HUMAN T LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1) ACCESSORY PROTEIN P30(II) MODULATES CELLULAR AND VIRAL GENE EXPRESSION

DISSERTATION

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by

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* * * * *

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ABSTRACT

Human T-lymphotropic virus type-1 (HTLV-1) is a deltaretrovirus that causes adult T-cell leukemia/lymphoma, and is implicated in a variety of lymphocyte-mediated disorders. In addition to structural and enzymatic proteins, the HTLV-1 provirus encodes various regulatory and accessory genes in four pX open reading frames. Our laboratory has elucidated the functional characteristics of the HTLV-1 accessory proteins, and it has become clear that these proteins are, in fact, essential for the virus life cycle and may determine disease outcome associated with HTLV-1 infection. We have identified the functional properties of the pX ORF-II encoded p30II, but the role of this viral protein in virus replication or pathogenesis remain incompletely defined.

Studies from our laboratory have demonstrated that pX ORF-II mutations diminish the ability of the virus to maintain high viral loads in vivo in our rabbit model of HTLV-1 infection. The ORF-II encoded p30II, a nuclear-localizing protein that interacts with the KIX domain of CREB-binding protein (CBP)/p300, disrupts CREB-Tax-CBP/p300 complexes bound to viral Tax-Responsive Element (TRE) repeats and differentially modulates CREB and TRE-mediated transcription. Herein, we have further characterized the role of p30II in regulation of cellular gene expression, using a stable p30II expression system employing lentiviral vectors to test cellular gene
expression with Affymetrix U133A arrays, representing ~33,000 human genes. Our data reveals alterations of interrelated pathways of cell proliferation, T-cell signaling, apoptosis and cell cycle in p30II expressing Jurkat T-lymphocytes. Moreover, we have verified our findings by reporter assays and RT-PCR in Jurkat and primary CD4+ T-lymphocytes. We are the first to test the overall effect of an HTLV-1 accessory protein, on cellular gene expression and demonstrate that p30II activates many key transcription factors involved in T-cell signaling/activation, such as nuclear factor of activated T-cells, nuclear factor-kappa B and activator protein-1. Collectively, these data indicate that this complex retrovirus, associated with lymphoproliferative diseases, rely upon accessory gene products to modify cellular environment to enhance clonal expansion of the virus genome and thus maintain proviral loads in vivo.

Next, we further characterized the role of p30II in regulation of viral gene expression, by identifying motifs critical in binding p300 and regulating TRE-mediated transcription in the absence or presence of provirus. Analysis of the amino acid domain of p30II (100-179) critical for repressing LTR mediated transcription, was used to identify a lysine residue at amino acid 106 (K3) of p30II, that is critical for repressing TRE-mediated transcription. Additionally, we have found that p300 reverses the p30II-dependent repression of TRE-mediated transcription, in the absence or presence of the provirus, in a dose-responsive manner. Our data confirms the role of p30II as a regulator
of viral gene transcription, in association with p300. However, in contrast to wildtype p300, p300 histone acetyl transferase (HAT) mutants only partially rescue p30II-mediated LTR repression. In addition, we also show that p30II is acetylated and that deacetylation enhances p30II-mediated HTLV-1 LTR repression. Inhibition of deacetylation decreased p30II-mediated LTR repression. We are the first to demonstrate that the HAT activity of p300 is crucial in modulating viral gene expression from HTLV-1 LTR by p30II. Overall, our data indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes to modulate viral gene expression in conjunction with host cell proteins in part, through the acetylation of p30II. Collectively, our data suggests that HTLV-1 enhances clonal expansion of the virus genome by regulating viral and cellular gene expression, avoids immune elimination by the host and thereby maintains proviral loads in vivo.
Dedicated to my wonderful parents, siblings and husband who gave me the freedom to be who I want to be and were there for me always, no matter what, and Vavlo who reminds me of the little joys in everyday life
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Review Publications


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CHAPTER 1

LITERATURE REVIEW: MOLECULAR MECHANISMS OF HTLV-1 INFECTION, LTR-MEDIATED GENE EXPRESSION AND ROLE OF ACCESSORY PROTEINS IN HTLV-1 PATHOGENESIS

1.1 First Recognition of Human T-Lymphotropic Virus Type 1 (HTLV-1) as Etiologic Agent of Human Disease

Takatsuki et al. reported a unique and aggressive CD4+ T cell malignancy called adult T cell leukemia (ATL) in a small geographically clustered group of patients with lymphoid neoplasms in Japan In 1977. Human T-lymphotropic virus type 1 was first detected and isolated as a type C retrovirus particle from two T cell lymphoblastoid cell lines, HUT 102 and CTCL-3 and fresh peripheral blood lymphocytes of a patient in the United States with cutaneous T cell lymphoma (mycosis fungoides) by Poisez, Gallo and their colleagues in 1980. This patient was diagnosed with cutaneous T cell lymphoma; however he had widespread systemic involvement typical of ATL and is now thought to have been possibly misdiagnosed and was in fact suffering from ATL. In 1981, Hinuma et al. demonstrated the presence of type C retroviral particles termed
adult T cell leukemia virus (ATLV) from a cell line termed MT-1, which was established from a patient diagnosed with ATL. About the same time, Miyoshi et al established another T cell line, MT-2, from an ATL patient which was later found to have type C retrovirus particles. Further sero-epidemiologic, immunologic, genetic and molecular studies showed that the viruses in these two cell lines were the same and helped to establish the close linkage between ATL and HTLV-1. Thus HTLV-1 was established as the first retrovirus associated with a human disease.

1.2 Geographical Distribution of HTLV-1

Although it is estimated that HTLV-1 infects 15 to 25 five million people worldwide, it is not uniform in its distribution, it is in fact found in geographical clusters. HTLV-1 infection is endemic in southern Japan, the Caribbean basin, Central and South America, among some Melanesian Islands in the Pacific basin and in regions of central Africa. It is also a serious public health problem among at-risk groups in Europe and North America and therefore a target of intervention policies including blood donor screening. HTLV-1 infection is also prevalent in regions such as southern India, northern Iran and northern Australia. The seroprevalence of HTLV-1 varies widely within endemic regions, between 0.1% and 30% of the population, depending on age and gender.

Recent studies indicate the presence of HTLV-1 sequences in a 1,500-year-old Andean mummy, indicating the ancient nature of HTLV-1 infections. Primate T lymphotrophic viruses are thought to have originated in Eastern Africa and spread...
worldwide. It is hypothesized that an early African lineage of the simian T lymphotrophic virus (STLV) evolved into STLV-1 in Asia and subsequently infected humans prior to their migration to Melanesia/Australia, resulting in the origin of "Melanesian" clad or subtype HTLV-1c, one of the 4 major widely-recognized clads of HTLV-1. The Asian STLV-1 strains possibly re-entered Africa at a much later stage, and multiple interspecies transmissions resulted in the establishment of the second clad of HTLV-1, the African clad, which can be further subdivided into five sub-clads (HTLV-1a, -b, -d, -e and -f). The third clad, referred to as the "Cosmopolitan" clad is considered to have originated from HTLV-1a, which spread throughout the world due to the increased human host mobility, particularly during the slave trade. Recent reports indicate the presence of subtype D in Central Africa, mainly in Pygmies. In addition, two other African HTLV-1 variants from Zaire (Efe1) and Gabon (Lib2) have been discovered and may be yet considered as the sole human prototypes of new subtypes (E and F).

