COMPARATIVE STUDIES ON MOLECULAR MECHANISMS UTILIZED BY
HTLV-1 AND HTLV-2 IN VIRAL REPLICATION AND INDUCTION OF
T-CELL TRANSFORMATION

DISSERTATION

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by

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ABSTRACT

Human T-cell leukemia virus (HTLV)-1 and HTLV-2 are closely related human retroviruses that have similar genetic organization and biological properties. However, they display distinct pathogenicity. HTLV-1 has been identified as a causal agent for two human diseases, adult T-cell leukemia (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), whereas HTLV-2 appears much less pathogenic without conclusive disease association. Studies in this thesis focus on the specific strategies utilized by HTLV-1 and/or HTLV-2 to replicate and induce cellular transformation.

HTLV cellular transformation and disease induction are dependent on expression of the viral Tax oncoprotein. HTLV-1 Tax (Tax-1), but not HTLV-2 Tax (Tax-2), contains a PDZ domain binding motif (PBM) that promotes Tax-1 interaction with several cellular PDZ proteins. PDZ is a modular protein interaction domain involved in cell polarity, synapse, and cellular proliferation. In Chapter 2 of this thesis, we investigate the contribution of the Tax-1 PBM in HTLV-induced proliferation and immortalization of primary T-cells in vitro and viral survival in an infectious rabbit animal model. We generated several HTLV-1 and HTLV-2 Tax viral mutants including HTLV-1ΔPBM, HTLV-2+C22(+PBM), and HTLV-2+C18(ΔPBM). Tax mutations did not alter the ability to activate the CREB/ATF or NFκB signaling pathways. Coculture microtiter
proliferation assays revealed that the Tax-1 PBM significantly increases both HTLV-1 and HTLV-2-induced primary T-cell proliferation. In addition, Tax-1 PBM was responsible for the micronuclei induction activity of Tax-1 relative to that of Tax-2. Viral infection and persistence were severely attenuated in rabbits inoculated with HTLV-1ΔPBM. Our results provide the first direct evidence that PBM-mediated associations between Tax-1 and cellular proteins play a key role in HTLV-induced cell proliferation and genetic instability \textit{in vitro} and facilitate viral spread and persistence \textit{in vivo}.

Previous studies have indicated that \textit{in vivo} HTLV-1 has a preferential tropism for CD4+ T cells, whereas HTLV-2 \textit{in vivo} tropism is less clear but appears to favor CD8+ T cells. This tropism difference has been recapitulated in \textit{in vitro} transformation assays. Taking advantage of the high homology between HTLV-1 and HTLV-2, we chose to generate chimeric HTLV proviral clones in order to identify the viral determinant(s) responsible for their distinct \textit{in vitro} transformation tropisms. Our previous studies have shown that Tax and its overlapping Rex sequence do not confer the distinct transformation tropisms between HTLV-1 and HTLV-2. In Chapter 3, two pairs of recombinant HTLV were generated, in which the viral LTR or \textit{env} gene was exchanged between HTLV-1 and HTLV-2. All the recombinants are functional based on the p19 Gag expression from proviral clones transfected into 293T cells. Stable transfectants expressing recombinant viruses were established, irradiated, and cocultured with human peripheral blood monocytes (PBMCs). All recombinants were competent to transform T-lymphocytes in \textit{in vitro} coculture transformation assays. Flow cytometry analysis of transformed PBMCs indicated that exchange of the viral LTR does not alter HTLV
transformation tropisms. However, when the env gene was exchanged, HTLV transformation tropism changed correspondingly, i.e. HTLV-2/Env1 preferentially transforms CD4+ T lymphocytes, and HTLV-1/Env2 has similar cellular tropism pattern with wild type HTLV-2 (CD8+ T cells). The results indicate that the env gene is a major viral determinant for HTLV T-cell transformation tropism and provide strong evidence implicating its contribution to the different pathobiologies of HTLV-1 versus HTLV-2.

Rex phosphoprotein functions to increase the level of viral structural and enzymatic gene products, which are expressed from the unspliced and incompletely-spliced viral mRNAs. Recently, it has been demonstrated that the phosphorylation of HTLV-2 Rex (Rex-2), particularly at serine residues 151 and 153 within the carboxy-terminal phosphorylation domain (CTPD), is critical for its function. Replacement of both serine residues with phosphomimetic aspartic acid (S151D/S153D) has been shown to lock Rex-2 in an active conformation. This mutant phenotype provides us with a unique reagent to evaluate the role of Rex-2 functional regulation in viral replication and cellular transformation in vitro and persistence in vivo. Since the mutation in RexS151D/S153D also affects viral oncoprotein Tax amino acid sequences and its transactivation activities, in Chapter 4 several additional Rex mutants in this region were generated and characterized to facilitate these studies, two aspartic acid substitution mutants, RexP152D and RexA157D and two carboxy-terminus deletion mutants, RexS151Term and RexS158Term. All mutants localized predominantly to the nucleus/nucleolus and display a locked active functional conformation similar to RexS151D/S153D. In short-term microtiter proliferation assays, RexA157D enhances HTLV-2-mediated cellular proliferation of human peripheral blood mononuclear cells (hPBMCs), whereas hPBMCs in coculture
with 729HTLV-2/RexS151Term or 729HTLV-2/RexS158Term display substantially decreased proliferation. In long-term immortalization assays, HTLV-2/RexP152D and HTLV-2/RexA157D have the capacity to immortalize primary T cells as efficiently as wtHTLV-2, whereas both HTLV-2/RexS151Term and HTLV-2/RexS158Term were deficient in immortalization of hPBMCs. Our studies indicate that constitutive active Rex-2, particularly the phosphomimetic mutants, can increase viral protein expression, contributing to the enhanced primary T-cell proliferation mediated by HTLV-2 infection. However, constitutive active Rex-2 mutants without CTPD impair HTLV-2-induced cellular proliferation and transformation, implying that the CTPD is required for proper functional regulation of Rex-2 in the context of the virus. Interestingly, HTLV-2/RexP152D was impaired in inoculated rabbits as compared to wtHTLV-2 supporting the conclusion that this domain plays an important regulatory role in vivo.

This is the first report of the critical role of HTLV Rex regulatory control in viral mediated cellular proliferation and immortalization, and provides insight into viral-host interaction in HTLV-2-infected cells.

Overall, the work described in this thesis employ methods that manipulate viral elements in the context of full-length proviral clones and analyze their function and mechanism of action in a system closely mimicking in vivo HTLV infection. In this way, our studies provide important insight into the molecular pathogenesis of HTLV-1 and HTLV-2.
Dedicated to:
My parents, Baoguo Xie and Guoyun Guo
       My husband, Haibin Huang
       And my son, Sunny (Shican) Huang
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CHAPTER 1

LITERATURE REVIEW

1.1 HTLV isolation and its disease association

1.1.1 Identification of HTLV-1 and Adult T-cell leukemia/lymphoma

Human T-cell leukemia virus type 1 (HTLV-1) was first isolated in 1980 from two T-lymphoblastoid cell lines, HUT 102 and CTCL-3, established from a patient with cutaneous T-cell lymphoma\(^1,2\). A year later, it was isolated from another cell line (MT-1), derived from a patient with adult T-cell leukemia (ATL). Further serological, immunologic, genetic and molecular studies have identified HTLV-1 as a causal agent for ATL\(^2-5\).

HTLV-1 infection is a prerequisite for ATL development. However, only a small proportion of HTLV-1-infected individuals, especially those who acquired the virus as a result of breast feeding, develop ATL after a long latent period\(^6,7\). It has been speculated that the development of ATL involves two stages, the initial polyclonal infection stage due to HTLV-1 replication and spread, and the clonal expansion stage of the infected T-lymphocyte\(^8,9\).

The clinical manifestations of ATL present as malaise, fever, dyspnea, lymph node enlargement, hepatosplenomegaly, jaundice, and drowsiness. In addition, abnormal
laboratory findings including marked leukocytosis, hypercalcemia, high serum levels of lactate dehydrogenase (LDH) and a soluble form of interleukin-2 receptor (sIL-2R) α-chain, and the appearance of characteristic leukemic cells with deeply convoluted or lobulated nuclei (flower cells). These morphologically abnormal T-cells are key marker of HTLV-1 infection and have a mature phenotype (CD2+CD3+CD4+CD8-CD25+ and HLA-DR+) \(^10\). One of the major complications of ATL is hypercalcemia found in about 20-30% of patients, which is classified as a humoral hypercalcemia of malignancy caused by parathyroid hormone-related protein (PTHrP) \(^11,12\). Another unique clinical characteristic of ATL is the massive infiltration of leukemic cells in the skin, liver, spleen, GI tract and lung. Constitutive overexpression of various cytokines and chemokines probably provide a basis for the lymphocytic infiltration. Immunosuppression and opportunistic infections by bacteria, fungi, protozoa and viruses have also been reported in ATL patients, contributing to their poor prognosis \(^13,14\).

Based on tumor lesions in various organs and clinical course, ATL is classified into four subtypes: acute, chronic, smoldering and lymphoma. The acute subtype has the highest incidence and shows the clinical characteristics listed above. The chronic and smoldering subtypes are less aggressive and may represent transitional states to the development of acute ATL. Patients with chronic ATL generally have elevated number of circulating ATL cells with mild symptoms, whereas patients with smoldering ATL present with skin lesions, lymph node enlargement and marrow involvement. The predominant feature of lymphoma type ATL is lymph node enlargement but not leukemic manifestations \(^15,16\).
1.1.2 HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

Five years after HTLV-1 identification, two different groups reported that a chronic inflammatory neurological disease, known as tropical spastic paraparesis, was seropositive for HTLV-1. Although the patients initially identified were from West India and Japan, to date HAM/TSP has been described in all areas of the world known to be endemic for HTLV-1. It has been demonstrated that high HTLV-1 proviral load and host genetic factors are two important contributing factors for HAM/TSP disease development.

Clinically, HAM/TSP is characterized by muscle weakness, spasticity in the lower extremities, hyperreflexia, urinary/fecal incontinence and mild peripheral sensory loss. Frequent clonal proliferation of HTLV-1-infected T cells, a phenomenon in ATL patients, was observed by Furukara et al in the peripheral blood or in the cerebrospinal fluid of HAM/TSP patients.

1.1.3 Other HTLV-1 associated diseases

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

1.1.4 Identification of HTLV-2 and its disease association

HTLV-2 was first isolated in Mo-T, a T-cell line established from the spleen of a patient (Mo) with a T-lymphocyte variant of hairy cell leukemia. Serum from Mo contained antibodies to the major internal core protein (p24) of HTLV-1 but with significantly different immunological cross-reactivity from all the HTLV-1 isolates. Therefore this virus was designated as HTLV-2. It appears that HTLV-2 is much less pathogenic in comparison to HTLV-1, and its etiological role in disease induction remains unclear. In addition to several cases of atypical hairy-cell leukemia, HTLV-2 has been implicated in a few cases of neurological disease.

1.2 HTLV transmission and epidemiology

Both HTLV-1 and HTLV-2 are highly cell-associated and efficient transmission requires cell-cell contact. They are primarily transmitted via three major routes: mother to child through breast feeding, male to female by sexual contact, and by way of infected blood such as blood transfusion or usage of contaminated needles.

HTLV-1 has been identified throughout the world, but is found predominantly in southwest Japan, the Caribbean basin, central Africa, South America, the Middle East, and parts of Melanesia. It has been estimated that there are ~15-25 million individuals infected with HTLV-1. HTLV-2 infection is less prevalent than that of HTLV-1 and tends to cluster in certain population groups, such as IV drug abusers.
1.3 HTLV genome organization and viral replication

HTLV is classified as deltaretroviruses, along with bovine leukemia virus (BLV) and simian T-cell leukemia virus (STLV). These viruses are referred to as complex retroviruses. As depicted in Figure 1.1, one of the cloned proviruses of HTLV-1 is about 9.0 kb and HTLV-2 is about 8.8 kb in length. They share a similar genomic organization, with about 65% homology at the nucleotide sequence level. Similar to other retroviruses, HTLV encodes the structural and enzymatic genes products including Gag, Pro, Pol and Env. In addition, there is a unique region called the pX at the 3’ end of HTLV genome that encodes several regulatory and accessory proteins. In HTLV virions the genomic information is carried by two copies of single-stranded positive sense RNA, enclosed by viral capsid protein and an envelope containing viral envelope protein and cellular membrane.

HTLV replicates through a complex life cycle. It begins with the adsorption of viral particles on the cell surface. Next the interaction between the SU component of Env and cellular receptor(s) triggers a series of events including SU conformational change to release TM component (fusion peptide), formation of fusion pores, and membrane fusion followed by the entry of virion contents into the cytoplasm. Once in the cytoplasm, the viral RNA genome undergoes reverse transcription to generate double stranded DNA (dsDNA) using virally encoded reverse transcriptase. The pre-integration complex containing the viral dsDNA is transported into the nucleus and randomly integrates into the host genome using viral encoded integrase. The DNA form of the viral genome is referred to as provirus, which contains direct long terminal repeats (LTR) at both the 5’-
and the 3’-end. Following viral gene transcription and translation mediated by host machinery, immature progeny virions are assembled at the plasma membrane and become mature after budding.

1.4 Viral LTR

As shown in Fig 1.1, the viral LTR is divided into three regions, U3, R, and U5. The U3 region contains viral transcription enhancer and promoter elements with binding sites for numerous cellular transcription factors. In particular, the three imperfect 21-nucleotide repeats, termed Tax response element-1 (TRE-1), are homologous to cellular cyclic AMP response element binding protein (CREB) binding sites (CREs). How the viral protein Tax activates viral transcription through interaction with CREB and CREB family members will be discussed further in section 1.7.2. Between the second (middle) and the third (promoter proximal) TRE-1, there is an additional Tax responsive element TRE-2 or Ets responsive region-1 (ERR-1), which can also mediate transactivation via the binding of many other cellular proteins such as Ets family proteins and c-Myb. *In vivo* footprinting on TRE-2 indicated that this region is bound by proteins in the presence or absence of Tax. Although Tax protein does not appear to bind directly to the viral promoter, it is proposed that Tax can stabilize the cellular protein-TRE complexes. On the other hand, the cooperative binding of Tax and some cellular proteins to the viral promoter may position Tax to interact with basal transcription factors CBP, TFIIA, and TFIID.

The viral LTR also contains some sequences that are important for viral replication. The mRNA termination or polyadenylation sequence begins at the end of R region in the
3’ LTR. The transcription start site is located at the U3/R junction. The *att* sites required for proviral integration are found in the 5’ terminal U3 and 3’ terminal U5 sequences. Downstream of the U5 region and upstream of the *gag* gene are the primer binding site (pbs) and the Psi or E sequence. The pbs is a 18nt sequence that can hybridize with host tRNA necessary for priming viral reverse transcription. The Psi or E sequence is the major signal for the encapsidation of the viral RNA into virion.

Although the 5’-LTR and the 3’-LTR are identical in sequence, CpG methylation has been implicated in viral gene regulation *in vivo*. Differential methylation predominantly silences the 3’-LTR, and selective hypermethylation of the 5’-LTR correlates with latency of viral gene expression $^{50-53}$.

Comparison of the LTRs between HTLV-1 and HTLV-2 indicated that they only share about 31% nucleotide sequence homology. However, those elements critical for viral gene expression (e.g., the TRE-1, polyadenylation signal, and TATA box) are well conserved $^{15}$.

### 1.5 HTLV structural and enzymatic gene products

HTLV expresses multiple gene products from the same coding region by employing strategies including alternative mRNA splicing, ribosomal frame shifting, polycistronic translation, and protease-mediated cleavage of polyprotein precursor into mature smaller polypeptides. As shown in Figure 1.1, three major mRNA species have been identified for HTLV. The full-length RNA is utilized as virion genomic RNA and for the synthesis of *gag*, *pro* and *pol* gene products. A single-spliced subgenomic mRNA encodes the *env* gene products and a completely spliced subgenomic mRNA encodes the two
transregulatory proteins, Tax and Rex. Several additional mRNA species have been detected by RT-PCR or RNase protection assays in cells isolated from patients with ATL or HAM/TSP. These RNAs encode accessory proteins that contribute to viral infectivity in quiescent T-lymphocytes and viral maintenance in vivo.

There are three mature gag (group-associated gene) gene products, p19 matrix (MA), p24 capsid (CA), and p15 nucleocapsid (NC), generated from the p55 precursor by protease-mediated cleavage. NH$_2$-terminus myristoylation of p19 targets the p55 Gag precursor to the inner surface of the cell membrane. Studies by Le Blanc et al indicated that the 11 basic amino acids of p19 MA plays an important role in viral particle assembly, budding and cell-cell transmission. In addition to assembling into virions, some Gag protein including p19, can be secreted out of infected cells. The amount of secreted p19 usually correlates with the amount of virion produced from the cells, thus p19 production is commonly used as a measure of virion production.

The viral protease is produced from the same gag precursor mRNA via ribosomal frameshifting. It is responsible for processing the mature Gag products as well as generating the mature form of itself by autocatalyzed self-cleavage. Another ribosomal frameshifting event is responsible for the translation of the pol gene, which encodes viral reverse transcriptase, integrase and RNaseH.

The product of the env gene is a glycoprotein of approximately 61-69Kd depending on different cell lines studied. Like those of other retroviruses, HTLV Env is synthesized in the endoplasmic reticulum (ER) as a precursor protein, glycosylated in the Golgi and cleaved into two mature products, a surface subunit (SU, gp46) and a transmembrane subunit (TM, gp21). The interaction(s) between Env and cellular
factors/receptors mediate the first step of viral entry, and may also contribute to HTLV cellular tropism.

1.6 HTLV accessory proteins

The unique pX region located at the 3’ portion of the HTLV genome comprises four (HTLV-1) or five (HTLV-2) open reading frames. ORF IV and III of both HTLV-1 and HTLV-2 encode two trans-regulatory proteins, Tax and Rex respectively, which will be reviewed in sections 1.7 and 1.8. In HTLV-1, alternatively spliced mRNAs into ORF I and II encode accessory proteins including p27\textsuperscript{I}, p12\textsuperscript{I}, p30\textsuperscript{II}, and p13\textsuperscript{II}. In HTLV-2, ORF I, II, and V are proposed to encode p10\textsuperscript{I}, p11\textsuperscript{V}, p28\textsuperscript{II} and its isoforms p22\textsuperscript{III}/p20\textsuperscript{III}. Although these proteins cannot be detected in HTLV infected cells, their mRNA species are detected by RT-PCR. In addition, cytotoxic T lymphocytes (CTLs) recognizing pX-I and pX-II peptides as well as serum antibodies against recombinant proteins/peptides representing these accessory proteins can be detected in infected patients. Accumulating studies highlight the importance of these accessory proteins in efficient viral infection, T-cell activation, and regulation of gene transcription.

1.6.1 p12\textsuperscript{I}

The p12\textsuperscript{I} accessory protein can be expressed from singly spliced mRNA pX-ORF I and from doubly spliced mRNA pX-rex-ORF I utilizing an internal methionine codon. The mRNA pX-rex-ORF I can also encode a 152 amino acid protein p27\textsuperscript{I}, which is highly hydrophobic and can stimulate specific CTLs response in HTLV-1-infected individuals. However, Koralinik et al demonstrated the preferential expression of p12\textsuperscript{I} from
pX-rex-ORF I mRNA by transfection of an HA-tagged expression plasmid 71.

p12I is a membrane associated hydrophobic protein made up of 99 amino acid residues 72. Structural and functional analyses revealed that p12I contains two potential transmembrane regions (TM-1 and TM-2), four SH3 binding motifs (PXXP), a leucine zipper region and a dileucine motif (DXXXLL) 73,74. Furthermore, it was demonstrated that p12I exhibits structural features and some biological behavior similar to those of latent membrane protein-1 (LMP-1) of Epstein-Barr virus, E5 transformation protein of bovine papillomavirus, and transmembrane proteins of the slowly transforming retroviruses 75,76.

Although initial studies indicated that abrogation of p12I had no effect on viral replication and immortalization of primary lymphocytes in vitro 77, later studies revealed the essential role of p12I in the establishment of persistent in vivo viral infection 78. Using multiple in vitro coculture assays, Albrecht et al demonstrated that p12I is required for optimal viral infectivity in quiescent but not interleukin-2 (IL-2) and phytohemagglutinin (PHA) -activated primary cells, which suggests a role for p12I in T-cell activation 79. Further studies from the same group indicate that p12I appears to induce calcineurin (a calcium-binding protein)-dependent nuclear factor of activated T-cells (NFAT) activation either indirectly by elevation of cytoplasmic calcium level or directly by p12I binding to calcineurin 80-82. In addition, p12I can enhance NFAT-mediated IL-2 production during T-cell activation 83.

Other studies from the Franchini laboratory suggest that p12I can activate T-cell independent of the NFAT pathway. In fact, p12I physically binds to the cytoplasmic domain of IL-2 receptor β and γ chains resulting in enhanced activation of signal
transducers and activators of transcription 5 (STAT5), which may account for the clonal proliferation of infected T cells \textit{in vivo} \textsuperscript{84,85}. On the other hand, p12\textsuperscript{I} can interact with free human major histocompatibility complex class I heavy chains (MHC-1-Hc) and subsequently target the heavy chains for proteasomal degradation. This finding implicates p12\textsuperscript{I} in the immune escape of HTLV-1-infected cells, probably through interference of \textit{in vivo} antigen presentation \textsuperscript{86}.

1.6.2 p30\textsuperscript{II} and p13\textsuperscript{II}

p30\textsuperscript{II} contains a serine/threonine-rich domain and shares homology with the transcriptional factors Oct-1/-2, Pit-1 and POU-M1 \textsuperscript{62}. In addition, a highly conserved bipartite NLS was identified in p30\textsuperscript{II}, which can functionally substitute for Rex NLS \textsuperscript{87}. Interestingly, p30\textsuperscript{II} was found to co-localize with p300 in the nucleus and physically interact with CREB binding protein (CBP)/p300 via the highly conserved KIX domain. HTLV-1 Tax, a viral transactivator, also interacts with CBP/p300 at the same domain \textsuperscript{71,88}. These features of p30\textsuperscript{II} suggest its involvement in both cellular and viral gene transcription. At high concentration, p30\textsuperscript{II} functions as a repressor of viral gene transcription by competing with Tax for CBP/p300. Similarly, p30\textsuperscript{II} may also repress cellular gene transcription from CREB-responsive promoters by sequestering the limited amount cellular CBP/p300 \textsuperscript{88,89}.

Recently, it has been shown that p30\textsuperscript{II} can inhibit virion production at the post-transcriptional level by binding to and retaining \textit{tax/rex} mRNA in the nucleus \textsuperscript{90,91}. As a negative regulator of viral gene expression, p30\textsuperscript{II} may serve to promote \textit{in vivo} viral persistence by reducing immune recognition of infected cells. In fact, when p30\textsuperscript{II}
expression is eliminated, an infectious HTLV-1 molecular clone failed to maintain high viral loads in vivo \textsuperscript{92,93}.

p13\textsuperscript{II} is initiated internally from full-length p30\textsuperscript{II} mRNA, and only represents the C-terminus of p30\textsuperscript{II}. Unlike p30\textsuperscript{II}, p13\textsuperscript{II} localizes to the mitochondria and alters its morphology by disrupting the inner membrane potential, implying that it is involved in apoptosis \textsuperscript{94,95}. Recent studies showed similarities between p13\textsuperscript{II} and G4 (a BLV oncoprotein), and highlighted the potential role of p13\textsuperscript{II} in oncogenesis \textsuperscript{96}. Although p13\textsuperscript{II} is dispensable for HTLV-1 infection and immortalization of PBMCs in vitro, it is required for maintaining high viral load in inoculated rabbits \textsuperscript{77,92}.

1.6.3 HBZ

Recently, a protein encoded by the complementary strand of HTLV-1 RNA genome was identified. Its expression is driven by a functional promoter present in the antisense strand of the 1.8-kb 3' terminus of the HTLV-1 proviral genome. This protein is designated HBZ (HTLV-1 bZIP factor), since it contains a leucine zipper motif (bZIP) in its C-terminus. HBZ can interact with CREB-2 via their bZIP domains, and thus interfere with the association between CREB-2 and Tax, leading to downregulation of viral transcription. More recent studies suggest that HBZ can also form heterodimers with cellular transcription factors belonging to the activating protein-1 (AP-1) family, and modulate their transcriptional activities \textsuperscript{97-101}.

1.6.4 HTLV-2 accessory proteins

As with HTLV-1, the proximal pX region of HTLV-2 between env and the last exon
of \textit{tax/rex} is not required for \textit{in vitro} viral replication and cellular transformation, but plays an important role in maintaining high viral load and viral persistence \textit{in vivo} \cite{102,103}.

\textit{p10}I and \textit{p11}V are two HTLV-2 accessory proteins expressed from the same mRNA. A previous study found that the MHC class I heavy chain is a common cellular target of HTLV-2 \textit{p10}I, \textit{p11}V and HTLV-1 \textit{p12}I. This study proposed the potential role of \textit{p10}I and \textit{p11}V in interference of HTLV-2 viral antigen presentation \cite{104}.

Similar to \textit{p30}II, HTLV-2 \textit{p28}II localizes to the nucleus and specifically inhibits the cytoplasmic export of \textit{tax/rex} mRNA in the context of the provirus. Therefore it functions as a post-transcriptional repressor of virion production and potentially promotes viral persistence \textit{in vivo} \cite{91}. There is no report about the function of the truncated Rex isoforms \textit{p22}III/\textit{p20}III to date.

\section*{1.7 HTLV Tax, transcriptional activator of pX region}

HTLV Tax is encoded by pX-ORF-IV, which largely overlaps pX-ORF-III encoding Rex. Originally it was identified as a trans-acting transcription activator of viral gene expression in response to the viral promoter in the LTR \cite{105,106}. Further studies indicated that Tax is a pleiotropic protein. In addition to the viral LTR, Tax can transactivate many cellular promoters via activation of a number of different cellular transcription factors including CREB/ATF, NFxB/Rel, SRF, and basic helix-loop-helix (bHLH) proteins. Tax can also modulate the activity of some cellular factors involved in DNA repair and cell cycle, such as p53 and p16\textsuperscript{INK4A}. These activities are all implicated in HTLV-mediated cellular transformation and pathogenesis \cite{15,107}. 

