according to the manufacturer’s protocol (Promega, Madison, WI). Briefly, cells were counted and plated at 1000 cells/well in 96-well round bottom plates on day zero and monitored over a five day time course. Cell Titer 96® reagent was added to each well, agitated slightly, and
incubated at 37°C, 5% CO₂ for two h. The optical density absorbance at 490nm was collected on an ELISA plate reader. To verify cellular viability over time, the same cell lines were plated in 96-well round bottom plates and counted by trypan blue exclusion over the same five day time course. For each cell line, data represent three independent experiments performed in triplicate.

**Mouse inoculation procedure and IL-2Rα ELISA**

Fifteen, seven week-old, female NOD.Cg-PrkdcSCIDIL2rgtm1Wjl/SzJ mice (pathogen free) were obtained from Jackson Laboratories (Bar Harbor, MN). The mice were inoculated subcutaneously in the lumbar (flank) region with 1 x 10^7 parental SLB-1, SLB-1 V4 lentivirus infected (HBZ knockdown), SLB-1 V5 lentivirus infected (scrambled), or Jurkat T-cell lines. Serum was drawn from the anterior facial vein at days 0, 7, 15, and 22. Mice were monitored daily, weight was recorded three times per week, and tumor size was measured manually using hand calipers. Mice were euthanized and necropsied at day 22. At necropsy, tumor was isolated from the underlying tissue and weighed. Tumors were flash frozen and remaining mouse tissues were fixed in 10% neutral buffered formalin, trimmed, paraffin embedded, sectioned, and stained using routine hematoxylin and eosin. This study protocol was approved by the University Laboratory Animal Resources (ULAR) of the Ohio State University. Since SLB-1 cells are known to produce interleukin-2 receptor alpha (IL-2Rα), mouse serum reactivity to (IL-2Rα) was used as a biomarker for SLB-1 cell proliferation *in vivo*. IL-2rα serum levels were detected using a commercial Quantikine IL-2Rα ELISA (R & D Systems, Minneapolis, MN) using mouse serum diluted (1:10) to obtain absorbance values in the
linear range of the assay. Western blot analysis was performed on homogenized tumor tissue cell lysates using antibodies as described above.

3.4 Results

**Hbz mRNA and HBZ protein expression in HTLV-1 cell lines**

Both spliced (major, SP1; minor, SP2) and unspliced *Hbz* transcripts with the potential to encode highly related protein isoforms have been detected in most ATLL cells (see Figure 3.1A). The spliced SP1 HBZ protein isoform previously was detected in HTLV-1-positive cell lines MT-4, C8166, SLB-1, and 729Ach. Satou *et al* also reported the successful knockdown of *Hbz* mRNA in HTLV-1 cell lines using a small interfering RNA (siRNA) approach. Overall, they concluded from their studies that the *Hbz* mRNA had a proliferative affect on T-cells independent of Tax. However, these studies did not evaluate HBZ protein or the potential for off target effects of *Hbz* siRNA on other viral mRNAs or proteins. Our first objective was to quantitate *Hbz* expression in a panel of HTLV-1 cell lines and correlate mRNA levels to HBZ protein expression. *Hbz*-specific mRNA expression was detected, but variable, in all HTLV-1 positive cell lines (Figure 3.1B). We observed the highest *Hbz* mRNA copy number (normalized to hGAPDH) in established SLB-1 and C8166 cell lines with the next highest level displayed by newly HTLV-1 immortalized IL-2-dependent T-lymphocytes (PBLACH). Furthermore, HBZ protein expression directly correlated with the *Hbz* mRNA levels in all positive samples (Figure 3.1B). However, we were surprised to detect HBZ protein in MT-2 cells since a previous study reported the detection of *Hbz* mRNA, but not protein in MT-2.
**shRNA lentiviral vector design and transient knockdown of HBZ protein expression**

To address the role of *Hbz* in HTLV-1 immortalized/transformed cells both in cell culture and *in vivo*, we employed an shRNA approach to knockdown *Hbz* mRNA and protein expression. Lentiviral vectors generating short hairpin RNAs (shRNAs) against various sequences of *Hbz* were generated. Vector (V2) and (V3) target *Hbz* sequences identical to previously described siRNA4 and siRNA31, respectively, and have the potential to target all known *Hbz* transcripts. V4 was designed specifically to target the *Hbz* major spliced transcript; this target sequence spans the splice junction. Two additional negative controls including the empty vector (V1) and a vector expressing a scrambled non-specific sequence (V5) also were generated. In order to determine if the lentiviral vector plasmid DNAs encoded the capacity to knockdown *Hbz* gene expression, we initially tested them in transient transfection. 293 T cells were cotransfected in duplicate with an HBZ cDNA expression vector and lentiviral vectors (V1-V5). HBZ protein expression was measured by Western blot at 24 and 48 h post transfection. GFP and β-actin protein expression was used as internal controls for loading and transfection efficiency. The three *Hbz*-specific target vectors (V2-V4) had the capacity to significantly knock down HBZ protein expression at 24 h and, to a greater extent, at 48 h post transfection (Figure 3.2). Our results with V2 are in contrast to those previously reported in which the identical target sequence was found not to suppress *Hbz* mRNA expression.188
Stable siRNA suppression of Hbz in SLB-1 cells does not alter tax/rex or gag/pol expression

Lentivirus particles encoding the shRNAs against Hbz were generated and used to transduce HTLV-1 SLB-1-transformed T-cells. We focused our studies on the SLB-1 cell line due to high Hbz expression levels relative to the other cell lines and the previous successful use of this cell line in the mouse tumorigenicity model.249 SLB-1 cells were infected with lentiviruses and stably transduced cells were selected and expanded for four weeks in geneticin-containing growth media. To determine if a stable knockdown of Hbz expression was achieved, total RNA was isolated from each transduced line and subjected to real-time RT-PCR to quantitate Hbz mRNA copy number relative to cellular hGAPDH control. Hbz mRNA expression was significantly knocked down (approximately three-fold) in SLB-1 cells transduced by lentiviruses V2, V3, or V4 as compared to wild-type SLB-1 and SLB-1 transduced by control lentiviruses V1 or V5 (Figure 3.3A). Western blot analysis indicated that HBZ protein expression was reduced, which correlated with the Hbz mRNA suppression (Figure 3.3B). Next, we wanted to be sure that V2, V3, and V4 generated shRNAs targeted Hbz specifically and did not have any off-target affects on other key viral genes. Total RNA was subjected to real time RT-PCR to quantitate both tax/rex and gag/pol mRNA copy number. Our results showed that Hbz knockdown had no significant affect on tax/rex or gag/pol mRNA expression (Figure 3.3C). Moreover, p19 Gag production in the viral supernatant also was unaffected (Figure 3.3D). Taken together, these results indicate that Hbz mRNA and protein are significantly and specifically suppressed by Hbz-targeting shRNAs.
Knockdown of *Hbz* gene expression significantly suppresses SLB-1 proliferation

We next evaluated whether *Hbz* knockdown altered the proliferation of our stable lentivirus-transduced SLB-1 cells. Cells were plated at low concentrations and monitored daily for five days by standard MTS assay. The data indicates that knockdown of *Hbz* results in a significant decrease in proliferation (~33%), compared to mock and transduced controls (Figure 3.4A). In addition, cells also were monitored and enumerated by trypan blue exclusion to verify that the decrease in proliferation rate was not attributed to increased cell death (Figure 3.4B). We confirmed that the proliferating cells evaluated in the growth assay maintained the stable knockdown of HBZ protein. As presented in Figure 3.4C, HBZ protein expression was consistently and significantly knocked down four-fold, whereas Tax protein expression was not affected. Together, the data indicate that *Hbz* expression significantly supported the proliferation of SLB-1 cells and that the decrease in proliferation in the *Hbz* knockdown cells was not attributable to apoptosis. In general support of this conclusion, shRNA suppression of *Hbz* in newly immortalized HTLV-1 T-lymphocytes (PBLACH) or MT-1 cells also resulted in growth suppression similar to SLB-1 (data not shown).

Knockdown of *Hbz* gene expression decreases tumor size in NOG mice

To evaluate the role that *Hbz* plays in tumor cell growth and organ infiltration *in vivo*, NOG mice were inoculated subcutaneously with wild-type SLB-1, SLB-V4 (*Hbz* knockdown), SLB-V5 control (scrambled), or Jurkat cell control (n = 5 each). Following inoculation, mice were observed on a daily basis for tumor engraftment, and weighed every other day.
Since IL-2Rα is secreted by SLB-1 cells, it can be used as a biomarker for cellular proliferation in vivo. Blood was drawn from each mouse just prior to inoculation (day 0) and at days 7, 15, and 22 post inoculation and IL-2Rα was quantitated in the serum by ELISA. We observed a statistically significant decrease (~35-40%) of IL-2Rα in the serum of mice inoculated with SLB-V4 Hbz knockdown cells. Consistent with the ELISA data, mice inoculated with SLB-V4 also displayed a significant 49-58% average decrease in overall tumor size and volume on the day of sacrifice (day 22) as compared to SLB-V5 control and parental SLB-1 (Table 3.1). Western blot on tumor tissue lysates from SLB-V4-inoculated mice revealed three-to-five-fold less HBZ protein as compared to tumor material from a representative SLB-1 inoculated mouse (M2) or SLB-V5 inoculated mice (Figure 3.5B). Tax protein expression was similar in tumors of all inoculated mice consistent with the conclusion that tumor cell growth directly correlated with Hbz expression and not the expression level of the Tax oncoprotein. In addition, the decrease in HBZ protein expression in the tumors from SLB-V4-inoculated mice was consistent with the level of HBZ protein knockdown observed in culture experiments. At sacrifice, mouse tissues were examined histologically by routine H&E staining and Ki67 expression for cell proliferation and tumor cell infiltration. Table 3.2 presents an average score for tumor cell infiltration in select tissues from each of the inoculated groups. Tumor infiltration to liver, heart, lung, kidney, and pancreas tissue was dramatically lower in SLB-V4-inoculated mice. Figure 3.5C shows representative Ki67 staining of liver from mice inoculated with SLB-1, SLB-V4, or SLB-V5. Results demonstrated a dramatic reduction in proliferation and infiltration of liver tissue by SLB-V4 (Hbz knockdown) cells as compared to SLB-1 or SLB-V5 controls. Taken together, our results
showed that knockdown in *Hbz* gene expression significantly decreases proliferation of SLB-1 cells *in vivo*. The decrease in proliferation of the SLB-1 *Hbz* knockdown cells *in vivo* correlated with a decrease in tumor size and infiltration of tumor cells to surrounding tissue.