1.3 Transmission of HTLV-1

HTLV-1 is a highly cell-associated virus and free HTLV-1 particles have low infectivity. Cell-cell contact through virus-infected T cells is required for efficient transmission. The most efficient route of HTLV-1 transmission is by exposure to infected blood or whole cell blood products, but not plasma (e.g., transfusion or through the use of contaminated needles), with a seroconversion rate of approximately 50% and an estimated median time for seroconversion of 51 days. In fact, sharing of
needles among intravenous drug users constitutes one of the most common modes of blood-to-blood transmission among endemic population in the United States\textsuperscript{153,154}. Transmission by infected blood products has also been a major problem in endemic areas, particularly in Japan\textsuperscript{221,268}. Nonetheless, most cases of HTLV-1 infections are due to transmission from mother to child through prolonged breastfeeding for more than 6 months (through infected milk-borne lymphocytes) or by sexual contact later in life\textsuperscript{120,121}. The probability of mother-to-infant transmission is estimated to be 18–30\% and the risk factors involved are maternal factors, including higher HTLV-1 antibody titer, prolonged ruptured membranes during delivery, and low socioeconomic status\textsuperscript{358}. The sexual route of transmission is less efficient than transmission involving blood, but has the potential to introduce infection into previously unexposed groups. Transmission from male to female through sexual contact is about four times as frequent as female to male transmission, at a rate of 4.9 per 100 person-years among females married to an infected male compared with 1.2 among males married to an infected female\textsuperscript{323}. Transmission risk to females is believed to be greater if the partner has high antibody titers or antibody to Tax proteins while the risk of female-to-male transmission is associated with penile sores/ulcers and diagnosis of syphilis among males\textsuperscript{251}.

1.4 HTLV-1-Associated Diseases

1.4.1 Adult T cell Leukemia/Lymphoma

Typically, 1-5 \% of the HTLV-1 infected individuals develop ATL after a latent period of 20-30 years\textsuperscript{50,371,372}. ATL is an aggressive T cell malignancy with a leukemic phase characterized by circulating, activated CD4+/CD25+ T cells\textsuperscript{343}. Infection early in
life is associated with the development of ATL and the estimated lifetime risk is about 5%
% in individuals infected before the age of 20 years \textsuperscript{50,359}. The incidence rate is 2–4 per
100,000 person-years and males have higher risk than females \textsuperscript{50,170}. The average age of
onset is 40 years in Jamaica, Trinidad, and Brazil and 60 years in Japan \textsuperscript{50,205}.

The clinical features of ATL are similar to non-Hodgkin’s lymphoma: malaise, fever, lymphoadenopathy, hepatosplenomegaly, jaundice, drowsiness, weight loss, and opportunistic infections. Typically widespread or localized skin involvement (in 40% of cases, there are large nodules, plaques, ulcers, and papular rash on the limbs, trunk, or face) and lytic bone lesions are noticed in these patients. Immunosuppression with bacterial and opportunistic infections such as \textit{Pneumocystis carinii} pneumonia, fungal infections, and strongyloidiasis has been reported in these patients, contributing to poor prognosis \textsuperscript{357}. Unique clinical chemistry characteristics of this condition include hypercalcemia, high serum concentrations of lactate dehydrogenase and elevated serum levels of soluble interleukin-2 (IL-2) receptor \(\alpha\) chain. Circulating ATL lymphocytes are morphologically unique abnormal T cells with characteristic convoluted multi-lobulated nuclei, which are defined as “flower cells” \textsuperscript{317}. Typical criteria to establish a diagnosis of ATL in patients include seropositivity for HTLV-1, marked leukocytosis, characteristic morphologic appearance of ATL cells, histology or cytology of malignant cells with a T cell immunophenotype, usually CD4+/CD3+, hypercalcemia and increased circulating levels of the IL-2 receptor \(\alpha\)-chain (IL-2R\(\alpha\)/CD25) and elevated serum lactate dehydrogenase (LDL) levels \textsuperscript{317}. Diagnostic tools for asymptomatic carriers include an immunoassay to detect HTLV-1 antibodies and polymerase chain reaction (PCR)-based
amplification of lymphocyte genomic DNA to detect provirus. Based on restriction fragment length polymorphism (RFLP) analysis, the proviral integration into host cells is believed to be polyclonal, but the RFLP analysis of leukemic cells isolated from ATL patients show a monoclonal proviral integration pattern, suggesting that a sub-population of infected cells are clonally selected to proliferate while rest of the infected lymphocytes either die or get eliminated.

Based on clinical and laboratory criteria, ATL is classified into four subtypes: acute, lymphoma, chronic or smoldering. Distribution of these subtypes vary depending on the geographic location and the median survival for ATL patients is 4-6 months depending on the subtype of ATL. While the acute subtype has the highest incidence and is characterized by increased ATL cells, skin lesions, systemic lymphoadenopathy, hepatosplenomegaly and poor prognosis, particularly with high level of serum LDH, calcium and bilirubin, the chronic subtype ATL is characterized by increased white blood cells counts, cough and skin lesions. Smouldering subtype ATL has the best prognosis among the four subtypes and is reported to have few ATL cells in the peripheral blood over a long time period.

The development of ATL has been the focus of many investigations, but the exact mechanism is not completely understood. After infecting CD4⁺CD25⁺ T cells, the HTLV-1 provirus is randomly integrated into the host genome, where it persists for years. Although HTLV-1 replicates via reverse transcription in vivo, HTLV-1 replication is thought to occur via mitosis rather than via reverse transcription. In ATL, tumor cell
clones were identified against a general background of oligo to polyclonal expansion of infected but non-transformed cells\textsuperscript{40}. Typically there is a progression from polyclonal to oligoclonal and then to monoclonal proliferation \textit{in vivo}, which is achieved while the cells become IL-2 independent \textsuperscript{85,125,378}. Based on the long clinical latency and the low percentage of individuals who develop ATL, T cell transformation is believed to occur after a series of cellular alterations and/or mutations. Transformation of infected lymphocytes is believed to be initiated through induction of cellular genes and alterations in critical cellular activation and death pathways by the viral transactivator Tax \textsuperscript{97}. Although the mechanism of cellular transformation by Tax is not fully understood, it is thought to involve dysregulation of cell cycle and suppression of DNA repair pathways, leading to the gradual accumulation of mutations over time and development of ATL. However, transformed cells often contain latent or defective proviruses \textsuperscript{87,88,175}, indicating the possibility that viral protein expression may not be essential to maintain the transformed phenotype.

Intriguingly, despite a strong immune response mounted against the HTLV-1, the virus is able to persist in the host. Several possible mechanisms have been suggested to explain this observation: Often the genome is partly deleted, resulting in defective virus which may provide a mechanism for escape from immune surveillance \textsuperscript{18-20}. Recent reports \textsuperscript{14} suggest that HTLV-1 may not be latent and the virus might be far more dynamic than previously thought. Based on \textit{in vitro} studies on the half-life of Tax-expressing cells, it is hypothesized that infected cells start expressing viral proteins as early as within one day after infection and might have a turn over rate of $\sim 10^9$ to $10^{11}$
infected cells per day. Cytotoxic T lymphocytes (CTLs) are thought to be highly efficient in significantly reducing the viral load \textit{in vitro} and possibly \textit{in vivo}, resulting in equilibrium between the rate of production and the rate of clearance of cells expressing viral proteins. Based on this proposed mechanism, cell-free virus, viral mRNA and viral protein may be often below detection limits and infectious transmission is limited, explaining the relative lack of sequence diversity\textsuperscript{14}.

\textbf{1.4.2 \textit{Tropical Spastic Paraparesis / HTLV-1-Associated Myelopathy (TSP/HAM)}}

In the mid-1980's HTLV-1 began to be associated through epidemiologic studies as a contributing factor in chronic neurodegenerative disorders. After detecting anti-HTLV antibodies in the serum and cerebrospinal fluid (CSF) of infected patients, HTLV-1 was associated with the development of a progressive neurological disease called tropical spastic paraparesis (TSP) in Martinique (French West Indies) in 1985. In 1986, Osame \textit{et al} reported similar clinical findings in HTLV-1-positive individuals in Japan and they named the syndrome HTLV-1-associated myelopathy (HAM)\textsuperscript{101,278}. Subsequently, in 1988, both syndromes were understood to be identical in clinical presentation and the World Health Organization (WHO) declared HAM and TSP as the same disease, now known collectively as HAM/TSP.