1.7.1 HTLV-1 Tax (Tax-1) and HTLV-2 Tax (Tax-2)

There are several isotypes for both HTLV-1 and HTLV-2. Interestingly, Tax-1 from all HTLV-1 isolates is quite homogenous with an equivalent number of amino acids. In contrast, Tax proteins from four different HTLV-2 isolates (A-D) are heterogeneous in length with divergent c-terminal sequences. Our review and studies are focused on the HTLV-2A isolate.

Both Tax-1 and Tax-2 are phosphoproteins of 40Kd and 37Kd respectively, sharing approximately 75% amino acid homology. Compared with Tax-1, Tax-2 lacks the c-terminal 22 amino acids as a stop codon truncates the protein at aa 331. Comparative studies between Tax-1 and Tax-2A indicated that although they share major functional similarity, there are some phenotypical differences that may account for the different pathogenesis between HTLV-1 and HTLV-2.

Structure/function studies revealed at least four functional domains for Tax-1: the N-terminal activation domain encompassing a nuclear localization signal, the Zinc-binding domain (Zinc finger) located to the N-terminus aa23-aa49, the central structural domain, and the c-terminal activation domain. Tax-2 has a highly similar functional domain structure with Tax-1, but with subtle differences in the distribution of the NFκB and CREB/ATF activation domains.

Tax-1 and Tax-2A also display different capacities to transactivate viral LTRs. Some studies indicate that Tax-1 is more efficient than Tax-2A in transactivating both HTLV-1 and HTLV-2 LTRs, whereas in other studies Tax-2A displays a greater capacity than Tax-1 in transactivating HTLV-2 LTR. Different cell types and Tax expression plasmid used in those studies may account for the observed discrepancy.
Originally both Tax-1 and Tax-2 were regarded as nuclear proteins that co-localized primarily with RNA polymerase II, splicing complexes and specific transcription factors\textsuperscript{127-129}. More recently, studies have shown that Tax-1 is a shuttling protein with a primary localization at the nuclear speckled structure (Tax speckled structure, TSS)\textsuperscript{130,131}. In contrast, Tax-2A, as well as other Tax-2 subtypes, is mainly localized in the cytoplasm based on a recent study from Meertens \textit{et al}. This unexpected subcellular localization is not linked to Tax-2 NLS sequence, which is still functional. Using a series of Tax-1 and Tax-2 chimeric constructs, the authors indicated that another region (aa90-100) in Tax-2 is necessary for conferring its predominant cytoplasmic localization\textsuperscript{132}.

In addition, Tax-1, but not Tax-2, inhibits p53 function efficiently, displays significant micronuclei inductive activity, induces ICAM-1 gene transcription in T-cells, and perturbs development and maturation of human pluripotent hematopoietic progenitor (CD34+) cells \textit{in vitro}\textsuperscript{126,133-135}. More importantly, Tax-1 morphologically transforms rat fibroblasts with higher efficiency than Tax-2A. Recent studies attributed this different transformation efficiency to the c-terminus PDZ domain-binding motif (PBM) in Tax-1, which is absent in Tax-2A\textsuperscript{136-139}. Data in Chapter 2 of this thesis further demonstrates the important role of the Tax-1 PBM in micronuclei induction, HTLV-1-induced T-cell proliferation, and \textit{in vivo} viral spread/persistence.

\textbf{1.7.2 Tax and transcriptional activation of viral LTR (CREB/ATF pathway)}

As discussed in section 1.5, the three imperfect 21bp repeats in the U3 region, termed TRE-1, are necessary for Tax activation of viral gene transcription\textsuperscript{140-143}. To activate the viral LTR, Tax requires at least two 21b repeats containing an imperfect CRE
which binds several cellular transcriptional factors, including CREB, cyclic AMP response element modulator (CREM), activating transcriptional factors (ATFs), Tax-responsive element binding proteins (TREBs) and 21bp binding proteins (HEBs). Under normal cellular conditions, phosphorylation of serine133 in the KID domain is required to activate CREB, which then binds with the CREB binding protein (CBP/p300) and brings it to the DNA. The histone acetyltransferase (HAT) activity of CBP can thus unwind the nucleosome structure around the transcriptional start site, resulting in activation of transcription. Tax can bypass this regulated system by inducing assembly of CREB and CBP/p300 into active complexes without specific phosphorylation. An additional protein, p300/CBP associated factor (P/CAF) has also been identified in complexes with Tax and p300, which is required for Tax mediated transcriptional activation. In these complexes, Tax does not directly bind with TRE-1 or TRE-2. Rather, direct binding of Tax to GC-rich sequences flanking TRE-1 at a region within the CBP/p300 binding domain has been shown to be important for Tax transactivation of the viral LTR.

1.7.3 Tax and regulation of cellular gene expression

**CREB/ATF pathway.** As a transcriptional factor, Tax can also modulate the expression or activity of cellular genes. Tax can activate transcription of some CRE-containing cellular genes. ATF-4 (CREB-2), a transcription factor which belongs to the CREB/ATF family, can also bind to Tax and is implicated in Tax-mediated transactivation of the viral promoter. ATF-4 preferentially heterodimerizes with members of the activator protein-1 (AP-1) family and the C/EBP family of proteins.
However, it appears that these AP-1 family members are dispensable for Tax transactivation of the LTR in vivo. More likely, they are stimulated by Tax and subsequently activate the downstream genes, such as IL-8\textsuperscript{160-162}. In addition, Tax can regulate cellular transcription through binding to other transcription factors, such as NFκB and serum responsive factor (SRF).

**NFκB pathway.** The NFκB family consists of at least five related proteins, p105/p50 (NFκB-1), p100/p52 (NFκB-2), c-Rel, RelA (p65), and RelB. All of the proteins contain an N-terminal DNA binding/dimerization/nuclear targeting domain called the Rel homology domain. They usually function as homodimers or heterodimers, with the most common combination being p50-RelA, which is referred to as NFκB\textsuperscript{163-165}. Tax can bind to multiple members of the NFκB family and activate NFκB-dependent transcription via a mechanism similar to activation of CRE-dependent transcription\textsuperscript{166-169}. In quiescent cells, NFκB is sequestered in the cytoplasm as an inactive complex with IκB, an inhibitor of NFκB\textsuperscript{163}. Upon stimulation, IκB is phosphorylated by IKK proteins (IκB kinases), leading to its proteosome-mediated degradation and release/translocation of NFκB into the nucleus\textsuperscript{170}. Tax can destabilize a subunit of IκB (IκBα), and promote nuclear translocation of NFκB by at least two strategies. First, Tax interacts with both the proteasome and IκBα and acts as a molecular bridge for IκBα degradation\textsuperscript{171-174}. Second, Tax promotes constitutive IKK kinase activity either by interacting with MEKK1, which normally phosphorylates the IKKs in a regulated fashion, or by directly interacting with IKKγ to increase their kinase activity\textsuperscript{175,176}. Therefore, Tax can function at multiple steps in the NFκB signaling pathway and ultimately lead to constitutive NFκB-responsive gene
expression, including growth factors (IL-2), apoptosis-related proteins (p53, Bcl-2, Bcl-X), and especially cytokines involved in immune response\textsuperscript{107}.

**SRF pathway.** SRF was first identified as a transcription factor activated in response to serum\textsuperscript{177}. Further studies indicated that SRF activated the gene expression involved in the early stages of development\textsuperscript{178,179}. Following mitogenic stimulation, SRF is phosphorylated and recruits other transcription factors of the Ets family. Tax does not directly bind to the serum response element, but interacts with both SRF and the Ets factors. Once the Tax-SRF-DNA ternary complex forms, Tax interacts with the TATA box binding protein (TBP) and enhances the transactivation of the early growth response genes, such as *c-fos* and *c-egr*\textsuperscript{49,180-182}. CBP/p300 has also been involved in this pathway, and interaction between CBP/p300 and Tax is essential for Tax activation of SRE\textsuperscript{183}.

In addition to gene activation, Tax represses a number of cellular genes, such as c-Myc, lck, and β-polymerase\textsuperscript{107}. Three potential mechanisms of Tax-mediated trans-repression have been proposed. First, Tax interacts with CBP/p300 at the KIX domain, as well as the CR2 domain, resulting in the competition between Tax and some transcription factors for the limited cellular CBP/p300. For example, Tax competes with c-Myb and p53 for the KIX domain of CBP/p300, and competes with steroid receptor coactivator (SRC) transcription factors for the CR2 domain of CBP/p300\textsuperscript{184-186}. Second, studies from the Nyborg group suggested that Tax represses gene expression through the bHLH family of transcription factors. The bHLH proteins bind to a consensus DNA sequence called the E box, which exists in the promoters of most genes repressed by Tax including DNA polymerase β. The mechanism of bHLH mediated repression is not clear, but probably also involves the competition for CBP/p300 between Tax and bHLH.
proteins. The third possibility comes from a recent finding that histone deacetylase (HDAC-1) interacts with Tax. It was proposed that HDACs function as a negative regulator for the transcriptional activation by Tax, as inhibition of these proteins increased viral transcription. Interestingly, HDAC-1, 2, and 3 were all detected at the viral promoter in vivo, and the mutually exclusive binding of Tax and HDACS to the proviral promoter in part contributes to the regulation of HTLV transcription.

1.7.4 Tax transactivation in the context of chromatin structure

Since the HTLV proviral genome and cellular genes are present in the context of chromatin structure, the importance of chromatin structure modulation in Tax transactivation activities must be taken into account. A study from Okada et al demonstrated that activation of transiently transfected HTLV-1 LTR plasmid DNA had different requirements for transcription factors/cofactors compared with the fully chromatinized integrated LTR. Recently, several groups have focused their studies on the regulation of Tax transactivation activities in the context of chromatin structure.

Tax utilizes cellular factors to modify the chromatin structure. A well-known example is CBP/p300, the co-transcription factor recruited by Tax in multiple transactivation pathways. CBP/p300 harbors intrinsic histone acetyltransferase (HAT) activity, which can acetylate the N-terminal lysine-rich tail of four histones, H2A, H2B, H3, and H4. This modification of histones results in the relaxation of chromatin structure and allows transcriptional machinery access to promoter sequences. As indicated by previous studies, the presence of p300 facilitated the recruitment of TFIID
and RNA polymerase II to chromatinized LTR and enhanced activated transcription by Tax/CREB\textsuperscript{194,195}.

In addition to HATs, Tax can complex with BRG1, a highly conserved ATPase subunit in mammalian chromatin remodeling SWI-SNF complexes\textsuperscript{196}. The SWI-SNF complex functions by altering nucleosome structure through movement/sliding of nucleosomes to allow the access of transcription factors\textsuperscript{197}. The interaction of BRG1 and Tax has been shown to improve the viral transcription by Tax\textsuperscript{196}.

HDACs have been shown to bind to the viral promoters \textit{in vivo}. However, the binding is not evenly distributed between the upstream promoter in the 5’-LTR and the downstream promoter in the 3’-LTR. This asymmetrical binding may in part contribute to the silencing of the downstream promoter, as well as regulated expression of integrated HTLV provirus\textsuperscript{198}.

1.7.5 Tax and cell cycle deregulation

Tax can deregulate cell growth through a variety of mechanisms. As discussed above, Tax is a potent transcription factor that is able to activate as well as repress a wide array of cellular genes, including those implicated in cell cycle control. In addition, Tax can modulate the activities of some cellular proteins to disrupt the tightly regulated cell cycle progression.

\textbf{G1/S transition.} It is notable that the G1/S cell cycle transition is altered in HTLV infected cells, which is mainly mediated by Tax. One mechanism employed by Tax to bypass this restriction point is to deregulate the cyclinD-cdk4/p16\textsuperscript{INK4A}/Rb/E2F pathway at multiple points. Tax has been shown to upregulate the expression of at least two cyclins,
cyclin D1 and D2 through the NFκB pathway. Also the kinase activity of cdk2/4/6 can be enhanced by Tax, either through increased protein expression or through increased protein association between cyclin D and cdks. On the other hand, Tax has been shown to differentially regulate the cdk inhibitors (CDKIs). Overall, Tax alters the activity of various cyclin/cdk complexes leading to increased phosphorylation of Rb and increased E2F dependent transcription. Alternatively, Tax may target the hypophosphorylated Rb to the proteosome for degradation, leading to enhanced E2F-dependent transcription. Through these two pathways, Tax expression eventually results in abnormal G1/S cell cycle progression.

Transforming growth factor (TGF)-β is another important mediator of the G1/S checkpoint especially for hematopoietic cells. Tax has been shown to counteract TGF-β induced G1 arrest by inhibiting the downstream mediators in the TGF-β signaling pathway. Tax also upregulates the expression and secretion of TGF-β from HTLV-1 infected cells. The secreted TGF-β can act on adjacent cells, resulting in impaired cytolytic T cell activity and enhanced angiogenesis.

Recently, a unique PDZ domain-binding motif (PBM) was identified at the c-terminus of Tax-1, but not Tax-2. The PBM mediates the association of Tax-1 with some PDZ domain-containing cellular proteins, including the human homolog of the Drosophila discs large protein (hDlg). It has been shown that hDlg forms a complex with adenomatous polyposis coli (APC) tumor suppressor protein via one of its PDZ domains, and this complex plays an important role in inducing the APC-mediated blocking of G0/G1 to S phase transition. The PBM of Tax-1 competes with APC to bind to the same PDZ domain of hDlg. Furthermore, it appears that the interaction
between Tax and hDlg induces hyperphosphorylation of hDlg. Through these two effects, Tax is able to perturb the cytostatic effect of hDlg, which may contribute to HTLV-1-mediated T-cell immortalization. Based on the above studies, we performed experiments detailed in chapter 2 to address the functional relevance of the Tax-1 PBM in HTLV-1 mediated T-cell proliferation and transformation.

S/G2/M progression. Recently, Tax has been shown to aberrantly target and activate APC complex before cellular entry into mitosis, whereas in normal cells the APC becomes active specifically during mitosis. The APC functions as an E3 ubiquitin ligase and induces proteasome-mediated degradation of mitotic cyclin B and securin, a protein related to sister chromatid separation. This unscheduled degradation of cyclin B and securin causes a cell cycle delay in G2/M transition, accompanied by severe chromosome aneuploidy.

Consistently, Tax has been shown to associate with Chk2 and induce G2 cell cycle checkpoint. Some important cell cycle checkpoints are triggered by DNA damages, such as G2 checkpoint. The activation of G2 checkpoint involves a series of phosphorylation events. In this pathway, DNA damage first triggers the phosphorylation of ATM or ATR, the protein kinases that in turn (directly or indirectly) activates downstream kinase Chk2 or Chk1 through phosphorylation. The activated Chk2/Chk1 can then phosphorylates and inactivates Cdc25, which is required for the onset of M phase, leading to G2 arrest. Some nuclear abnormalities were observed in the cells blocked in G2 phase, including multinucleation and formation of decondensed, highly convoluted and lobulated nuclei.
By contrast, others reported Tax-mediated attenuation of G2 cell cycle arrest in HTLV-1 infected cells upon γ-irradiation treatment. In addition, the transactivation of Cdc25 and upregulation of mitotic cyclins were also observed in HTLV-1 infected cells, which indicate a faster progression through G2 phase. These findings appear in conflict with the above studies. Different experimental conditions or different cell lines tested may be the potential factors resulting in this conflict.

**Mitotic checkpoints.** During mitosis, a spindle checkpoint regulates nuclear division by preventing the onset of anaphase until all chromosomes are aligned properly at the bipolar spindles. As the spindle checkpoint factors, the mitotic arrest deficient (MAD) protein 1 and 2 form heterodimers which enforce metaphase arrest in response to DNA/spindle damage. Tax binds to MAD1 at the domain necessary for its dimerization. In addition, Tax may impact MAD1 activity by inactivating p53 which regulates MAD1 transcription. Since MAD1 is required for the localization of MAD1-MAD2 heterodimer to the kinetochore and the block of the anaphase-promoting complex (APC) activity, Tax interference of MAD1 may eventually promote cell cycle progression through mitosis even in the presence of chromosome lesions.

### 1.7.6 Tax and apoptosis

The effect of Tax on apoptosis pathways in HTLV infected cells is quite intriguing yet unclear. Several studies suggested that Tax can induce apoptosis. It has been shown that Tax upregulates FasL gene expression and the CD95/Fas-signaling pathway plays an important role in Tax-induced apoptosis. Conversely, some other studies suggest that the TNF-related apoptosis ligand (TRAIL), but not the FasL pathway, is important
for Tax induced apoptosis. In addition, both Tax interaction with CBP/p300 and activation of NFκB are implicated in apoptosis.

In contrast, there is data indicating that Tax inhibits apoptosis. Tax can transactivate the human Bcl-xL promoter through both the NFκB and the CREB pathways, which contributes to increased resistance to apoptosis. Furthermore, the contribution of the NFκB pathway to Tax inhibition of apoptosis was demonstrated using anti-inflammatory inhibitor and other NFκB inhibitors.

Based on all of the data, it appears that Tax does not directly induce or inhibit apoptosis. Rather, Tax affects apoptosis through other factors that it regulates, such as NFκB. Some of the discrepancy discussed above may be attributed to the different cell cycle phases, different cellular environment, or other HTLV encoded proteins, as suggested by a study done by de la Fuente et al.

1.7.7 Tax and DNA repair

Although Tax affects a wide range of cellular functions, there is no evidence that Tax directly induces DNA damage. Rather, Tax appears to repress the DNA repair ability of cells, which results in increased mutation frequency. Based on the type of damage recognized and repaired and the factors involved, DNA repair processes can be classified into four pathways: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and recombination repair. Tax has been implicated in two of these pathways, NER and BER.

Damaged bases, along with apurinic/apyrimidinic sites, are repaired by BER. During BER, nucleotides are inserted by DNA polymerase-β (pol-β). Tax can suppress
expression of DNA pol-β, and thus interfere with BER\textsuperscript{244}. In addition, Tax was shown to induce overexpression of proliferating cell nuclear antigen (PCNA), which is correlated with disruption of the NER pathway \textsuperscript{245-247}. PCNA is an auxiliary protein for DNA polymerase δ and ε, which function in both DNA replication and DNA repair\textsuperscript{248}. When the transcription machinery encounters DNA damage, CDKI p21/waf expression is upregulated and interacts with PCNA, thus blocking its role in DNA replication but not DNA repair\textsuperscript{249}. It is proposed that Tax-induced overexpression of PCNA can overcome the p21/Waf1 induced block, enabling replication in the presence of damage\textsuperscript{246}.

Suzuki et al showed that Tax binds and inhibits the catalytic activity of DNA topoisomerase I, resulting in inhibition of DNA relaxation that may affect the access of DNA repair enzymes to the damaged DNA sequences\textsuperscript{250}. Tax expression also results in increased free OH-containing DNA fragments, which are uncapped and labile to degradation. These unstabilized DNA breaks may interfere with prompt DNA repair and contribute to the genomic instability induced by Tax\textsuperscript{240}.

Notably, Tax can inhibit the function of some tumor suppressor proteins, including Rb and p53. The inhibition of Rb is mainly involved in G1/S cell cycle dysregulation as discussed earlier. In HTLV-1-infected cells, p53 is found to be stabilized and functionally impaired in a Tax-1-dependent manner. The inhibition of p53 has been implicated in HTLV-1 induced abnormal cell cycle progression, anti-apoptosis and induction of genomic instability\textsuperscript{251}.

Consistent with its interference with cell cycle regulation, apoptosis, and DNA repair, Tax, particularly Tax-1, induces genomic instability as indicated by increased micronuclei (MN) formation in Tax-1 expressing cells\textsuperscript{240,252}. MN are chromosome
fragments that are induced by chromosome breakage or dysfunction of the mitotic apparatus and fail to incorporate into the nucleus at cell division. Formation of MN is an important, but not the only, mechanism for chromosome aneuploidy, which is a hallmark of malignant cells. MN formation can be easily detected by fluorescence in situ hybridization (FISH) using α-satellite DNA probes or using anti-kinetochore antibodies. Therefore, it is commonly used as a measure of genomic instability\(^2\). In comparison with Tax-1, Tax-2 has limited micronuclei inductive activity\(^1\). One of our studies suggests that the Tax-1 PBM, which is absent in Tax-2, is a key factor responsible for this difference.

1.7.8 Tax and HTLV mediated cellular transformation and pathogenesis

Tax can modulate the expression or activity of numerous cellular factors through transcriptional pathways, protein-protein interaction or post-translational modification, eventually resulting in inhibition of tumor suppressor proteins, disruption of cell cycle progression, repression of DNA repair processes and alteration of apoptosis. Many or all of these activities of Tax likely cooperate and crosstalk with each other, contributing to its transforming activity (Figure 1.2). Unlike traditional oncoproteins, Tax is a viral oncoprotein without an identified cellular homologue.

Tax alone is sufficient to establish and maintain the transformation phenotype. It was shown that rat fibroblast cells (Rat) or NIH3T3 cells can be morphologically transformed \textit{in vitro} either by Tax alone or in combination with Ras\(^2\). Transgenic mice expressing Tax-1 develop several tumors. Tax-1 expression under the control of the HTLV-1 LTR induces neurofibromas\(^2\). Tax-1 expression under the control of the
human granzyme B promoter induces primary peripheral lymphomas, consisting primarily of CD8+ T and NK cells.

Although the mechanism of HTLV transformation has not been clearly elucidated, it has been demonstrated that Tax is essential for HTLV mediated \textit{in vitro} cellular transformation. Depletion of Tax expression by specific mutation of the Tax initiation codon eliminated the transforming potential of \textit{Herpesvirus saimiri} recombinant virus containing the 3’ portion of HTLV-1 genome. An HTLV-1 proviral clone with mutated Tax (M22), which is defective for NFκB activation, failed to immortalize/transform primary T-lymphocytes \textit{in vitro} compared to wild type HTLV-1. In a study by Ross et al, a Tax knock-out chimeric HTLV-2, termed HTLVC-enh Tax, was generated by replacing the viral promoter with the cytomegalovirus immediate-early promoter enhancer (C-enh). Transfection and infection studies indicated that HTLVC-enh Tax is competent for replication but unable to transform primary T lymphocytes.

A distinguishing feature of HTLV infection and pathogenesis is that \textit{in vivo} viral gene expression, including Tax, is undetectable or found at a very low level. However, once cells are put into culture, viral gene expression can be detected shortly thereafter. Therefore, the role of low Tax expression in the HTLV pathogenesis is an intriguing topic. As proposed in a model by Yoshida \textit{et al}, at early stage of infection Tax expression induces cellular proliferation of infected cells. Because of host immune surveillance, the continuous expression of viral proteins including that of Tax, is limited in a subset of infected cells at certain time points responsive to specific stimulation. Simultaneously, Rex may control the viral replication by feedback regulation of Tax.
expression in order to evade the immune system. During the long-term infection, such events may take place repeatedly and facilitate clonal expansion of certain infected cell populations. In addition, pleiotropic effects of Tax function to accumulate mutations and prevent apoptosis of malignant transformation. In this respect, Tax likely acts as a tumor initiator as well as a tumor promoter for HTLV leukemogenesis. However, it is necessary to note that Tax alone cannot explain the clonal expansion of infected cells in ATL patients, and some other viral or cellular factors as well as molecular events may also play important roles\textsuperscript{265}.

1.8 HTLV Rex, regulator of viral protein expression

The full-length and incompletely spliced mRNAs encode essential structural and enzymatic proteins of HTLV. However, these intron-containing mRNAs are usually recognized by the cell as pre-mRNAs and retained in the nucleus until they are completely spliced or degraded. In order to overcome this problem, specific regulatory proteins are employed by HTLV as well as other complex retroviruses to facilitate the export and translation of these mRNA species. As indicated by internally deleted proviruses and complementation assays, Rex is required for the efficient accumulation of incompletely spliced viral mRNA in the cytoplasm\textsuperscript{266-269}.

1.8.1 Rex protein: structural and functional domains

Rex is expressed from the same doubly spliced mRNA that expresses Tax. HTLV-1 Rex (Rex-1) contains 189aa, and HTLV-2 Rex is composed of 170aa. At the amino acid level, Rex-1 and Rex-2 share about 61% homology. Originally, two protein species were
detected for both Rex-1 (p27 and p21) and Rex-2 (p24 and p26), as analyzed by SDS-PAGE.\textsuperscript{270-272} p21\textsuperscript{Rex1} is a truncated form of p27\textsuperscript{Rex1}, whereas the two isoforms of Rex-2, p24 and p26, have the same amino acid backbone and differ by conformational change induced by serine phosphorylation.\textsuperscript{273,274} Truncated Rex-2 forms, p22/20, were also identified. The truncated Rex-1/Rex-2 proteins are proposed to be trans-dominant and to interfere with Rex function by titrating cellular export factors.\textsuperscript{275-277}

Mutational analyses of Rex-1 have defined several domains critical for its function, including an arginine-rich N-terminal RNA binding domain (RBD) that overlaps with a nuclear localization signal (NLS), a leucine-rich activation domain encompassing a nuclear export signal (NES), and two multimerization domains flanking the NES (Figure 1.3). Similar domain structure is found in Rex-2. In addition, a unique c-terminus phosphorylation domain has been identified for Rex-2. Mutations in this phosphorylation domain also impair the nuclear-to-cytoplasmic shuttling of Rex-2 proteins.\textsuperscript{274,287}

The Rex NLS and RBD map to the N-terminal 19 residues. Importantly, substitution of all seven arginines with positively charged lysine in this region does not affect Rex-1 subcellular localization to the nucleus, but seems to affect the nucleolar distribution pattern of Rex-1. This finding implied that it is the positive charges and charge distribution within this domain rather than specific amino acid residues that play an important role in Rex nuclear import.\textsuperscript{280,284} However, multiple arginine residues in this region are essential for RNA binding as well as functional activity, and are possibly involved in forming hydrogen bonds with Rex responsive element (RxRE).\textsuperscript{288}

The functional activity of Rex requires two specific cis-acting sequences in the viral LTR, RxRE and cis-acting repressive sequence (CRS). The proposed working model for
these elements is that the CRS retains the unspliced mRNA in the nucleus and probably prevents it from degradation until Rex binds to the RxRE. The Rex/RxRE interaction overcomes the inhibitory effect of CRS, and stimulates the mRNA export to the cytoplasm. In HTLV-1, RxRE, comprised of 205 nucleotides, is located in the U3/R region of the 3’-LTR and at least partly in the 5’-LTR. The CRS is located in the U5 region. Interestingly, HTLV-2 RxRE, a sequence composed of 226 nucleotides, is mapped to the R/U5 region that also contains the CRS. Substitution and mutational analysis revealed that the proper secondary structure of RxRE is essential for Rex function, and RxRE-2 is functionally interchangeable with RxRE-1.