### 3.5 Discussion

The role of the HTLV-1 negative strand *Hbz* gene in viral replication and/or pathogenesis remains to be fully defined. In the context of an HTLV-1 molecular clone HBZ protein is dispensable for transformation/immortalization of T-lymphocytes in culture, but disruption of HBZ dramatically reduced viral infectivity and persistence in inoculated rabbits. Exogenously over-expressed HBZ interacts with cellular transcription factors of the Jun and CREB family of proteins and negatively regulates Tax-mediated and AP-1-mediated transcription. Interestingly, in addition to the functional properties of the HBZ protein, the secondary structure of the *Hbz* mRNA has been reported to support the proliferation of T-cells. Both spliced and unspliced *Hbz* transcripts with the potential to encode highly related protein isoforms have been described. The *Hbz* major spliced transcript (SP1) is the most abundant and is detected in multiple HTLV-1-infected cell lines and most all ATLL cells. In addition, our group and Murata *et al*, recently demonstrated by Western blot analyses and immunochemistry that *in vitro* transformed HTLV-1 and ATLL cells predominantly express the protein isoform of the major spliced transcript. In this study, we detected *Hbz* mRNA and protein in all the HTLV-1 positive cell lines tested (Figure 3.1B). We noted one inconsistency from a previous report in which HBZ protein was not
detectable in MT-2 cells. Differential detection between the two studies could be attributed to the different HBZ antibodies utilized or to the use of variants of the MT-2 cell line. SLB-1 became the cell line of choice for our studies for several reasons. (1) SLB-1 is a well characterized and established HTLV-1-transformed T-cell line that consistently expresses the highest level of \( Hbz \) mRNA and protein. (2) SLB-1 secretes IL-2R\( \alpha \), thus providing a biomarker for cellular proliferation that can be measured easily by quantitative ELISA. (3) Previous experiments indicated that SLB-1 cells form solid clustered subcutaneous tumor masses in NOG mice, whereas other cells such as MT-1 form a dispersed tumor pattern which is difficult to monitor and newly HTLV-1 immortalized PBLACHs do not engraft (data not shown).

We infected HTLV-1-positive SLB-1 cells with lentiviruses expressing shRNAs targeting \( Hbz \) sequences to determine the role of \( Hbz \) in global viral gene expression and cell proliferation. We clearly showed that the lentiviral plasmid vectors targeting \( Hbz \) sequences significantly knocked down \( Hbz \) gene expression both transiently (Figure 3.2) and long term in stably transduced SLB-1 cells (Figure 3.3A and 3.3B). SLB-1 cells with suppressed \( Hbz \) gene expression showed a significant decrease in cellular proliferation with no apparent loss of viability or increase in cell death (Figure 3.4A and 3.4B). Furthermore, we extended the direct correlation of \( Hbz \) expression and proliferation to additional cell lines including MT-1 and more relevant newly HTLV-1-immortalized T-lymphocytes, PBLACH (data not shown). It is interesting to speculate that \( Hbz \) V2 and V3 target sequences could have the potential for off-target affects on viral positive strand mRNAs that might include \( gag/pol \), p12, or p30. Importantly, we show for the first time that suppressing \( Hbz \) gene expression has no significant affect on the levels of \( gag/pol \)
and *tax/rex* mRNA and p19 Gag and Tax protein (Figure 3.3C, 3.3D, and 3.5C). The fact that *tax* and other quantified viral mRNAs are not altered in SLB-1 *Hbz* knockdown cells may be contradictory to the current dogma for the function of the HBZ protein. For example, the loss of HBZ expression in the context of a molecular clone resulted in an increase in Tax transactivation and overall viral gene transcription.\(^{187}\) However, in those studies, HBZ protein was completely knocked out, whereas in the present study, *Hbz* was down-regulated but not knocked out. Thus, we speculate that there could be a threshold level of HBZ protein required for its repressive affects on Tax-mediated transactivation.

We utilized the immune compromised NOG mouse to determine the role of *Hbz* gene expression in cell growth and tumor infiltration *in vivo*.\(^{253,254}\) Our results demonstrated that the high level of *Hbz* expression in SLB-1 cells was not required for tumor cell engraftment, but *Hbz* expression significantly contributed to tumor growth and organ infiltration. Moreover, we showed that serum levels of IL-2R\(\alpha\) in animals challenged with SLB-V4 *Hbz* knockdown cells was significantly decreased throughout the study relative to controls. These results indicated that *Hbz* expression contributes significantly to the proliferation of cells in the NOG mouse, thereby facilitating tumor growth as measured by increased tumor weight and volume (Table 3.1). Western blots on tumor lysates confirmed that the *Hbz* repression was maintained and HBZ protein levels directly correlated with tumor growth (Figure 3.5B). Furthermore, tumor growth appears to be independent of the Tax oncoprotein, since Tax expression was not affected significantly in any mouse tumors, irrespective of HBZ levels (Figure 3.5B). The animals challenged with the SLB-V4 *Hbz* knockdown cells also displayed a reduced pattern of infiltrating tumor cells (Table 3.2 and Figure 3.5C).
Based on the data that HBZ protein represses Tax-mediated transcription and attenuates AP-1 activity, we hypothesized that HBZ might function with other viral gene products to tightly regulate viral replication and/or promote infected cell survival. The down-regulation of Tax clearly would be beneficial to the infected cell by enabling it to evade host immune surveillance since Tax elicits a strong CTL response \textit{in vivo} and Tax-expressing cells would be rapidly targeted for elimination. However, without Tax expression, the infected cell would lose key growth and survival signals critical during the early stages of viral infectivity, replication, transformation, and persistence.\textsuperscript{77,83,114} One such pathway involves Tax-mediated degradation of the tumor suppressor retinoblastoma (Rb), which leads to the release and activation of E2F1, a key regulator of cell progression from G1 to S phase.\textsuperscript{136,230,232} A recent finding showed that the \textit{Hbz} mRNA enhances \textit{E2F1} gene expression. This observation suggests an alternate mechanism by which HTLV-1 infected cells, independent of Tax, can maintain the deregulation of \textit{E2F1} leading to cell cycle transition and continued cell proliferation. HBZ protein is detectable in all HTLV-1-positive cell lines tested in this study as well as in most all ATLL cells, suggesting that at least one of the activities of \textit{Hbz} is required late in the leukemogenic process. In this regard, the cellular protein \textit{hTERT} has been shown to be up-regulated in ATLL cells and many cancers. A recent publication reported that HBZ protein enhances transcription of \textit{hTERT} to its highest levels most notably in the absence of Tax.\textsuperscript{186} thus, providing further support for both the mRNA and protein forms of \textit{Hbz} in the dysregulation of cellular activities. Current studies are underway to determine if the \textit{Hbz} mRNA and the HBZ protein synergistically affect cellular genes within the infected cell. Taken together, it seems likely that Tax and \textit{Hbz} function cooperatively: Tax, early
and Hbz late in the process of HTLV-1 tumorigenesis.

In summary, we show that knockdown of Hbz gene expression in SLB-1 cells had no affect on Tax expression but significantly altered the growth properties of these cells in culture and their ability to develop solid tumors and infiltrate/spread to other tissues in the NOG mouse. These results are consistent with the maintenance and expression of Hbz in most all ATLL cells and support an important role for Hbz in leukemogenesis. It appears that HTLV-1 has evolved to use HBZ (protein and mRNA) in unique ways to exploit the host cell machinery and enhance the growth of the infected cell. It is possible that the Hbz mRNA and HBZ protein function synergistically in infected cells to support leukemogenesis. Further studies may provide new targets for therapy to disrupt virus replication and the proliferation and survival of infected cells.

Acknowledgments

We thank Cecilia Machado-Parrulla and Matthew Kesic for technical assistance, Kate Hayes-Ozello for editorial comments on the manuscript, and Tim Vogt for figure preparations.
Figures