HAM/TSP is characterized as a progressive chronic myelopathy, with preferential damage of the thoracic spinal cord\textsuperscript{2,95,149,167,253}. In affected patients the myelopathy presents as paraparesis with spasticity in the lower extremities, hyporeflexia, muscle
weakness, sphincter disorders including dysfunction of the urinary bladder and intestines. Less frequently it may precede, or give rise to, a cerebellar syndrome with ataxia and intention tremor 39. Some HAM/TSP patients exhibit a predominance of sympathetic nervous system dysfunction 6. Magnetic resonance imaging (MRI) of HAM/TSP patients reveals the presence of multiple white matter lesions in both the spinal cord and the brain involving perivascular demyelination and axonal degeneration 93,104,162,163.

Within the spinal cord, regions mainly affected are the so-called ‘watershed’ zones, suggesting a nonrandom distribution 132. Lesions include degeneration of lateral corticospinal tract and spinocerebellar or spinothalamic tract of the lateral column as well as perivascular and parenchymal cellular infiltration, consisting of mainly T cells (both CD4+ and CD8+ T cells), macrophages, astrocytes, and glial cells 276. Typically, cellular destruction and inflammation is a feature of HAM/TSP. CD4+ T cells, CD8+ T cells, and activated macrophages are present within white matter lesions of HAM/TSP patients 133. Peripheral blood and the CSF of HAM/TSP patients have high levels of proinflammatory cytokines, such as IFN-γ, TNF-α, IL-1 and IL-6, suggesting the importance of these proinflammatory cytokines in HAM/TSP 15,150,179,227. Additionally, CSF of these patients contains activated lymphocytes, suggesting that activated lymphocytes and macrophages also play a role in the development of HAM/TSP 133.

The lifetime risk of developing HAM/TSP among carriers is estimated to be 0.23%, based on multiple risk factors 148. The progression to HAM/TSP is influenced by multiple risk factors such as host genetic factors 254, host immune response, high HTLV-1
Provisional load \textsuperscript{19,20,166,264,374}, route of infection (exposure via blood transfusion) \textsuperscript{277} and perhaps by specific viral characteristics such as variations in HTLV-1 \textit{tax} \textsuperscript{19,65,82,106,212,306}.

HAM/TSP patients develop a vigorous expansion of CD8+ T cells \textsuperscript{307} and their CSF contain high levels of anti-HTLV-1 antibodies \textsuperscript{100} and HTLV-1 Tax specific CTLs \textsuperscript{176}. These CTLs are implicated in the cellular destruction and inflammation within the central nervous system (CNS) \textsuperscript{176}, probably by directly killing of HTLV-1 infected cells expressing Tax, CNS cells expressing a cross-reactive cellular determinant and neighboring uninfected neuronal cells by apoptosis mediated via the toxic inflammatory cytokines \textsuperscript{21}. Although the pathogenesis of HAM/TSP is not completely resolved, immune mechanism is widely accepted due to the increased cellular and humoral immune responses in HAM/TSP patients. Autoimmune mechanism has also been proposed due to the presence of a unique T cell receptor CDR3 motif in infiltrating lymphocytes in the spinal cord lesions of HAM/TSP patients, as in brain lesions of MS and experimental autoimmune encephalomyelitis patients\textsuperscript{116}. Moreover, antibodies to hnRNP-A1 may cross-react with HTLV-1-Tax, during the immune response associated with HAM/TSP, leading to a recent theory that molecular mimicry between HTLV-1 and autoantigens in CNS play a role in the pathogenesis of HAM/TSP \textsuperscript{195,196}.

1.4.3 \textit{Other Diseases Associated with HTLV-1-Infection}

HTLV-1 infection has been linked to many other immune-mediated diseases, although some of these remain controversial. A high incidence of idiopathic uvetis with a high rate of anti-HTLV-1 antibodies in HTLV-1 endemic areas of Kyushu, Japan led to the speculation that HTLV-1 might be the etiological agent \textsuperscript{165,239}. Proviral DNA was
detected in cells from the patients’ aqueous humor. Moreover, peripheral blood leukocytes of these patients had higher proviral loads than HAM/TSP patients. Interestingly, proviral loads showed significant positive correlations with the severity of disease among uveitis patients with a previous history of Grave's disease. Immunological and virological studies further confirmed these findings. Moreover, all patients with HTLV-1-associated uveitis had hyperthyroidism preceding the onset of uveitis, implying a potential role for hyperthyroidism in the onset of HTLV-1-associated uveitis.

In 1990, HTLV-1 was associated with infective dermatitis, characterized by watery nasal discharge with crusting of the anterior nares and severe exudative dermatitis in scalp, neck, ears, axillae and groin. Later, these infective dermatitis patients were confirmed to be seropositive for HTLV-1, however the pathogenesis is not yet understood. Interestingly, the presence of infective dermatitis in children may predispose them to subsequent development of ATL or TSP/HAM.

HTLV-1 has also been associated with cutaneous T cell lymphoma including mycosis fungoides and Sezary syndrome, supported by the detection of HTLV-1 DNA in a minority of cutaneous T cell lymphoma patients. Although cutaneous T cell lymphoma is clinically and histologically similar to ATL, its association with HTLV-1 is debated, due to the failure to detect HTLV-1 proviral DNA in patients, HTLV-1 seronegative nature in most of these patients and uncertainty on whether HTLV-1 specific sequences in these patients are intact, full-length proviral genomes or non-functional deleted proviruses.
HTLV-1 has been linked to Sjögren’s syndrome based on epidemiological evidence in southwest Japan, a high endemic region of HTLV-1 infection. Sjögren's syndrome is a chronic inflammatory disorder characterized by lacrimal and salivary gland insufficiency, due to high HTLV-1 \(^{337}\). These patients also had high serum titers of HTLV-1 antibodies \(^{223}\) but low viral load and only tax sequences were detected in their labial salivary glands \(^{222}\). The pathogenesis and the causative role of HTLV-1 in Sjögren's syndrome remains a topic of debate.

HTLV-1 has also been linked to HTLV-1 associated arthropathy \(^{263}\). However, whether HTLV-1 associated arthropathy represents a separate clinical entity remains controversial. HTLV-1 infection is also associated with a variety of additional autoimmune disorders such as polymyositis \(^{194}\), T lymphocyte alveolitis \(^{324}\), lymphadenitis \(^{267}\), chronic respiratory disease \(^{159}\), acute myeloid leukemia \(^{367}\) and conditions such as systemic sarcoidosis as well as *Strongyloides stercoralis* infection \(^{368}\). Since the evidence linking these diseases to HTLV-1 infection are not as strong as those for ATL and HAM/TSP, the etiological role of HTLV-1 in these diseases remains controversial. Nonetheless, the association of HTLV-1 to an array of clinical disorders indicates the huge public-health impact of this human pathogen.

### 1.5 Genetic Organization and Replication of HTLV-1

Based on its morphologic appearance, HTLV-1 is a C-type retroviral particle, which is typically assembled directly at the cell membrane and does not mature until after budding from the infected cell \(^{51}\). It is a complex retrovirus and a member of the
deltaretrovirus group, which includes bovine leukemia virus (BLV) and STLV. Unlike simple retroviruses, complex retroviruses carry additional genes to encode for several regulatory and accessory proteins. Mature HTLV-1 particles are spherical and have a diameter about 110 to 140 nm with an outer envelope composed of both viral and cellular proteins and lipids. The core of the virion is composed of the ribonucleoprotein complex consisting of two copies of an identical ~9 kb RNA genome that contains the m$^7$Gppp capping group, the primer tRNAPro of host cell origin, coding regions and 3’ poly(A) sequences with structural and enzymatic proteins, including nucleocapsid, capsid, matrix, reverse transcriptase, integrase and protease.