The activation domain (AD) of Rex-1 maps to residue 79-99. Rex-2 AD maps to a similar region encompassing residues 81-94. Subsequent studies revealed that the NES is a minimal activation domain. First identified in HIV Rev, this type of leucine-rich NES has been found in many cellular and viral proteins with shuttling properties, such as adenovirus E4, IκBα, and protein kinase inhibitor (PKI). Several cellular proteins have been shown to associate with Rex through the NES and to mediate Rex-dependent export of viral mRNA, including chromosome region maintenance interacting protein 1 (CRM1), eukaryotic translational initiation factor-5A (eIF-5A), and human nucleoporin-like protein (hRIP/Rab). It has been proposed that Rex multimerizes and binds to a single mRNA molecule prior to cytoplasmic export. There are two regions in both Rex-1 and Rex-2 that function as multimerization domains, approximately aa57-66 and aa106-124 in Rex-1, and aa60-70 and aa120-130 in Rex-2. This multimerization event appears critical for Rex function, as multimerization mutants act as trans-dominants and interfere
with wild type Rex. However, it has been shown that Rex-1 can form homo-oligomers in the absence of RxRE in vivo. Furthermore, Rex mutants deficient in multimerization can still efficiently shuttle between the nucleus and the cytoplasm. The latter two studies suggest that multimerization may not be essential for Rex-dependent mRNA export.

1.8.2 Rex function and a proposed working model

The mechanisms by which Rex facilitates the expression of viral structural and enzymatic proteins are not clearly understood. Previous data suggest that Rex functions at three levels. Several studies support the first possibility that Rex can actively transport the incompletely spliced HTLV-1 or HIV-1 mRNA to the cytoplasm for translation through its interaction with viral RNA and cellular export machinery. Other studies propose that Rex can inhibit the splicing and degradation of RxRE-containing mRNA in the nucleus, and this mRNA is regarded as processed and is efficiently exported to the cytoplasm. A third possibility is that Rex can increase the translational efficiency of mRNA containing the RxRE. The interaction between Rex-1 and eIF5A shed more light on this hypothesis.

Rex containing an NLS is actively imported into the nucleus by binding directly to transport receptor importin β. Subsequently, Rex is released and the exposed RBD, which overlaps with the NLS, is available to RxRE-containing mRNA. Binding of Rex to the mRNA increases mRNA stability and in turn facilitates its export to the cytoplasm. Alternatively, this interaction with Rex may inhibit the splicing of intron-containing mRNA. Next the cellular factor CRM1, also called exportin 1, can interact
directly with the Rex/RxRE complex. CRM1 plays an important role in the export of leucine rich NES containing proteins. The complex Rex/RxRE/CRM1 is then recognized and bound by Ran-GTP, leading to passage of the transport complex through the nuclear pore and exit into the cytoplasm. Once in the cytoplasm, the hydrolysis of Ran-GTP into Ran-GDP results in the dissociation of the transport complex and the release of mRNA. Unbound Rex and CRM1 can shuttle back into the nucleus for further transport cycles, whereas the mRNA can now be translated into viral structural and enzymatic proteins.

1.8.3 Functional regulation of Rex

Both Rex-1 and Rex-2 are phosphoproteins, and phosphorylation is essential for their functions. The phosphorylation sites of Rex-1 were identified as serines 70 and 177 and threonine 174. The importance of phosphorylation to Rex-1 function is demonstrated in a study by Adachi et al., in which HTLV-1 infected cells treated with protein kinase C inhibitor resulted in a decrease in Rex-mediated viral unspliced mRNA accumulation in the cytoplasm.

In HTLV-2, the two isoforms of Rex differ by phosphorylation, with p26 as the phosphorylated form of p24. Cell fractionation analysis as well as immunofluorescence assay showed that p24 is predominantly cytoplasmic, while p26 has nuclear/nucleolar localization, suggesting that the active form of Rex-2 is phosphorylated. Further mutational analysis of Rex-2 targeting all serines and threonines revealed that phosphorylation of at least two serines in the c-terminus (S151, S153) is critical for Rex-2 function. Alanine substitution of these serines renders Rex-2 functionally inactive, whereas replacing the two serine residues with aspartic acids locks the protein into a
constitutively active state. It was proposed that phosphorylation of these two serines introduces negative charges to the c-terminus of Rex, which likely causes conformational change of Rex into an unstable active form. Further phosphorylation events on additional sites may be necessary to stabilize this active form. Consistently, in our following studies deletion of the C-terminus of Rex-2 also locks it into an active form (Unpublished data in this thesis). In addition, the C-terminus of Rex, including the two key phosphorylation serines, is involved in nucleocytoplasmic shuttling of the protein. The phosphorylation events of Rex provide additional regulatory control for HTLV gene expression in infected cells and may be essential for the virus to adapt to the cellular environment. Therefore, disruption of the phosphorylation regulation of Rex may affect the balance of viral replication and ultimately interfere with HTLV-mediated transformation. This question is addressed in chapter 4.

Furthermore, it should be note that the full-length viral RNA also serves as genome RNA for packaging into infectious virions other than mRNA for synthesis of viral proteins. The unspliced mRNA and virion genome RNA are physically indistinguishable. For some retroviruses such as murine leukemia virus, the full-length transcripts segregate into two functionally distinct populations of mRNA for translation or virion genome RNA for encapsidation. In contrast, for some retroviruses including HIV-1, the unspliced RNA constitutes a single functional pool that can function interchangeably as mRNA and as virion genome RNA. Therefore, constitutively active Rex may cause aberrantly enhanced viral protein translation and potentially interfere with the assembly of genome RNA and production of infectious virions.
1.8.4 Rex, viral infectivity, and cellular transformation

Two previous reports showed that Rex-deficient HTLV-1 and HTLV-2 are still able to produce low but detectable levels of Gag proteins (p19 and p24), which suggests that Rex may not be absolutely required for HTLV viral replication \(^{269,323}\). However, as discussed above, numerous studies have indicated that Rex plays a critical role in efficient viral replication. Therefore, it is reasonable to propose that Rex may regulate the transition from the early latent phase to the late productive phase of HTLV infection, hence Rex is essential for efficient viral infectivity and likely for transformation.

Previous studies by Grassmann et al demonstrated that in the context of Herpesvirus Saimiri vector, Rex expression is not able to immortalize primary human CD4+ cord blood lymphocytes \(^{259}\). Consistently, a recent study by Ye et al further addressed this question using a Rex-deleted HTLV-1 proviral clone (HTLV-1/Rex-) \(^{323}\). In the in vitro immortalization/transformation system, human peripheral blood monocytes (hPBMCs) cocultured with irradiated HTLV-1/Rex- producer cells were able to sustain IL-2-dependent long-term growth, but with a delayed immortalization phenotype in comparison with those cocultured with wtHTLV-1 producer cells. This data suggests that Rex is not essential for, but may facilitate, in vitro HTLV infection and cellular immortalization. When inoculated into immune competent rabbits, HTLV-1/Rex- failed to infect/spread or induce a detectable serum antibody response, indicating that Rex expression is essential for the establishment of in vivo viral infection.
1.9 HTLV tropism

1.9.1 Distinct cellular tropism between HTLV-1 and HTLV-2

HTLV-1 and HTLV-2 display distinct pathogenic properties. HTLV-1 has been identified as the etiological agent for ATL, a CD4$^+$ T cell malignancy, as well as HAM/TSP, a neurological disorder$^{324-327}$. In contrast, HTLV-2 is much less pathogenic, and only a few cases of variant hairy cell leukemia (CD8$^+$ T-cell origin) and several cases of neurological disease have been reported$^{24,26,328}$. Consistently, studies on both asymptomatic carriers and patients with ATL and neurological diseases have indicated that HTLV-1 infection takes place preferentially in CD4$^+$ T lymphocytes, whereas HTLV-2 infection seems to be more restricted to CD8$^+$ T lymphocytes$^{326,329,330,331,332}$. For example, in 107 patients with ATL, 81% of infected cells were found to be CD4$^+$ T cells while only 4% were CD8$^+$ T cells$^{326}$. Moreover, nine out of eleven HTLV-1 positive cell clones isolated from different HAM/TSP patients were identified as CD4$^+$ and the other two were identified as CD8$^+$ T lymphocytes$^{330}$. On the other hand, Ijichi et al isolated PBMCs from HTLV-2 infected individuals and analyzed the presence of provirus in different cell types by PCR. In 8 out of 9 patients, the HTLV-2 provirus was detected exclusively in CD8$^+$ T cells, whereas in one patient, the provirus was detected in both CD4$^+$ and CD8$^+$ T lymphocytes$^{332}$.

However, some studies suggest that both HTLV-1 and HTLV-2 may possess a broader in vivo tropism for PBMCs. Recently, CD8$^+$ T cells were identified as another in vivo reservoir for HTLV-1$^{333}$. Interestingly, HTLV-1 infected CD4$^+$ T cells are usually monoclonal with respect to the integration site of the provirus, whereas polyclonal expansion occurs more frequently in infected CD8$^+$ T cells$^{334,335,331}$. Similarly, after
examination of peripheral blood lymphocytes from 35 HTLV-2 infected individuals, Lal et al concluded that both CD4+ and CD8+ T cells can be infected by HTLV-2, with a higher proviral load detected in CD8+ T cells. Occasionally, both HTLV-1 and HTLV-2 infection have been found in non-T cells in vivo.

In contrast with their restricted in vivo tropism, when put into in vitro cultures, HTLV-1/2 can rapidly propagate in numerous cell types, including T cells, B cells, endothelial cells, glial cells, and monocytes of both human and nonhuman origin. However, as demonstrated by in vitro coculture immortalization/transformation assays as well as studies of patients, HTLV-1 and HTLV-2 display their transforming or pathogenic activity only in T cells. In the coculture assay, lethally irradiated HTLV producer cells are cocultivated with freshly isolated human PBMCs, so that HTLV can transmit via cell-cell contact and efficiently infect hPBMCs, leading to ultimate transformation. It has been shown that the in vitro transformed cells share many similarities with leukemic cells. Taking advantage of this well-established in vitro transformation assay, previous studies have recapitulated the distinct in vivo HTLV-1 and HTLV-2 distinct transformation tropism, showing that HTLV-1 primarily transforms CD4+ T cells and HTLV-2 preferentially transforms CD8+ T cells.

Overall, many factors, including host factors, viral proteins, and the different stages during the oncogenic/pathogenic process, may contribute to the distinct but not exclusively defined cellular tropism between HTLV-1 and HTLV-2. In our effort to identify the viral determinant(s) for HTLV cellular tropism, it has been shown that tax and overlapping rex sequences do not confer the distinct transformation tropism between HTLV-1 and HTLV-2. Herein, one of our studies is to further identify the potential
viral determinant(s) of HTLV tropisms, mainly focusing on viral LTR sequences and the env gene.

1.9.2 Potential viral determinants for HTLV tropisms

**Tax.** The viral oncoprotein Tax possesses transforming activity and is essential for HTLV-mediated cellular transformation and oncogenesis. Therefore, it is very likely that the interaction(s) between Tax-1/2 and specific cellular factor(s) in different CD4⁺/CD8⁺ T-cell subsets may contribute/determine the distinct transformation tropisms. Newbound et al found that the ability of Tax-1 to activate HTLV-1 viral transcription is greatly enhanced in purified CD4⁺ T cells compared with that in purified CD8⁺ T cells, suggesting that Tax transactivation of the viral LTR may affect HTLV tropism 350. A HTLV-1 proviral clone containing the Tax M47 mutations, which is deficient in the CREB/ATF activation pathway, retains the ability to immortalize T lymphocytes, but with a higher frequency of CD8⁺ T cells *versus* CD4⁺ T cells for wild type virus 261. Therefore, it is speculated that Tax may contribute to HTLV-1 tropism via the CREB/ATF pathway. However, a recent study from Ye et al indicated that chimeric HTLV-1 with Tax-2 and overlapping Rex sequences (HTLV-1/TR2) still preferentially transforms CD4⁺ T cells similar to wtHTLV-1. Also PBMCs transformed by chimeric HTLV-2 with Tax-1 and overlapping Rex sequences (HTLV-2/TR1) exhibit a similar phenotype pattern as those transformed by wtHTLV-2. Therefore, Tax and overlapping Rex do not confer the distinct HTLV transformation tropism 124.

**Viral LTR.** The precedence for LTR-mediated cellular tropism has been clearly demonstrated with murine leukemia virus (MuLV). Moloney MuLV (M-MuLV) induces
T-cell lymphoma, whereas Friend MuLV (F-MuLV) induces erythroleukemia when injected into newborn NFS mice\(^\text{351,352}\). However, when the viral LTR was exchanged between these two types of MuLV, the tumor types generated in the mice were altered, with each corresponding to the LTR but not the parental viruses\(^\text{353,354}\). Further studies indicated that specific enhancer sequences within the M-MuLV LTR in cooperation with unique T-cell transcription factors are the primary determinants for cell tropism\(^\text{355,356}\).

Comparative studies between HTLV-1 LTR and HTLV-2 LTR indicate that they are the least homologous regions between HTLV-1 and HTLV-2, with about 31% similarity at the nucleotide sequence level. Preliminary data from the Fan laboratory suggested that the loading profile of transcriptional factors on LTR-1 differs from that on LTR-2. By footprinting, they found that in HTLV-2 (Mo-T cells), the predominant location of factor binding is at the distal TRE1 and adjoining PU.1 site (a binding site for the Ets family proteins), while for LTR-1 (MT-2 cells), the predominant factor binding site is at the proximal TRE1 and the adjoining TRE2 sequences (Personal communication). Therefore, it is possible that the LTR may be a viral determinant(s) for HTLV transformation tropism.

**Envelope and HTLV receptor(s).** The interaction between the cellular receptor(s) and the viral envelope (Env) mediate viral entry into specific cell types. Therefore, it is likely that Env is involved in the cellular tropism of viral infection, such as in the case of HIV-1 and feline leukemia viruses\(^\text{357,358}\). However, since both HTLV-1 and HTLV-2 Env-mediated infection can be established in all tested vertebrate cell lines, it is speculated that HTLV-1 and HTLV-2 have the same primary receptor, and that this receptor is ubiquitous and expressed in numerous cell types\(^\text{359,360}\).
Several cell surface molecules have been implicated in Env-mediated membrane fusion and virus transmission. Based on the cross-reactivity between HTLV Env and monoclonal antibodies against HLA molecules and human interleukin-2 (hIL-2), cellular factors that interact with class I histocompatibility antigen as well as the hIL-2 receptor were suspected as the HTLV receptor \textsuperscript{361,362}. Env SU derived peptides were used to screen for inhibition of syncytia formation in order to identify the HTLV receptor, and heat shock cognate protein (HSP 70) was proposed as a candidate receptor. However, HSP 70 is likely involved in post-entry fusion instead of an entry event \textsuperscript{363,364}. HTLV infection is largely dependent on cell-cell contact and diverse adhesion molecules have been found to be involved in HTLV transmission, including E-selectin, LFA-1, VLA-44, L-selectin, CD44, ICAM-1, ICAM-3 and VCAM-1 \textsuperscript{365-367}. Some non-protein molecules, such as palmitoyl(16:0)-oleoyl(18:1)-phosphatidylglycerol, lipid raft, and heparin sulfate proteoglycans, are also implicated in HTLV Env-mediated fusion and syncytium formation \textsuperscript{368,369}. Most recently, a multimembrane-spanning molecule, glucose transporter GLUT-1, was shown to convey the susceptibility of Env-mediated fusion for both HTLV-1 and HTLV-2 \textsuperscript{370-372}.

Although there has been no conclusive finding to date, these studies suggest that HTLV may have multiple receptor/co-receptors, some are universal, some are cell type-specific, and some of these cell surface molecules may have different expression profiles in different tissue/cells. In this respect HTLV Env, similar to HIV-1, may affect the cellular tropism via interaction with specific cellular factors (co-receptors), in which their densities on specific cell surfaces or their affinities to specific envelope probably play an important role \textsuperscript{373,374}. 

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On the other hand, previous studies have demonstrated that HTLV-1 Env gp46, in conjunction with the CD2/LFA-3 activation pathway, is mitogenic to resting T lymphocytes \(^{375-377}\). Furthermore, it has been shown that expression of the HTLV receptor is induced upon T cell activation \(^{370,378,379}\). More intriguingly, both Env-1-mediated syncytium formation and T-cell antigen-receptor signaling require the presence of lipid rafts, which are distinct cell membrane structures formed by dynamic clustering of sphingolipids and cholesterol. The lipid rafts are enriched in many proteins involved in numerous cell signalling pathways, such as glycosyl-phosphatidylinositol (GPI)-anchored proteins, proteins of the src family kinases, protein kinase C, heterotrimeric G proteins, actin and actin binding proteins \(^{368,380-381}\). Therefore, Env may affect HTLV cellular tropism via post-entry signaling events that modulate cellular environment, facilitate viral replication, and eventually promote virus induced cellular transformation.

**Accessory proteins.** Although it was shown that the accessory proteins are not required for *in vitro* cellular transformation induced by both HTLV-1 and HTLV-2 \(^{77,103}\), it remains possible that they contribute to the transformation tropism. As mentioned above, p12 is implicated in T-cell activation, and p30 may also function to modulate transcription of viral genes as well as cellular genes. Therefore they could act to provide an optimal cellular environment for viral replication and to ultimately facilitate cellular transformation.

In order to further identify the viral determinant(s) of transformation tropism, one of our studies utilized recombinant HTLV in which specific gene/sequences were exchanged between HTLV-1 and HTLV-2. Through analysis of the phenotypes of
hPBMCs transformed by these recombinant HTLVs, we determined the genetic determinant of the distinct transformation tropism between HTLV-1 and HTLV-2.

1.10 Postscript

Since the initial isolation of HTLV-1 and HTLV-2 over two decades ago, tremendous studies have been carried out in order to decode the mystery of their pathology, including the clinical manifestation of associated diseases, epidemiology, and molecular pathogenesis. These studies greatly extend our knowledge on HTLV in the above areas. However, most of the studies on the molecular mechanisms of HTLV pathogenesis are performed in over-expression systems, in which the viral genes are expressed from unrelated promoters and are isolated from the full-length infectious viral backbone. In addition, the lack of a good short-term experimental animal model potentially hinders the progress of HTLV studies. Therefore, studies on in vitro transformation of primary human T lymphocytes mediated by HTLV infection has been widely accepted as a good model, since the transformation process correlates with the initial stage of HTLV disease development. In our well-established in vitro coculture system, freshly isolated human PBMCs can be efficiently infected and transformed in coculture with HTLV producer cells. Moreover, the viral genes are manipulated in the context of full-length proviral clones in our system, and are analyzed in in vitro transformation/immortalization assays to understand their working mechanism. In this way, our studies will provide more pertinent and direct insight into HTLV molecular oncogenesis.
On the other hand, HTLV-1 and HTLV-2 are closely related human retroviruses that have similar genetic organization and biological properties. However, they display distinct pathogenicity. HTLV-1 has been identified as a causal agent for two fatal human diseases, ATL and HAM/TSP, whereas HTLV-2 appears much less pathogenic without conclusive disease association. Comparative studies between HTLV-1 and HTLV-2 and their genetic elements will reveal critical factors that are responsible for their distinct pathogenicity, eventually enlarging our knowledge on the malignancy of HTLV-1 and on the medical treatment for HTLV-1-associated diseases. Data in this thesis mainly focuses on the distinct molecular mechanisms utilized by HTLV-1 and HTLV-2 to undergo efficient viral replication and to induce cellular proliferation and transformation.
Figure 1.1  Genome organization of HTLV-1 and HTLV-2 and their gene products. The provirus is flanked by direct long terminal repeats and consists of typical retroviral gag, pro, pol and env genes and additional genes of the pX region, encoding Tax, Rex, and other accessory proteins. (A) HTLV-1 proviral genome is approximately 9000bp long. In addition to the structural and enzymatic proteins (Gag, Pro, Pol, Env) and the trans-regulatory proteins (Tax and Rex), several accessory proteins have been identified, including p27, p12, p30, p13 and an antisense HBZ. p21 is a truncated form of p27ex. (B) Eight major mRNA species expressed from HTLV-1 are depicted, the numbers below correspond to their exon splicing acceptor and donor sites. Two splice donor sites (nt119 and nt 4831) and five splice acceptor sites (nt4641, nt6383, nt6478, nt6875, and nt6950) are also shown in the proviral genome as solid or empty arrows, respectively. The tentative antisense mRNA for HBZ is also indicated. (C) HTLV-2 has similar genome structure with HTLV-1 with differences in the pX ORF I, II, and V. At least seven HTLV-2 mRNA species have been identified as shown, the numbers below correspond to their exon splice acceptor and donor sites. Two splice donor sites (nt134 and nt 4868) and four splice acceptors sites (nt4729, nt6492, nt6629, and nt6899) are shown in the proviral genome as solid or empty arrows, respectively. In both panels (B and C), exons are represented as solid lines, and introns are represented as dotted lines. In addition, the positions of the Rex response element (RxRE) and cis acting repressive sequence (CRS) are indicated by horizontal bars; nucleotide numbering starts at the beginning of R region.
To be continued

Figure 1.1
Figure 1.1 continued

B

env
tax/rex
p21\textsuperscript{res}
p12\textsuperscript{i}
p27\textsuperscript{i}
p30\textsuperscript{u}
p13\textsuperscript{u}
HBZ?

C

env
tax/rex
p28\textsuperscript{u}, p22\textsuperscript{u}/p20\textsuperscript{u}
p10\textsuperscript{i}, p11\textsuperscript{v}
?
**Figure 1.2** Tax and HTLV-mediated cellular transformation. (A) In HTLV-infected cells, in addition to transactivating viral gene expression, Tax can also activate numerous cellular gene expression through cellular transcriptional factors, including CREB/ATF pathway, NF-kB pathway, and SRF pathway. Tax can also repress some cellular gene expression mainly by competition of limited cellular CBP/p300. (B) Tax can deregulate cell cycle progression, particularly G1/S transition. As depicted, Tax can modulate the expression or activity of cdks, cyclins, cdk inhibitors, leading the hyperphosphorylation of Rb. In addition, Tax can target Rb to proteasome mediated degradation. Both result in the release of E2F and activation of E2F-responsive gene transcription. Tax-1 can uniquely interact with hDlg and perturb the complex APC/hDlg blocking of G1/S transition. (C) Tax can repress the DNA repair processes through transrepression or transactivation of some DNA repair factors, such as DNA polymeraseβ, PCNA, and tumor suppressor protein p53. (D) Tax can disrupt mitotic checkpoint through two mechanisms including alteration of MAD1 dependent pathways and degradation of cyclin B. Tax induced cell cycle deregulation and DNA repair repression both contribute to genomic instability, which is a critical factor in tumorigenesis. These pleiotropic activities of Tax likely culminate in HTLV induced cellular transformation.
Figure 1.3 Domain structure of the HTLV-1 and HTLV-2 Rex. The functional domains or regions of the 189aa Rex-1 and 170aa Rex-2 proteins are depicted, including the N-terminal RNA binding domain (RBD) and nuclear localization signal (NLS), the central activation domain (AD) encompassing the nuclear export signal (NES), and two multimerization domains. In addition, a unique C-terminal domain in Rex-2, containing two key phosphorylation sites (Ser151, Ser153), has been identified to be important for efficient function. Mutations in this region were also found to be impaired for nucleo-cytoplasmic shuttling (modified from Younis et al., Frontiers in Bioscience, 2005).
THE PDZ BINDING MOTIF OF HTLV-1 TAX PROMOTES VIRUS MEDIATED T-CELL PROLIFERATION IN VITRO AND PERSISTENCE IN VIVO

2.1 Abstract

HTLV-1 cellular transformation and disease induction is dependent on expression of the viral Tax oncoprotein. PDZ is a modular protein interaction domain used in organizing signaling complexes in eukaryotic cells through recognition of a specific binding motif in partner proteins. Tax-1, but not Tax-2, contains a PDZ-binding domain motif (PBM) that promotes the interaction with several cellular PDZ proteins. Herein, we investigate the contribution of the Tax-1 PBM in HTLV-induced proliferation and immortalization of primary T-cells in vitro and viral survival in an infectious rabbit animal model. We generated several HTLV-1 and HTLV-2 Tax viral mutants including HTLV-1∆PBM, HTLV-2+C22(+PBM), and HTLV-2+C18(∆PBM). Tax mutations did not alter the ability to activate the CREB/ATF or NFκB signaling pathways. Coculture microtiter proliferation assays revealed that the Tax-1 PBM significantly increases both HTLV-1 and HTLV-2-induced primary T-cell proliferation. In addition, Tax-1 PBM was responsible for the micronuclei induction activity of Tax-1 relative to that of Tax-2. Viral infection and persistence were severely attenuated in rabbits inoculated with HTLV-1 PBM. Our results provide the first direct evidence that PBM-mediated
associations between Tax-1 and cellular proteins play a key role in HTLV-induced cell proliferation and genetic instability in vitro and facilitate viral spread and persistence in vivo.