**Figure 3.1:** HTLV-1 genome and detection of *Hbz* mRNA and protein in HTLV-1 cell lines. (A) HTLV-1 proviral genome highlighting the HTLV-1 protein open reading frames (ORFs). Detailed below is *Hbz* including the unspliced mRNA transcript and ORF and the major spliced transcript (SP1) and ORF. Black line denotes mRNA and grey line in SP1 denotes multiple transcription start sites. The location of the *Hbz* short hairpin target sequences used in this study (Vector 2-4) relative to positive strand proviral clone numbering are indicated above the *Hbz* transcript. (B) Total RNA was isolated from HTLV-1 cell lines and negative control Jurkat T-cells and subjected to real-time Taqman® RT-PCR to quantify the HBZ major spliced transcript (80-90% of *Hbz*-specific mRNA \(^{181,187}\)). Copy numbers of *Hbz* mRNA are normalized to 1 x 10^6 copies of the hGAPDH and the average values from three independent experiments are presented; error bars denote standard deviation (SD). Below shows Western blot analysis of HBZ protein expression.
**Figure 3.2:** *Hbz*-specific shRNA lentiviral plasmid vectors knockdown HBZ protein expression transiently. 293T cells (1 x 10^5) were cotransfected with 0.2 ug HBZ cDNA expression vector and 1.0 ug of shRNA lentiviral vector as indicated (V1-V5). Cellular lysates were harvested at 24 and 48 h time points and subjected to Western blot analysis to detect HBZ, GFP and β-actin. β-actin and GFP levels were used as internal loading and transfection normalization controls, respectively.
Figure 3.3: Characterization of HTLV-1 gene expression in SLB-1 cells stably infected with shRNA lentiviral vectors. (A) Total RNA was isolated from parental SLB-1, stably transduced SLB-1 (V1-V5), and control Jurkat cells and subjected to real-time RT-PCR to quantify \textit{Hbz} transcripts. Numbers are normalized to 10^6 copies of hGAPDH. Cells
transduced with *Hbz*-specific shRNA vectors (V2-4) have a significant 3-3.5 fold decrease in *Hbz* mRNA levels compared to controls (*). (B) Western blot analysis on total cellular lysates from indicated cells show a significant 3.0 fold decrease in HBZ protein expression (normalized HBZ densitometry numbers) in cells transduced with *Hbz*-specific shRNA vectors (V2-4). Protein reduction correlates directly with mRNA reduction. (C) Quantitation of *tax/rex* and *gag/pol* mRNA expression in total cellular RNA by real-time RT-PCR (normalized to $10^6$ GAPDH) and (D) p19 Gag expression in culture supernatant by ELISA. The data presented in panels A, C, and D are the average values from three independent experiments; error bars denote standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey’s test.
Figure 3.4: Hbz supports cell proliferation in culture. (A) $1 \times 10^3$ SLB-1 or stable SLB-1 lentiviral infected cells (V1-V5) were plated in normal growth medium in 96 well plates and MTS assays were performed on triplicate wells at 24 h intervals for five days. The average absorbance numbers are plotted and error bars denote SD. The significant difference in proliferation in Hbz-specific shRNA transduced cells compared to controls.
is denoted by (*). (B) The same cell lines in panel A were subjected to trypan blue exclusion to assess cell viability. A total of five wells per cell line were enumerated per day and presented as average percent of viable cells with error bars denoting SD. (C) Western blot analysis was performed on total cell lysates as indicated. HBZ and Tax levels were quantified and normalized to β-actin control by densitometry. Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey’s test.
Figure 3.5: *Hbz* knockdown cell proliferation is significantly reduced in NOG mice. NOG mice were inoculated with $10^7$ parental SLB-1, stable lentiviral infected SLB-1 derivatives (V4,V5), or Jurkat cell control (5 mice each). Since SLB-1 secretes IL-2Rα mouse serum levels of IL-2Rα were measured by ELISA as a biomarker for SLB-1 proliferation in vivo. Each dot represents the average absorbance value of a single inoculated mouse at 0, 7, 15, and 22 days post inoculation within each group. The horizontal line represents the average of the mouse group at each weekly time point and the dotted line represents negative absorbance values. Significant differences from
controls are denoted by (*). Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey’s test. (B) Western blot analysis was performed on total tumor cell lysates of individual mice as indicated. HBZ and Tax levels were quantified and normalized to β-actin control by densitometry. Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey’s test. (C) Immunohistochemistry was performed on representative mouse liver tissue stained for Ki67 human antigen; a marker for proliferation.
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<th>Ave. Tumor Size mm</th>
<th>Std. Tumor Size mm</th>
<th>Ave. Tumor Volume cm^3</th>
<th>Std. Tumor Volume cm^3</th>
<th>Tumor Weight gm Ave.</th>
<th>Tumor Weight gm Std.</th>
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</table>

Table 3.1. Knockdown of Hbx gene expression significantly decreased tumor size in vivo. Mouse tumor size was measured in millimeters as length x width with the average and standard deviations indicated. Mouse tumor volumes were calculated by $3.142 \times$ radius$^2$. Mouse solid tumor weights in grams were taken at day of sacrifice. M5 and M8 were sacrificed on day 17 due to necrosis and are not included in the final averaged numbers (-). M14 died on day 22 due to complications from peritoneal injection and also was not included in the final average numbers (-). N/A; not applicable, because no tumor mass at day 22 in any of the Jurkat cell inoculated animals. * denotes statistically significant from SLB-1 inoculated mice.
<table>
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<tr>
<th>Tissue</th>
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<th>SLB-V4</th>
<th>SLB-V5</th>
<th>Jurkat</th>
</tr>
</thead>
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<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
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<tr>
<td>Pancreas</td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>-</td>
</tr>
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Table 3. 2 Mouse tissues examined histologically by routine H&E and Ki67 staining. Tissues were fixed in 10% neutral buffered formalin, trimmed, paraffin embedded, sectioned, and stained using routine hematoxylin and eosin. Scores represent the average values of each animal group. All tissues were scored using a scale consisting of: no neoplastic cells (-), minimal or + corresponds to an infiltrate of 0-5 cells in radius, mild or ++ corresponds to an infiltrate of 6-10 cells in radius, moderate or +++ corresponds to an infiltrate of 11-15 cells in radius, or ++++ corresponds to an infiltrate of 16 or greater cells in radius. The data represents the average form the mouse groups of tumor cell infiltration in each tissue.
CHAPTER 4

HTLV-1 HBZ FACTOR mRNA AND PROTEIN INDEPENDENTLY ENHANCE CELLULAR PROLIFERATION IN CULTURE

4.1 Abstract

Emerging data in the field suggests that in addition to HBZ protein suppression of basal level and Tax-mediated transactivation via disruption of transcription factor binding (CREB-2, AP-1) at the viral promoter, Hbz mRNA has the capacity to promote T-lymphocyte proliferation. The goal of this study was to determine the contribution of the Hbz mRNA and HBZ protein in supporting cellular proliferation in cell culture. Our data indicates that Hbz mRNA has the capacity to enhance cellular growth. More directly for the first time, we show that the Hbz mRNA and HBZ protein display a synergistic additive effect enhancing proliferation in overexpression studies and in the context of a replicating virus. Together, these novel findings support the hypothesis that Hbz, a unique HTLV-1 nonstructural gene, has multiple functions in two distinct molecular forms within the host cell that manipulate the cellular microenvironment facilitating malignant cell growth.
4.2 Introduction

The human T-cell leukemia virus type-1 (HTLV-1) is a highly cell-associated virus and infection is primarily spread horizontally via sexual transmission, exposure to contaminated blood products, or vertically via breast milk.\textsuperscript{111} Once in the host following the initial burst of replication, HTLV primarily increases its copy number by proliferation of the infected cells. Infection with HTLV-1 has clearly been linked to the development of adult T-cell leukemia/lymphoma (ATLL), an aggressive CD4+ T-lymphocyte malignancy, and various lymphocyte-mediated inflammatory diseases including HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, infectious dermatitis, and arthropathy.\textsuperscript{255-257}

HTLV-1 is a complex retrovirus that encodes the structural and enzymatic genes \textit{gag}, \textit{pol}, and \textit{env}. HTLV-1 also encodes positive regulatory genes \textit{tax} and \textit{rex} as well as accessory genes \textit{p12}, \textit{p13}, \textit{p30}, and \textit{Hbz}.\textsuperscript{165,172,220} Tax, the transcriptional activator, is an important modulator of both viral and cellular gene expression\textsuperscript{221} and its expression is essential for HTLV-mediated cellular transformation of T lymphocytes.\textsuperscript{77,83,222,182,258} In the past six years the \textit{Hbz} gene has been under intense study since its discovery\textsuperscript{177} and initial functional characterization.\textsuperscript{176} Initially, exogenously over-expressed HBZ was found to down-regulate Tax-induced HTLV-1 transcription and interact with and disrupt the DNA binding activity of JunB and c-Jun (AP-1 components).\textsuperscript{183,233} As presented in Chapter 2, we were the first to evaluate the contribution of HBZ to HTLV-1 biology in the context of an infectious molecular clone. We showed that HBZ protein in the context of a replicating virus is dispensable for transformation and immortalization \textit{in vitro} but is required for efficient infectivity in infected rabbits.\textsuperscript{187} Additional work from Satou \textit{et al},
suggested that the secondary structure of the *Hbz* mRNA supports cellular proliferation of infected cells in culture and altered lymphocyte compartments in transgeneic mice.\textsuperscript{188} Furthermore, data presented in Chapter 3 showed that the *Hbz* gene is responsible for enhancing HTLV-1 positive SLB-1 T-cell growth in culture and facilitates the growth and spread of tumors in NOG mice (Chapter 3). Collectively these findings suggest that the *Hbz* gene and its protein product may have unique roles in two distinct molecular forms where each form manipulates the cellular environment in a balance to other viral genes favoring infectious cell and thus virus survival.

Previous work has identified that the *Hbz* mRNA supports the growth of infected cells in culture; although one important caveat to this study is that the protein was never detected or considered to contribute to the cell growth phenotypes.\textsuperscript{188} Several studies over the years have shown that the HTLV-1 positive regulatory protein Tax and accessory proteins p13, and p30 viral proteins have pleiotropic functions within the infected host.\textsuperscript{111,163,173,259,260} Since other HTLV-1 viral proteins have many functional roles within the infected cell, we hypothesize that the HBZ protein has multiple functions in addition to negatively regulating Tax-mediated viral transcription. The primary goal of this study was to determine if the *Hbz* mRNA and HBZ protein both contribute to cell growth in culture. In an attempt to identify if the *Hbz* mRNA and HBZ protein are both facilitating cell growth we used several molecular approaches: 1) lentiviral expression vectors transcribing short hairpin RNAs (shRNAs) specific to *Hbz* sequences, 2) 729ACH B-cell lines that expresses *Hbz* mRNA, but are deficient for HBZ protein expression, 3) and overexpression studies using Jurkat T-cells employing lentiviral vectors carrying *Hbz* as a transgene. We show that the *Hbz* mRNA directly supports the
growth of infected cells culture. More importantly for the first time, we show that the \textit{Hbz} gene in both its mRNA and protein molecular forms display additive properties on the infected cell positively enhancing growth.

4.3 Material and Methods

Cells

293T cells, 729 B-cell lines, 729ACH, and 729ΔHBZ stable transfected cell lines were previously described \cite{187}, and Jurkat T cells were maintained in Dulbecco modified Eagle and Iscove medium, respectively. The growth media were supplemented to contain 10\% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Immortalized PBLACH cells were previously described \cite{78,99} and maintained in Iscove growth medium in the presence of recombinant hIL-2 at 10U/mL.