The HTLV-1 life cycle starts when viral particles attach to the cell surface through an interaction between envelope and HTLV-1 receptors, (which will be discussed in later section) which are not yet fully characterized. After the outer membrane of the viral particles fuse with cell membrane, the contents of virions enter the cytoplasm and the viral genomic RNA is reverse transcribed to generate double-stranded DNA (dsDNA) using the virally encoded reverse transcriptase (RNA-dependent/ DNA-dependent polymerase). Viral dsDNA traffics to the nucleus and randomly integrates into the host genome using the viral encoded integrase (the genome is now referred to as the provirus). Host cellular machinery drives transcription of the proviral DNA using host cell RNA polymerase II to produce viral mRNA. The unspliced full-length (~9 kb) viral genomic RNA is used to generate progeny virus particles (virions) and to generate Gag (internal group specific antigen), Pol (polymerase, reverse transcriptase and integrase enzymes needed for viral replication and maturation) and Pro (protease enzyme required
for maturation of structural and enzymatic proteins) proteins while the singly spliced subgenomic ~4.3 kb mRNA is used to generate Env protein (envelope glycoprotein) and doubly-spliced (two introns removed) RNA (~ 2.1 kb) is used to translate the regulatory proteins Tax (open reading frame (ORF) IV) and Rex (ORF III). Immature virus particles are assembled at the plasma membrane and become mature only after budding. Additionally, by alternatively splicing, several accessory proteins are also produced from pX ORF I and II, which will be discussed further in the following sections.

HTLV-1 proviral genome is 9032 nucleotide long, has 5’ and 3’ long terminal repeats (LTR). The LTR is 755 nucleotides long and contain cis-acting regulatory sequences necessary for viral gene expression and replication, including sequences essential in transcription start and termination, splicing, mRNA polyadenylation, strand transfer during reverse transcription and RNA transport. LTR is divided into three regions, termed U3, R and U5. The U3 region contains the viral transcriptional enhancer and promoter elements, including three imperfect 21-base-pair repeats, known as Tax-responsive elements-1 (TRE-1). The TRE-1 in 5’ LTR of HTLV-1 proviral genome at positions –251 to –231 (promoter distal repeat, pd), -203 to –183 (promoter central repeat, pc) and –103 to –83 (promoter proximal repeat, pp) relative to the transcription start site, and is necessary for transactivation. The TRE-1 regions contain a conserved TGACG sequence, with consensus to the TGACG cyclic adenosine monophosphate (cAMP) response element (CRE), present in the promoters of several cellular genes. Thus, TRE-1 elements are in fact defective/ imperfect CREs, which bind multiple transcription factors.
such as cyclic AMP response element binding protein (CREB)\textsuperscript{326,382}, cAMP response element modulator (CREM)\textsuperscript{326}, activating transcription factors (ATFs)\textsuperscript{111} and other cellular proteins that facilitate transcription including TRE binding proteins (TREB-1\textsuperscript{335}, TREB-5\textsuperscript{379}, TREB-7\textsuperscript{379}, c-Jun\textsuperscript{135}, c-Fos\textsuperscript{91}, activator protein-2 (AP-2)\textsuperscript{244,266}, HTLV enhancer binding protein (HEB)-1p67 and HEB-1p94\textsuperscript{205}. Between the pc and pp repeats is another second enhancer element, namely, Tax-responsive element 2 (TRE-2), also critical in viral transcription\textsuperscript{224,336}. In addition, many cellular proteins such as Ets family proteins (Ets-1, Ets-2, Elf-1 and TIF-1) and c-Myb bind to TRE-2\textsuperscript{32,33,49,53,340}. Based on sequence analysis, NF-κB, AP-2 and PU.1 are also thought to potentially interact with TRE-2\textsuperscript{69}. Additionally, inducible cAMP early repressor protein (ICER) binds TRE-1 and/or TRE-2 and decreases transcription from the LTR. Newbound et al\textsuperscript{257} reported that Tax-mediated transcription can be repressed by ICER protein in peripheral blood mononuclear cells, with possible significance in decreasing viral gene expression, leading to immune evasion and subsequent viral persistence. Tax mediated transactivation through TRE is discussed further in the following sections. In addition, many cellular proteins such as Sp-1\textsuperscript{266} and nuclear factor-1 (NF-1)\textsuperscript{266} bind the LTR at regions outside TRE and enhance transcription. Moreover, LTR also contains the Rex-responsive element (RxRE). RxRE secondary structure directly interacts with Rex and is essential for proper polyadenylation of HTLV-1 transcripts and Rex-mediated cytoplasmic expression of incompletely-spliced RNAs. R and U5 provide the 1-119 leader sequence for HTLV-1 transcripts\textsuperscript{3,4}. 
1.6 Structural Proteins of HTLV-1

HTLV-1 uses ribosomal frame shifting and alternative splicing mechanisms to produce an array of viral proteins from the same coding region \(^{51}\). Ribosomal frame shifting is the predominant mechanism employed to generate the structural and enzymatic proteins and precursor proteins. First, the \(gag\) gene encoding the viral structural proteins is translated from an unspliced, full-length mRNA as a single precursor polyprotein p55(Gag) \(^{275}\), which is myristoylated at its N-terminus, targeted to inner plasma membrane \(^{271}\) and later gets proteolytically cleaved by viral protease into three final products of 19 kDa (matrix), 24 kDa (capsid) and 15 kDa (nucleocapsid) proteins \(^{119,275}\). The p19 matrix protein remains myristoylated and is localized to the inner surface of the viral envelope \(^{256}\). HTLV-1 Gag protein alone has been found to be adequate for the assembly and release of virus particles \(^{188}\). The p19 matrix protein (MA) contains eleven basic amino acids, critical in the release of HTLV-1 virus particles and cell-to-cell transmission \(^{189}\).

The \(pol\) gene message is translated into the Gag/Pol precursor from the same genomic mRNA as the Gag precursor, but in a different reading frame through a ribosomal frame shift \(^{255}\). This precursor is available in lower levels than the Gag precursor and is subsequently cleaved by the protease to produce reverse transcriptase (RT), integrase (IN) and the remaining structural Gag proteins \(^{256}\). Pol also has RNase H activity that is required during the process of reverse transcription \(^{52}\).
Protease (PR) is encoded by a reading frame extending between 3' part of gag and 5' part of pol and is made by another frame shift\textsuperscript{256}. Due to the absence of a methionine codon at the 5' end of the pro ORF, an mRNA processing event or translational frame shifting is thought to occur prior to the synthesis of protease\textsuperscript{256}. In addition, protease is synthesized in an immature form that subsequently undergoes self-cleavage to generate the active form\textsuperscript{110}.

The viral envelope glycoprotein (Env) is synthesized from a ~4.3 kb singly-spliced mRNA transcript as a gp61 envelope precursor in the endoplasmic reticulum (ER), gets heavily glycosylated and cleaved into the mature surface protein (SU) gp46 and a transmembrane (TM) gp21 protein\textsuperscript{289}. SU and TM proteins form a heterodimer necessary for receptor binding and the fusion events involving the virus and host cell\textsuperscript{109}. The SU protein contains two functionally well-defined regions\textsuperscript{71} while the TM protein contains a leucine-zipper like region required for fusion\textsuperscript{169,304} and the region between C terminus of TM ectodomain to the end of the cytoplasmic domain critical for viral transmission, likely for post-fusion steps in viral infection\textsuperscript{72,139}. In addition, TM protein is recognized by neutralizing antibodies\textsuperscript{75}. Although Env has been extensively investigated, the identity of HTLV-1 receptor that facilitates its spread has remained elusive until recently. Transferrin receptor, an inducible receptor which plays a crucial role in the regulation of iron uptake and cell growth, is overexpressed in ATL cells\textsuperscript{285} and a neutralizing monoclonal antibody directed against transferrin receptor induced apoptosis of ATL cells in patients\textsuperscript{243}. More importantly, Manel \textit{et al} recently identified GLUT-1, a ubiquitous glucose transport protein, as a receptor for HTLV-1\textsuperscript{215}. It is not
clear how interactions of the HTLV-1 envelope with GLUT-1 affect cell-to-cell spread of
the virus, however if GLUT-1 is essential for the spread of both cell-free and cell-
associated HTLV-1, further characterization of this receptor may be valuable in
understanding of the complexities of HTLV-1 pathogenesis and in designing strategies to
prevent HTLV-1 transmission.279

1.7 Regulatory Proteins of HTLV-1: Tax and Rex

As a complex retrovirus, HTLV-1 contains regulatory genes in pX region, located
between env and 3’ LTR. By means of alternative splicing, utilizing sequences within the
5’ LTR, pol and pX region and internal initiation codons, virus produces regulatory and
accessory proteins from four open reading frames in the pX region. ORF IV and ORF III
encode regulatory proteins Tax and Rex respectively 168,313 while ORF I and ORF II
encode four accessory proteins, p12I, p27I, p30II and p13II 24,47,94,173,41 (Fig. 1.1).