2.2 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 are highly related complex retroviruses that immortalize and transform T-lymphocytes in cell culture and persist in infected individuals. However, the clinical manifestations of infection with these two viruses differ. HTLV-1 is associated with adult T-cell leukemia (ATL) and a variety of immune mediated disorders including the chronic neurological disease termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)\(^{10,15,382,383}\). In contrast, HTLV-2 is much less pathogenic with only a few cases of variant hairy cell leukemia and neurological disease associated with infection\(^ {26,384-387}\).

Both HTLV-1 and HTLV-2 encode the essential Tax protein. Tax acts in trans to activate transcription initiation from the viral promoter\(^ {36,105}\). In addition, Tax modulates the expression or activity of various cellular factors involved in growth and differentiation and disrupts cell cycle control and DNA repair processes\(^ {69,107,265}\). Strong evidence in several experimental systems suggests that these pleiotropic effects of Tax on cellular processes are required for the transforming or oncogenic capacity of HTLV\(^ {388}\). Indeed, mutational analysis directly demonstrated that Tax of both HTLV-1 and HTLV-2 is essential for viral mediated cellular transformation of primary human T-cells\(^ {261,262,389}\).

Comparative studies of Tax-1 and Tax-2 revealed that these proteins display many similarities but also some major differences. Tax-1 has a higher intrinsic transactivation
activity for the viral promoter than Tax-2. Tax-1, but not Tax-2, is a potent inducer of micronuclei formation, which is a marker of genetic instability. Tax-2, in contrast to Tax-1, fails to suppress the maturation of CD34+ cells in vitro. Tax-1 has been shown to inhibit p53 function more efficiently than Tax-2. Tax-1 morphologically transforms rat fibroblasts (Rat-1) with higher efficiency than Tax-2. This phenotypic property recently was attributed to a C-terminal PDZ binding motif (PBM) that is present in Tax-1 but not Tax-2. Therefore, the Tax-1 PBM could be a major determinant of the differences in pathogenicity of HTLV-1 and HTLV-2.

The PDZ domain was named after the first identified PDZ-containing proteins, post-synaptic density protein (PSD-95), 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. A PDZ domain usually coexists in the same polypeptide either with one or multiple PDZ domains or with other protein domains such as SH3 and guanylate kinase-like domains. Through recognition of the specific carboxyl-terminal binding motif in its partner protein, PDZ domain-containing proteins play a key role in recruiting and organizing the appropriate proteins to sites of cellular signaling, as well as polar sites of cell-cell communication.

The human homologue of Drosophila discs large tumor suppressor protein (hDlg), a scaffolding protein containing three PDZ domains, has been identified as a common target for human virus oncoproteins, including HTLV-1 Tax, adenovirus type 9 E4ORF1, and human papillomavirus E6. E6 targets hDlg for proteasome-mediated degradation, which is necessary for its transforming activity. In contrast, Tax-1 and E4ORF1 are thought to interfere with the binding of hDlg to the adenomatous polyposis
coli (APC) tumor suppressor protein via competition for the same PDZ domain of hDlg. This binding leads to increased cell proliferation mediated by increased signaling through APC. A recent study indicated that the interaction between Tax-1 and hDlg is responsible for the higher colony forming efficiency of Rat-1 cells by Tax-1 relative to Tax-2. Additional studies have implicated other cellular PDZ domain-containing proteins as Tax-1 targets, such as precursor of interleukin-16 (pro-IL-16) and a membrane-associated guanylate kinase (MAGUK) with inverted orientation (MAGI)-3. Pro-IL-16 is an abundant protein constitutively expressed in human peripheral blood T-cells that can induce cell growth arrest. MAGI-3 belongs to the same MAGUK family as hDlg and has been implicated in several cellular signaling pathways involved in cell survival as well as cell polarity. Together, these studies imply that the PBM of Tax-1 and its interacting partners, the cellular PDZ domain containing proteins, could be a major determinant of the differences in pathogenicity of HTLV-1 and HTLV-2.

In this study we used full-length infectious viral clones to address the role of Tax-1 PBM in the HTLV-mediated T-cell transformation process and virus survival in the rabbit model of infection. Several Tax-1 and Tax-2 viral mutants were generated including HTLV-1ΔPBM, HTLV-2+C22 (Tax-2 + the last 22 amino acids of Tax-1), and HTLV-2+C18 (ΔPBM). Tax mutations did not significantly affect activation of CREB/ATF or NFκB signaling pathways. Additional analysis indicated that Tax-1 PBM is responsible for the micronuclei induction activity (genetic instability) of Tax-1 relative to that of Tax-2. Using an in vitro coculture assay, we demonstrated that Tax-1 PBM promotes proliferation of infected T-cells. Furthermore, we show that Tax-1 PBM is necessary for efficient viral infectivity, spread, and persistence in a rabbit model of
infection. Overall, our data suggest that the interaction between Tax-1 PBM and some cellular proteins facilitates in vivo viral survival, stimulates abnormal cell proliferation, induces genetic instability, and ultimately, promotes HTLV-1 mediated T-cell transformation and pathogenesis.

2.3 Materials and methods

Cells. 293T cell, 729 human B cell, and Jurkat T cell lines were maintained in Dulbecco's modified Eagle's medium, Iscove's medium, and RPMI 1640 medium, respectively. Medium was 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 (PBMCs) were isolated from blood of normal donors by centrifugation over Ficoll-Paque (Amersham, Piscataway, NJ) and cultured in RPMI 1640 medium supplemented with 20% FCS, 10 U/mL IL-2 (Boehringer Mannheim, Manheim Germany), 2 mM glutamine and antibiotics.

Plasmids. Construction of the Tax-1 cDNA expression vector SE356 and the Tax-2 cDNA expression vector BC20.2Sph was described previously. Mutations in the tax-1 and tax-2 genes were introduced by PCR mutagenesis using SE356 and BC20.2 as templates. Tax-1ΔPBM contains a 12 nucleotide deletion of the tax-1 C-terminus resulting in loss of the last four amino acids of Tax-1 (ETEV), the consensus PBM. Tax-2+C22 was generated by adding 66 nucleotides of the tax-1 coding sequence in frame to the end of tax-2, thus adding the last 22 amino acids of Tax-1 to the shorter (231 amino acids) Tax-2A. Tax-2+C18 was generated from Tax-2+C22 by removing the last four amino acids (PBM). LTR-1-Luc, LTR-2-Luc, and κB-Luc reporter plasmids and the
TK-Renilla transfection efficiency control plasmid were described previously 124,323. The wild-type (wt) HTLV-1 proviral clone (Ach) 402 and the wtHTLV-2 proviral clone pH6neo 33 were used to generate Tax mutant viruses. The unique Mlu-I site (nt 7479, HTLV-1 and nt 7392, HTLV-2) and a newly generated EcoRV site at the stop codon of Tax in both proviruses 124 were used to exchange fragments containing the altered tax genes. All mutations were confirmed by DNA sequencing.

**Transfection, luciferase assay, and p19 Gag ELISA.** To measure Tax CREB/ATF (LTR) activating function, 2x10^5 293T cells were transfected using Lipofectamine® PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. The total amount of DNA was kept constant and was composed of 2 µg of Tax expression vector or a negative control, 0.02µg TK-Renilla, and 0.1 µg of LTR-1- or LTR-2-Luc. To determine Tax NFκB activation capacity, 4x10^6 Jurkat cells were transfected using the Nucleofector® method (Amaxa, Gaithersburg, MD) with 3µg of Tax expression vector or a negative control, 0.05µg TK-Renilla, and 0.25 µg of κB-Luc. Cell lysates were harvested 48 h post-transfection and subjected to a dual luciferase assay (Promega, Madison, WI). All experiments were performed independently three times in triplicate, and results were normalized for transfection efficiency using Renilla-Luc. To generate stable transfectants, proviral plasmid clones containing the Neo^R gene were introduced into cells by electroporation as described previously 103,403. Stable transfectants containing the desired proviral clones were isolated following incubation in 24-well culture plates in medium containing 1 mg/ml of Geneticin. After four-to-five weeks of selection, viable cells were single cell cloned, expanded, and maintained in culture for further analysis. The clones were screened by Gag p19 expression in the cell
supernatant using a commercially available enzyme-linked immunosorbent assay (ELISA; Zeptometrix, Buffalo, NY).

**Western blotting.** Cells expressing Tax proteins were lysed with modified RIPA buffer (50mM Tris-Cl [pH8.0], 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2.0mM phenylmethanesulfonyl fluoride, 20µg/ml aprotinin, 1.0 mM Na₃VO₄, and 1mM NaF) on ice for 30 min. After centrifugation, the cell lysates were subjected to 10% SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell Biosciences, Keene, NH). The membrane was incubated in 1x TBS-T (20mM Tris-Cl [pH 7.6], 137mM NaCl, 0.1% Tween) with 5% defatted milk for 1 h at room temperature. The membrane then was incubated with rabbit anti-Tax-1 or rabbit anti-Tax-2 antibody for 1 hr at room temperature, washed, and followed by incubation with secondary antibody goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Tax protein was visualized using the ECL Western blotting analysis system (Santa Cruz Biotechnology, Santa Cruz, CA).

**DNA preparation and PCR.** Genomic DNA was isolated from permanently transfected cell clones or from immortalized PBMCs using PUREGENE® DNA purification system (Gentra, Minneapolis, Minn.). One microgram of genomic DNA was subjected to 30-cycle PCR as described previously. Primer pair Tax8290S (³8290GAGCCCCAAATATCACC³8308) and TRE-AS (³8612CACGCTTTTATAGACTC³8593) was used to amplify a 323 bp fragment from wtHTLV-1 and a 311 bp fragment from HTLV-1 PBM and primer pair KK1 (³8071CCCTCCTATCTACT³8089) and LTRII-AS (³8939CGGGAAGACAATGCTC³8916) was used to amplify a 868 bp fragment.
from wtHTLV-2, a 934bp fragment from HTLV-2+C22, and a 922bp fragment from HTLV-2+C18. The PCR-amplified products were separated on agarose gels and visualized by ethidium bromide staining.

**Short-term coculture microtiter proliferation and long-term immortalization assays.** Short-term microtiter proliferation assays were performed as described previously. Briefly, freshly isolated human PBMCs were prestimulated with 2µg/ml phytohemagglutinin (PHA) and 10U/ml IL-2 (Roche, Indianapolis, IN) for three days. 729 HTLV producer cells (2000) were irradiated with 10,000 rads and cocultured with 10^4 prestimulated PBMCs in the presence of IL-2 in 96-well round bottom plates. Wells were enumerated for growth and split 1:3 at weekly intervals. At week seven, cell proliferation was confirmed by MTS assays using CellTiter 96® Aqueous One Solution Reagent as recommended by the manufacturer (Promega, Madison, WI). For the long-term immortalization assays, 10^6 irradiated 729 producer cells were cocultivated with 2 × 10^6 freshly isolated PBMCs with 10U/ml IL-2 in 24-well culture plates. The presence of HTLV expression was confirmed by detection of p19 Gag protein in the culture supernatant using an ELISA at weekly intervals. Viable cells were counted weekly by trypan blue exclusion. Cells inoculated with HTLV-1/2 that continued to produce p19 Gag antigen and proliferate 12 weeks post-coculture in the presence of exogenous interleukin-2 (IL-2) were identified as HTLV immortalized.

**Micronuclei assay and cell cycle analysis.** Assay was performed essentially as described. Briefly, HeLa cells grown in Iscove's medium (Invitrogen) containing 10% fetal calf serum and 1% penicillin-streptomycin, were transiently transfected with 10µg of wtTax-1, Tax-1ΔPBM, wtTax-2, Tax-2+C22 expression plasmids, or pUC19 control
using the calcium phosphate transfection procedure. After 24 h, the media was supplemented to contain 3 µg/ml cytochalasin B (Sigma, St Louis, MO), a cytokinesis blocking agent, and grown for an additional 30 hr. Cells then were fixed with cold methanol/acetic acid (3:1) and air dried for 1 hr. The nuclear material was stained with a 10µg/ml acridine orange solution (40µg/ml in PBS pH6.8) for 1 min and rinsed five times with dH₂O. Cells were immunostained with CREST for kinetochore identification and analyzed using a Zeiss LSM510 confocal microscope. Approximately 1000 cells were counted in two separate experiments.

For cell cycle analysis, HeLa cells were transiently transfected with 10µg wtTax-1, Tax-1ΔPBM, wtTax-2, Tax-2+C22, or pUC19 control and incubated 48 hr. After harvesting using trypsin, the cells were fixed in ice-cold 70% ethanol for 24 hr at 4°C. Cells were washed and resuspended in 1 ml of propidium iodide solution (PBS, 50µg/ml propidium iodide and 100 U/ml RNase A) and incubated for 30 min at room temperature. Following the PBS wash, cells were resuspended in 0.5 ml of flow buffer (PBS, 1% fetal bovine serum, and 1% sodium azide), and subjected to DNA flow cytometry analysis using a BD Biosciences FACScan with MODFIT software.

**Rabbit inoculation procedures.** Twelve-week-old specific pathogen-free New Zealand White rabbits (Hazelton, Kalamazoo, MI) were inoculated via the lateral ear vein with 1 x 10⁷ gamma-irradiated (7500 rad) 729wtHTLV-1, 729HTLV-1ΔPBM, or 729 uninfected control cells (five rabbits per group). At weeks 0, 2, 4, 6, and 8 after inoculation, 10 mL of blood was drawn from the central ear artery of each animal. Serum reactivity to specific viral antigenic determinants was detected using a commercial HTLV-1 Western blot assay (ZeptoMetrix, Buffalo, NY) adapted for rabbit plasma by use
of avidin-conjugated goat anti-rabbit IgG (1:200 dilution) (Sigma, St Louis, MO). Serum showing reactivity to Gag (p24 or p19) and Env (gp21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity. To detect integrated proviruses, genomic DNA was harvested using the PUREGENE® DNA purification system (Gentra, Minneapolis, MN) and 1µg of DNA was subjected to 40-cycle PCR using primers 670 and 671 to amplify a 159-bp fragment specific for the HTLV-1/2 tax/rex region. The maintenance of the HTLV-1ΔPBM mutation in positive rabbits was confirmed by PCR using specific primers followed by restriction enzyme digestion and nucleotide sequencing.

2.4 Results

Tax-1 PDZ-binding motif (PBM) is dispensable for Tax transcriptional activation through CREB/ATF and NFκB signaling pathways. It has been hypothesized that a PBM present at the C-terminus of Tax-1 (ETEV), but not Tax-2, may be important for the distinct pathogenic properties of HTLV-1 and HTLV-2. We generated several Tax mutants to assess directly the importance of the Tax-1 PBM in Tax function and viral oncogenic activity (Fig. 2.1A). Tax-1ΔPBM contains a deletion of the four C-terminal amino acids, resulting in removal of the PBM. Tax-2+C22 encodes a Tax-2/Tax-1 hybrid protein that contains the C-terminal 22 amino acids of Tax-1 added to the shorter Tax-2A resulting in a protein with the equivalent length of wtTax-1. Tax-2+C18 was generated as a control and contains a deletion of the four C-terminal amino acids from Tax-2+C22, thereby removing the PBM. All the mutations were confirmed by diagnostic restriction enzyme digestion and nucleotide sequencing. To
confirm stable protein expression by each of the mutants, expression vectors were
transiently transfected into 293T cells and Tax protein expression was examined by
Western blotting using rabbit Tax-1- or Tax-2-specific antisera. Our results showed that
the Tax mutants were expressed efficiently, with sizes consistent with peptide deletion or
addition. Tax-1ΔPBM showed slightly increased mobility compared to wtTax-1 and both
Tax-2 mutants showed slower mobility than wtTax-2 (Fig 2.1B). Since Tax activation of
both CREB/ATF and NFκB signaling pathways plays a critical role in efficient viral
replication and HTLV-mediated cellular transformation, we next tested the
transactivation activity of these Tax mutants on CREB/ATF or NFκB responsive
promoters. Tax cDNA expression vectors were cotransfected with LTR-Luc or κB-Luc
reporter constructs into cells and functional levels of Tax were assessed by measuring
luciferase activity. Our results demonstrated that neither the deletion of the PBM from
Tax-1 (Tax-1ΔPBM) nor the addition of the PBM to Tax-2 (Tax-2+C22) resulted in
significant alteration of luciferase activities as compared to wtTax-1 or wtTax-2, except
that deletion of PBM from Tax-1 result in about 50% deduction of NFκB transactivation.
(Fig. 2.2 A&B). These data are consistent with the conclusion that the Tax-1 PBM is
dispensable for Tax transcriptional activation via the CREB/ATF and NFκB signal
pathways.

**Establishment and characterization of stable HTLV producer cell lines.** To
determine the effect of Tax PBM on virus biology, mutations were transferred to their
respective HTLV proviral clones resulting in the generation of HTLV-1ΔPBM, 
HTLV-2+C22 and HTLV-2+C18. We first assessed the effect of the PBM on p19 Gag
production in the supernatant of transfected cells. The concentration of p19 Gag in the
culture supernatant typically is used as a measure of virion production. As would be predicted from the results of our transcriptional assays, all mutant HTLV proviral clones had the capacity to produce high levels of p19 Gag, similar to the wild-type proviral clones (Fig 2.3). Since efficient HTLV transmission is dependent on cell-to-cell contact, 729 B-cell stable transfectants expressing HTLV-1ΔPBM, HTLV-2+C22 and HTLV-2+C18 were generated and further characterized. Each of the stable transfectants contained complete copies of the provirus, and the presence of tax gene sequences with expected mutations was confirmed by diagnostic PCR. To monitor the production of viral protein in these stable transfectants, the concentration of p19 Gag in the culture supernatant of several cell clones was quantified by ELISA. As shown in Figure 2.4A, the amount of p19 Gag expression from each stable cell line tested was variable. This likely is attributable to the chromosomal location of proviral sequences and the overall proviral copy number in the cell clones. We selected stable producer lines with p19 Gag production similar to that of our well characterized HTLV-1 and HTLV-2 producer cell lines, 729Achneo and 729pH6neo, for assessing the ability of these mutant viruses to induce cellular proliferation and immortalization (Fig 2.4A*). Furthermore, using Western blot, we confirmed that these lines expressed similar amounts of Tax protein and showed the corresponding gel mobility consistent with their variable amino acid length (Fig. 2.4B).

The Tax-1 PBM promotes HTLV-1-induced proliferation of human PBMCs. We next determined whether the Tax PBM contributed to the ability of the virus to infect and induce primary human PBMCs to proliferate. To quantify the infection and proliferation of PBMCs, 96-well microtiter infectivity assays were performed\textsuperscript{349}. In each
well, 2000 irradiated 729 stable producer cells were cocultured with 10,000 prestimulated PBMCs in the presence of IL-2. The 729 producer cell lines used for the assays included 729HTLV-1, 729HTLV-1ΔPBM, 729HTLV-2, 729HTLV-2+C22, and 729HTLV-2+C18. HTLV negative 729 cells were used as a control. At weekly intervals, individual wells were assayed for proliferation as measured microscopically by increased cell number or by MTS assay, and then were split 1:3. It is important to note that our coculture assays used freshly isolated PBMCs and cell-associated virus transmission designed to mimic the in vivo infection. However, subjecting the cells to weekly splits is more stringent than our standard, more traditional liquid culture immortalization/transformation assays, which maintain a relatively high cell concentration. A Kaplan-Meir plot of HTLV-1-induced T-cell proliferation indicated that HTLV-1ΔPBM is significantly attenuated in its ability to induce primary T-cell proliferation (Fig. 2.5A). The percentage of wells containing proliferating lymphocytes was substantially decreased compared with cells infected with the parental HTLV-1 (5% for HTLV-1ΔPBM vs 42% for HTLV-1) at seven weeks post-plating. Consistently, the Kaplan-Meir plot indicated that HTLV-2-mediated T-cell proliferation was significantly enhanced by adding the Tax-1 PBM to the HTLV-2 Tax C-terminus (see curve for HTLV-2+C22 vs HTLV-2+C18 and wtHTLV-2 in Fig. 2.5B). Taken together, these results show that the Tax-1 PBM (ETEV) plays a critical role in HTLV-1 induced T-cell proliferation in vitro.

**Tax-1 PBM is not required for HTLV-mediated immortalization of human PBMCs.** In order to address the role of the Tax-1 PBM in HTLV-mediated human T-cell immortalization, we performed long-term coculture immortalization assays. Viable cell
numbers were recorded at weekly intervals to monitor the immortalization process and the characteristic expansion of infected PBMCs. Viral replication also was examined by quantification of p19 Gag production in the culture supernatant starting at three weeks postcultivation, a time point by which the viral protein production from residual irradiated viral producer cells becomes negligible. As shown in Figure 2.6A, PBMCs cocultured with 729HTLV-1ΔPBM consistently displayed impaired or delayed growth that reached a crucial point after 6-7 weeks cocultivation, which is consistent with the data in the short-term proliferation assay. However, the majority of the T-cell cultures survived this death crisis and eventually became immortalized. In addition, the Gag p19 production from the infected PBMCs did not fluctuate with the growth curve, and deletion of PBM did not affect viral protein production (Fig. 2.6B). HTLV-2+C22 stimulated a higher growth burst in the PBMC cocultures than wtHTLV-2 or HTLV-2+C18, especially at early infection time points (Fig. 2.6C). It is worthy to note that microscopically, HTLV-2+C22 infected PBMCs appeared as either large tight cell clusters or syncytium-like giant cells, which was not observed in PBMCs infected with wtHTLV-2 or HTLV-2+C18. One possible explanation for this phenotype could be that in addition to increased cellular proliferation, the PBM present in HTLV-2+C22 Tax also could induce enhanced cell-cell fusion of infected PBMCs. The enhanced cell-cell fusion leads to increased syncytium formation and eventually cell death, which may counteract the increased proliferation. In contrast to what we observed in HTLV-1-infected PBMCs, cells infected with HTLV-2+C22 continuously produced higher amounts of Gag p19 than those infected with wtHTLV-2 or with HTLV-2+C18 (Fig. 2.6D). In order to address whether the Tax-1 PBM affects viral protein expression, we quantified p19 Gag
production from two cell lines newly immortalized by mutant or wild-type viruses. As shown in Figure 2.6E, our results demonstrated that p19 Gag expression was not significantly affected by either the absence of PBM in HTLV-1 or by the presence of the Tax-1 PBM in HTLV-2. We confirmed the presence of HTLV sequences and the expected Tax mutations in immortalized PBMC lines using diagnostic genome DNA PCR (Fig 6F and 6G). In summary, our data from short-term proliferation assays and long-term immortalization assays indicated that Tax-1 PBM can facilitate HTLV-mediated PBMC proliferation, which does not appear to alter the ability of the virus to immortalize cells.

**Tax-1 PBM plays an important role in Tax induction of micronuclei and disruption of normal cell cycle timing.** Genetic alterations that affect the function of critical cellular machinery can result in deregulated cell cycle progression and abnormal cellular proliferation, eventually contributing to cellular transformation. Micronuclei (MN) induction has been used as an indicator of genomic instability. It has been shown that Tax-1 can rapidly induce MN in transfected cells, whereas Tax-2 lacks or has very limited MN induction capacity\(^\text{126,252}\). Our data demonstrated that Tax-1 PBM can facilitate HTLV-1-induced cellular proliferation. We next compared the relative potency of MN induction by our Tax-1 and Tax-2 mutants to determine if the PBM is responsible for the induction of genomic instability. HeLa cells were transiently transfected with wtTax1, Tax1ΔPBM, wtTax2 and Tax-2+C22 and MN were counted. MN induction by wtTax-1 and wtTax-2 was consistent with previous reports showing 4-5 fold and 2 fold induction, respectively, over background (Fig. 2.7A). Deletion of PBM significantly decreased Tax-1 MN induction activity to near background levels, whereas the addition
of the PBM to Tax-2 resulted in a modest increase in MN but not to wtTax-1 levels (Fig. 2.7A). This result suggested that another domain of Tax-1 that is absent in Tax-2 may enhance the MN activity. Furthermore, and consistent with the MN induction/genetic instability results, cell cycle profiles indicated that wtTax-1 expression led to the accumulation of cells in G₂/M phase, whereas this G₂/M phase cell population was reduced significantly upon deletion of PBM from Tax-1 (Fig. 2.7B). Overall, our results are consistent with the conclusion that the interaction of Tax-1 with some PDZ-containing cellular proteins can induce genetic instability and contribute to HTLV-1-induced T-cell proliferation and ultimately, malignant transformation.

**HTLV-1ΔPBM is deficient in viral spread and persistence in rabbits.** To evaluate the role of the Tax-1 PBM in vivo, we compared the abilities of 729, 729HTLV-1, 729HTLV-1ΔPBM cell lines to establish infection and persistence in our rabbit model. Rabbits were inoculated with lethally irradiated cell lines and rabbit blood was drawn at weeks 0, 2, 4, 6, and 8 after inoculation. Rabbit PBMC were isolated from blood to determine viral DNA integration by PCR, and rabbit serum was assessed for anti-HTLV-1 antibody response by Western blot. The representative seroconversion patterns from each of the inoculated groups are shown in Figure 2.8A. Seroconversion was detected in the 729HTLV-1-inoculated rabbits starting at week two, and antibody titers rose over the time course of the experiment. However, we were unable to detect any antibody response in rabbits inoculated with 729HTLV-1ΔPBM or 729 control. To determine the infection status of the inoculated rabbits, amplification of specific HTLV-1 genomic fragments from rabbit PBMC was performed using PCR. We detected viral DNA integration in all five 729HTLV-1-inoculated rabbits starting at week two after
inoculation, and the viral DNA was consistently detected until the end of the experiment (Fig. 2.8B). However, viral DNA was detected only transiently in two out of five 729HTLV-1ΔPBM-inoculated rabbits at week four and week six. Taken together, our results demonstrated that Tax-1 PBM is required for the establishment and maintenance of persistent infection in rabbits.