Plasmids

\textit{HBZ} cDNA expression vector (based on the \textit{Hbz} major transcript was previously described). \cite{181,187} \textit{ΔHBZ} cDNA expression vector was generated by introducing a G to A point mutation that resulted in termination of the \textit{HBZ} reading frame at amino acid 8 of the \textit{Hbz} major transcript previously described. \cite{181,187} Wild-type \textit{HBZ} and \textit{ΔHBZ} cDNA expression vectors (pME vector based) were generated from the ACH proviral clone. Empty pME- mock plasmid cDNA was previously described. \cite{187} The third generation (pRNAT-U6.2/Lenti) lentiviral vector was purchased from the Genscript Corporation (Piscataway, NJ). The shRNA lentiviral vectors V4 and V5 were derived from the (pRNAT-U6.2/Lenti) plasmid previously described in (Chapter 3 materials and methods)
(See Figure 4.3). The pCDH-MCS-copGFP lentiviral expression plasmid was purchased from System Biosciences (Mountain View, CA). Nhel and BamHl restriction endonuclease sites and a KOZAK consensus sequence GCCACC was cloned in frame by polymerase chain reaction linker cloning upstream of the ATG codon of the wild-type HBZ or ΔHBZ cDNA expression plasmids respectively. These Nhel to BamHl PCR products were then ligated in the pCDH-MCS-copGFP plasmid to generate the HBZ and ΔHBZ lentiviral vector plasmids. The original CMV immediate early promoter from the pCDH vector was removed by Clal to Nhel restriction digest and replaced by elongation factor 1 alpha (EF1α) promoter to enhance transcription in T-lymphocyte cell lines.

**Transient Transfections**

To measure cDNA expression vector plasmids ability to express HBZ protein and mRNA, 1.5 x 10^5 293T cells were transfected using Lipofectamine Reagent (Invitrogen, Carlsbad, CA). The amount of DNA was kept constant and was composed of 1.0 µg of cDNA for control and each respective vector plasmid independently or empty plasmid DNA as negative control. Three independent wells were transfected for each sample and harvested, mixed together, and lysed in 1X passive lysis buffer (Promega) at 24, 48, 72h time points. All experiments were performed independently three times in triplicate. Transfection efficiency in 293T transfections was measured by cotransfection of 10 ng TK-Renilla. 3.0x10^6 Jurkat T-cells were transfected with 4µg of pDCH-MCS-copGF, pCDH-HBZ-copGFP, and pCDH-ΔHBZ-copGFP independently by Amaza Nuclefector® V-kit Reagent according to manufacturer’s instructions (Gaithersburg, MD).
Western blot and p19 Gag Matrix ELISA

Western blot analysis was performed as described\textsuperscript{170} on transiently transfected 293T, Jurkat T, 729HTLV-1 stable cells, and stable (Geneticin, Gibco) selected PBLACH cell clones total cellular protein lysates using rabbit anti-pHBZ polyclonal antiserum (1:500) and goat anti rabbit-conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). GFP and β-actin were detected using (1:1000) anti-GFP rabbit polyclonal antibody (Santa Cruz) and anti-β-actin rabbit monoclonal antibody, and goat-anti rabbit conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). HTLV-1 Tax was detected using (1:1000) mouse hybridoma monoclonal antibody and HRP-conjugated goat anti-mouse secondary antibody (HIH anti-Tax repository # 1318 AM110405). Proteins were visualized using the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ). Stable HTLV-1 729 transfectants containing proviral clones were isolated as described\textsuperscript{99} and cell clones were screened by p19 Gag expression in the cell supernatant by ELISA.

Virus particle production and cell infection

Plasmid lentiviral vectors V4 and V5 were isolated from bacterial cultures and sent to the Ohio State University Comprehensive Cancer Center viral vector core located in Cincinnati Ohio for virus particle production. Vectors 4 and 5 were previously described in Chapter 3. Lentiviral vector supernatants were produced by transient cotransfection of lentiviral plasmid vectors and packaging plasmids pCDNA3.g/p.4xCTE, pRSV-rev, and M75-VSV-G. 293T cells were transfected using the calcium phosphate method (Sigma Aldrich) in DMEM/10% FBS in the presence of
25 mM chloroquine. 12-18 h post-transfection, the media was changed, and supernatants were collected at four 10-14 hour intervals, 0.45 micron filtered and frozen at -80°C. Supernatants were concentrated by size exclusion using a 100 kDa filter (VivaScience) and infectious titers were determined by expression of GFP on HT1080 cells 4 days post-transduction. PBLACH cells were infected with an multiplicity of infection (MOI) of 15 in supplemented Iscove’s media along with 8μg/mL polybrene (Sigma) in the presence of recombinant hIL-2 at 10U/mL. 48 h post infection virus inoculums were removed and infected cells were selected by growth in 1μg/ml Geneticin (Gibco, Carlsbad, CA). Stable clones were isolated in bulk cultures 4 weeks following initial selection.

**RT-PCR and quantitative Taqman real-time RT-PCR**

Total RNA was isolated from non-transfected and transfected 293T, Jurkat T-cells, as well as non-infected PBLACH or stable lentiviral infected PBLACH V4 and V5, cell lines using the RNeasy kit and DNase treated column (Quiagen, Valencia, CA.). 2μg of total RNA was subjected to reverse transcriptase (RT)-PCR using (1μM) oligo DT primer for first strand synthesis to generate cDNA libraries (Invitrogen). To detect Hbz mRNA transcript, the cDNAs then were subjected to standard PCR using the Hbz HTLV-1-specific primer pair as described.252 PCR amplified fragments were separated on a 1% agarose gel and visualized by ethidium bromide staining. Forty cycles of real-time Taqman® PCR were conducted to quantitate mRNA transcripts from cDNA libraries as indicated. cDNA was amplified in duplicate using the primers and probes for Hbz, tax/rex, and gag/pol sequences as described.252 The final numbers were averaged and transcript quantity was determined based on a standard curve generated from duplicate
samples of log_{10} dilutions of a plasmid containing the \textit{Hbz}, \textit{tax/rex}, and \textit{gag/pol} DNA sequences. The copy of each transcript was normalized to $1.0 \times 10^6$ hGAPDH. hGAPDH was amplified using primers previously described.\textsuperscript{92}

**DNA preparation for viral load**

DNA was isolated from 729 producer cells using PURGENE\textsuperscript{®} DNA purification system (Gentra, Minneapolis, MN). 500ng of genomic DNA was subjected to forty cycles of real-time Taqman\textsuperscript{®} PCR to quantify proviral copy number per cell as previously described.\textsuperscript{252}

**Proliferation assay and cell viability**

The CellTiter 96\textsuperscript{®} AQ\textsubscript{ueous} Non-Radioactive Cell Proliferation Assay\textsuperscript{®} is a homogeneous, colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The CellTiter 96\textsuperscript{®} AQ\textsubscript{ueous} Assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt; MTS\textsuperscript{(a)}] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan product at 490nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. Briefly, cells were counted and
plated at 1.0X10³ cells/well in 96 well round bottom plates on day one and monitored over a 5 day time course. Cell Titer 96® reagent was added to each well agitated slightly and incubated at 37 °C 5% CO₂ for 2 hour. Optical density absorbance at 490nm was measured on an ELISA plate reader. Absorbance numbers are the average of 8 replicates from 2-3 experiments for each cell line. To verify cellular viability over time, the same cell lines were plated on a separate 96 well round bottom plates and counted by trypan blue exclusion over time (Data not shown).

4.4 Results

Transient expression of Hbz mRNA and protein support cell proliferation

It has been suggested that the secondary structure of the Hbz mRNA supports the proliferation of cultured cells however HBZ protein contributions were not directly addressed. To address this issue we first performed transient transfections in 293T cells. 293T cells were transfected with either 1 ug of pME-mock, wild-type HBZ (wt.HBZ cDNA), and ΔHBZ cDNA plasmid constructs along with 10ug of TK-Renilla for transfection efficiency. The wt. HBZ and ΔHBZ cDNA expression plasmids were described in the materials and method section of this chapter. Twenty-four hours post transfection; cells from triplicate independent transfections were mixed together and plated at 1.0x10³ in 8 replicate wells in 96 well round bottom microtiter plates. Cells were monitored for at 24 hour time-points for 3 consecutive days by MTS assay. Consistent with a previous report, the transfected ΔHBZ cDNA plasmid, clearly supports the proliferation of 293T cells in MTS assays compared to controls. (Figure 4.1A) For the first time, our data shows that the wt. HBZ cDNA expression plasmid significantly
enhanced proliferation of 293T cells when compared to the ΔHBZ cDNA (Figure 1A). Both wild-type and mutant HBZ constructs promoted significant increases in proliferation above pME-mock transfected and untransfected negative controls (Figure 4.1A). We performed Western blot and RT-PCR analysis on total protein and total mRNA respectively to verify HBZ protein and mRNA expression (Figure 4.1B, and 4.1C). Together, our results indicate that in transient transfections the \( Hbz \) mRNA and protein are both positively enhancing the proliferation of 293T cells in culture (Figure 4.1A).

**Hbz mRNA and protein supports the proliferation of cells in the context of a replicating virus**

The HBZ protein, in the context of the molecular clone, is dispensable for immortalization and transformation of T-lymphocytes in culture.\(^{187}\) However, in the context of a replicating virus, the contribution of the HBZ protein supporting cell growth in culture has never been evaluated. Using previously characterized 729 B-cell stable transfectants, 729ACH and 729ΔHBZ we evaluated the contribution of the HBZ protein on cellular proliferation in culture.\(^{187}\) 729ACH, 729ΔHBZ, and mock 729B-cell lines were plated in a 96 well micro titer plate on day zero and assessed for 5 consecutive days by MTS assay. Our results indicate that the 729ACH stable cell line proliferates at a higher rate compared to 729ΔHBZ (Figure 4.2A). We also observed an increased growth phenotype of the 729ΔHBZ cells relative to mock 729 B-cells (Figure 4.2A). We next addressed the possibility that the different growth phenotypes between the cell lines might be related to HTLV-1 proviral copy number. Total cellular genomic DNA was
isolated from each cell line and quantitative Taqman real-time PCR was performed. The quantitative real-time PCR data indicates that the proviral load in copies per cell were statistically comparable between the wild-type and HBZ mutant cell lines (Figure 4.2B). Consistent with the loss of HBZ protein, we observed an increase in p19 matrix antigen by p19 Gag ELISA and Tax protein expression by western blot in 729ΔHBZ stable cell lines (Figure 4.2C and 4.2D). Together the data indicate that in the context of a replicating virus, both HBZ protein and mRNA have the capacity to support the proliferation of stably transfected 729 B-cells.