1.7.1 Tax and Transactivation of the HTLV-1 LTR

Tax, (Transcriptional Activator of pX region) is a 40 kd nuclear protein, produced
from a doubly spliced ~2.1 kb mRNA, and is known as the viral transactivator, which
regulates viral gene expression from the HTLV-1 LTR 37,83,92,320,321. As discussed in a
previous section, HTLV-1 gene expression is dependent on three 21-bp imperfectly
repeated elements, termed TRE-1 34,107,164,339,355,356. Among the 3 repeats within TRE-1,
at least two have been demonstrated to be necessary for Tax transactivation of LTR. Tax
does not directly bind to TRE-1 or TRE-2 sequences, but by protein-protein interactions, Tax increases the DNA-binding activity of CREB/ATF proteins to the TRE-1 and several basic leucine zipper (bZIP) proteins to the TRE-2. Interaction of Tax with CREB and serum response factor (SRF) enhances the stability of the transcription enhancers - DNA complexes. However, Tax transactivation of the LTR requires direct binding of Tax to GC-rich sequences flanking the TRE-1, at a region within the CREB binding protein (CBP/p300) binding domain. Once cells are stimulated, protein kinase A (PKA) phosphorylate CREB, which then binds CBP/p300. Tax recruits CBP/p300 to the DNA bound CREB, allowing CBP/p300 to further associate with DNA and facilitate transcription. By binding both CREB and CBP, Tax acts as a bridge between these two proteins without the need for CREB phosphorylation, enabling the virus to maintain constitutive activation (without CREB phosphorylation) through TRE-1 in HTLV-1 infected cells. However, CBP/p300 recruitment by Tax is not sufficient for transactivation and requires CBP/p300 associated factor (P/CAF), which associates with the C-terminal activation domain of Tax.

1.7.2 Tax and Regulation of Cellular Gene Expression

Tax regulates the gene expression of numerous cellular genes as well, mostly by induction of the transcription factors NF-κB and SRF, independent of CREB activation. An increasing list of cellular genes activated by Tax include cytokines such as IL-2, IL-3, IL-4, IL-6, IL-8, IL-2Rα, IL-1, GM-CSF, TNFα, TNFβ, transcription factors such as c-myc, c-fos, c-sis, erg-1, c-rel, as well as...
genes involve in apoptosis Bcl-xL\textsuperscript{261,342} and DNA repair (proliferating cell nuclear antigen or PCNA)\textsuperscript{300}. Tax has also been shown to repress expression of β-polymerase (an enzyme involved in DNA repair), lck, and bax\textsuperscript{35,136,192}.

ATL cells have characteristic IL-2R\(\alpha\) overexpression\textsuperscript{338}, which is believed to be induced by Tax-mediated NF-κB activation\textsuperscript{128}. Tax directly binds multiple NF-κB family proteins such as p50, p65, c-Rel\textsuperscript{327,329} and lyt10\textsuperscript{248}. Since NF-κB proteins and Tax both bind CBP/p300\textsuperscript{99,283,383}, it is possible that Tax links NF-κB and CBP/p300 to enhance the stability of the DNA-protein complex\textsuperscript{25}. In addition, Tax localizes in the cytoplasm and associates with IκB\(\alpha\), the inhibitor of NF-κB, destabilizes IκB\(\alpha\), thereby allowing NF-κB to translocate to the nucleus\textsuperscript{210,328}. Moreover, Tax interacts with and induces the activation of cytokine-inducible IκB kinase (IKK), which contains IKK\(\alpha\), IKK\(\beta\) and IKK\(\gamma\) (NEMO) subunit, resulting in dephosphorylation and degradation of IκB\(\alpha\) and subsequent NF-κB activation\textsuperscript{201}. Additionally, Tax activates cellular gene promoters controlled by the serum response element (SRE), such as c-fos. Similar to activation of CREB, Tax induces dimerization of the serum response factor (SRF) protein p67\textsuperscript{SRE} in a complex with the SRE\textsuperscript{10,90}.

Tax has a significant effect on cell cycle regulation, by means of trans-repression of multiple cellular genes including p18INK4c, p53 and Bax\textsuperscript{35}. By sequestering CBP/p300, Tax downregulates the promoter activity of p18INK4c, an inhibitor of CDK4 that can arrest the cells at G1 phase of the cell cycle\textsuperscript{181,333}. Although similar mechanism has been implicated in Tax-mediated repression of p53\textsuperscript{12,345}, it is not consistent with the
elevated p53 levels detected in HTLV-1 transformed and Tax immortalized cell lines. This led to the hypothesis that Tax does not impair the expression of p53, instead impairs its function, supported by the detection of hyperphosphorylated and inactive p53 in HTLV-1 infected cells. In addition, Mulloy et al. found CREB binding domain of Tax to cause Tax-mediated p53 repression while Pise-Masison et al. suggested an NF-κB dependent pathway for p53 inactivation by Tax. In addition, Tax associates with other cell cycle regulators, such as p16INK4a and p15INK4b, negatively influences their inhibitive functions on cyclin dependent kinase 4/6 (CDK4/6), leading to Rb phosphorylation and induction of G1/S phase transition. Tax also binds to HsMAD1, a component of mitotic checkpoint that prevents anaphase until chromosomal alignment is completed and may abrogate the function of HsMAD1 to facilitate cell division. Furthermore, Tax expression leads to accumulation of DNA damage expression, by inhibition of topoisomerase I and DNA polymerase β. Overall, Tax suppresses DNA repair, enhances DNA replication and cell-cycle progression, thereby increase cellular mutation frequency and contribute to HTLV-1-mediated cellular transformation.

1.7.3 HTLV-1 Rex: Post-transcriptional Regulator

Rex, a nucleolar localizing 27KDa protein encoded by pX ORF III, is responsible for viral post-transcriptional regulation. Rex facilitates transport of unspliced RNA (gag/pol) and singly spliced RNA (env) from nucleus to cytoplasm and inhibits the splicing, transport of doubly spliced RNAs that encode the regulatory proteins in the pX region, which include Rex itself. Overall, Rex is necessary for viral replication.
by regulating the balance between messages encoding for viral structural proteins and messages encoding regulatory/accessory proteins. Rex functions by binding to the Rex response element (RxRE), a highly stable RNA stem-loop structure in the U3/R region of the HTLV-1 3’LTR \cite{17,22,26,27,156,314}, critical in polyadenylation of viral RNAs \cite{3,73}. RxRE is present in doubly spliced, singly spliced and unspliced messages, and thus other cis-acting sequences possibly determine the selectivity of Rex on various messages. In fact, cis-acting repressive sequence (CRS) in the U5 region of the viral LTR negatively regulate Rex \cite{28}. Additionally, Rex may inhibit mRNA splicing by altering early splicesome assembly and stabilizing the mRNA of cellular gene, IL-2Rα chain by acting in trans on its coding sequence \cite{147}.