2.5 Discussion

Investigators have undertaken comparative studies between HTLV-1 and HTLV-2 and their gene products in an effort to understand how and why leukemogenesis is induced by HTLV-1 and only rarely by HTLV-2. Recent studies have focused on the PBM of Tax-1, not present in Tax-2, as one possible key factor in the differences in pathogenesis between HTLV-1 and HTLV-2. Indeed, the PBM of Tax-1 relative to Tax-2 has been implicated in the higher transforming activity of HTLV-1 in Rat-1 fibroblasts. This increased transforming activity has been attributed to the interaction between the Tax-1 PBM and the tumor suppressor Dlg \textsuperscript{136,137}, but it remains to be determined whether other PDZ-containing proteins also may contribute. In this study, we used full-length infectious viral clones to determine the contribution of Tax-1 PBM in the HTLV-mediated T-cell transformation process and virus survival in the rabbit model of infection. Our data showed that deletion of the Tax-1 PBM severely disrupted the proliferation of HTLV-1-infected T lymphocytes, which also correlated with a delayed immortalization phenotype as measured in long-term coculture assays. The addition of the Tax-1 PBM to the C-terminus of Tax-2 resulted in enhanced HTLV-2-induced T-cell proliferation. Furthermore, MN induction analysis revealed that the PBM is the major
determinant of Tax-1 induced genomic instability, which correlated with an abnormal increase in the G₂/M cell cycle profile. Lastly, in vivo studies using our reproducible rabbit model of infection demonstrated the essential role of the Tax-1 PBM in efficient HTLV-1 infection and persistence.

To date, three PDZ-containing proteins, hDlg, MAGI-3, and pro-IL-16, which have been implicated in either tumor suppression or cell cycle regulation, have been demonstrated to interact with Tax-1 via their respective PDZ domains. The hDlg interacts with adenomatous polyposis coli (APC) and a functional APC-hDlg complex is required to efficiently block cell cycle progression from G₀/G₁ to S phase. Tax-1 has been shown to bind to the same PDZ domain of hDlg as APC, thereby competing with APC for hDlg binding and releasing the cell cycle inhibition by this complex. A recent study by Wu et al. suggested that the PDZ domain-mediated association between MAGI-3 and a tumor suppressor, ‘phosphatase with tensin homology mutated in multiple advanced cancers’ (PTEN/MMAC), performs a critical role in bringing PTEN/MMAC to proper subcellular sites, allowing for enhanced regulation of certain signaling pathway involved with cell survival. Similarly, Tax-1 PBM could disrupt the function of PTEN/MMAC by competing for binding to the MAGI-3 PDZ-domain. Pro-IL-16, which is expressed constitutively in human CD4+ T-cells (the target for HTLV-1 malignancy), contains three PDZ motifs that are highly homologous to hDlg. Tax-1 interacts with the first PDZ domain of Pro-IL-16 and eliminates the G₀/G₁ cell cycle arrest function mediated by pro-IL-16. These results, taken together with ours, strongly support the conclusion that the Tax-1 PBM enhances T-cell proliferation, viral spread, and survival in the infected host by disrupting cell
partners important for cell cycle and apoptosis regulatory control.

In our transfection and coculture experiments, we observed increased syncytia or tight cell clusters induced by HTLV-2+C22 infection, which were not present in wtHTLV-2 or HTLV-2+C18-infected cultures. This suggested that Tax-1 PBM also may enhance cell-cell fusion and/or possibly facilitate viral transmission. The underlying mechanism for syncytium induction is not yet known. However, several lines of evidence suggest that hDlg and MAGI-3 may participate in this activity. First, both hDlg and MAGI-3 belong to MAGUK, a family of proteins with multiple protein domains/motifs that act as scaffolding proteins to mediate recruiting and clustering various macromolecular complexes at the plasma membrane \(^3\). Furthermore, hDlg has been found in T-lymphocytes in addition to neurons and epithelial cells. Upon cell-cell contact by experimental crosslinking of CD2 adhesion molecules, diffused hDlg has been shown to translocate to the plasma membrane particularly at cell-cell adhesion sites \(^4\). MAGI-3 expression also has been detected in HTLV-1-infected T-cell lines and can be induced by Tax \(^1\). Second, a recent report from Blot et al. indicated that both HTLV-1 and HTLV-2 Env can complex with hDlg via their conserved PBMs and constitute platforms at restricted areas of infected T-cell plasma membrane for viral assembly and transmission \(^5\). Therefore, it is possible that the Tax-1 PBM may interact directly with these cellular factors to facilitate viral spread. We speculate that this is the most likely explanation for the deficient in vivo infection by HTLV-1ΔPBM. In the absence of PBM, cell-cell fusion is impaired resulting in clearance of the infected cells by the host immune system before efficient viral transmission can occur. Moreover, the impaired ability of HTLV-1ΔPBM to induce PBMC to proliferate also may contribute to the failure of the virus to persist.
In *in vitro* immortalization assays, HTLV-1ΔPBM cocultured PBMC consistently showed a decreased growth pattern compared to those cocultured with wtHTLV-1. In contrast, the Tax-1 PBM in the context of HTLV-2 (HTLV-2+C22) resulted in increased growth of infected PBMCs at the early stage of infection. Furthermore, addition of the Tax-1 PBM to Tax-2 (Tax-2+C22) could not completely rescue its micronuclei induction activity compared with wtTax-1. One explanation for the immortalization data could be the formation of syncytia or tight cell clusters in HTLV-2+C22 coculture system as mentioned above. Another possibility is that the function of Tax-1 PBM is different in the context of Tax-2 *versus* in the context of Tax1, corresponding to their divergent subcellular distribution. In the context of Tax-1, which is a shuttling protein that is present both in the nucleus and cytoplasm, PBM can associate with some nuclear proteins as well as with membrane-associated cellular proteins and play an important role in both cellular proliferation and cell-cell communication, whereas in the context of Tax-2, which is localized predominantly in the cytoplasm, PBM mainly complexes with some membrane-associated cellular proteins and facilitates cell-cell fusion. However, this difference is not exclusive, since HTLV-2-induced cell proliferation was significantly improved in the presence of PBM in the short-term proliferation assay.

In summary, the associations between Tax-1 and cellular proteins mediated by PBM can induce genetic instability, stimulate abnormal cell cycle progression and cellular proliferation, facilitate *in vivo* viral infection, and ultimately promote HTLV-1-induced T-cell transformation and pathogenesis.
**Figure 2.1** Structure and expression of wtTax-1, wtTax-2 and their mutant proteins. (A) Schematic representation of the Tax-1 (gray) and Tax-2A (black) constructs used in this study. The amino acid length of the proteins and sequence of the Tax-1 PBM (ETEV) are indicated. (B) Western blot of Tax-1 and Tax-2 expressed in transiently transfected 293T cells. Proteins were detected using rabbit Tax-1/Tax-2-specific antisera.
Figure 2.2  Tax transcriptional activation of CREB/ATF- and NFκB-dependent reporter genes. (A) 293T-cells (2 x 10^5) were cotransfected with 2 µg of Tax expression vector or a negative control, 0.02µg TK-Renilla, and 0.1 µg of LTR-1-Luc. Cell lysates were harvested 48 h post-transfection and subjected to a dual luciferase assay. The histogram presents the average fold activation over control values for three independent experiments and error bars denote standard deviations. (B) Jurkat T cells (4x10^6) were cotransfected with 3µg of Tax expression vector or a negative control, 0.05µg TK-Renilla and 0.25 µg of κB-Luc, and luciferase activity was measured as presented in panel A.
Figure 2.3 p19 Gag production in transient transfected 293T cells. 293T cells (2x10^5) were transfected with 2µg of proviral DNAs, as well as negative control. At 48h posttransfection, p19 Gag production was measured in the supernatant by ELISA. The values, which represent p19 Gag levels for three independent experiments are normalized for transfection efficiency. Error bars indicated standard deviations. The data indicate that all PBM mutant HTLV proviral clones are competent to produce high levels of p19 Gag, with similar efficiency to wild type proviral clones.
Figure 2.4 Viral protein expression in permanent transfectants. (A) Three 729 stable transfectants were isolated for HTLV-1ΔPBM, HTLV-2+C22, and HTLV-2+C18 as described in Materials and Methods. Our well-established 729Achneo and 729pH6neo cell clones were used as wtHTLV-1 and wtHTLV-2 stable producer cell lines, respectively. Culture supernatants were harvested at 48 h and tested for p19 Gag production by ELISA. Clones indicated by asterisks, which produce similar quantities of p19 Gag, were further characterized by Western blot for Tax protein expression using specific antibodies against Tax-1 (B) or Tax-2 (C), and these clones were used in subsequent experiments.
Figure 2.5 Representative Kaplan-Meir plots for T-lymphocyte proliferation in short-term microtiter assay. Prestimulated PBMCs ($10^4$) were cocultured with 2000 irradiated 729 stable producer cells in 96 well plates. The percentages of proliferating wells were plotted as a function of time (wks). (A) Kaplan-Meir plots for wtHTLV-1 and HTLV-1ΔPBM, and uninfected 729 control. (B) Kaplan-Meir plots for wtHTLV-2, HTLV-2+C22 and HTLV-2+C18, uninfected 729 as control.
To be continued
Figure 2.6 continued

C

Number of Viable Cells (10⁶)

Weeks post-plating

D

p19 Expression (pg/ml)

Weeks Post-plating

To be continued
Figure 2.6 continued

**Figure 2.6** HTLV T-lymphocyte immortalization assays. Human PBMCs were isolated by Ficoll/Paque and cocultivated with irradiated (10,000 rads) 729 stable cell lines. PBMCs ($2 \times 10^6$) were cultured with irradiated donor cells ($1 \times 10^6$) in 24 well plates as indicated. Representative growth curves for HTLV-1 (A) and HTLV-2 (C) infected cells are shown. Cell viability was determined weekly by trypan blue exclusion (0-11 wks post-cocultivation). The mean and standard deviation of each time point was determined from three independent samples. The presence of HTLV-1 (B) and HTLV-2 (D) gene expression was confirmed by detection of structural Gag protein in the culture supernatant by p19 Gag ELISA at 3, 4, 5, 6, 7, 8, and 9 weeks post-cocultivation. The mean and standard deviation for each time point were determined from three independent samples. (E) Quantification of viral protein expression from several immortalized T-lymphocyte lines established from panels A and B (20 wks in culture). HTLV-immortalized T-cells were plated in 24-well plates at $10^6$ cells/ml/well. Culture supernatants were collected at 24 h and tested for p19 Gag output by ELISA. The values represent p19 production per cell per day. The mean and standard deviation were determined from four replicates of two independent samples. (F/G) Detection of HTLV sequences in immortalized T-lymphocytes by DNA PCR. Although fragment size is consistent with the expected deletion or insertion, diagnostic restriction enzyme digestion and DNA sequencing also were performed to confirm the presence of the mutations (data not shown).
Figure 2.7 Tax-1 and Tax-2 induction of micronuclei and deregulation of cell cycle. (A) Micronuclei induction was evaluated in HeLa cells transiently transfected with cDNA expression vectors Tax-1, Tax-1ΔPBM, Tax-2, Tax-2+C22, or vector control. Data is presented as micronuclei counts per 1200 cells from two independent experiments. (B) Representative cell cycle profiles in HeLa cells transiently transfected with cDNA expression vectors Tax-1, Tax-1ΔPBM, Tax-2, Tax-2+C22, or vector control. The percentages of cell population in G2-M phase are marked.

To be continued
Figure 2.7 continued
Figure 2.8 Assessment of HTLV-1 infection in inoculated rabbits. Rabbits were inoculated with approximately $1 \times 10^7$ irradiated 729HTLV-1, 729HTLV-1\(\Delta\)PBM-infected cells, or uninfected control cells. At weeks 0, 2, 4, 6, 8 post-inoculation, rabbit PBMCs and sera were isolated from blood. A) HTLV-1-specific serologic response. Sera from inoculated rabbits were tested for reactivity to specific HTLV-1 proteins by Western blot. A representative rabbit from the positive wtHTLV-1 inoculated group (R1-R5) and negative control group (R18-R22), and three HTLV-1\(\Delta\)PBM inoculated rabbits (R40-R42) are shown. Viral proteins and 729 cell-specific proteins (**) are labeled on the left. B) Detection of HTLV-1 specific sequences in rabbits. Genomic DNA was isolated from rabbit PBMCs and subjected to PCR using HTLV-1-specific primers (670-671).
Figure 2.8 continued

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CHAPTER 3

ENVELOPE IS A MAJOR VIRAL DETERMINANT OF THE DISTINCT IN VITRO CELLULAR TROPISM OF HTLV-1 AND HTLV-2

3.1 Abstract

HTLV-1 and HTLV-2 are related deltaretroviruses, but are distinct in their disease inducing capacity. Studies have indicated that HTLV-1 has a preferential tropism for CD4⁺ T-cells in vivo and is associated with the development of leukemia and neurological disease. Conversely, the in vivo T-cell tropism of HTLV-2 is less clear, although it appears that CD8⁺ T-cells are infected preferentially with only a few cases of disease association. The difference in T-cell transformation tropism has been confirmed in vitro as shown by the preferential transformation of CD4⁺ T-cells by HTLV-1 versus the transformation of CD8⁺ T-cells by HTLV-2. Our previous studies showed that Tax and overlapping Rex do not confer the distinct T-cell transformation tropisms between HTLV-1 and HTLV-2. Therefore, for this study HTLV-1 and HTLV-2 recombinants were generated to assess the contribution of LTR and env sequences in T-cell transformation tropism. Both sets of proviral recombinants expressed p19 Gag following transfection into cells. Furthermore, recombinant viruses were replication competent and had the capacity to transform T-lymphocytes. Our data showed that exchange of the env
gene resulted in altered T-cell transformation tropism as compared to wild type virus, while exchange of LTR sequences had no significant effect. HTLV-2/Env1 preferentially transformed CD4\(^+\) T-cells similar to wtHTLV-1, whereas HTLV-1/Env2 had a transformation tropism similar to wtHTLV-2 (CD8\(^+\) T-cells). These results indicate that the env is a major viral determinant for HTLV T-cell transformation tropism in vitro and provides strong evidence implicating its contribution to the distinct pathogenesis resulting from HTLV-1 versus HTLV-2 infection.

### 3.2 Introduction

Human T-cell leukemia virus (HTLV) type 1 and type 2 are related but distinct pathogenic complex retroviruses. HTLV-1 has been identified as the etiologic agent of adult T-cell leukemia/lymphoma (ATL), a malignancy of CD4\(^+\) T-lymphocytes, as well as a chronic progressive neurological disorder, termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) \(^{324-327}\). In contrast, HTLV-2 has not been conclusively associated with disease; to date only a few cases of variant hairy cell leukemia (CD8\(^+\) T-cell origin) and several cases of neurological disease have been reported \(^{24,26,328}\).

It has been shown that HTLV-1 and HTLV-2 exhibit distinct in vivo T-cell tropisms. HTLV-1 has a preferential tropism for CD4\(^+\) T-lymphocytes in asymptomatic patients and those with leukemia and neurological disease \(^{326,329,330}\). However, CD8\(^+\) T-cells from HAM/TSP patients were identified as an additional reservoir for HTLV-1 \(^{333}\). In contrast, HTLV-2 in vivo tropism is less clear but seems to favor CD8\(^+\) T-lymphocytes. Proviral sequences were detected predominantly in CD8\(^+\) 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 transformation/immortalization assays in which irradiated 729 producer cells were cocultured with freshly isolated human peripheral blood mononuclear cells (PBMCs). Results from these studies showed that the majority of cells transformed by HTLV-1 in vitro were CD4+ T-lymphocytes. In addition, Tax-mediated HTLV-1 transcription was increased significantly in purified CD4+ versus CD8+ T-cell subsets, suggesting that an enhanced rate of viral transcription may be responsible for the preferential transformation of CD4+ T-cells by HTLV-1. Conversely, purified CD4+ and CD8+ T-cells were shown to be equally susceptible to HTLV-2 infection and subsequent viral gene expression. However, coculture of HTLV-2 producer cells with freshly isolated, non-stimulated PBMCs or purified T-cell subsets resulted in preferential transformation of CD8+ T-cells. Since Tax has been shown to be critical for cellular transformation in vitro and interacts with numerous cellular processes involving cell growth and differentiation, cell cycle regulation and DNA repair it has been hypothesized that the Tax would encode the viral determinant for transformation tropism. However, recent studies using recombinant HTLVs indicated that Tax and overlapping Rex did not confer the distinct HTLV-1 and HTLV-2 transformation tropism in vitro. This suggests that other viral genes or sequences are responsible for the differential ability to transform CD4+ or CD8+ T-cells.

Herein, we generated and evaluated recombinant viruses in which the LTR and env sequences were exchanged between HTLV-1 and HTLV-2. Our results indicated that the
exchange of LTR sequences did not alter HTLV transformation tropisms. Interestingly, we identified Env as a major viral factor that confers the *in vitro* HTLV transformation tropism; wtHTLV-2 and HTLV-1 with Env-2 (HTLV-1/Env2) preferentially transforms CD8\(^+\) T-cells, whereas wtHTLV-1 and HTLV-2/Env-1 displayed a preferred transformation tropism for CD4\(^+\) T-cells. This study provides the first biological evidence that the envelope gene may play an important role in the distinct pathogenesis resulting from HTLV-1 and HTLV-2 infection.

3.3 Materials and Methods

**Cells.** 293T cells and human osteogenic sarcoma cells (HOS) were maintained in Dulbecco's modified Eagle's medium. The 729 and BJAB human B-cell lines were maintained in Iscove's medium. Jurkat T-cells were maintained in RPMI 1640 medium. All 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 (PBMCs) were isolated from the blood of normal donors by centrifugation over Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) and were cultured in RPMI 1640 medium supplemented with 20% FBS, 2 mM glutamine and antibiotics. In selected experiments, transformed T-lymphocytes (10 wks post-coculture) were treated with 10 U/ml of human interleukin-2 (IL-2) (Roche, Indianapolis, IN) to enhance the short-term growth of cells required for molecular analysis.

**Plasmids.** The wild-type (wt) HTLV-1 proviral clone ACH\(^{402}\) and wtHTLV-2 proviral clone pH6neo\(^{33}\) were used to generate recombinant proviral clones for this study. To assist in the generation of the recombinant HTLV proviral clones, several restriction
enzyme sites were introduced using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specifically, an EcoRI site was generated between the end of the 5’ LTR and the start codon of Gag in the HTLV-1 provirus (786TTATTC791 to GAATTC); an EcoRI site already is present at the identical location in the HTLV-2 provirus. An EcoRV site was generated 3’ to the stop codon of tax-1 in the HTLV-1 provirus (8356TGAAAG8362 to TGATATC) and 3’ tax-2 in the HTLV-2 provirus (8203TAGCCTCC8210 to TAGATATC) 124. The HTLV-1/LTR2 and HTLV-2/LTR1 recombinants, where both the 5’-LTR and 3’-LTR were exchanged between HTLV-1 and HTLV-2, were generated by exchanging the EcoRI-EcoRV fragments. In order to exchange the env genes, an NheI site was generated downstream of the stop codon of env-1 in the HTLV-1 provirus (6651GCACAC6656 to GCTAGC), which corresponds to the location of an identical site already present in the HTLV-2 provirus. HTLV-2/Env1 and HTLV-1/Env2 were generated by exchanging the HTLV-1 and HTLV-2 NcoI (HTLV-1 nt5177; HTLV-2 nt5178) -NheI fragment containing the entire env gene.

**Transfection and p19 Gag ELISA.** In order to measure virion production from the recombinant HTLV proviral clones, 2 x 10^5 293T cells were transfected using Lipofectamine® PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. The total amount of DNA was kept constant and contained 2 µg of HTLV proviral plasmid DNA or a negative control and 0.1 µg of CMV-luciferase. After 48 h of growth, culture supernatants were collected and assayed for p19 matrix antigen using a commercially available enzyme-linked immunosorbent assay (ELISA; Zeptometrix, Buffalo, NY). The cell lysates also were harvested and assayed for luciferase activity to monitor the transfection efficiency. p19 Gag calibration curves
were generated using HTLV-1 p19 antigen standards as described by the manufacturer, with a detection sensitivity of 25 pg/ml. All experiments were performed in triplicate. To generate stable transfectants, proviral plasmid clones containing the Neo’ gene were introduced into cells by electroporation as described previously \cite{103,403}. Stable transfectants containing the desired proviral clones were isolated following incubation in 24-well culture plates in medium containing 1 mg/ml of Geneticin. After four-to-five weeks of selection, viable cells were single-cell cloned, expanded, and maintained in culture for further analysis. The clones were screened for p19 Gag expression in the cell supernatants by ELISA.

**DNA preparation and PCR.** Genomic DNA was isolated from permanently transfected cell clones or from transformed PBMCs using the PUREGENE® DNA purification system (Gentra, Minneapolis, Minn.). One microgram of genomic DNA was subjected to 30-cycle PCR analysis. The PCR-amplified product was separated on an agarose gel and visualized by ethidium bromide staining. The primer pair TRE-1(S) $^{74(8351)}$AAGTCTGAAAAGGCAGGG$^{92(8369)}$ and TRE-1(AS) $^{335(8612)}$CACGCTTTTATAGACTCCTG$^{316(8593)}$ was used to amplify a 262 bp fragment specific for the HTLV-1 LTR, and the primer pair $^{8(8197)}$GGCGACTAGCTCCTCCAAGCCAG$^{29(8218)}$ and $^{750(8938)}$CGGGAAGCAATGCTCCTAGGGCG$^{727(8916)}$ was used to amplify a 743 bp fragment specific for the HTLV-2 LTR (numbers and numbers in parentheses represent proviral nucleotide position in the 5’ LTR and 3’ LTR, respectively). The primer pair $^{8182}$GAGCGGATGACAATGGCGAC$^{8202}$ (Tax-2/LTR-2 specific) and TRE-1 (AS) was used to amplify a 279 bp fragment and specifically detect a hybrid LTR-2/1 that
results from the *tax*-2 gene being part of the LTR. The degenerative primer pair
\[
\begin{align*}
5276(5265) & \quad \text{TCTCCTCMTACCACTCYA} \quad 5293(5282) \\
6346(6335) & \quad \text{CTTGYTCCAGAAYAGGAG} \quad 6328(6317)
\end{align*}
\]
was designed to amplify a 1071 bp product from both \textit{env}-1 and \textit{env}-2 sequences (numbers and numbers in parentheses represent proviral nucleotide position in HTLV-1 and HTLV-2, respectively).

**Syncytium and transformation assays.** Syncytium and transformation assays were performed as described previously \textsuperscript{103,389}. Briefly, 729 producer cells were irradiated with 10,000 rads and then cocultured with BJAB or HOS cells. Syncytia in BJAB cocultures were enumerated microscopically 2 to 5 days post-plating. In HOS cocultures, plates were washed after 24 h incubation, Wright's stained, and scored for syncytia containing at least four nuclei per cell as described previously \textsuperscript{78}.

For the \textit{in vitro} transformation assays, \textnum{10}^6 irradiated 729 producer cells were cocultured with \textnum{2} \times \textnum{10}^6 freshly isolated PBMCs in the absence of IL-2 in 24-well culture plates. Transformed cells were defined as cells with continuous proliferation 8 wks post-coculture in the absence of IL-2. HTLV expression was confirmed by detection of p19 Gag protein in culture supernatants using an ELISA at weekly intervals. Viable cells were counted weekly by trypan blue exclusion. Wells containing transformed T-cells were enumerated and phenotyped by fluorescence-activated cell sorter analysis (FACS). Cells were stained with anti-human CD3 antibody-fluorescein isothiocyanate (FITC), anti-human CD4 antibody-phycocerythrin (PE), and anti-human CD8 antibody-PE-Cy5 (BD PharMingen, San Diego, CA) and analyzed using a Coulter Epics Elite flow cytometer.
3.4 Results

**Construction of recombinant HTLV-1 and HTLV-2 proviral clones.** We constructed recombinant proviruses in which the LTRs or the env genes of HTLV-1 and HTLV-2 were exchanged to determine if these sequences might be responsible for the different biological properties of these two related viruses, with specific emphasis on transformation tropism in vitro. Figure 3.1 shows the genome structure of HTLV and schematics of the wild type (wt) and recombinant proviruses. The wtHTLV-1 molecular clone Ach and wtHTLV-2 molecular clone pH6neo were the parental clones used in these studies. Upon transfection into cells, both clones were capable of virion synthesis, resulting in infection and transformation of human PBMCs as determined by coculture assay. Specific restriction enzyme sites were generated by site-directed mutagenesis and were used to exchange the LTRs or env genes between HTLV-1 and HTLV-2 (Fig. 3.1B, see Materials and Methods). There is approximately 65% homology between HTLV-1 and HTLV-2 at the nucleotide sequence level, with the lowest homology in the LTR region (31%). The amino acid homology between Env-1 and Env-2 are 61% for the surface glycoprotein (SU) and 84% for the transmembrane protein (TM), respectively.

**p19 Gag production by recombinant proviruses.** Although HTLV-1 and HTLV-2 genome structure is relatively similar and highly homologous, the ability of regions or genes, excluding tax/rex, to substitute for each other in a proviral context that results in virion production has not been assessed directly. Efficient p19 Gag production from proviral clones requires a functional viral promoter as well as viral trans-regulatory proteins Tax and Rex; the concentration of p19 Gag in the supernatant of transfected cells
has been used as a measure of virion production. The parental and LTR or env recombinant proviral clones were transfected into 293T cells and p19 Gag production in the culture supernatant was quantified by ELISA. As shown in Figure 3.2, all recombinant proviral clones produced p19 Gag. Consistent with our previous studies, p19 Gag production from wtHTLV-1-transfected cells was approximately four-fold higher than wtHTLV-2- transfected cells. Cells transfected with HTLV-1/LTR2 produced slightly lower levels of p19 Gag as compared to wtHTLV-1, and p19 Gag production from HTLV-2/LTR1 was approximately four-fold less than wtHTLV-2. These differences suggest that Tax-1 can activate both LTR-1 and LTR-2 at a similar level, whereas Tax-2 displays a greater transactivation capacity on LTR-2 than on LTR-1. As would be predicted, exchange of the env gene did not significantly alter p19 Gag production as compared to wt proviruses. Taken together, these results indicate that HTLV-1 and HTLV-2 LTR sequences or env genes are functionally interchangeable. However, gene expression levels from the LTR recombinants were altered likely due to differences in promoter/enhancer sequences and/or intrinsic Tax-1 and Tax-2 transactivation activities.