**Hbz enhances proliferation of HTLV-1 immortalized PBMCs**

In previous experiments we have constitutively down-regulated *Hbz* expression using a lentiviral vector system transcribing short hairpin RNAs specific to *Hbz* sequences (Chapter 3). We made use of the same lentivirus vector system by infecting HTLV-1 immortalized human (IL-2 dependent) T-lymphocytes referred to as PBLACH, which were generated by cocultivation of 729ACH with human peripheral blood mononuclear cells (PBMCs). Cocultivating PBMCs with HTLV-1 producer cells have been used extensively to study immortalization and transformation properties of HTLV in culture. In these experiments, separate populations isolated from bulk cultures of immortalized PBLACH cells were infected with lentiviral viral particles at an MOI of 15: V5 (Scrambled), or V4 (HBZKnockdown) (Figure 4.3A). 48 hours post-infection virus inoculums were removed and cells were placed under G418 selection for 4 weeks. After 4 weeks stable bulk populations of lentiviral infected PBLACH cells were characterized for gene expression by real-time RT-PCR and growth phenotypes by MTS assays. Total
mRNA was isolated from each cell line and subjected to Taqman real-time RT-PCR to quantitate Hbz, tax/rex, and gag/pol mRNA levels. PBLACH V4 infected cells have showed a significant knockdown of Hbz mRNA by real-time RT-PCR, relative to controls. Tax/rex and gag/pol mRNAs were unaffected indicating the shRNAs target Hbz specifically consistent with the results in Chapter 3.

Primary T-lymphocytes are susceptible to immortalization/transformation and this has historically represented an accepted experimental system for exploring the early events associated with malignancy. HTLV is a highly cell-associated virus; cell-free infection is very inefficient and efficient infection of cells requires co-cultivation of target cells, primary PBMCs, with irradiated HTLV-producer cells. Immortalization is defined as continuous growth of T-lymphocytes in the presence of exogenous IL-2. In the next set of experiments we characterized the growth properties of the lentiviral infected PBLACH cell lines in the presence and absence of exogenous IL-2. Briefly, cells were plated on day zero and monitored for 5 consecutive days for proliferation by MTS assay in the presence or absence of recombinant IL-2. Our results indicate that the expression of Hbz significantly supports the proliferation of PBLACH cells in culture even in the absence of IL-2 compared to the Hbz down-regulated PBLACHV4 cells and controls (Figure 4.4A). In our previous study when Hbz was down-regulated by shRNAs in the context of a replicating virus, we did not observe an increase in Tax-mediated transcription (Chapter 3). We speculate that the down regulation of Hbz, which is not a protein knockout, is not sufficient enough abolish the repressive affects of the HBZ protein on Tax-mediated viral transcription. However, in these experiments, the down regulation of Hbz is sufficient to negatively affect cell proliferation, but not enough to alter the stoichiometry of HBZ.
protein to Tax protein which would alleviate the attenuation of Tax thus increasing transactivation. In support of these results, the Tax protein expression levels by Western blot analysis were unaffected by the down regulation of *Hbz* (Figure 4.4B). In conclusion, these findings are consistent with our previous report and indicate the down regulation of *Hbz* did not meet the threshold level needed to alleviate HBZ’s attenuation on Tax-mediated transcription. (Figure 4.4B and Chapter 3)

**Overexpression of *Hbz* mRNA and protein enhances proliferation of Jurkat T-cells**

Using the well characterized Jurkat T-cell line we transfected plasmid cDNA pDCH-MCS-copGF (mock vector), pCDH-HBZ-copGFP (wt. HBZ vector), or pCDH-ΔHBZ-copGFP (ΔHBZ vector) into separate Jurkat cell populations. The wt. HBZ vector plasmid expresses the *Hbz* mRNA and protein, whereas the ΔHBZ vector is deficient for full length HBZ protein expression, but continues to expresses the *Hbz* mRNA. Forty eight hours after transfection, Jurkat cells were sorted by flow assisted cell sorting (FACS) for GFP fluorescence. After FACS sorting, live cell populations were expanded for 3 days in culture. A fraction of proliferating cells were then divided into three populations to characterize *Hbz* mRNA expression by RT-PCR (Figure 4.5C), HBZ protein expression by Western blot (Figure 4.5B), and to monitor growth properties in MTS assays (Figure 4.5A). RT-PCR analysis on total mRNA from transfected cells using primers specific to *Hbz* sequences were used to detect *Hbz* message (Figure 4.5C). Next, we performed Western blot analysis on total protein lysates to confirm the expression of the HBZ protein in the transfected cells (Figure 4.5B). Our data clearly shows that Jurkat cells transfected with wt HBZ vector express *Hbz* mRNA and protein,
whereas the ΔHBZ vector only expressed the \( Hbz \) mRNA as expected. Lastly, GFP sorted Jurkat T-cells were plated in 96 well microtiter plates and monitored 5 days post-plating for proliferation by MTS assays. The mock vector transfected Jurkat T-cells display a slower growth rate comparable to the non transfected cells. However, the wt HBZ vector transfected cells proliferate at a substantially higher rate than the mock transfected cells (Figure 4.5A). Additionally, the ΔHBZ vector transfected cells proliferate at a higher rate above mock controls but significantly lower than cells transfected with the wt HBZ vector (Figure 4.5A). Taken together the data indicates that overexpression of the \( Hbz \) mRNA and HBZ protein are synergistically enhancing proliferation of Jurkat T-cells

### 4.5 Discussion

HTLV-1 Tax protein is a major target of CTL response \textit{in vivo}.\textsuperscript{257} It is not surprising to find that the majority of ATLL cell lines have epigenetic changes, mutations in the Tax ORF, and hypermethylation of the 5’LTR to suppress Tax expression allowing the virus to evade immune surveillance.\textsuperscript{261} The transformation process of HTLV-1 infected cells is not completely understood but thought to be initiated by Tax early on in infection. The current conundrum in the HTLV field is that most ATLL cells, in which \textit{tax} gene expression is undetectable, still display deregulated growth, chromosomal instability, and a multitude of additional problems initially caused by Tax.\textsuperscript{111,146,262} Recent studies have shown that in all ATLL and HTLV-1 positive cells characterized to date \( Hbz \) gene expression is detectable by RT-PCR.\textsuperscript{188} Thus, if \textit{tax} is silenced, \( Hbz \) is still detectable, leading us to speculate that \( Hbz \) is contributing to the neoplastic outcome resulting from HTLV-1 infection. Recently, Kulhmann and colleagues have indicated that
the HBZ protein enhances the expression of the human Telomerase reverse transcriptase (hTERT) gene which has been found to be upregulated in many cancers including ATLL. Interestingly, telomerase activity in HTLV-1 infected cells varies in function in the presence of Tax and consequently in relation to the cellular phenotype (preleukemic vs. leukemic). Telomere shortening represents a cell autonomous mechanism that restricts the proliferative capacity of normal cells. Ectopic expression of the reverse transcriptase hTERT subunit in primary human cells causes lifespan extension and facilitates immortalization. On the other hand, telomerase activity is decreased in Tax-activated T-cells where Tax repressed hTERT transcription via an E-box element present in its promoter. The mechanism of repression was probably through the competition for p300/CBP as described for another E-box binding factor, MyoD. Additionally, the tumor suppressor p53 plays an important role in cell cycle progression. p53 is activated in response to DNA damage, and its induction results in either cell cycle arrest in G1 or cellular apoptosis. The accumulation of DNA damage in HTLV-1 transformed cells has been associated with Tax expression suggesting that Tax itself might inhibit p53 function. By repressing telomerase and the p53 tumor suppressor activity, Tax favors accumulation of chromosomal rearrangements and then the transformation of the infected cells toward the malignant phenotype. Subsequent repression of Tax expression in the leukemic cells might then allow telomerase reactivation enhanced by Hbz expression, preventing critical telomere shortening, stimulating the proliferative potential of infected cells, and thus leading to ATLL. The reactivation of telomerase activity is a key event in the progression toward ATLL and has been suggested to be a useful prognostic marker.

Our previous work has shown that in the context of a replicating virus, the HBZ
蛋白是所需要的，以实现有效的感染和在接种的兔子中保持持久性。我们推测HBZ蛋白在细胞增殖早期是必需的，以建立有效的持久性感染。在我们实验室的一个未发表的试点研究中，我们通过实时RT-PCR量化了兔子感染HTLV-1后PBMCs中的Hbz mRNA水平。数据表明Hbz mRNA转录本在6至8周后可检测到，且水平高于或接近主要病毒基因gag/pol和tax/rex的水平，这表明Hbz是在支持受感染细胞的生存（数据未显示）。最后，在一个更近期的研究中，我们使用了基因工程改变的NOG小鼠，发现Hbz显著贡献于肿瘤生长（Chapter 3）。这些发现共同支持了Hbz基因及其产物支持细胞增殖的假设，表明Hbz在促进HTLV-1感染动物的细胞生长中起作用，暗示Hbz可能在ATLL的发展中发挥作用。

在这篇报告中，我们支持了Satou et al的最近发现，其中Hbz mRNA支持细胞在体外的增殖。188在过表达实验中，我们首次证明Hbz mRNA和HBZ蛋白都支持细胞增殖（图4.1A）。在使用729ΔHBZ细胞的实验中，我们观察到HBZ蛋白表达的729ACH细胞中的增殖显著下降，与HBZ蛋白表达的729ACH细胞形成对比（图4.2A和4.2B）。此外，通过shRNAs下调Hbz在新 immortalized PLBACH细胞中引起生长模式的减弱，而与对照组相比，这些发现直接表明，Hbz在细胞增殖中起作用。显然，在过表达实验中使用Jurkat T细胞在外源性背景下，
of the virus, we found that the *Hbz* mRNA and protein exert additive positive affects contributing to cellular growth. We speculate that *Hbz* may be a positive regulator of an autocrine feedback loop by affecting cellular genes favoring growth and survival. Future experiments using gene microarrays may be utilized to best understand how these two molecular forms, mRNA and protein, are differentially affecting cellular growth and cellular gene expression.