Rex contains both an N-terminal located NLS responsible for nuclear localization and RxRE binding \cite{265,318}, as well as a nuclear export sequence (NES) located in the middle, with similarity to human immunodeficiency virus (HIV) Rev, influenza virus NS2, herpes simplex virus type 1 (HSV-1) ICP27 protein \cite{61} and cellular protein IκBα \cite{140}. The molecular mechanism of Rex-mediated RNA export is not completely elucidated, but current understanding involves the CRM1/exportin1 pathway \cite{31,112}, which is also employed by HIV Rev to facilitate viral RNA transport \cite{61,294}. Interestingly, Rex can functionally substitute for HIV Rev \cite{131,301}. Rex and Rev directly bind both the viral mRNA and hCRM1 in the presence of RanGTP via their RNA binding domains and NES, respectively, acting as adaptor proteins that bridge the target mRNA and the export receptor. Interestingly, multimerization of Rex/Rev along the viral mRNA is necessary for RNA export \cite{67,209,354}, possibly because multimerization allows a number of Rex/Rev
proteins to shield RNA, preventing the attachment of factors involved in splicing and nuclear retention of RNA and thus increasing the number of CRM1 molecules that associate with the complex. The association of multiple CRM1 molecules may be important in overcoming the factors that retain RNA in the nucleus and allow RNA to pass efficiently through NPCs. Human CRM1 (hCRM1) functions in the Rex-mediated mRNA export of HTLV-1 as an export receptor and as an inducing factor for Rex multimerization on its cognate RNA\textsuperscript{113}. Recently, Ye \textit{et al}\textsuperscript{376} reported that Rex is critical for efficient infection of cells and persistence \textit{in vivo}, but its function to modulate viral gene expression and virion production is not required for \textit{in vitro} immortalization by HTLV-1.

Interestingly, a variety of HTLV-1-infected cell lines including MT-1, MT-2, TL-Su, H582, HUT-102 contain mRNA and protein for a truncated form of Rex (p21\textsuperscript{Rex})\textsuperscript{41,94,272}, which can be generated from both the doubly-spliced message encoding Tax and Rex, as well as the message singly spliced from nt 119 directly to the splice acceptor at nt 6875 and only encoding p21\textsuperscript{Rex}. The second type of message is highly expressed in primary cells isolated from asymptomatic HTLV-1 carriers, ATL and HAM/TSP patients\textsuperscript{94,274}. This protein contains only the NES sequence and not the NLS sequence and when coexpressed with the full-length Rex, it repressed the shuttle function of the full-length protein and increased the nuclear accumulation of full-length protein\textsuperscript{177}. HTLV-2 and bovine leukemia virus (BLV) have p21\textsuperscript{Rex} homolog mRNAs\textsuperscript{273}, however the exact function of p21\textsuperscript{Rex} in viral pathogenesis remains to be elucidated.
1.8 HTLV-1 Accessory Proteins

The HTLV-1 pX genome region includes ORF I and II that produce alternatively spliced forms of mRNA, which encode four accessory proteins, p12\textsuperscript{I}, p27\textsuperscript{I}, p13\textsuperscript{II}, and p30\textsuperscript{II} (Fig. 1.1)\textsuperscript{24,46,172,173}. pX ORF I mRNA is produced by alternative splicing events that combine the second exon of Tax with additional downstream sequences and encodes p27\textsuperscript{I} (152 amino acids long) and p12\textsuperscript{I} (99 amino acid long). p12\textsuperscript{I} can be translated from a singly spliced message produced by direct splicing of nucleotide 119 to the splice acceptor at position 6383 or by initiation at an internal methionine codon in the p27\textsuperscript{I} ORF\textsuperscript{47}. p12\textsuperscript{I} is thought to be preferentially expressed from the p27\textsuperscript{I} mRNA since transfection of expression plasmids containing HA1-tagged versions of either the full-length p27\textsuperscript{I} cDNA or the cDNA for the singly spliced p12\textsuperscript{I} produced only the smaller p12\textsuperscript{I} protein\textsuperscript{173}. However, using in vitro transcription-translation systems, Ciminale \textit{et al} \textsuperscript{47} produced p27\textsuperscript{I} from the doubly spliced mRNA. Therefore, removal of the internal p12\textsuperscript{I} AUG start codon could yield detectable levels of p27\textsuperscript{I}. Interestingly, Pique \textit{et al} \textsuperscript{290} demonstrated production of cytotoxic T cells in HTLV-1-infected subjects that were reactive against peptides representing all putative pX accessory proteins, including p27\textsuperscript{I}.

The accessory proteins encoded by pX ORF II of HTLV-1 are produced from two alternatively spliced mRNAs. The larger protein, p30\textsuperscript{II}, is encoded by a doubly spliced message including the first and second exon of Tax spliced to the splice acceptor site at position 6478\textsuperscript{24}. p13\textsuperscript{II}, the smaller protein contains the C-terminal 87 amino acids of p30\textsuperscript{II} and is produced from a singly spliced message by splicing of the first Tax exon.
directly to the splice acceptor at position 6875 or translated from an internal methionine codon within p30\textsuperscript{II24,173}. HTLV-1 accessory proteins were originally thought to be dispensable for viral replication \textsuperscript{74}. However, recent investigations performed by our laboratory and others have shed light into the role of the HTLV-1 accessory proteins in viral infectivity, maintenance of high viral loads, host cell activation, and regulation of gene transcription \textsuperscript{7,8,23,48,55,64,70,76,141,157,186,341,380,381}. There is evidence that pX ORFs I and II mRNAs and proteins are expressed in infected cell lines and freshly isolated cells from asymptomatic carriers, ATL and HAM/TSP patients \textsuperscript{173,41}. In addition, HTLV-1 infected patients and asymptomatic carriers have antibodies \textsuperscript{43,70} and cytotoxic T cells\textsuperscript{290} against recombinant proteins or peptides of the pX ORF I and II proteins. However, to date, there are no conclusive reports on the temporal expression of Tax, Rex and the four accessory proteins messages in terms of their specific quantities or relative levels. Chronically infected cell lines were found to have pX-tax/rex mRNA at 500-fold to 2500-fold higher levels than pX tax-ORF II mRNA and 1000-fold higher levels than pX-rex-ORF I mRNA\textsuperscript{296}.

Interestingly, analogous gene regions encoding the accessory proteins, especially the pX ORF I-encoded p12\textsuperscript{1}, are highly conserved in the closely related virus HTLV-2 and the nonhuman primate counterpart, simian T cell lymphotropic virus type 1 (STLV-1)\textsuperscript{9}. Further illustration of the conserved nature of these gene regions comes from studies of another member of the deltaretroviruses, bovine leukemia virus (BLV). BLV, like HTLV-1, contains an X region between the env sequences and the 3’ long terminal repeat. Two proteins are expressed from this region of BLV: the protein R3, which
shares a common NLS with the Rex protein of HTLV-1, and G4, an arginine rich protein that may exist as two isoforms following protease processing \(^{152,191}\). Similar to HTLV-1, deletion of homologous sequences from BLV infectious molecular clones encoding these accessory proteins, R4 and G3, results in decreased viral loads in the experimental sheep model \(^{152,190,360}\). Collectively these studies illustrate that these retroviruses, which are all associated with lymphoproliferative disorders, during the course of their evolution have retained conserved gene regions that apparently serve analogous functional roles.

1.8.1 pX ORF I p12\(^{1}\)

1.8.1.1 *Biochemical Characteristics of p12\(^{1}\): Features of a Signaling Molecule*

HTLV-1 p12\(^{1}\) is a highly hydrophobic protein, which contains a significant percentage of leucine (32\%) and proline (17\%) residues \(^{173}\), a minimal number of soluble regions and two putative transmembrane domains (aa 12 to 30 and aa 48 to 67) \(^{77,341}\), which overlap with two predicted leucine zipper motifs that form alpha-helices. These distinct structural features (Fig. 1.2) could contribute to membrane localization and dimerization \(^{341}\). Additionally, p12\(^{1}\) contains four predicted SH3-binding motifs (Fig. 1.2), which are typically proline rich cellular signaling proteins with a minimal core of PXXP \(^{38}\). Interestingly, aa 8 to 11 and 70 to 74, encoding the first and third PXXP motifs of p12\(^{1}\) are highly conserved among viral strains, suggesting a possible role for these domains in the function of p12\(^{1}\). In addition, there is a conserved sequence (PSP(L/I)T) extending from aa 48 to 99 in p12\(^{1}\), highly homologous to the PXI\(\text{AT}\) calcineurin-binding motif of nuclear factor of activated \(\text{T}\) cells (NFAT) and found to be critical for the
interaction between p12\textsuperscript{I} and calcineurin\textsuperscript{157}. In addition, p12\textsuperscript{I} contains a dileucine motif (DXXXLL) at amino acid positions 26 to 31, with no functional role ascertained yet (Fig. 1.2). However, dileucine motifs have been described in viral proteins such as HIV Nef and are commonly involved in directing protein trafficking through endosomal compartments by mediating association of the protein with adapter protein 1 (AP-1) to AP-3\textsuperscript{60}. Sequence analysis of p12\textsuperscript{I} suggests possible post-translational modifications of the protein due to the presence of potential phosphorylation sites, glycosylation sites and a ubiquitynation motif surrounding the lysine at position 88, which might significantly enhance the half-life of the protein\textsuperscript{341} are also present in p12\textsuperscript{I}. However, p12\textsuperscript{I} was determined to be not a glycoprotein or a phosphoprotein \textsuperscript{77}.