**Establishment of 729 stable transfectants for recombinant HTLV production.**

To determine the capacity of recombinant proviral clones to synthesize viral proteins, direct viral replication, and to induce cellular transformation, permanent 729 B- cell transfectants expressing wt and recombinant proviral clones were generated and further characterized. Each of the stable transfectants contained complete copies of the provirus and the presence of the expected LTR or env sequences was confirmed by diagnostic DNA PCR. To monitor the production of viral protein in these stable transfectants, the
concentration of p19 Gag in the culture supernatants were quantified by ELISA. As shown in Figure 3, representative stable cell clones selected for this study had p19 Gag expression similar to levels observed in transient transfected cells (compare Figs. 3.2 and 3.3).

To evaluate the capacity of the stable transfectants to produce infectious progeny virions, the transfectants were irradiated and cocultured with HOS or BJAB cells. Productive HTLV infection of these cells results in the rapid induction of syncytia. Interestingly, infection of HOS cells by either HTLV-1 and HTLV-2 results in induction of syncytia, whereas efficient syncytium induction following infection of BJAB cells generally is restricted to HTLV-2 infection. Syncytium formation is an indirect measure of viral Env expression such that the presence of infectious virus capable of spreading throughout the culture dramatically reduces the time required for syncytium induction. As summarized in Table 3.1, coculture of $10^6$ 729wtHTLV-2, 729HTLV-2/LTR1, or 729HTLV-1/Env2 with $5 \times 10^5$ BJAB cells resulted in syncytium formation as early as two days post-plating. To address the efficiency with which the viruses could replicate and induce syncytia, 10-fold serial dilutions of irradiated producer cells were cocultured with BJAB cells. Syncytia were induced with as few as 100 irradiated producer cells (Table 3.1), and there was no apparent difference in the time course of syncytium induction between wtHTLV-2 and recombinant viruses containing the HTLV-2 Env. Stable transfectants expressing the HTLV-1 Env failed to produce appreciable syncytia in BJAB cells up to five days post-plating. Consistent with previous studies, stable cell clones expressing either wtHTLV-1, wtHTLV-2 or recombinant viruses irrespective of LTR or Env were competent to induce syncytia in HOS cells.
These results demonstrated that the wt and recombinant viruses could replicate and spread efficiently throughout the culture.

**Recombinant viruses can transform PBMCs.** We determined whether the recombinant viruses had the capacity to transform human PBMCs. These experiments used a stringent transformation assay that closely mimics the *in vivo* infection. Irradiated 729 stable producer cells were cocultured with freshly isolated, nonstimulated PBMCs in the absence of exogenous lectins or IL-2. Cell number and viability were examined at weekly intervals to monitor the transformation process and the characteristic expansion of cells from the PBMC mixed cell population. A growth curve from a representative experiment is depicted in Figure 3.4A. When cocultured with irradiated uninfected 729 cells, PBMCs showed a progressive loss of viable cells over time and eventually died off approximately 5-6 weeks postplating. In contrast, the transformation process of PBMCs was apparent in coculture wells containing producer cells of wtHTLV-1, wtHTLV-2, and both LTR and Env recombinant viruses. Cell number and viability were similar for all virus-producing cells throughout the experiment. It is noteworthy that although HTLV-2/LTR1 expression, as determined in transfected cells and stable cell clones, was consistently lower than expression of other proviruses, its transformation capacity of PBMCs was not significantly different. Viral replication was assessed by quantitation of p19 Gag production in the culture supernatant starting at three weeks post cocultivation. This is the time point at which productively HTLV-infected PBMCs typically produce viral particles and the particle production from residual irradiated viral producer cells becomes negligible (Fig. 3.4B). Our results indicated that the recombinant viruses, like the
parental viruses, were capable of productively infecting PBMCs and inducing sustained proliferation or transformation of the cells in the absence of exogenous cytokines.

We assessed the presence of HTLV sequences in PBMCs transformed by both wt and recombinant viruses. Diagnostic DNA PCR was performed to determine if transformed cells contained the expected viral sequences. Figure 3.5 shows that high molecular weight DNA from cells transformed by all parental and recombinant viruses contain HTLV sequences. Sets of specific primer pairs were used to confirm the presence of the expected viral sequences. LTR-specific primer pairs distinguished between LTR-1, LTR-2, or an LTR-2/LTR-1 that results from the 3’ portion of the tax gene being part of the LTR (Fig 3.5A). The appropriate env sequences were confirmed using a degenerative set of primers that detects both env-1 and env-2. The amplified fragment then was digested with BamHI and ClaI, which distinguishes env-1 from env-2 (Fig. 3.5B). Together these results indicated that the expected wild type and recombinant proviral sequences were present in transformed PBMCs.

**Envelope is a major viral determinant for HTLV transformation tropism.** To determine if the exchange of LTR or env sequences altered transformation tropism, we evaluated phenotypes of cells transformed by wtHTLV-1, wtHTLV-2, HTLV-1/LTR2, HTLV-2/LTR1, HTLV-1/Env2, and HTLV-2/Env1. Since it has been well documented that HTLV transforms only T-lymphocytes, individual wells of cells at 10 weeks post coculture were stained with anti-CD3-FITC, anti-CD4-RPE, and anti-CD8-SPRD and subjected to the flow cytometry analysis. The data from multiple wells from at least three independent experiments are summarized in Figure 3.6. Results with wtHTLV-1 and
wtHTLV-2 were consistent with previous reports by us and others in that wtHTLV-1 preferentially transforms CD4\(^+\) T-cells \textit{in vitro} and that wtHTLV-2 has a preferential transformation tropism for CD8\(^+\) T-cells \textsuperscript{124,261,262,347}. Importantly, we showed that exchange of the \textit{env} gene significantly alters the transformation tropism (p<0.0001). HTLV CD4\(^+\) T-cell transformation tropism correlated with Env1 and HTLV CD8\(^+\) transformation tropism correlated with Env2. Exchange of the LTRs had no significant effect on transformation tropism (Fig. 3.6). Overall, our results indicated that the viral envelope is a major viral determinant of the distinct transformation tropism of HTLV-1 and HTLV-2 \textit{in vitro}.

### 3.5 Discussion

Consistent with their disease association, HTLV-1 and HTLV-2 display distinct \textit{in vivo} and \textit{in vitro} cellular transformation tropisms. Therefore, identification of the viral determinant(s) of cellular transformation tropism may provide the basis for understanding HTLV pathogenesis. In a recent study, we showed that the viral oncoprotein Tax and overlapping Rex did not confer the distinct difference in transformation tropism between HTLV-1 and HTLV-2. In this study, we assessed the role of the viral LTR and \textit{env} gene in HTLV-1 and HTLV-2 cellular transformation tropism \textit{in vitro}. Our results revealed that LTR or \textit{env} gene recombinant viruses were replication competent and could transform primary human T-lymphocytes. Flow cytometry analysis indicated that wtHTLV-1, HTLV-1/LTR2, and HTLV-2/Env1 had a preferential transformation tropism for CD4\(^+\) T-cells and that wtHTLV-2,
HTLV-2/LTR1, and HTLV-1/Env2 had a preferential tropism for CD8$^+$ T-cells. We conclude from our study that the *env* gene is a major viral determinant of the distinct differences in transformation tropism between HTLV-1 and HTLV-2.

The precedent for LTR-mediated cellular tropism and disease induction has been clearly demonstrated for the murine leukemia viruses (MuLV). Moloney MuLV (M-MuLV) induces T-cell lymphomas, whereas Friend MuLV (F-MuLV) induces erythroleukemia when injected into newborn NFS mice $^{351,408}$. Using recombinant viruses, disease tropism was mapped to the viral LTR $^{353,354}$. Further studies demonstrated that specific enhancer sequences within the M-MuLV LTR in cooperation with unique T-cell transcription factors were the primary determinants for the distinct cell tropism $^{355,356}$. Sequence comparison of the LTRs between HTLV-1 (LTR-1) and HTLV-2 (LTR-2) revealed that they share approximately 31% homology at the nucleotide sequence level indicating that the LTRs are the least homologous viral region between HTLV-1 and HTLV-2. The HTLV LTR contains viral transcriptional enhancer and promoter elements with binding sites for numerous cellular transcriptional factors $^{15}$ and studies suggest that there are differences in cellular transcription factor loading between LTR-1 and LTR-2 $^{41}$ and Hung Fan personal communication). Interestingly, an HTLV-2 mutant in which the three imperfect 21-nucleotide repeats in the U3 region of LTR were replaced with the CMV immediate-early enhancer preferentially transformed CD8$^+$ T-cells, similar to wtHTLV-2 $^{262}$. This finding is consistent with the results of the present study where we showed that exchange of the LTR does not confer the differential cellular transformation tropism of HTLV-1 and HTLV-2.
The interactions between viral Env and cellular receptors mediate viral entry into specific cell types. Evidence suggests that HTLV-1 and HTLV-2 have the same primary receptor, which is expressed ubiquitously on the surface of numerous cell types. In contrast to their restricted in vivo and in vitro transformation tropism, in vitro infection with both HTLV-1 and HTLV-2 can be established in many vertebrate cell lines, including T-cells, B-cells, endothelial cells, glial cells, and monocytes. Therefore, it would seem unlikely that the HTLV Env would be responsible for the distinct cellular transformation tropism between HTLV-1 and HTLV-2. Studies of other retroviruses have suggested that Env may affect cellular tropism by utilization of specific co-receptors and/or modulation of the intracellular environment through post-entry events. In fact, different HIV-1 stains utilize different co-receptors to preferentially infect specific cell types, CXCR4 for T-cells and CCR5 for macrophages. Moreover, it was found that multiple sequences in the Env surface unit (SU), including those outside of the receptor-binding domain, dictate the T-cell tropism and cytopathic properties of feline leukemic virus (FeLV).

In spite of the fact that both HTLV-1 and HTLV-2 can productively infect numerous cell types of different species and likely employ the same primary receptor, this study indicates that Env plays an important role in determining the major T-cell population transformed by HTLV-1 versus HTLV-2. Further investigation of the underlying mechanisms utilized by HTLV Env will be a most worthy pursuit. To date, many cellular factors have been implicated in Env-mediated HTLV infection and syncytia formation, including heat shock protein (HSP-70), various adhesion molecules (VCAM-1, ICAM-1), membrane glycoprotein C33, HLA A2 receptor, IL-2 receptor, lipid rafts, and the glucose
The failure to identify a conclusive receptor for HTLV suggests that more than one cell-surface molecule may play a critical role in HTLV entry. This raises the possibility that these molecules may have different expression levels or subcellular distribution in certain cell types, or possess different affinities to HTLV-1 Env or HTLV-2 Env. Therefore, the susceptibility of particular cells to HTLV-1 or HTLV-2 infection may be modulated by different receptor density and/or affinity, contributing to distinct transformation tropism phenotypes. The important role of receptor density in efficient viral infection of different cell types has been demonstrated in HIV\textsuperscript{373,374}. In support of this theory is the observation by us (in this study) and others of the differences in syncytium induction between Env-1 and Env-2\textsuperscript{78,406,411,412}. HTLV-2 Env expression induces efficient syncytium formation in both BJAB cells and HOS cells; whereas Env-1 mediates syncytium formation upon coculture with HOS cells only.

Another plausible explanation is that Env-1 and Env-2 may trigger specific post-entry signaling pathway(s) that promote HTLV-mediated cellular transformation in different T-cell types. Env-1 gp46, in conjunction with the CD2/LFA-3 activation pathway, is mitogenic to resting T-lymphocytes\textsuperscript{375-377}. Furthermore, two recent reports indicated that HTLV receptor expression is induced by T-cell activation and possibly plays a role in the immunobiology of activated T-cells\textsuperscript{378,379}. Interestingly, both Env-1-mediated syncytium formation and T-cell antigen-receptor signaling require the presence of lipid rafts\textsuperscript{368,380}. Lipid rafts are distinct cell membrane structures formed by dynamic clustering of sphingolipids and cholesterol, which are enriched in many glycosyl-phosphatidylinositol (GPI)-anchored proteins, as well as src family kinases,
protein kinase C, heterotrimeric G proteins, actin, and actin binding proteins \(^{381,413,414}\). Therefore, it is possible that differences in the interactions between Env-1 and Env-2 and cellular receptor(s) in certain cell membrane microenvironments may induce unique downstream signaling events, leading to distinct transformation tropism.

In conclusion, we have identified the \(env\) gene as a major viral genetic determinant of distinct T-cell transformation tropism between HTLV-1 and HTLV-2. With this knowledge, we can search for its cellular partner(s) that play a critical role in HTLV cellular tropism to ultimately gain important insights into the mechanisms of HTLV pathogenesis.
Stable transfectants | Syncytium induction in BJAB cells<sup>a</sup> | Syncytium induction in HOS cells<sup>b</sup>
---|---|---
HTLV-1 | − | +
HTLV-2 | + (100, 2 days) | +
HTLV-1/LTR2 | − | +
HTLV-2/LTR1 | + (100, 2 days) | +
HTLV-1/Env2 | + (100, 2 days) | +
HTLV-2/Env1 | − | +
Untransfected 729 | − | −

<sup>a</sup> 5x10⁵ BJAB cells were cocultivated with serial 10-fold dilutions of irradiated 729 stable producer cells (10⁶, 10⁵, 10⁴, 10³, 10², 10¹). Syncytia were counted 2-5 days post-plating. Numbers in the parentheses indicate minimum number of irradiated 729 producer cells and number of days required for significant microscopic syncytia formation, respectively.

<sup>b</sup> Confluent HOS cells were cocultured with 10⁶ irradiated 729 stable producer cells. Syncytia were counted after 24 hours post-plating.

**Table 3.1** HTLV induction of syncytia in BJAB and HOS cells
Figure 3.1 Organization of HTLV genome and the exchanged regions in recombinant proviral clones. (A) The complete HTLV proviral genome is shown schematically. LTRs are depicted with their U3, R, and U5 regions. The locations of gag, pro, pol, env, tax, and rex genes and their corresponding reading frames are indicated, along with orf-I and orf-II of HTLV-1. (B) Genomic organizations of the parental and recombinant proviral clones used in this study. Black boxes indicate HTLV-1 origin. Grey boxes indicated HTLV-2 origin. Specific restriction endonucleases used in the construction of rHTLV are shown on wtHTLV-1 genome.
Figure 3.2 p19 Gag expression in transiently transfected 293T cells. 293T cells (2x10^5) were transfected with 2µg of proviral DNA using Lipofectamine® PLUS reagent. At 72 h post-transfection, p19 Gag expression was measured in the culture medium by ELISA. The values, which represent p19 Gag levels for three independent experiments, are normalized for transfection efficiency. Error bars indicate standard deviations.
Figure 3.3 p19 Gag expression in permanent transfectants. 729 stable transfectants containing wtHTLV-1, wtHTLV-2, HTLV-2/LTR1, HTLV-1/LTR2, HTLV-1/Env2, or HTLV-2/Env1 were isolated as described in the Materials and Methods. A representative of each cell clone used in syncytia and transformation assays is shown. Culture supernatants were harvested after 48 h of growth and tested for p19 Gag production by ELISA. In general, p19 Gag production from these stable producer cell lines showed a level similar to transient transfected 293T cells.
Figure 3.4 Growth curve for HTLV T-lymphocyte transformation assay. Human PBMCs were isolated by Ficoll/Paque and cocultivated with irradiated (10,000 rads) 729 producer cells (729-wtHTLV-1, 729-wtHTLV-2, 729-HTLV-2/LTR1, 729-HTLV-1/LTR2, 729-HTLV-1/Env2, or 729-HTLV-2/Env1) or 729 uninfected control cells as indicated. PBMCs (2x10^6) were cultured with donor cells (1x10^6) in 24 well plates. Cells were fed once per week with medium containing 20% FBS. (A) Cell viability was determined weekly by trypsin blue exclusion from 0-8 wks post-cocultivation. The mean and standard deviation for each time point were determined from three independent samples. (B) The presence of HTLV gene expression was confirmed by detection of structural Gag protein in the culture supernatant by p19 ELISA at 3, 4, 5, 6, 7, 8 wks post co-cultivation. The mean and standard deviation for each time point were determined from three independent samples.
Figure 3.4 continued

B

- 729-wtHTLV-1
- 729-wtHTLV-2
- 729-HTLV-1/LTR2
- 729-HTLV-2/LTR1
- 729-HTLV-1/Env2
- 729-HTLV-2/Env1
- 729 Control

p19 Expression (pg/ml)

Weeks Post-plating

3 4 5 6 7 8
To be continued
Figure 3.5 Transformed PBMCs contain the expected HTLV-1 or HTLV-2 LTR and env sequences. (A) Specific HTLV-1 and HTLV-2 LTR sequences were PCR amplified from high molecular weight DNA obtained from wtHTLV-1, wtHTLV-2, HTLV-2/LTR1, HTLV-1/LTR2, HTLV-1/Env2, or HTLV-2/Env1 transformed PBMCs. Primers were designed to specifically amplify LTR-1 262 bp, LTR-2 (743 bp), or LTR-2/1 hybrid (279 bp) as indicated. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. (B) HTLV-1/2 env sequences were PCR amplified from transformed PBMC DNA as indicated. The PCR amplified product (1071 bp) was incubated in the presence or absence of BamHI and Clal. Products were separated on a 2% agarose gel and visualized by ethidium bromide staining. The location of the BamHI and Clal restriction sites that distinguishes Env-1 from Env-2 is shown below.
Figure 3.6. Cell surface phenotype of HTLV transformed cells. Transformation assays were performed as described in the legend to Fig. 3.4. Wells containing transformed T cells, defined as cells with continuous growth of at least 8 weeks post-plating in the absence of exogenous IL-2, were stained with anti-CD3 antibody-FITC, anti-CD4 antibody-PE and anti-CD8 antibody-PE-Cy5 and analyzed on a Coulter Epics Elite flow cytometer. The percentage of transformed CD4$^+$ and CD8$^+$ cells in individual wells from three independent experiments for wtHTLV-1 (n=50), wtHTLV-2 (n=50), HTLV-1/LTR2 (n=44), and HTLV-2/LTR1 (n=50), HTLV-1/Env2 (n=42), and HTLV-2/Env1 (n=42) are plotted. Mean values for CD4$^+$ and CD8$^+$ viral transformants are indicated. Statistics analyses using ANOVA and Tukey’s studentized range (HSD) test indicate that there is significant difference between means of readings from different groups (p<0.0001). Specifically, there is significant difference between wtHTLV-1 mean and each of the means of wtHTLV-2, HTLV-2/LTR1, and HTLV-1/Env2. In addition, there is significant difference between wtHTLV-2 mean and each of the means of wtHTLV-1, HTLV-1/LTR2, and HTLV-2/Env1. The data indicates that env is the genetic determinant responsible for the distinct transformation tropism between HTLV-1 and HTLV-2.
4.1 Abstract

Human T-cell leukemia virus (HTLV) Rex phosphoprotein functions to increase the level of viral structural and enzymatic gene products, which are expressed from the unspliced and incompletely-spliced viral mRNAs. Recently, it has been demonstrated that the phosphorylation of HTLV-2 Rex (Rex-2), particularly at serine residues 151 and 153 within the carboxy-terminal phosphorylation domain (CTPD), is critical for its function. Replacement of both serine residues with phosphomimetic aspartic acid (S151D/S153D) has been shown to lock Rex-2 in an active conformation. This mutant phenotype provides us with a unique reagent to evaluate the role of Rex-2 functional regulation in viral replication and cellular transformation in vitro and persistence in vivo. Since the mutation in RexS151D/S153D also affects viral oncoprotein Tax amino acid sequences and its transactivation activities, four additional Rex mutants in this region were generated and characterized to facilitate these studies; two aspartic acid substitution mutants, RexP152D and RexA157D and two carboxy-terminus deletion mutants,
RexS151Term and RexS158Term. All mutants localized predominantly to the nucleus/nucleolus and display a locked active functional conformation similar to RexS151D/S153D. In short-term microtiter proliferation assays, RexA157D enhances HTLV-2-mediated cellular proliferation of human peripheral blood mononuclear cells (hPBMCs), whereas hPBMCs in coculture with 729HTLV-2/RexS151Term or 729HTLV-2/RexS158Term display substantially decreased proliferation. In long-term immortalization assays, HTLV-2/RexP152D and HTLV-2/RexA157D have the capacity to immortalize primary T cells as efficiently as wtHTLV-2, whereas both HTLV-2/RexS151Term and HTLV-2/RexS158Term were deficient in immortalization of hPBMCs. Our studies indicate that constitutively active Rex-2, particularly the phosphomimetic mutants, can increase viral protein expression, contributing to the enhanced primary T-cell proliferation mediated by HTLV-2 infection. However, constitutively active Rex-2 mutants without CTPD impair HTLV-2-induced cellular proliferation and transformation, implying that the CTPD is required for proper functional regulation of Rex-2 in the context of the virus. Interestingly, the viral persistence of HTLV-2/RexP152D was impaired in inoculated rabbits as compared to wtHTLV-2 supporting the conclusion that this domain plays an important regulatory role in vivo. This is the first report of the critical role of HTLV Rex regulatory control in viral mediated cellular proliferation and immortalization, and provides insight into viral-host interaction in HTLV-2-infected cells.
4.2 Introduction

Human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are related complex oncogenic retroviruses that transform primary human T cells in culture and are associated with leukemia and neurological disorders in humans. In addition to the structural and enzymatic genes *gag*, *pol*, and *env*, the unique pX region of HTLV encodes two trans-regulatory gene products, Tax and Rex, in partially overlapping open reading frames (ORFs) 15.

Tax increases the rate of transcription from the viral long terminal repeat (LTR). In addition, Tax can modulate the expression and/or activities of numerous cellular proteins involved in cell proliferation and differentiation, cell cycle regulation and DNA repair processes 69,107,265. These pleiotropic effects of Tax on cellular processes are likely required for its transforming or oncogenic capacity 261,262,388,389. However, the fact that efficient transformation requires Tax expression in the context of full length viruses suggests that other gene products or the regulated expression of specific gene product(s) are involved in the HTLV transformation process.

Rex, a key regulator of viral replication, functions at a post-transcriptional level to induce cytoplasmic expression of unspliced and incompletely-spliced viral mRNAs encoding the viral structural and enzymatic proteins 266,269. Rex function is mediated by a *cis*-acting RNA Rex response element (RxRE) located in the R region of viral LTR 279,296,311. Previous studies suggested that HTLV-1 Rex (Rex-1), HTLV-2 Rex (Rex-2) and their RNA response elements are structurally similar and functionally interchangeable 295. Mutational analyses of Rex-1 and Rex-2 have defined several
domains critical for their function. These include the arginine-rich N-terminal sequences that serve both as an RNA binding domain and as a nuclear localization signal, the central leucine-rich activation domain encompassing the nuclear export signal (NES), and the multimerization domain composed of two regions flanking the NES 278-282,284-287,415.

Both Rex-1 and Rex-2 are phosphoproteins and phosphorylation has been shown to be critical for their function 319. Rex-1 is phosphorylated at serines 70 and 177 and threonine 174 320. In HTLV-2-infected cells, as well as in cell lines transfected with Rex-2 expression plasmids, two major species of Rex-2 have been detected, p24\textsuperscript{rex} and p26\textsuperscript{rex}. Both Rex-2 species have the same amino acid backbone and differ by conformational change induced by serine phosphorylation 15,273,274. p24\textsuperscript{rex} is found primarily in the cytoplasm, whereas the phosphorylated form p26\textsuperscript{rex} is predominantly localized to the nucleus and nucleolus 276,294. In addition, phosphorylation of Rex-2 correlates to its binding to the RxRE and its inhibition of mRNA splicing 313,318. Recently, mutational analyses of Rex-2 targeting all serines and threonines revealed a novel C-terminal phosphorylation domain (CTPD) containing two critical phosphorylated residues at serine 151 and 153. Rex-2 mutants with alanine substitution of these two serines (S151A, S153A) displayed reduced phosphorylation, impaired RNA binding capacity, diffused cytoplasmic localization, and decreased functional activity. In contrast, replacement of both serine residues with phosphomimetic aspartic acid (S151D, S153D) resulted in detection of only the p26\textsuperscript{rex} in cells, enhanced the RNA binding capacity of Rex-2, and resulted in a predominant intense nucleus/nucleolus localization. This mutant Rex-2 was locked in a phosphorylated active conformation since it could not be altered by phosphatase treatment \textit{in vitro}. Moreover, several mutations within Rex-2 CTPD,
including S151D, S153D, partially impaired Rex-2 nucleocytoplasmic shuttling, implying the important role of CTPD in Rex-2 functional regulation.²⁷⁴,²⁸⁷

It has been proposed that the regulation of Rex through phosphorylation allows the regulation of HTLV-2 replication in response to regulatory signals of infected cells.²⁷⁴,²⁹⁰ Therefore, in the context of full length HTLV, constitutively active Rex may promote viral replication and viral spread, leading to improved cellular proliferation and transformation. On the other hand, loss of regulatory control of Rex may disrupt the balance of viral gene expression and interfere with the crosstalk between virus and host cells, leading to deficient cellular proliferation and transformation and ultimately virus survival in vivo.

The goal of this study is to understand the importance of Rex functional regulation in HTLV-2 cellular immortalization/transformation and virus survival in vivo. Since the constitutively active Rex mutant S151D/S153D affected the amino acid sequence and transactivation activities of the viral oncoprotein Tax, several new Rex mutants in this region were generated that do not significantly affect Tax. These mutants include two phosphomimetic mutants P152D and A157D and two CTPD deletion mutants, S151Term and S158Term. All mutants predominantly localize to the nucleus/nucleolus, display a locked p26³⁶ active conformation, and are fully functional compared with wtRex-2. In comparison to wtHTLV-2, HTLV-2/RexA157D induced enhanced proliferation of hPBMCs, whereas HTLV-2/RexS151Term and HTLV-2/RexS158Term infected hPBMCs displayed substantially attenuated proliferation. In vitro long-term immortalization assays indicated that HTLV-2/RexP152D and HTLV-2/RexA157D can immortalize PBMCs with efficiency comparable with wtHTLV-2. However, both
HTLV-2/RexS151Term and HTLV-2/RexS158Term are deficient in immortalization of hPBMCs. Lastly, and consistent with the important role of this regulatory domain in the biology of HTLV, the viral persistence of HTLV-2/RexP152D was impaired in inoculated rabbits as compared to wtHTLV-2.