The data collected from the gene microarray experiments may divulge a mechanism to how HTLV-1 is utilizing an antisense mRNA transcript and protein at multiple levels within the infected cell not only to regulate viral replication, but support the growth of cells through the manipulation of cellular genes. Our findings suggest HTLV-1 is utilizing a novel way utilizing two molecular forms of the same gene to generate a survival stimulus. We believe that the *Hbz* mRNA and protein are functioning synergistically to manipulate the cellular microenvironment to promote cell survival as a balance to the positive regulator Tax. Silic-Benussi and colleagues, using another HTLV-1 accessory gene *p13*, observed that p13 protein negatively influences cell proliferation at high density *in vitro* and interferes with tumor growth *in vivo*. Their results suggest that the antiproliferative effects of p13 could be thought of as a safeguard that limits the oncogenic potential of HTLV-1 in general and of the Tax transactivator in particular, resulting in enhanced long-term coexistence with its host, a hallmark of the natural history of survival might also be relevant in the context of HAM/TSP.

Emerging evidence indicates that the HTLV-1 accessory protein p30 has important roles in the viral and cellular gene expression at both the transcriptional and the post translational level. p30 acts as a negative regulator of viral gene expression by
binding to the *tax/rex* mRNA and retaining it in the nucleus.\textsuperscript{170} Additional data from Datta *et al*, indicate that HTLV-1 p30 expression modulates regulatory cell cycle control in T-cells resulting in a delay in G2 exit, which would enhance viral replication by prolonging cell survival or contribute to cell transformation.\textsuperscript{171} Based on these findings p13, and p30 may work in concert to act in opposition to *Hbz* at many more levels to regulate virus pathobiology than previously predicted.
Figure 4.1: Transient transfection of wild-type or protein deficient ΔHBZ cDNA enhances proliferation of 293T cells. (A) 293T cells were plated at 1.5x10^5 cells per well in 6 well plate. 1 ug of pME-mock, wt HBZ, or mutant ΔHBZ plasmid cDNA expression vectors were transfected independently in triplicate. 24 hours post-tranfection cells were mixed and plated at 1.0x10^3 per well in microtiter plates. MTS assays were performed at 24, 48, and 72 hour time-points. Total protein lysates and mRNA was isolated from remaining cells at each time point. (*) Indicates a statistically significantly slower proliferation rate of mock and negative control cells vs. wt HBZ and ΔHBZ cDNA transfected cells by paired T-test (p<.0023) Significant increase in wt HBZ cDNA vs. ΔHBZ cDNA (p<.0052) (B) Western blot analysis was performed on transfected cell lysates to detect HBZ protein using rabbit specific antisera. β-actin was used as internal loading control (C) Standard RT-PCR was performed on total mRNA using primers specific to detect Hbz mRNA at indicated time points post transfection.
A

![Graph showing OD Absorbance vs. time for different cell lines](image)

B

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Proviral Copy Number per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>729Mock</td>
<td>0.000844</td>
</tr>
<tr>
<td>729ACH</td>
<td>1.961367</td>
</tr>
<tr>
<td>729AHBZ</td>
<td>1.818722</td>
</tr>
</tbody>
</table>

C

![Bar graph showing β1 integrin expression](image)

D

![Western blot images showing HHZ, β-Actin, and TAX expression](image)

To be continued
Figure 4.2: HBZ protein and mRNA independently support the proliferation HTLV-1 729 stable transfectants. (A) 729 stable producer cell lines were plated in triplicate on day zero and monitored over 5 days. MTS assay was performed on each day. The data indicates a significant increase in proliferation determined by paired T-test (*) in 729ACH cells and 729ΔHBZ vs. control 729Mock B-cells (p<.0068) (729ACH vs. 729ΔHBZ, p<.0289) (B) Integrated proviral copy number per cell quantified by real-time Taqman PCR in genomic DNA isolated from the indicated cell lines. (C) 729 stable producer cells were plated at 5.0x10^5 in triplicate. At 48h post plating cellular supernatant was collect and p19 Matrix ELISA was performed. (*) Indicates a significant increase in p19 production by paired T-test (p<.0015) (D) Western blot analysis for HBZ, Tax, and β-Actin loading control was performed on total cellular lysates. Tax protein expression from the Western blot analysis was quantified by densitometry where 729ΔHBZ is set to 100.00. The data indicates a significant increase in Tax protein in 729ΔHBZ cells consistent with the p19 expression and loss of HBZ.
Figure 4.3: Hbz mRNA is downregulated in lentiviral infected PBLACH cell lines. (A) A schematic representation of the third generation lentiviral vector used in the Hbz down regulation experiments. The Hbz shRNAs are expressed from the U6.2 promoter, GFP by a CMV promoter, and Neo from the SV40 promoter. Indicated is the lentiviral vector containing the shRNA sequence target for the Hbz mRNA. V4 generates shRNA to Hbz target sequences. A cartoon representation of the HBZ open reading frame and spliced major transcript. (B) Total RNA was isolated from, stably transduced PBLACH T-cell lines, non transduced PBLACH, and negative control naive Jurkat T-cells. Real-time Taqman® RT-PCR was performed on the total RNA to quantify the Hbz, tax/rex, and gag/pol transcripts normalized to 1.0x10^6 copies of hGAPDH in the indicated cells lines. In the vector transduced PBLACH V4, show a significant decrease in Hbz mRNA levels compared to controls. (*) Statistical significance was determined by paired T- test (p<.0062)
Figure 4.4: *Hbz* supports the proliferation of HTLV-1 immortalized PBLACH cell lines in the presence or absence of IL-2. (A) Cell lines were plated on day one, an MTS assay was performed on each cell line. Optical density values were taken from the average of 8 samples in 3 replicate experiments from each cell line plated at 1.0x10^3 cell/well with or without exogenous IL-2 on each day. (*) Indicates a significant decrease in proliferation at day 5 in *Hbz* down-regulated PBLACH cells compared to controls determined by paired T-test (with IL-2, p<.0008, without IL-2 p<.0031) (B) Western blots for Tax, GFP, and β-actin were performed on cellular lysates of the indicated cell lines.
Figure 4.5: *Hbz* gene expression enhances proliferation of transfected Jurkat T-cells (A) 3.0X10^6 Jurkat T-cells were transfected with 5ug of pCDH-MCS-copGFP (mock vector), pCDH-HBZ-copGFP (wt. HBZ vector), or pCDH-ΔHBZ-copGFP (ΔHBZ vector) cDNA lentiviral expression plasmids. A fraction of GFP FACS sorted cells were lysed for total protein. Western blot analysis was performed to detect HBZ protein expression. β-Actin was used as a loading control. (B) Standard RT-PCR was performed on total RNA from each cell line to detect *Hbz* mRNA. The *Hbz* message and GAPDH loading controls were
visualized on EtBr agarose gel. (C) GFP sorted Jurkat T-cell lines were plated in 8 wells of a microtiter plate on day zero at 1.0x10^3 cells/well. An MTS assay was performed for 5 consecutive days. Optical density absorbance values represent the average from 2 independent experiments. (*) Indicates a significantly lower rate of proliferation compared to wt HBZ and ΔHBZ expressing Jurkat T-cells. Statistical significance was determined by paired T-test (p<.0024). A significant increase in proliferation in wt HBZ vector expressing Jurkat T-cells vs. ΔHBZ vector expressing cells (p<.011).
CHAPTER 5

SYNOPSIS AND FUTURE PERSPECTIVES

5.1 Introduction

HTLV-1 is a pathogenic human retrovirus that has the capacity to transform primary human T lymphocytes in vitro and in vivo. After nearly 30 years of research, the exact mechanism by which HTLV-1 induces cellular transformation and ultimately disease still remains elusive. In vitro coculture assays have identified Tax as the major viral determinant and is essential to the HTLV-1-mediated T-cell transformation process, whereas the accessory proteins p12, p13, and p30 are dispensable. However, these proteins play an important role in viral infectivity and persistence in vivo. In this dissertation, we sought to broaden our knowledge of HTLV-1 using in vitro culture assays and two HTLV-1 animal model systems focusing on the contribution of Hbz in virus biology. The work in this dissertation utilizing these systems has allowed us to better understand the contribution of Hbz in virus infectivity, how it supports infected cell growth, and its involvement in the development of leukemogenesis. Chapter 1 focused on important aspects of HTLV-1 pathobiology and highlighted insightful comparative studies between HTLV-1 and HTLV-2. It has become clear from studies utilizing infectious molecular clones in primary human T-cells in cell culture and relevant animal model systems that other viral proteins play a supportive role. Chapters 2 and 3 of this
dissertation we used the rabbit model of infection and a genetically altered NOG mouse tumorigenicity model to study the contribution of \textit{Hbz} in HTLV-1 infection, persistence, and tumor cell growth. Chapter 4 expanded on Chapter 3 to show that the HTLV-1 \textit{Hbz} gene supports host cell growth in culture at two molecular levels: mRNA and protein. We speculate that HTLV-1 has evolved a novel undefined mechanism to manipulate the cellular environment where it utilizes the novel antisense encoded \textit{Hbz} gene and its protein product to support the growth of infected cells. The studies in this dissertation combined with future perspectives have been and will be instrumental to dissect the virus/host interactions associated with HTLV-1 infection, proliferation/survival of infected cells, and the development of disease.

5.2 The role of HBZ in early infection

The antisense transcript \textit{Hbz} mRNA was first identified by Larocca \textit{et al}, in HTLV-1 positive SLB-1 T-cells.\textsuperscript{177} Over 13 years later, Guadray \textit{et al}, identified the first functional role of HBZ to negatively regulate Tax-mediated transcription.\textsuperscript{176} Since the initial characterization of HBZ, most studies to date have been conducted in overexpression systems outside the context of the replicating virus. Mentioned previously, the accessory proteins of HTLV-1 collectively have been shown to be dispensable for cellular transformation/immortalization in cell culture, yet the specific contribution of HBZ in this process had not been tested.