1.8.1.2 Subcellular Localization of p12\textsuperscript{I} and Interactions with Cellular Proteins

HTLV-1 p12\textsuperscript{I} was originally reported to localize in cellular endomembranes, which was suggested by a spider-like staining of cells expressing the viral protein \textsuperscript{172}. Subsequent immunofluorescent confocal microscopy, electron microscopy and subcellular fractionation studies confirmed that p12\textsuperscript{I} accumulates in the ER and cis-Golgi apparatus. In addition, p12\textsuperscript{I} directly binds calreticulin and calnexin, two resident ER-proteins which regulate calcium storage and calcium-mediated cell signaling, suggesting a role in the establishment of HTLV-1 infection by activation of host cells \textsuperscript{77}.

HTLV-1 p12\textsuperscript{I} shares sequence homology with the bovine papilloma virus (BPV) E5 protein and Epstein-Barr virus (EBV) LMP-1 protein \textsuperscript{86}. The region of highest
homology starts after the first transmembrane domain and extends into the second. Like E5, p12\(\text{I}\) also binds to the 16 kD subunit of the vacuolar H\(^+\)-ATPase (16K). Although this association appears to be required for the E5-mediated transformation of epithelial cells,\(^{86,174}\) additional studies are necessary to determine its functional significance. Interestingly, Nef, a key accessory protein of simian immunodeficiency virus (SIV) and HIV, binds the catalytic subunit NBP-1 of the ATPase\(^{206}\). NBP-1 association of Nef mediated by the Nef C-terminal flexible loop is critical for Nef-dependent internalization of CD4 and viral infectivity\(^{214}\).

Analogous to E5, p12\(\text{I}\) also associates with immature form of IL-2 receptor \(\beta\) and \(\gamma\) chain when transiently co-expressed, resulting in reduced surface expression of the receptor chains\(^{246}\). The p12\(\text{I}\)-binding site on the IL-2R chain overlaps with the binding site for Janus kinases, JAK 1 and 3 and the adapter protein Shc. p12\(\text{I}\) does not influence JAK3 kinase activity directly, however it is considered to be accountable for the modest increase in STAT5 DNA binding activity\(^{262}\). As a consequence, p12\(\text{I}\)-expressing cells displayed a decreased requirement for IL-2 to induce proliferation during suboptimal stimulation with anti-CD3 and anti-CD28 antibodies\(^{262}\). However, peripheral blood derived lymphocyte cell lines immortalized by a HTLV-1 proviral clone ablated for pX ORF I expression, ACH.p12\(\text{I}\), have intact IL-2 receptor signaling pathways\(^{54}\). A possible explanation for these conflicting observations is that p12\(\text{I}\) may modestly activate IL-2 receptor pathways during the early stages of HTLV-1 infection before immortalization. Nevertheless, p12\(\text{I}\) does not appear to be necessary for the activation of the IL-2R-associated JAK1 and JAK3, or their downstream effectors STAT3 and STAT5, after
immortalization. Collectively, these studies indicate that p12\textsuperscript{I} may induce STAT activity to confer a growth advantage to infected cells during the early stages of infection, before immortalization. Future studies are necessary to elucidate the JAK3-independent pathway p12\textsuperscript{I} uses to induce STAT5 activation.

HTLV-1 p12\textsuperscript{I} was reported to associate with immature forms of the major histocompatibility complex class-I (MHC-I), interfere with the interaction of MHC-I with $\beta_2$-microglobulin, decrease the surface expression of MHC-I and direct its degradation in the proteasome\textsuperscript{143,143}, suggesting that p12\textsuperscript{I} might help the virus escape immune surveillance. However, levels of MHC-I and II were similar between T-lymphocytes immortalized with the wild type and p12\textsuperscript{I}-mutant HTLV-1 molecular clones (ACH and ACH.p12 respectively), indicating that p12\textsuperscript{I}-mediated modulation of MHC-I surface expression likely occurs only during the early stages of infection\textsuperscript{54}. Intriguingly, the accessory proteins p10\textsuperscript{I} and p11\textsuperscript{V} of HTLV-2 also associate with MHC-I, however these do not bind to either 16K or IL-2R$\beta$ or $\gamma$\textsuperscript{142}. Additionally, HIV-1 Nef also binds to and down regulates the cell surface expression of MHC-I and is believed to contribute to immune evasion by HIV-1\textsuperscript{287}. However, down regulation of MHC-I of virus-infected cells does not explain the early loss of infectivity of a molecular clone of HTLV-1 that lacks ORF I expression, as virus infection is blocked as early as 1 week post-inoculation, before a possible active immune response occurs\textsuperscript{55}. Future studies of early virus replication immediately after inoculation of virus infected cells in animal models might provide evidence to whether p12\textsuperscript{I} indeed down regulates MHC-I expression on infected peripheral blood mononuclear cells (PBMC) \textit{in vivo} and actively contributes to viral spread or persistence.
In addition, p12\textsuperscript{I} associates with two ER-resident calcium-binding proteins, calreticulin and calnexin\textsuperscript{77} and the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin \textsuperscript{157}. Calreticulin, a highly conserved and ubiquitous protein, serves as one of the major calcium-binding proteins in the ER, participates in calcium signaling, and has been linked to activation of the transcription factor NFA\textsuperscript{T}\textsuperscript{230}. Through protein-protein interactions, calcineurin regulates transcriptional activation of NFAT by triggering the dephosphorylation and subsequent nuclear translocation of NFAT, which results in transactivation of NFAT-inducible cytokine genes including IL-2\textsuperscript{178,228,385}. It would be advantageous for a virus to target such conserved proteins to dysregulate calcium signaling pathways to activate and modulate NFAT in infected T lymphocytes.

1.8.1.3 Role of p12\textsuperscript{I} in Regulation of Viral Infectivity In vitro and In vivo

Earlier reports showed that deletion of pX ORF I from HTLV-1 infectious molecular clones did not affect the viral infectivity and primary lymphocyte transformation mediated by HTLV-1 infection \textit{in vitro}\textsuperscript{74,302}. In contrast, our research group demonstrated that selective elimination of pX ORF I from the molecular clone ACH resulted in dramatically reduced viral infectivity \textit{in vivo}\textsuperscript{55}. Rabbits inoculated with ACH,p12, harboring selective mutations that abolish the expression of pX ORF I mRNA, failed to establish persistent infection as indicated by reduced anti-HTLV-1 antibody responses, failure to demonstrate viral p19 antigen production in PBMC cultures, and only transient detection of provirus by PCR\textsuperscript{55}. A major difference between these \textit{in vitro} and \textit{in vivo} studies is that standard \textit{in vitro} co-culture techniques used to transmit virus to
naive PBMC utilize target cells stimulated by IL-2 and mitogen. However, in vivo the majority of circulating and tissue-associated lymphocytes are non-dividing.