4.3 Materials and Methods

Cells. 293T cell, 729 human B cell, and Jurkat T cell lines were maintained in Dulbecco's modified Eagle's medium, Iscove's medium, and RPMI 1640 medium, respectively. Medium was 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 (PBMCs) were isolated from blood of normal donors by centrifugation over Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) and cultured in RPMI 1640 medium supplemented with 20% FBS, 2 mM glutamine and antibiotics.

Plasmids. The Rex expression vector BC20.2, containing the HTLV-2 tax/rex cDNA expressed from the cytomegalovirus (CMV) immediate-early gene promoter, has been described previously \(^{273,403}\). Various rex mutants were generated in BC20.2 or in wild-type (wt) HTLV-2 proviral clone pH6neo \(^{33}\) using QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing. The human immunodeficiency virus type 1 (HIV-1) Tat expression vector, pctat and the Rex-2 reporter plasmid (pCgagRxRE-II) were described previously \(^{274,276}\). LTR-2-luciferase Tax reporter plasmid, κB-Luc Tax reporter plasmid, CMV-luciferase (firefly) plasmid and Thymidine kinase-Renilla luciferase plasmid were described
previously\textsuperscript{323}. Wild type and mutant Rex-2-GFP constructs were generated by inserting Rex-2 sequences into EGFP-N3 vector (Promega) upstream of GFP open reading frame.

**Transfection, luciferase assay, Gag p19 and p24 ELISA.** To measure Tax CREB/ATF (LTR) activating function, 2x10\textsuperscript{5} 293T cells were transfected using Lipofectamine\textsuperscript{®} PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. The total amount of DNA was kept constant and was composed of 2 µg of Tax expression vector or a negative control, 0.02 µg TK-Renilla, and 0.1 µg of LTR-2-Luc. To determine Tax NF\textkappa B activation capacity, 4x10\textsuperscript{6} Jurkat cells were transfected using the Nucleofector\textsuperscript{®} method (Amaxa, Gaithersburg, MD) with 3µg of Tax expression vector or a negative control, 0.05 µg TK-Renilla, and 0.25 µg of κB-Luc. Cell lysates were harvested 48 h post-transfection and subjected to a dual luciferase assay (Promega, Madison, WI). All experiments were performed independently three times in triplicate, and results were normalized for transfection efficiency using Renilla-Luc.

In order to check the capacity of virion production of HTLV proviral clones, 2 x 10\textsuperscript{5} 293T cells were transfected using Lipofectamine\textsuperscript{®} PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation. The total amount of DNA was kept constant and was composed of 2 µg of HTLV constructs or a negative control and 0.1 µg of CMV-luciferase. After 48 h of growth, conditioned medium were collected and assayed for p19 matrix antigen using a commercially available enzyme-linked immunosorbent assay (ELISA; ZeptoMetrix, Buffalo, NY). The Rex functional assay was performed as described previously\textsuperscript{91,274}. Briefly, 0.5 µg of an empty plasmid, Rex cDNA expression plasmid, or HTLV-2 proviral clone was cotransfected with 0.1 µg of pcTat and 0.3 µg of the Rex reporter plasmid pCgag-RxRE. Cell lysates were prepared in
passive lysis buffer at 48 h posttransfection, and luciferase activity was determined to control for transfection efficiency. HIV-1 p24 Gag levels in the cell lysates were determined by ELISA (Beckman-Coulter). All transfection experiments were performed in triplicate for at least three times.

To generate stable transfectants, proviral plasmid clones containing the Neo gene were introduced into cells by electroporation as described previously \(^{103,403}\). Stable transfectants containing the desired proviral clones were isolated following incubation in 24-well culture plates in medium containing 1 mg/ml of Geneticin. After four-to-five weeks of selection, viable cells were single cell cloned, expanded, and maintained in culture for further analysis. The clones were screened by p19 Gag expression in the cell supernatant by ELISA.

**DNA preparation and PCR.** Genomic DNA was isolated from permanently transfected cell clones or from immortalized PBMCs using PUREGENE® DNA purification system (Gentra, Minneapolis, Minn.). One microgram of genomic DNA was subjected to 30-cycle PCR. The primers pair 670\(^{269}\) and PG201\(^{7684}\)GCTGGTATAGGTATAGGCAT\(^{7665}\) were used to amplify a specific 437-bp fragment from the HTLV-2 tax/rex region. The PCR-amplified product was separated on agarose gels and visualized by ethidium bromide staining. Mutations were confirmed by DNA sequencing.

**Western blot.** Cells were lysed with modified RIPA buffer (50mM Tris-Cl [pH8.0], 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2.0mM phenylmethanesulfonyl fluoride, 20µg/ml aprotinin, 1.0 mM Na\(_3\)VO\(_4\), and 1mM NaF) on ice for 30 min. After centrifugation, the cell lysates were subjected to
12% SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell Biosciences, Keene, NH). The membrane was incubated in 1x TBS-T (20mM Tris-Cl [pH 7.6], 137mM NaCl, 0.1% Tween) with 5% defatted milk for 1 h at room temperature. The membrane then was incubated with primary antibodies for 1 h at room temperature, washed, and followed by incubation with secondary antibody conjugated with horseradish peroxidase. Rex protein was visualized using the ECL Western blotting analysis system (Santa Cruz Biotechnology).

**Short-term coculture microtiter proliferation and long-term immortalization assays.** Short-term microtiter proliferation assays were performed as described previously with some modifications. Briefly, freshly isolated human PBMCs were prestimulated with 2µg/ml phytohemagglutinin (PHA) and 10U/ml IL-2 (Roche Diagnostic Corporation, Indianapolis, IN) for three days. 729 HTLV producer cells (100 or 1000) were irradiated with 10,000 rads and cocultured with 10⁴ prestimulated PBMCs in the presence of IL-2 in round bottom 96-well plates. Wells were enumerated for growth and split 1:3 at weekly intervals. At week seven, cell proliferation was confirmed by MTS assays using CellTiter 96® Aqueous One Solution Reagent as recommended by the manufacturer (Promega, Madison, WI). For the long-term immortalization assays, 5x10⁵ irradiated 729 producer cells were cocultivated with 2 × 10⁶ freshly isolated PBMCs with or without 10U/ml IL-2 in 24-well culture plates. The presence of HTLV expression was confirmed by detection of p19 Gag protein in the culture supernatant using an ELISA at weekly intervals. Viable cells were counted weekly by trypan blue exclusion. Cells inoculated with HTLV-1 or HTLV-2 that continued to produce p19 Gag
antigen and proliferate 12 weeks post-coculture in the presence of exogenous interleukin-2 (IL-2) were identified as immortalized.

**Rabbit inoculation procedures.** Twelve-week-old specific pathogen-free New Zealand White rabbits (Hazelton, Kalamazoo, MI) were inoculated via the lateral ear vein with $1 \times 10^7$ gamma-irradiated (7500 rad) 729wtHTLV-2, 729HTLV-2/Rex P152D, or 729 uninfected control cells (five rabbits per group). At weeks 0, 2, 4, 6, and 8 after inoculation, 10 mL of blood was drawn from the central ear artery of each animal. Serum reactivity to specific viral antigenic determinants was detected using a commercial ELISA (BioMerieux, Inc., Durham, N.C.) and HTLV Western blot assay (ZeptoMetrix) adapted for rabbit plasma by use of avidin-conjugated goat anti-rabbit IgG (1:3000 and 1:200 dilution respectively) (Sigma, St Louis, MO). Serum showing reactivity to Gag (p24 or p19) and Env (gp21 or gp46) antigens was classified as positive for HTLV-2 seroreactivity. To detect integrated proviruses, genomic DNA was harvested using the PUREGENE® DNA purification system (Gentra, Minneapolis, Minn.) and 1µg of DNA was subjected to 40-cycle PCR using primers 670 and 671 to amplify a 159-bp fragment specific for the HTLV-1 and HTLV-2 tax/rex region. The maintenance of the HTLV-2 Rex mutations in positive rabbits was confirmed by PCR using specific primers followed by restriction enzyme digestion as well as sequencing.

### 4.4 Results

**Generation of Rex-2 mutants.** It has been previously reported that Rex-2 function is regulated by phosphorylation. In particular, serine 151 and serine 153 were identified
as two *in vivo* phosphorylation sites that play a critical role in the functional regulation of Rex-2. Moreover, replacement of serine 151 and/or serine 153 with phosphomimetic aspartic acid (S151D/S153D) locked Rex-2 in a phosphorylated active conformation. Herein, we address whether the ability to regulate Rex function is important for HTLV-2-mediated cellular proliferation and transformation *in vitro* and survival in a rabbit model of infection. Numerous studies have demonstrated the essential role of the viral oncoprotein Tax in HTLV-mediated cellular proliferation and transformation. Since the pX-ORF IV encoding Tax is largely overlapping with the pX-ORF III encoding Rex, mutations in *rex* could alter the amino acid sequence of Tax disrupting critical transcriptional activities required for replication and cellular transformation. Indeed, the well characterized RexS151D/S153D mutation altered Tax coding sequence and significantly disrupted Tax CREB/ATF and NFκB transactivation activities (Table 4.1).

It has been well documented that a common consequence of phosphorylation is a charge-induced protein conformational alteration leading to a change in protein function. Therefore, we hypothesized that introducing a phosphomimetic amino acid into the Rex-2 carboxy terminus, but not necessarily at serine 151 and/or serine 153, might result in the same conformational alteration as reported for RexS151D/S153D. Based on this hypothesis, three Rex-2 “phosphomimetic” mutants were generated in the 170 amino acid Rex-2 polypeptide, including G145E, P152D, and A157D. RexG145E and RexA157D mutants do not result in alteration in the Tax amino acid sequence; RexP152D results in a single amino acid change in Tax (A132G). Using a LTR-Luc and κB-Luc Tax reporter assays this mutation in Tax resulted in a 50% reduction in CREB/ATF transactivation activity with very little affect on the capacity to activate
NFκB (Table 4.1). Our working model is that the C-terminus of Rex-2, when unphosphorylated, is inhibitory to Rex-2 function and positively regulated or activated by phosphorylation events. Therefore, if our model is correct, deletion of the C-terminal phosphorylation domain (CTPD) would be expected to result in a constitutively active Rex-2. In order to test this hypothesis, we generated three serial deletion mutants in which the codon for serine 144, serine 151, or serine 158 were mutated to a termination codon. The overlapping Tax-2 amino acid sequence is not altered in these CTPD deletion mutants (Table 4.1).

Rex-2 is locked in an active conformation by introducing phosphomimetic amino acids into the CTPD or by deletion of CTPD. The stable expression of Rex-2 mutants in transfected 293T cells was determined by Western blot using rabbit anti-Rex antisera. As expected, in the cells transfected with our wild type rex-2 expression construct, the p24Rex and the phosphorylated p26Rex conformation were detected (Fig 4.1A). Consistent with our previous report, expression of RexS151D or RexS153D resulted in detection of only the phosphorylated p26Rex conformation. Mutants that substitute a phosphomimetic or charged residue within this region, RexP152D, and RexA157D, also exhibit only the phosphorylated p26Rex conformation. Interestingly, mutant RexG144E results in a significant increase in the p26Rex conformation as compared to wtRex-2, but on shorter exposure also reveals the presence of the p24Rex form. Two CTPD deletion mutants, RexS151Term and RexS158Term, display a single protein form with the gel mobility corresponding to their predicted molecular weights. However, a third CTPD deletion mutant, S144Term, migrated as two protein species of
reduced molecular weight that likely correlates to the \( \text{wtRex-2 p26}^{\text{Rex}} \) and \( \text{p24}^{\text{Rex}} \) species (Fig 4.1A).

Rex-2 CTPD mutants were next tested for their ability to function in our quantitative bioassay using reporter plasmid pCgagRxRE-II. This plasmid contains the HIV-1 LTR and \( \text{gag} \) gene linked to the RxRE of HTLV-2 \(^{276}\). Efficient expression of Gag is dependent on Tat-mediated transcription and functional Rex binding to the RxRE sequences. 293T cells were cotransfected with pCgagRxRE-II, and wt or mutant Rex expression vectors and p24 Gag production was monitored using a Gag antigen capture assay. The results indicate that all these mutants are functional (Fig 4.1B). RexG145E and RexA157D showed significantly higher activity than \( \text{wtRex-2} \). We speculate that the increased expression level of these two mutants may contribute to the higher functional activity that we observed. Together, these results support the conclusion that introducing phosphate groups or negative charges into Rex-2 CTPD or deletion of Rex-2 CTPD can result in a functionally active form of Rex-2. Moreover, these Rex-2 mutants would be constitutively active and not regulated by phosphorylation status. The remainder of our studies focus on Rex mutants P152D, A157D, S151Term, and S158Term since these mutants are observed to be in a single functionally active conformation in cells.

Subcellular localization of Rex-2 is not affected by phosphomimetic mutations or C-terminus deletions. Previous studies indicated that the phosphorylated form of Rex-2, \( \text{p26}^{\text{Rex}} \), is primarily found in the nucleus and nucleolus, whereas the inactive form \( \text{p24}^{\text{Rex}} \) displays diffused cytoplasmic and nuclear localization, suggesting that the subcellular localization of Rex-2 correlates with its functional activity \(^{276,294,313,318}\). To
address whether the phosphomimetic mutations and C-terminus deletions affect Rex-2 subcellular localization, Rex-2-GFP fusion proteins using EGFP were generated. The subcellular localization of Rex-2 wild type and mutant-GFP proteins were evaluated in Hela-Tat cells. GFP alone displays a bright, diffused staining throughout the cytoplasm as well as nucleus. In contrast, all of the Rex-2-GFP fusion proteins tested exhibit predominant nuclear and nucleolar staining with weak cytoplasmic staining (Fig 4.2A). This finding is consistent with a previous report of the sublocalization distribution of wtRex-2 and RexS151D, S153D mutants. We next examined the expression level and function in these Rex-GFP fusion proteins by Western blot and HIV p24 Rex reporter assays, respectively. Detection of Rex-2-GFP fusion proteins using either Rex or GFP specific antisera indicate that they run as expected with mobility corresponding to their predicted molecular weights (Fig 4.2B and not shown). As shown in Figure 2C, the functional activities of these Rex-2 mutant GFP fusion proteins are comparable to the wtRex-2-GFP and display a similar relative activity as the untagged proteins (compare Fig. 4.1B and Fig. 4.2B).

**Rex-2 mutants increase HTLV virion production in transient transfected 293T cells.** In order to address the role of the Rex-2 CTPD and its phosphorylation in viral replication as well as HTLV-mediated cellular transformation, we generated mutant HTLV-2 proviral clones including HTLV-2/RexP152D, HTLV-2/RexA157D, HTLV-2/RexS151Term, and HTLV-2/RexS158Term. The virion production from these HTLV-2 mutants was first assessed in transient transfected 293T cells by p19 Gag ELISA. The concentration of p19 Gag production in the supernatant is commonly used as a measure of virion production. Our results indicate that HTLV-2/RexA157D,
HTLV-2/RexS151Term, and HTLV-2/RexS158Term produce levels of p19 Gag higher than wtHTLV-2 (Fig 4.3). This increased p19 Gag production is consistent with constitutively active phenotype of these Rex CTPD mutants observed in cDNA over-expression assays. However, in contrast p19 Gag production from HTLV-2/RexP152D is consistently lower than wtHTLV-2. We attribute this reduction to the impaired Tax transactivation activity on the CREB/ATF pathway, thus lowering overall viral transcription.

Establishment of stable producer cell lines of HTLV-2 Rex mutants. To determine the capacity of HTLV-2/RexP152D, HTLV-2/RexA157D, HTLV-2/RexS151Term, and HTLV-2/RexS158Term proviral clones to synthesize viral proteins, direct viral replication, and induce cellular immortalization/transformation, permanent 729 B cell transfectants expressing the proviral clones were isolated and characterized. For each of the stable transfectants, the presence of tax/rex gene sequences with the expected mutations was confirmed by diagnostic genomic DNA PCR and DNA sequencing. To monitor the production of viral protein in these stable transfectants, the concentration of p19 Gag in the culture supernatant of several cell clones was quantified by ELISA. As shown in Figure 4.4A, the amount of p19 Gag expression from each stable cell clone tested is variable. This is likely attributed to the chromosomal location of proviral sequences and the overall proviral copy number in the cell clones. We next analyzed the expression of viral proteins in representative stable cell lines (marked with * in Fig 4.4A) using Western blot. Rex protein from all the tested cell lines migrated corresponding to their predicted molecular weight. Interestingly, in spite of the comparable amount of p19 Gag output from 729HTLV-2/RexS151Term#2 and our
well-characterized wtHTLV-2 producer cell line, Rex expression was reduced substantially in 729HTLV-2/RexS151Term#2, implying the important role of the CTPD and/or the phosphorylation of the CTPD in Rex-2 protein expression or stability (Fig 4.4B, band indicated with *). In contrast, Tax protein expression from all the tested cell lines was comparable to that from 729wtHTLV-2, except for 729HTLV-2/RexS151Term (Fig 4.4B). Since both Tax and Rex are expressed from the same doubly spliced viral mRNA, it is unlikely that the altered protein levels are attributed to proviral copy number or transcriptional regulation in the stable cell clones. Rather, either the translational efficiency or the protein stability in these mutants is affected. Lastly, since viral envelope mediates virus entry into host cells and plays an important role in viral infectivity, we also examined envelope expression in the established 729 stable cell lines by Western blot (Fig 4.4B). Monoclonal antibody specific to surface protein gp46 was used and the multiple bands detected are likely due to glycosylation. Overall, we characterized viral genetic mutations and viral protein expression from the established stable cell lines using diagnostic PCR, p19 ELISA, and Western-blot. These viral proteins have been demonstrated to play an important role in efficient viral replication, viral infectivity and viral induced cellular transformation.

**HTLV-2 induced cell proliferation is promoted by Rex phosphomimetic mutants but impaired by Rex C-terminus deletion mutants.** In order to determine whether the constitutively active Rex mutants affect the viral infectivity and the ability of the virus to induce the proliferation and transformation of human PBMCs, short-term microtiter proliferation assays and long-term immortalization assays were performed. These coculture assays use freshly isolated PBMCs and cell-associated virus.
transmission designed to mimic the *in vivo* infection. The short-term microtiter proliferation assays were carried out in 96-well plates, in which 100 or 1000 irradiated 729 stable producer cells were cocultured with 10,000 prestimulated PBMCs per well in the presence of IL-2. At weekly intervals, individual wells were assayed for proliferation as measured microscopically by increased cell number or by MTS assay. In addition, the cells in individual wells were split at a 1:3 ratio weekly. The 729 producer cell lines used for the assays include 729HTLV-2, 729HTLV-2/RexA157D, 729HTLV-2/RexS151Term, and 729HTLV-2/RexS158Term. HTLV negative 729 cells were used as a control. The Kaplan-Meir plots of HTLV-2-induced T-cell proliferation from a representative experiment are shown in Figure 4.5. Using 1000 irradiated 729 stable producer cells per well, PBMCs cocultured with 729HTLV-2/RexA157D displayed similar proliferation with those cocultivated with 729wtHTLV-2 (100% v.s. 99%). However, using 100 cells/well, PBMCs cocultured with 729HTLV-2/RexA157D displayed greater proliferation than those cocultivated with 729wtHTLV-2 (97% v.s. 74%). These results suggest that HTLV-2/RexA157D can induce greater cellular proliferation of primary cells than wtHTLV-2, especially with low virus input. In contrast, HTLV-2/RexS151Term and HTLV-2/RexS158Term are both significantly attenuated in their ability to induce cellular proliferation. There were at most 1-3% of wells that continue proliferating for 8 weeks with HTLV-2/RexS151Term or HTLV-2/RexS158Term. Taken together, we show that introducing phosphomimetic amino acid into the CTPD and deletion of the CTPD can both lock Rex in the active conformation with the capacity to facilitate incompletely spliced viral mRNA expression,
but the CTPD itself is required for complete function of Rex-2 in the context of full-length infectious virus.

**HTLV-2 proviral clones with Rex phosphomimetic mutations but not Rex CTPD deletions are competent to immortalize human PBMCs.** The *in vitro* long-term coculture immortalization assays were carried out in 24-well plates, in order to monitor the immortalization process and the characteristic expansion of infected PBMCs. The viable cell numbers were recorded at weekly intervals. Moreover, viral protein production from infected cells was examined by quantification of p19 Gag production in the culture supernatant starting at three weeks postcultivation, a time point by which the viral protein production from residual irradiated viral producer cells becomes negligible. As shown in Figure 4.6A, HTLV-2/RexA157D infected PBMCs exhibit a similar growth curve to those infected with wtHTLV-2, suggesting that HTLV-2/RexA157D and wtHTLV-2 are able to induce cellular immortalization with comparable efficiency. In addition, although the infected cells exhibited slightly reduced proliferation, HTLV-2/RexP152D is able to efficiently immortalize PBMCs (Table 4.2). wtHTLV-2, HTLV-2/RexA157D, or HTLV-2/RexP152D had the capacity to immortalize PBMCs and cells continued proliferating and producing viral protein until the experiment was terminated at 12 weeks. In contrast, the cellular immortalization ability of HTLV-2/RexS151Term and HTLV-2/RexS158Term was abrogated, as none of the cocultured cells in any wells became immortalized, as tested in four independent experiments using three different 729 stable producer cell lines (Table 4.2).

The viral protein production from the infected PBMCs was also quantified by Gag p19 ELISA (Fig 4.6B). As expected, HTLV-2/RexA157D infected PBMCs continuously
produce more p19 Gag than those infected with wtHTLV-2 due to the constitutively active Rex in HTLV-2/RexA157D. Similarly, the p19 Gag production from HTLV-2/RexP152D infected cells was less than that from wtHTLV-2 infected cells, likely due to the impaired Tax transactivation activity on CREB/ATF pathway. There was no detectable p19 Gag production from PBMCs cocultured with 729HTLV-2/RexS151Term or with 729HTLV-2/RexS158Term, possibly because the majority of those PBMCs are either not productively infected or the appropriate proliferative signals are disrupted. Our data from the long-term immortalization assays is consistent with that from the short-term proliferation assays, implying that deletion of the CTPD disrupts important regulator control and function of Rex and thus is detrimental to viral replication and/or viral-induced cellular proliferation.

**The effect of constitutively active Rex-2 on the in vivo HTLV-2 spread and persistence in inoculated rabbit model.** Our *in vitro* data indicated that maintenance of Rex-2 in the active state by introducing phosphomimetic amino acids into the CTPD can increase the cellular proliferation of HTLV-2 infected cells. In order to evaluate the role of the regulatory functional control of Rex on HTLV-2 infection *in vivo*, we inoculated rabbits with untransfected 729 control, 729wtHTLV-2, or 729HTLV-2/RexP152D cells. We choose HTLV-2/RexP152D because it is one of the two HTLV-2 mutants that harbor constitutively active Rex and is competent to infect and immortalize PBMCs *in vitro*. Rabbits were inoculated with lethally irradiated cell lines and rabbit blood was sampled at weeks 0, 2, 4, 6, and 8 post-inoculation. Rabbit PBMC were isolated from blood to determine viral DNA integration by PCR, and rabbit serum was assessed for anti-HTLV-2 antibody response by Western blot. The representative seroconversion
patterns from each of the inoculated groups are shown in Figure 4.7. As expected, we did not detect any antibody response in rabbits inoculated with 729 control cells. In contrast, seroconversion can be detected in all the 729wtHTLV-2-inoculated rabbits and in three out of five 729HTLV-2/Rex P152D-inoculated rabbits starting at week two, with antibody titers rising over the time course of the experiment. The antibody responses against different viral proteins appear variable among individual rabbits. Further quantitative ELISA analysis will be performed to determine whether there are significant different antibody responses between 729wtHTLV-2-inoculated rabbits and 729HTLV-2/Rex P152D-inoculated rabbits.

To determine the infection status of the inoculated rabbits, amplification of specific HTLV-2 genomic fragments from rabbit PBMCs was performed using PCR. We detected viral DNA integration in all five 729wtHTLV-2-inoculated rabbits and in three out of five 729HTLV-2/Rex P152D-inoculated rabbits, which is consistent with the seroconversion data (Table 4.3). The maintenance of viral genetic mutation in positive rabbits was confirmed by restriction enzyme digestion and DNA sequencing. Real-time PCR will be performed to quantify the proviral load in infected rabbits. Our preliminary data indicates that HTLV-2/Rex P152D exhibits decreased in vivo viral spread and persistence in comparison with wtHTLV-2. Either the impaired Tax transactivation activities or the constitutively active Rex could be responsible for this decreased viral infectivity and persistence. Further studies using 729HTLV-2/RexA157D-inoculated rabbits will be required before we can make a more definitive conclusion.
4.5 Discussion

In this study, we assessed the role of the Rex-2 C-terminus phosphorylation domain (CTPD) on known Rex function, viral replication, and HTLV-2-induced cellular proliferation and immortalization. Two sets of Rex-2 mutants were evaluated; phosphomimetic mutants that increase charge to the region (RexP152D and RexA157D) and the C-terminus deletion mutants (RexS151Term and RexS158Term). We show that both sets of mutants can lock Rex-2 in the functional active p26-like conformation and result in increased viral protein expression in transient transfection assays. Our in vitro coculture (infection) assay system revealed that these two sets of Rex-2 mutants displayed sharply different biological properties. HTLV-2 proviral clones with Rex phosphomimetic mutations can efficiently infect PBMCs, undergo viral replication and induce cellular proliferation and immortalization of infected cells. In contrast, HTLV-2 proviruses with Rex CTPD deletions showed limited ability to infect PBMCs or to induce cellular proliferation of infected cells. No immortalized cell lines have been observed in coculture assays using hPBMCs and either 729HTLV-2/RexS151Term or 729HTLV-2/Rex S158Term. This data suggests that deletion of Rex-2 CTPD either significantly attenuates or completely disrupts the oncogenic potential of HTLV-2, which could be attributed to either decreased viral infectivity and/or impaired cellular proliferation of infected cells due to lose of concerted viral gene expression and appropriate signals.