In Chapter 2 we investigated the contribution of HBZ to HTLV-1-mediated immortalization of primary T-lymphocytes \textit{in vitro} and HTLV-1 infection and persistence in a rabbit animal model.\textsuperscript{187} HTLV-1 HBZ mutant viruses were generated and evaluated
for viral gene expression, protein production, and immortalization capacity. Biological properties of HBZ mutant viruses in vitro were indistinguishable from wild type HTLV-1 provides the first direct evidence that the HBZ protein is dispensable for viral replication and cellular immortalization. We then used a reproducible rabbit model that has been successfully used to study HTLV-1 and HTLV-2 transmission, infection, and persistence. The rabbit model is widely accepted as an appropriate model of the persistent asymptomatic infection in humans. Rabbits inoculated with irradiated cells expressing HTLV-1 HBZ mutant viruses became persistently infected. However, these rabbits displayed a decreased antibody response to viral gene products and reduced proviral copies in peripheral blood mononuclear cells (PBMCs) as compared to wild type HTLV-1 infected animals. Collectively, our findings were the first to indicate that HBZ was not required for in vitro cellular immortalization, but is required for enhanced infectivity and persistence in inoculated rabbits. This study demonstrated that a human retrovirus utilizes a negative strand-encoded protein in the establishment of chronic viral infections.

In this study we found a decreased antibody response in immune competent rabbits inoculated with HBZ mutant viruses and significantly lower proviral load numbers in rabbit PBMCs. Our data indicate that the HBZ knockout viruses were able to infect and persist, but at lower levels as compared to wild-type HTLV-1 infected controls. The low level of detection could be attributed to lack of efficient infectivity early on in infection, where the HBZ protein may be required to establish a persistent infection. It remains to be determined if the in vivo requirement of HBZ is dependent on this specific function (i.e. the repression of Tax-mediated transactivation) or whether
HBZ, like other retroviral proteins, is pleiotropic and has other direct or indirect roles in maintaining viral load and the infected cells’ ability to persistence in vivo; such as regulating the innate immune response. A more detailed understanding of the in vivo role of HBZ especially early after infection is further needed. Our data leads us to believe that the HBZ protein is needed early in infection for some undefined mechanism for virus survival. We hypothesized that the biological function of HBZ and its role in HTLV-1 biology is not likely during the early phase of viral infection. However, future experiments to quantitatively assess viral infectivity of rabbits at 1-2 days post inoculation are required to definitively rule out an early block to infection in vivo. Combined with the data that HTLV-1 HBZ represses Tax-mediated transcription and attenuates AP-1 activity, we speculate that HBZ might function in concert with other viral gene products to tightly regulate viral replication and/or influence the infected lymphocyte to ultimately promote cell survival, viral spread, and assist in establishment of persistent infection.

To date HTLV-1 researchers have not successfully epitope tagged any HTLV-1 protein in the context of a replicating virus without compromising function of other viral genes. This task has been proven to be very challenging due to the complex HTLV-1 genomic organization of overlapping open reading frames. Previously, we have shown that the HBZ protein is detectable by Western blot analysis in stably transfected 729ACH and most HTLV-1 positive cell lines. Interestingly, the C-terminus of HBZ does not overlap any positive strand open reading frame within the provirus. In theory this would be an ideal location to epitope tag HBZ with the green fluorescent protein (GFP) in the context of the pACHneo molecular clone. Implementing this cloning strategy and
employing diagnostic fluorescence imaging systems would allow detection of the HBZ-GFP protein in a timely manner early after viral infection, thus evaluating the potential temporal expression of HBZ following infection of cells both in cell culture and inoculated rabbits.

5.3 Hbz supports the growth of infected cells in vitro and in vivo

Chapter 3 shows that Hbz supports the proliferation of T-lymphocytes in culture. We made use of a lentiviral vector delivery system transcribing specific short hairpin RNAs (shRNAs) to Hbz nucleotide sequences. HTLV-1 positive SLB-1 T-cells were infected with the shRNA viruses targeting Hbz. We found that these shRNAs were specific to Hbz and did not affect global viral gene expression, but were capable of down regulating Hbz mRNA and protein expressions greater than 3 fold as compared to controls. Hbz knockdown correlated with significant decrease in T-cell proliferation in culture. Both SLB-1 and SLB-1-Hbz knockdown cells engrafted into inoculated NOD/SCIDγchain-/− mice to form solid clustered tumor masses that infiltrated into surrounding tissues. However, tumor formation and tumor cell organ tissue progression was significantly decreased in animals challenged with SLB-1-Hbz knockdown cells. Taken together, our data indicate that Hbz expression enhances the proliferative capacity of HTLV-1 infected T-cells playing a critical role in cell survival and ultimately HTLV-1 tumorigenesis in the infected host.

A study conducted by Satou et al, using transgenic mice expressing the Hbz gene under the control of the mouse CD4 promoter found that Hbz enhanced the proliferation of T-lymphocytes in vivo.188 Furthermore, data from an unpublished pilot study
conducted in our lab suggests the *Hbz* gene is supporting infected cell growth in HTLV-1 infected rabbits. At weekly time-points post challenge, rabbit PBMCs were isolated and quantitative real-time RT-PCR was performed on total cellular RNA. The data indicates that the *Hbz* message is the only accessory gene found at detectable levels and over an 8 week time-course these levels are comparable or higher than that of *gag/pol* and *tax/rex* messages. Collectively, the findings from these studies indicate that *Hbz* is playing a significant role in virus biology supporting infected cell growth.

The *Hbz* message can be detected in nearly all ATLL cells characterized to date, whereas *tax* expression is silenced in the majority of cases. With support from the data in Chapters 3 and 4 of this dissertation, we speculate that *Hbz* is largely involved in the leukemic process. *Hbz* and its protein may serve as an equilibrator to the functions of Tax, p13, and p30 proteins to balance survival signals thus allowing the infected cell to remain hidden from immune surveillance. Additionally when *tax* and other major viral genes are suppressed we believe HTLV-1 has evolved a unique function utilizing *Hbz* to promote cellular growth. Studies in Chapter 4 support a novel hypothesis that *Hbz* is functioning in two molecular forms: mRNA and protein. Collectively, our data shows for the first time that in the context of a replicating virus, *Hbz* mRNA in combination with the HBZ protein supports the growth of in cells culture. More directly, when *Hbz* is down-regulated by shRNAs in HTLV-1 immortalized PBMCs (PBLACH), we show a suppressed growth phenotype in presence and absence of exogenous recombinant hIL-2. Finally, in long term overexpression experiments using Jurkat T-cells, the *Hbz* mRNA promotes proliferation of the Jurkats and an additive effect in growth is seen when mRNA and protein are expressed together.
One of our future goals is to expand our preliminary data regarding a role of the\( Hbz \) mRNA and protein differentially regulating T-cell gene expression. To better understand the affects of \( Hbz \) mRNA and protein on cellular genes, we will utilize infectious lentiviral particles characterized in Chapter 4 to infect the natural host cell of HTLV-1, a purified population of CD4+ primary T-lymphocytes. Purified CD4+ primary T-lymphocytes will be infected and sorted by FACS analysis for GFP which is also encoded by the lentiviral expression vector. The GFP sorted cell populations will be characterized for \( Hbz \) gene expression and analyzed in MTS proliferation assays. The long-term goal is to perform separate gene microarrays on CD4+ primary T-lymphocytes which have been infected with an HBZ wild-type expression virus and a virus where the protein has been knocked out. A comparative analysis of the gene microarrays performed on each cell line will hopefully identify unique patterns of cellular genes that are affected by the mRNA or in conjunction with the protein resulting in the growth phenotypes observed \textit{in vitro}.

5.4 Identification and characterization of post-translational modifications involved in the functional regulation of HBZ

A better understanding of HBZ and its cellular binding partners is important to identify other potential functional roles of the HBZ protein. Thus, one of the future directions is to isolate and analyze the composition of complexes or binding partners associated with HBZ using mass spectrometry (MS). Proteins identified in these complexes under different conditions will not only increase our knowledge of how HBZ regulates viral transcription and affects cellular genes, but may also provide new insights
into different functions of HBZ. Based on the knowledge of other retroviral proteins, we and others propose that Hbz is multifunctional not only at the protein level, but based on our data presented in Chapter 4 also at the mRNA level. An answer may lie in biochemical approaches using MS in detecting new binding partners using an overexpression system where wild-type spliced HBZ open reading frame has been tagged at the amino terminus with S-epitope tag (Figure 5.1). Preliminary experiments indicate that following transfection in 293T cells the Stag-HBZ is functionally equivalent to exogenously overexpressed wild-type HBZ cDNA in repressing Tax-mediated viral transcription and p19 matrix antigen (Figure 5.2 A and 5.2B). Western blot analysis performed on protein lysates shows that wild-type HBZ and Stag-HBZ proteins are detectable using rabbit antisera or S-protein conjugated antibodies (Figure 5.2C) An important control for these proposed binding studies would be to cotransfect the HBZ knockout proviral clone (characterized in Chapter 2) along with the Stag-HBZ cDNA. This is a critical control because in the absence of the other viral proteins the cellular binding partner interactions with HBZ may not be accurate.

The difference between the predicted molecular weight ~ 26.0 kDa and the actual molecular weight of HBZ 30 kDa suggest that the protein is post-translationally modified. This begs the question of whether HBZ itself is regulated by phosphorylation or other modifications, and if this regulation is critical for its function. A preliminary experiment in which the Stag-HBZ cDNA was transfected into 293T cells and purified using S-protein beads shows that HBZ is phosphorylated. The purified Stag-HBZ protein was purified and run and an SDS-PAGE gel followed by a ProQ Diamond staining that only detects phosphorylated proteins. In Figure 5.3 the purified Stag-HBZ is detected by
the ProQ stain indicating HBZ is phosphorylated. To confirm and expand these findings our next step will be to purify this Stag-HBZ protein and subject it to MS analysis to specifically identify phosphorylated amino acid residues. Similar proteomic approaches can be done to identify some other biological posttranslational modifications including acetylation, sumoylation, and ubiquitination. If HBZ is proven to be post-translationally modified in multiple ways, then one could hypothesize that it acts as a sensor of environmental and cellular cues. That is, the viral gene expression would not be inhibited by HBZ; however only when the later picks up a cellular signal in the form of specific post-translational modification. Such control could be of paramount importance for viral survival in the host. Several domains of HBZ have been identified by mutational analysis and localization studies. Our work in Chapter 2 revealed that the leucine zipper is a key functional domain of HBZ. The amino acid sequence of HBZ (Figure 5.4) indicates that there are 11 possible residues that could be phosphorylated. Some of these potential phosphorylation sites are located in important domains of HBZ including the leucine zipper, activation domain, and the basic region. Posttranslational modifications, specifically phosphorylations of these residues, could be essential to proper function of the HBZ protein. If these suspect residues prove to be phosphorylated we will next perform mutational analysis making amino acid substitutions to phosphorylated residues. After these HBZ point mutations are cloned, the mutants will be tested by cotransfections in 293T cells using a Tax-mediated repressional reporter assay for loss or gain function.

A bioinformatics approach was taken to analyze the HBZ amino acid sequence using NetPhos 2.0 server. Based on the server results and the amino acid consensus sequences of HBZ, serines 29, 49, 54, 150, 174 and threonines 73, 165 have a 97-92%
probability of being phosphorylated by Casine-Kinase II or Protein Kinase C (Figure 5.4). In ideal conditions the \textit{in vivo} binding study mentioned above could be a good approach to identify the cellular kinase(s) responsible for the phosphorylation of HBZ. Additional experiments using specific kinase inhibitors might prevent the phosphorylation of HBZ and provide correlative support to the kinases identified in the binding study. Once the kinase(s) is identified and purified, identification of phosphatases will likely be easier and can be pursued using similar strategies. Additionally, the suspect kinases that potentially phosphorylate HBZ could be tested in an \textit{in vitro} kinase assay.

5.5 Characterization and transcriptional regulation of the 3’LTR

It has been reported by several groups that there are multiple \textit{Hbz} mRNA transcripts coded on the minus strand of the provirus: an unspliced \textit{Hbz} transcript and two spliced \textit{Hbz} transcripts.\textsuperscript{181,182,188} Interestingly, the \textit{Hbz} major spliced and \textit{Hbz} unspliced transcript is detectable by RT-PCR methods in all ATLL cells characterized to date. Yet the spliced isoform of the HBZ protein is the only form detected in HTLV-1 positive cells.\textsuperscript{182} Mentioned previously in this dissertation, several transcription initiation sites for \textit{Hbz} have been identified by nucleotide sequencing of the 5’RACE amplified products from total RNA of ATLL cells. To date the promoter region regulating the different transcripts has not been elucidated. These preliminary observations suggest that positive transcriptional regulatory elements could be confined to the region of \( \sim 180 \) nucleotides in the 3’LTR U5 region 5’ to transcription initiation. The first step to determine if there are consensuses sequences in this region of DNA would be to use bioinformatics
approach to search for potential transcription factor binding sites. If such binding sites are
revealed, site directed mutagenesis could be used to alter the sequences of these binding
factor sites thereby inhibiting the transcription factor binding. Next, chromatin
immunoprecipitation assays could be preformed to identify the transcription factor
binding or loss of binding to DNA. These experiments should be performed in the
context of the provirus using the molecular clone of HTLV-1. Specifically, quantitative
real time RT-PCR will allow us to precisely quantify and identify the different \( Hbz \)
transcript mRNA levels whereby identifying the essential upstream regulators of
antisense viral gene transcription.

5.6 \( Hbz \) gene in ATLL development

Accumulating evidence suggests that the development of ATLL requires two
HTLV-1 genes Tax and \( Hbz \), whose expression is respectively controlled by the 5’ and 3’
LTRs respectively.\(^{111,261}\) (Figure 5.5) In the initial stage of infection by HTLV-1, \( Tax \)
expression is high, depends on the ATF/CREB pathway, and via a positive feedback
stimulates protein viral synthesis and virus production. However, cells highly expressing
HTLV-1 proteins are eliminated by the humoral immune response and host cell CTL
activity. At this stage, \( Hbz \) plays a critical role by down regulating the 5’-LTR-dependent
viral transcription and may allow infected cells to evade immune surveillance. After
evasion, clonal expansion of infected T cells is stimulated through activation of the NF-
\( \text{KB} \) and AP-1 transcription factors by Tax whose level is controlled by HBZ. The balance
between proliferation of HTLV-1 infected cells and their elimination by ant-Tax CTL
response favors the establishment of a persistent infection. However, during this lengthy
period of time, complex interactions between Tax and host cells (including mitosis, stimulation, cell death suppression, and mutations in tumor suppressor genes) are critical events which may contribute to the emergence of transformed cells. Moreover, suppression of Tax expression, reactivation of telomerase activity, and activation of E2F1 by HBZ, in addition to an immunosuppressed state in the HTLV-1 infected host, might favor the evasion of the transformed cell to the immune system, thus resulting in ATLL.

A blockade of viral protein functions has been shown to be a good strategy in the development of anticancer treatments, as it has been developed against the human papilloma virus (HPV) E6 and E7 proteins. However, Tax does not appear to be a good candidate for the development of such a strategy in the treatment of ATLL. Tax-induced transformation corresponds with a “hit-and-run” mechanism, that is, Tax mediates cellular transformation through an initial hit while the maintenance of the transformed state is paralleled with the loss of its expression in the cell. As mentioned before, the majority of ATLL clones do not express detectable levels of Tax. On the other hand, the presence of Hbz in leukemic cells still remains unclear. It would be of high interest to determine its exact function. For instance, ATLL shows poor prognosis mainly because of its resistance to conventional chemotherapy through the possible resistance of transformed cells to induce apoptosis. Hbz could directly affect apoptosis by inhibiting p53 activity or indirectly by stimulating expression of genes involved in apoptosis inhibition. Another important question is what is the mechanism involved in the activation of JunD by HBZ? JunD was initially considered to be an inhibitor of cellular transformation, and for this reason it has been suggested that ATLL cells might rather be expressing mutated forms of JunD. However, the capacity of HBZ to activate this
cellular factor suggests that JunD may act as a positive regulator of cell transformation in the presence of HBZ. Interestingly, it has been shown that the absence of transforming activity of JunD may be due to its instability to form highly stable homodimers \textit{in vivo}. In fact, the replacement of the dimerization domain of JunD with the heterologous homodimerization domain of the Eptesine-Barr virus increased the transcriptional activity of its transforming activity. In a similar manner, JunD switches from a growth suppressor to a growth promoter when it no longer interacts with the tumor suppressor, menin. Characterization of the HBZ/JunD heterodimer is also an important step to determining the exact function of $Hbz$ in the development of ATLL.

In conclusion, it is currently unclear whether interfering with $Hbz$ may be a useful strategy for the prevention and/or treatment of ATLL. Currently it is still too early to affirm that $Hbz$ could represent a potential target for treatment strategies as we have not answered the question: Why is $Hbz$ expressed in ATLL cells? Appropriate answers to this question represent a very important challenge for the future development of anti-ATLL therapies. Thorough investigations of these future directions highlighted in this chapter have implications on several fronts. First and foremost, they will expand our limited understanding on the roles of natural antisense transcripts in basic retroviral biology. Second, HTLV-associated malignancies remain a significant problem in endemic regions around the world. Thus, the need to develop a vaccine is urgent. Understanding the exact role of this accessory protein, how it regulates viral replication, and it is regulated might prove to be critical for the development of vaccines or novel therapeutic strategies.
Figure 5.1: A cartoon representation of the N-terminal epitope tagged HBZ. Wild-type HBZ cDNA was cloned inframe to the TriEx-Neo4 expression plasmid. Promoter, epitope tags, where appropriate are indicated.
Figure 5.2: Exogenously expressed Stag-HBZ results in dose-dependent repression of Tax-mediated transcription and p19 Gag production. 293T cells (1.5x10^5) were cotransfected with 1 µg of wtHTLV-1 proviral clone or negative control DNA, 0.1 µg of LTR-1-Luc and 0.01 µg of TK-Renilla, and concentrations 0.4 µg of wildtype HBZ or Stag-HBZ expression vectors as indicated. (A) Tax function was measured as firefly luciferase activity from LTR-Luc normalized to Renilla luciferase activity. RLU, relative light units. (B) Culture supernatant was collected from cells in panel A and assayed for p19 Gag production by ELISA. (C) Western blot analysis to confirm protein expression wild-type HBZ and Stag-HBZ used in panels A and B using rabbit antisera or S-protein conjugate. (*) represents statistically significant dose-dependent reduction of Tax transactivation activity or p19 Gag production. Statistical significance was determined by paired T- test (p<.0012).
**Figure 5.3:** HBZ is phosphorylated *in vivo*. (A) 293T cells were transiently transfected with Stag-HBZ cDNA expression vectors. 24 h post transfection total cellular protein lysates were harvested. A pull-down using S-protein beads was used to purify the Stag-HBZ protein. Purified protein was run on an SDS-PAGE. (A) ProQ diamond staining was used to detect only phosphorylated proteins. HBZ was detected by ProQ staining (Green) indicating the protein is phosphorylated. (B) Total protein was detected by Sypro Ruby Red or Gel Code Blue stain. (C) Western blot analysis using HBZ rabbit antisera or S-protein conjugate was performed on inputs prior to S-protein bead pull down.
Figure 5.4: The wild-type HBZ amino acid sequence. Highlighted are the 11 candidate serine, threonine, and tyrosine residues that may be phosphorylated. The underlined amino acids identify the basic region and leucine zipper domain.
Roles of *Tax* and *Hbz* in steps associated with transformation of infected T-cells

**S'LTR: Tax**

- Tax enhances viral expression:
  - HTLV-I-expressing cells are killed by host CTL activity
  - Tax can cause apoptosis

**3'LTR: Hbz**

- HBZ down regulates viral transcription:
  - Infected cells escape HTLV-I specific CTL killing
  - Limiting Tax expression

**Establishment of a persistent Infection**

- Tax stimulates expression of infected T-cells
  - Through the activation of NFKB and AP-1

- HBZ controls Tax expression

**Transformation of the infected T-cells**

- Tax inhibits Rb, p53, and hDLg inducing chromosomal rearrangements

- HBZ controls AP-1 activity
  - (c-Jun, JunB, & JunD)

**Leukemic clone escapes host immune control:**

- Development of ATL.
  - No more expression of Tax due to epigenetic modifications or deletion of the S'LTR

- HBZ activates *MTERT* and *E2F1* expression promoting cell survival

**Figure 5.5:** Roles of *Tax* and *Hbz*, in the different steps associated with the transformation of the infected T-cell. 5’ and 3’ LTRs, respectively, control expression of Tax and Hbz genes involved in different interactions with host cell that contribute to the development of ATLL.
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