A critical difference between the transfection assay and the infection assay is that the former does not require the assembly of mature virions for viral protein expression, which is a prerequisite for the latter. Complete viral assembly and maturation are
necessary for efficient HTLV infectivity, which requires direct cell-to-cell transmission via a virological synapse\textsuperscript{419,420}. The well-recognized function of Rex is to induce efficient cytoplasmic expression of unspliced and incompletely-spliced mRNA. However, the working mechanism of Rex needs further characterization. Previous studies suggest that Rex can facilitate the cytoplasmic transport, and/or inhibit the splicing, and/or increase the translation efficiency of its mRNA targets. In particular, Rex-2 has been shown to increase Gag protein expression by 130 fold, while the level of unspliced/incompletely viral mRNA in the cytoplasm increases by only 7 to 9 fold\textsuperscript{269,290}. For some complex retroviruses, such as HIV-1, the same unspliced mRNA pool serves both as mRNA templates for viral protein synthesis and as genomic RNAs for virion assembly\textsuperscript{322,421}. The constitutively active Rex-2 mutants could result in continuous increased translation of the unspliced viral mRNA, which in turn would disrupt the balance between the mRNA for viral structural protein synthesis and the genomic RNA for assembly into infectious viruses. Thus, a possible outcome of having a constitutively active Rex is that the majority of virions generated from mutant HTLV-2 producer cells may not be competent to undergo further replication without proper encapsidation of viral genomic RNA. Moreover, we only observed the decreased viral infectivity in HTLV-2 Rex-2 CTPD deletion mutants, but not in aspartic acid substitution mutants (HTLV-2/RexP152D and HTLV-2/RexA157D). Therefore, it is unlikely that the competition between viral protein synthesis and virion genome encapsidation can account for the low infectivity of HTLV-2/Rex CTPD deletion mutants. Rather, Rex-2 CTPD appears to play an essential role in the appropriate direction and assembly of viral genomic mRNA into progeny virions.
Alternatively, deletion of Rex-2 CTPD may affect viral infectivity through intervention of Gag protein processing and assembly. A report from Swanson et al suggests that the pre-translational event mRNA export can predetermine the fate of the mRNA encoded proteins, possibly through modulation of the mRNA structure or through remodeling of the composition of the mRNA associated protein complex, including the messenger ribonucleoproteins (mRNP). For example, replacement of Rev-responsive element (RRE) in the gag-pol mRNA with the constitutive transport element (CTE) promotes both the trafficking of Gag to cellular membranes and efficient HIV-1 assembly in non-permissive murine cells. Therefore, the alteration of mRNA export element or other factors related to the mRNA export pathways may interfere with the destiny of encoded proteins. In particular, deletion of the CTPD from HTLV-2 Rex could affect the structure of Rex-associated mRNA or disrupt the association of mRNA with certain critical cellular factor(s) required for its appropriate “fate”, leading to deficient processing of Gag protein and assembly of HTLV virions.

Our studies indicate that maintenance of Rex-2 in an active state can improve viral protein expression, which is consistent with the well-recognized Rex-2 function. Furthermore, it appears that the C-terminus of Rex-2 has additional functional activities other than as a phosphorylation regulatory domain. Our preliminary data indicated that the amount of Rex-2 protein detected by Western blot is increased in “phosphomimetic” mutants (G145E and A157D) but decreased in deletion mutants (S144Term and S151Term). This finding suggests that the CTPD or its phosphorylation can either increase Rex-2 expression or enhance the protein stability of Rex-2.
In addition, in the present study our data indicated that the CTPD of Rex-2 is essential for the viral infectivity and/or induction of cellular proliferation as well as immortalization. By contrast, in our previous study it has been shown that Rex-deleted HTLV-1 can still undergo viral replication and produce infectious virus progeny but with less efficiency than wtHTLV-1 \(^{323}\). However, it is worth noting that in the present study, deletion of the CTPD locks Rex-2 in an active state which may disrupt the balance of expression of all viral genes, viral replication, or the assembly of infectious viruses. Another possibility is that the CTPD-deleted Rex may directly interfere with the function of certain cellular factors through unknown mechanisms, leading to impaired cellular proliferation. Therefore, these two studies are not in discrepancy as they appear to be.

Overall, our studies revealed that the functional regulation of Rex-2 plays an important role in viral replication and viral induced cellular immortalization. In particular, the C-terminus of Rex-2 is essential for its overall function in the context of full-length infectious virus. This finding provides insight into the crosstalk between HTLV-2 and host cells. Further studies aim to identify the potential role and the working mechanism of Rex CTPD in HTLV-2 infectivity.
The Rex-2 mutants generated were named based on the mutated amino acid (aa) and their position within the 170-aa HTLV-2 Rex. For example, S151D indicates that serine residue at aa 151 was changed into aspartic acid.

S, serine; D, aspartic acid; G, glycine; E, glutamic acid; P, proline; A, alanine; L, leucine; F, phenylalainine; Term, termination codon.

Tax transactivation activity on CREB/ATF- and NFκB-responsive Luciferase reporter genes was examined using dual luciferase assay as described in the Materials and Methods. The data is based on three independent experiments and normalized to wtRex-2 (set as 1.0).

Table 4.1 Rex-2 mutants and their Tax transactivation activity
<table>
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<tr>
<th>HTLV-2 proviral clones</th>
<th>Wells immortalized/Total wells</th>
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<tr>
<td>wtHTLV-2</td>
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*5x10³* irradiated (10,000 R) 729 stable producer cells were cocultured with 2x10⁶ freshly isolated human PBMCs in the presence of exogenous IL-2. Cells were fed twice a week with RPMI 1640 supplemented with 20% FBS, antibiotics, and 10 U/ml IL-2. Viable cell numbers and viral protein expression were monitored at weekly intervals. The wells in which cells continued proliferating and producing viral protein for at least 12 weeks were defined as immortalized.

**Table 4.2** HTLV-2 immortalization of primary T cells
<table>
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<tr>
<th>Inoculum and rabbits</th>
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*Genomic DNA was isolated from rabbit PBMCs and subjected to PCR using HTLV-2-specific primers (670-671).

**Table 4.3** PCR detection of HTLV-2 proviral sequences in inoculated rabbit PBMCs
Figure 4.1 Expression and functional activity of generated Rex-2 mutants. (A) Western blot of Rex-2 proteins expressed from 293T cells transiently transfected with Rex-2 cDNA plasmids. Proteins were detected using rabbit Rex-2 specific antisera. (B) Functional activity of generated Rex-2 cDNA mutants was determined using the HIV-1 p24 Rex reporter assay. The values, which represent relative p24 Gag production for three independent experiments, are normalized and shown relative to wtRex-2. Error bars indicate standard deviations.
To be continued
Figure 4.2 Subcellular localization of Rex-2 mutants. HeLa-Tat cells were transfected with 1 µg of Rex-2-GFP using Lipofectamine® PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation, EGFP-N3 as negative control. (A) For Rex-2-GFP detection, cells were plated and visualized by using a Zeiss LSM 510 microscope. (B) Expression of Rex-2-GFP fusion proteins were detected by Western blot using rabbit Rex-2 antisera. Rex-2-GFP proteins (*) are indicated. (C) Rex-2-GFP fusion proteins are fully functional as determined by HIV p24 Gag reporter assay as outline in Figure 1B.
**Figure 4.3** p19 Gag expression of proviral clones in 293T cells. 2x10^5 293T cells were transfected with 2µg of wtHTLV-2, HTLV-2/RexP152D, HTLV-2/RexA157D, HTLV-2/RexS151Term, HTLV-2/RexS158Term, or expression control vector DNA. At 48 h post-transfection, p19 Gag production was measured in the supernatant by ELISA. The values represent p19 Gag production for three independent experiments. Error bars indicate standard deviations.
Figure 4.4 Viral protein expression in permanent transfectants. (A) Three independently isolated 729 stable producer cell clones for each HTLV proviral mutant (HTLV-2/RexP152D, HTLV-2/Rex157D, HTLV-2/RexS151Term, and HTLV-2/RexS158Term) were isolated as described in Materials and Methods. Gag p19 production was measured in 48 h culture supernatant by ELISA. Our well-characterized 729wtHTLV-2 was used as the wild type virus positive control. (B) Viral protein expression, including Rex, Tax, and Env, were characterized by Western blot for selective stable producer cell lines (marked with * in panel A) to potentially to be used in coculture analyses. The asterisk (*) indicates the specific band for RexS151Term. The asterisks (**) indicates the specific band for RexS158Term. The arrows indicated specific bands corresponding to the predicted gel mobilities of Rex-2 mutants. The envelope displays multiple bands due to glycosylation.
Figure 4.4 continued

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Figure 4.5 Representative Kaplan-Meir plots for T-lymphocyte proliferation in short-term microtiter assay. Prestimulated PBMCs (10^4) were cocultured with 1000 (A) or 100 (B) irradiated 729 stable producer cells in 96 well plates. Kaplan-Meir plots for wtHTLV-2, HTLV-2/RexA157D, HTLV-2/RexS151Term, HTLV-2/RexS158Term, and uninfected 729 as control. The percentage of proliferating wells were plotted as a function of time (wks).
Figure 4.6 HTLV T-lymphocyte immortalization assays. Human PBMCs were isolated by Ficoll/Paque and cocultivated with irradiated (10,000 rads) 729 stable cell lines. PBMCs (2x10⁶) were cultured with irradiated donor cells (1x10⁶) in 24 well plates as indicated. (A) Cell viability was determined by trypan blue exclusion staining at weekly intervals. Growth Curve from 0 to 9 weeks post-cocultivation is shown. The mean and standard deviation for each time point were determined from three independent wells in a representative experiment. (B) HTLV-2 gene expression was confirmed by detection of p19 Gag protein in the culture supernatant by ELISA at 3, 4, 5, 6, 7, 8 and 9 weeks post-cocultivation. The mean and standard deviation for each time point were determined from three independent samples in a representative experiment.
Figure 4.7 Assessment of HTLV-2-specific serologic response in inoculated rabbits. Rabbits were inoculated with approximately $1 \times 10^7$ irradiated 729HTLV-2, 729HTLV-2/RexP152D-infected cells, or uninfected control cells. At weeks 0, 2, 4, 6, 8 post-inoculation, rabbit PBMCs and sera were isolated from blood. Sera from inoculated rabbits were tested for reactivity to specific HTLV-2 proteins by Western blot. Two representative rabbits from the positive wtHTLV-2 inoculated group (R29-R33) and negative control group (R18-R22), and three HTLV-2/RexP152D inoculated rabbits (R34-R38) are shown. Viral proteins (arrow) and 729 cell-specific proteins (*) are indicated.
CHAPTER 5

SUMMARY AND PERSPECTIVES

5.1 Summary

HTLV-1 and HTLV-2 are closely related human oncogenic complex retroviruses that can transform human primary T cells \textit{in vitro} and \textit{in vivo}. Data in this thesis primarily focused on the role of several viral elements, including the viral LTR, Tax, Rex, and Env in HTLV replication and in HTLV-mediated cellular proliferation and transformation.

In addition to the structural and enzymatic proteins, HTLV encodes two trans-regulatory proteins, Tax and Rex. Both have been demonstrated to play a critical role in efficient viral replication and/or viral induced cellular transformation. Comparative studies have revealed a unique C-terminal PDZ-domain binding motif (PBM) in Tax-1, which is absent in Tax-2. In addition, a C-terminal phosphorylation domain (CTPD) has been described in Rex-2, but not in Rex-1. Using mutational analyses, our studies assessed the functional activities of these motifs/domains in the context of their corresponding proviruses. In studies detailed in Chapter 2, we show that the Tax-1 PBM plays an important role in Tax-1 induction of genetic instability, promotes HTLV-1-mediated cellular proliferation, and facilitates HTLV-1 \textit{in vivo} infectivity and persistence, thus likely contributing to HTLV-1 induced malignancy.
Studies in Chapter 4 addressed the important role of Rex-2 CTPD in regulation of HTLV-2 replication and the induction of cellular proliferation and transformation. Interestingly, our data indicates that Rex-2 can be locked in an active conformational state, either by introducing phosphomimetic amino acids (negative charges) into the CTPD or by deletion of the CTPD. The constitutively active Rex-2 can promote viral protein expression in transient transfection systems. However, these two classes of Rex-2 mutants display different biological properties in \textit{in vitro} coculture proliferation and immortalization assays. In the context of an infectious proviral clone, the phosphomimetic mutants can efficiently be transferred to the primary human T cells and induce the cellular proliferation of infected cells, whereas the Rex-2 mutants with CTPD deletion showed limited infectivity and substantially reduced induction of cellular proliferation. The underlying mechanism for this difference will be a focus of future studies.

HTLV-1 and HTLV-2 are related viruses that display differing \textit{in vitro} and \textit{in vivo} tropism. In addition, their clinical manifestations are distinct with disease association only clearly demonstrated for HTLV-1. Taking advantage of the high homology between HTLV-1 and HTLV-2, we chose to generate chimeric HTLV proviral clones in order to identify the viral determinant(s) responsible for their distinct \textit{in vitro} transformation tropisms. The identification of a viral tropism determinant will be an important initial step toward the elucidation of HTLV pathogenesis. As highlighted in Chapter 3, we showed that Env is a major viral factor that conveys HTLV transformation tropism in addition to its role in viral entry into host cells.
5.2 The role of the PBM in Tax-1 intervention of cell cycle regulation.

Our studies in Chapter 2 indicate that deletion of the PBM from Tax-1 attenuates HTLV-1 induced cellular proliferation and reduces Tax-1 micronuclei inductive activity. In addition, flow cytometry analysis was performed to assess the cell cycle profile in Tax-1 expressed cells. Interestingly, the G2/M accumulation mediated by Tax-1 expression is reduced by 50% upon deletion of the PBM. This data suggests that the PBM is important in Tax-1 cell cycle deregulation. Previous reports showed that naïve cells either transfected with a Tax-1 expression plasmid or transduced with a retroviral vector encoding Tax-1 exhibited increased G2/M accumulation. At the same time, nuclear abnormalities were observed in these cells including multinucleation and the formation of decondensed, highly convoluted and lobulated nuclei. Therefore, this abnormal cell cycle profile was correlated with Tax-1 induction of genomic instability. Further studies by Haoudi et al attributed these cell cycle alterations to the association between Tax-1 and the ATM/Chk2 pathway, which is involved in DNA damage recognition and cell cycle checkpoint activation. It has been shown that Tax-1 colocalizes with Chk2 in Tax speckled structures (TSS), and that Tax induces the activation of Chk2. The activation of Chk2 further targeted Cdc25c for degradation, inactivated cdc2 (cdk1), and downregulated the expression level of mitotic cyclin B leading to G2 arrest.

In contrast, microarray expression analysis revealed the upregulation of cyclin B and cdk1, and the transactivation of Cdc25c in HTLV-1 infected cells, which potentially led to a faster progression through G2 phase of the cell cycle. Another study indicated that in HTLV-1 infected cells, Tax can attenuate the γ-irradiation-induced G2-arrest through association with Chk1 and inhibition of Chk1, but not Chk2 kinase activity.
Although this finding appears to be in conflict with those from Haoudi et al., it is worth noting that the G2/M accumulation was observed in the naïve cells with de novo Tax-1 expression in both studies, while the attenuation of DNA damage induced G2-arrest was identified in HTLV-1 transformed cell lines derived from in vitro coculture assays (C81) or established from ATL patients (HUT 102 and MT-2). Therefore, we hypothesize that the distinct cell cycle alteration observed may be related to the different Tax functional activities at different HTLV-1 infection stages.

At least two important questions need to be addressed by our future studies. First, a time course of Tax-1 induced cell cycle alteration during HTLV-1 mediated cellular immortalization/transformation process is needed. For example, we could analyze the cell cycle profiles of HTLV-1 cocultured with hPBMCs at 0, 2, 4, 6, 8, 12, 16, 20 weeks post-plating. This would allow us to determine whether the G2-arrest is an early event in response to HTLV infection, and whether the resistance to DNA damage induced G2-arrest is a unique phenotype of HTLV transformed/immortalized cells. Secondly, it would be important to determine whether the Tax-1 PBM is involved in the association between Tax-1 and Chk1 and/or Chk2, as well as determine the effect of the PBM on Tax-1 induced cell cycle dysregulation. Co-immunoprecipitation could be performed to determine whether the Tax-1/Chk1 or Tax-1/Chk2 association is affected upon deletion of the PBM either in transiently transfected cells or in immortalized T-cell lines. Cell cycle profiles could also be analyzed and compared between HTLV-1∆PBM-infected PBMCs and wtHTLV-1-infected PBMCs.
5.3 The implication of Tax-1 PBM in HTLV-1 cell-to-cell transmission.

In Chapter 2, we showed that the *in vivo* proviral load and persistence of HTLV-1 was significantly attenuated by deletion of Tax-1 PBM. In addition, we observed increased syncytia or tight cell clusters induced by HTLV-2+C22 (with Tax-1 PBM) infection *in vitro*. These results are consistent with the hypothesis that Tax-1 PBM plays a role in cell-cell fusion of HTLV-1 infected cells and possibly facilitates viral transmission. The PBM has been shown to mediate the association between Tax-1 and several cellular proteins belonging to MAGUK family, such as hDlg and MAGI-3. MAGUK proteins typically consist of three domains, a Src homology 3 (SH3) domain; a domain with homology to the enzyme guanylate kinase (GUK), and a PDZ domain named after three MAGUK family members PSD-95, Dlg, and ZO-1. The MAGUKs act as scaffolding molecules that connect the extracellular environment to the intracellular signaling pathways and cytoskeleton, and thus play an important role in cell signaling, tight junctions, and synapses.

It has been proposed that HTLV transmits through virus synapse (VS), a specialized contact region from infected cells to uninfected cells. Upon cell-cell contact, HTLV-1-infected cell can rapidly induce the re-orientation of its microtubule organizing center (MTOC) towards the cell contact region. This allows the accumulation of HTLV-1 core complex and genome at the cell-cell junction, followed by viral transmission to the adjacent uninfected cells. Recently, studies from the same group suggest that the intercellular adhesion molecule-1 (ICAM-1) plays an important role in HTLV-1 induced VS formation. It has been proposed that the positive feedback loop involving HTLV-1 Tax protein and ICAM-1 upregulates ICAM-1 expression, activates HTLV-1 gene
expression, and facilitates the formation of VS\textsuperscript{425,426}. Moreover, in wtTax-1 expressing cells, hDlg and MAGI-3 were found to colocalize with Tax-1 predominantly in the detergent insoluble fraction (hDlg) or perinuclear/cytoplasm (MAGI-3). In contrast, the above colocalization was minimally observed in cells transfected with Tax-1ΔPBM or HTLV-2 infected cells\textsuperscript{137,401}.

Our hypothesis is that the PBM participates in the feedback loop involving Tax-1 protein and ICAM-1 and contributes to HTLV-1 cell-cell transmission, possibly through its interaction with some PDZ domain-containing cellular proteins such as MAGUK proteins. A possible working model is that upon cell-cell contact ICAM-1 on HTLV-1 infected cell surface interacts with its partner lymphocyte function antigen-1 (LFA-1) on the adjacent cells, triggering a specific cell signal to membrane bound MAGUKs. On one hand, this signal can be delivered to Tax-1 through its association with the MAGUK proteins, leading to enhanced transactivation of HTLV-1 and related cellular gene expression, such as ICAM-1. In addition, MAGUKs can transmit this signal to the cytoskeleton, stimulate the MTOC reorganization, and direct the transportation of Gag protein and viral genome to the cell-cell contact region. The above events are synergistic resulting in the formation of VS and subsequent HTLV-1 transmission into uninfected cells.

Yamamoto et al reported that cross-linking of ICAM-1 on the cell surface upregulates HTLV-1 gene expression\textsuperscript{426}. Moreover, Tax-1 strongly transactivates ICAM-1 in T-cell lines, whereas Tax-2 (no PBM) transactivation of ICAM-1 has not been observed in T cells\textsuperscript{134,427}. The differential capacities of Tax-1 and Tax-2 to
transactivate ICAM-1 shed more light on the potential role of PBM in HTLV transmission.

It would be interesting to address whether HTLV-1ΔPBM-infected cells and HTLV-2 (which does not have a Tax PBM)-infected cells can efficiently form viral synapse upon coculture with freshly isolated hPBMCs or purified CD4⁺ or CD8⁺ T cells. Either 729 stable producer cells or immortalized primary T-cell lines established from in vitro coculture assay could be analyzed. If a decreased accumulation of viral Gag protein and/or viral genome at the cell-cell contact region was observed, it would be beneficial to determine whether this deficiency in VS formation involves ICAM-1, or MAGUK proteins, or other cellular factors.

5.4 To identify the viral and cellular determinants of HTLV transformation tropism.

Using recombinant proviral clones between HTLV-1 and HTLV-2, we showed that viral Env plays an important role in determining HTLV-1 and HTLV-2 distinct transformation tropism. The next series of experiments would be to identify the specific domain(s) in the Env that are responsible for distinct tropisms between HTLV-1 and HTLV-2. This would require the generation of additional recombinant proviruses in which specific region(s) or domain(s) in the env gene are exchanged. Initial experiments should focus on exchange of the Env SU and the Env TM region. If for example the exchange of SU region alters HTLV tropism as compared to the parental virus, then more recombinants within this region will be generated and characterized. At the same time, sequence alignment and the domain structure revealed by previous studies will help us to
identify the potential domains or determinant(s). It should be noted that the viral determinant may span multiple domains within Env. In this respect, the knowledge or prediction of Env three-dimensional structure will be appreciated.

In addition, the yeast two-hybrid system could be employed to identify cellular counterpart of viral Env (CCE). As previous studies suggest, it is possible that the CCE can interact with both Env1 and Env2, but with different affinity, or the CCE may exhibit differential subcellular distribution within CD4+ or CD8+ T cells. Therefore, further analyses would be required to confirm the role of the identified CCE candidates. For instance, fluorescence in situ hybridization (FISH) or immunofluorescence could be used to detect the different subcellular localizations of the identified CCE candidates. Surface plasmon resonance could be carried out using a BIAcore instrument to delineate the kinetics and affinity of protein-protein interaction between Env and the CCE candidate(s). Furthermore, if the CCE is not essential for T-cell growth, CCE-knockout T-cell lines would be instrumental in characterizing further parameters of HTLV-1 or HTLV-2 transformation.

5.5 The role of Rex-2 CTPD in the assembly of infectious virions.

As proposed in Chapter 4, deletion of the CTPD from Rex-2 may potentially disrupt the assembly of infectious HTLV virions, either through abrogating the supply of full-length genomic mRNA for encapsidatation or through disrupting the membrane trafficking/assembly of Gag. First, the genomic mRNA in the virions could be detected by reverse transcription-polymerase chain reaction (RT-PCR) and quantified by real-time RT-PCR. Furthermore, encapsidation efficiency could be determined by RNase
protection assay (RPA) with $^{32}$P-labeled HTLV specific anti-sense RNA probes. Based on the above assays, we expect to be able to deduce whether the encapsidation of viral genomic mRNA is decreased in HTLV-2/RexΔCTPD infected cells in comparison to wtHTLV-2 infected cells. Radio-immunoprecipitation assay (RIPA) could be performed to define the onset and duration of accumulation of newly synthesized Gag in cell lysates, in conditioned medium and in virions. The same number of HTLV-2/RexΔCTPD infected cells and wtHTLV-2 infected cells would be used for each experiment. One possibility is that the ratio of Gag protein detected from virions versus from cell lysates and conditioned medium would be decreased in HTLV-2/RexΔCTPD infected cells, allowing us to conclude that the membrane trafficking and assembly of Gag is impaired upon deletion of Rex-2 CTPD.

Alternatively, as discussed in section 5.3, deletion of Rex-2 CTPD could interfere with HTLV-2 cell-cell transmission. Experiments discussed in section 5.3 could be used to test this hypothesis.


60. Seiki M, Hikikoshi A, Tanquichi T, Yoshida M. Expression of the px gene of


178. Arsenian S, Weinhold B, Oelgeschlager M, Ruther U, Nordheim A. Serum


202. Li J, Li H, Tsai MD. Direct binding of the N-terminus of HTLV-1 tax oncoprotein to cyclin-dependent kinase 4 is a dominant path to stimulate the kinase activity. Biochemistry. 2003;42:6921-6928.


213. Lee SS, Weiss RS, Javier RT. Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor


226. Chun AC, Jin DY. Transcriptional regulation of mitotic checkpoint gene MAD1


265. Yoshida M. Multiple viral strategies of HTLV-1 for dysregulation of cell growth


268. Inoue JI, Yoshida M, Seiki M. Transcriptional (p40x) and post-transcriptional (p27XIII) regulators are required for the expression and replication of human T-cell leukemia virus type I genes. Proc Natl Acad Sci USA. 1987;84:3653-3657.


278. Bogerd H, Greene WC. Dominant negative mutants of human T-cell leukemia


280. Hammes SR, Green WC. Multiple arginine residues within the basic domain of HTLV-I Rex are required for specific RNA binding and function. Virology. 1993;193:41-49.


290. Younis I, Green PL. The human T-cell leukemia virus Rex protein. Frontiers in


301. Dobbelstein M, Roth J, Kimberly WT, Levine AJ, Shenk T. Nuclear export of the
176


313. Bakker ALX, Ruland CT, Stephens DW, Black AC, Rosenblatt JD. Human T-cell leukemia virus type 2 Rex inhibits pre-mRNA splicing in vitro at an early stage of


417. Smith CL, Debouck C, Rosenberg M, Culp JS. Phosphorylation of serine residue