In support of these findings, using co-culture assays that would allow transmission of the virus to resting primary lymphocytes, our group demonstrated that pX ORF I mRNA is critical for viral infectivity in non-activated/quiescent PBMCs in vitro. More importantly, viral infectivity was restored upon addition of IL-2 and mitogen to the co-cultured PBMCs. These data provided the first evidence that HTLV-1 p12 is required for optimal viral infectivity in non-dividing primary lymphocytes and suggested a role of p12 in T-lymphocyte activation and in the early stage of viral infection. Analogously, studies of HIV-1 Nef indicate that the accessory protein is dispensable for transmission of the virus to activated target cells in vitro but is required for viral infectivity in nondividing lymphocytes.

1.8.1.4 Role of p12 in Calcium-Mediated T Cell Activation

Reports from our laboratory illustrated that p12 expression in Jurkat cells results in ~ 20-fold activation of NFAT dependent gene expression, while AP-1 or NF-κB-mediated transcription remained unchanged. p12 specifically activates NFAT in synergy with Ras/MAP kinase activation stimulated by the phorbol ester, PMA. By inhibition of proximal signaling molecules, such as phospholipase C-γ (PLC-γ) and LAT, as well as distal signals, including calcineurin and NFAT, the function of p12 was mapped to be between PLC-γ and calcineurin. p12 mediated NFAT activation was dependent on cytosolic calcium since this function was abolished by BAPTA-AM.
Importantly, p12I functionally substituted for thapsigargin, which specifically depletes the ER calcium store by blocking the ER calcium ATPase. Therefore, HTLV-1 p12I was found to activate NFAT-mediated transcription in lymphoid cells in a calcium-dependent manner. Indeed, p12I expression increases the base-line cytoplasmic calcium concentration, possibly secondary to reduced ER stores calcium content and subsequently higher extracellular calcium entry. Both the calcium channel on ER membranes, IP3R, and the calcium channels on plasma membranes, calcium release activated calcium channels (CRAC), contribute to the p12I mediated NFAT activation, strongly indicating the modulation role of p12I on calcium homeostasis. Induction of calcium release from the ER by p12I to activate NFAT would be an advantage for the virus during the early stages of HTLV-1 infection. Interestingly, this is similar to the function of a cellular protein CAML (Ca2+-modulating cyclophilin ligand), which also contain two putative transmembrane domains like p12I, colocalizes with calreticulin in the ER, induces calcium release from the ER and leads to NFAT activation. In addition, p12I mediated increase in NFAT activity could cause complete activation of cellular stimuli that would normally induce only partial activation of T cells (e.g., through AP-1). These stimuli could be triggered by cytokines or chemokines released from infected neighboring cells or by direct contact between viral envelope proteins and certain cell surface receptors on newly targeted lymphocytes prior to viral entry. Although localization of p12I to the ER appears to be essential for NFAT activation, direct binding between calreticulin and p12I does not correlate with the NFAT activation and future studies are necessary to identify the biological significance of p12I-calreticulin interaction in HTLV-1 infection and pathogenesis.
Expression of NFAT induces a highly permissive state to overcome the blockade at reverse transcription and permitted HIV replication in primary CD4\(^+\) T cells, therefore it is possible that p12\(^I\) causes T cells to be hypersensitive to T cell receptor and CD28 stimulation and thus highly permissive for subsequent viral infection. Interestingly, susceptibility of these cells to HIV infection could be restored by mitogen treatment, likely due to the phytohemagglutinin-induced upregulation of NFAT activity. This is similar to earlier reports from our laboratory that addition of mitogens can rescue the infectivity of a p12\(^I\) mutant viral clone in resting PBMC\(^7\), likely by overriding the requirement for p12\(^I\)-induced activation of NFAT.

Other retroviruses also encode proteins regulating Ca\(^{2+}\)-related signals by analogous or different mechanisms in T lymphocytes or other cell types. HIV accessory protein, Nef, also activates NFAT in synergy with Ras/MAPK pathway in a calcium dependent fashion\(^{217-219}\) and is dispensable for viral infection of activated target cells \textit{in vitro}, but required for viral infectivity in quiescent lymphocytes\(^{216,352,364,384}\). Importantly p12\(^I\) appears to enhance the production of a downstream gene of NFAT activation, IL-2 in Jurkat T cells and primary lymphocytes, which could be abrogated by calcium chelator BAPTA-AM and calcineurin inhibitor, cyclosporin A, suggesting the effect is calcium pathway-dependent\(^{78}\). This increase in IL-2 could account for the decrease in requirement for the cytokine in proliferation of human primary lymphocytes in the presence of p12\(^{1262}\). Overall, p12\(^I\) expression promotes T cell activation and likely facilitates the viral replication and productive infection, which correlates with our previous finding that p12\(^I\) is necessary for viral infectivity in a rabbit model of infection\(^{45}\).
Recently, p12\textsuperscript{I} was found to contain two positive (aa 33-47, aa 87-99) and two negative (aa 1-14, aa 70-86) regions containing individual PXXP motif, that regulate the NFAT activation\textsuperscript{78}. In a recent report from our laboratory, proline residues in these motifs were mutated into alanine residues to test the role of PXXP motifs in p12\textsuperscript{I}-mediated NFAT activation. Interestingly, the third SH3 binding domain (aa 70-73) was responsible for the negative effect of region aa 70-86 on NFAT activation\textsuperscript{78,157}. Besides, mutations in the first two PXXP motifs (aa 8-11 and 35-38) caused only minimal changes in NFAT activation, while mutations in the last PXXP motif (aa 90-93) in the second positive region (aa 86-99), enhanced the NFAT activation. In addition, p12\textsuperscript{I} competes with NFAT for calcineurin binding. More strikingly, alanine substitution mutations in PSLP(I/L) caused more NFAT nuclear translocation and increased NFAT transcriptional activity (~2-fold) than wild type p12\textsuperscript{I}\textsuperscript{157}. Interestingly, PSLP(I/L)T calcineurin binding site is within the aa 70–86 negative region and third PXXP motif which was responsible for the negative effect of region aa 70-86 on NFAT activation. Additionally, NFAT-inhibitory function of the PSLP(I/L)T motif in p12\textsuperscript{I} was verified to be not from the inhibition of calcineurin phosphatase activity. PSLP(I/L)T is homologous to the conserved functionally critical calcineurin-binding motif (PXLXIT) in the N-terminal regulatory domain of NFAT, which binds both inactivated and activated calcineurin\textsuperscript{11}. Many calcineurin-binding proteins such as the anti-apoptotic protein Bcl-2, calcineurin B homologous protein, a kinase anchoring protein AKAP79, and myocyte-enriched calcineurin-interacting protein 1 inhibit either calcineurin phosphatase activity or its substrate NFAT transcriptional activity\textsuperscript{59,197,350}. However, PXLXIT motif mediated binding itself does not inhibit the catalytic activity of calcineurin, because NFAT
activation requires enzymatic activity of calcineurin as well as binding via this motif. Not surprisingly, p12I binding to calcineurin via a motif similar to PXLXIT did not inhibit calcineurin catalytic activity but instead influenced NFAT and calcineurin interaction by competing for binding with NFAT similar to artificial peptides representing this motif \(^{11}\).

Due to the existence of a calcineurin-binding motif in p12I, it may have at least two regulatory actions to modulate NFAT activation: (1) positive modulation by increasing cytosolic calcium concentration from ER stores and (2) negative modulation by calcineurin binding. It is unclear why p12I has two regulatory functions for NFAT transcriptional activity. It is notable that Bcl-2 has these similar properties like p12I, however the functional relationship between calcium release from the ER and calcineurin binding of Bcl-2 is also still unresolved. Bcl-2 maintains calcium homeostasis and prevents apoptosis by localizing not only at the mitochondrial membrane but also in the ER membranes \(^{1,288}\). p12I may function like Bcl-2 at the ER membrane, acting like an ion channel protein to increase ER calcium permeability. Like Bcl-2, p12I may affect apoptosis in HTLV-1-infected T cells. Another ER membrane protein, CAML that activates NFAT by increasing calcium flux binds with calcineurin indirectly, through its association with cyclophilin\(^{351}\). Overall, p12I interacts with calcineurin, an important regulator of NFAT signaling, via a highly conserved PSLP(I/L)T motif, to further T cell activation, an important antecedent to effective viral infection, via a calcium/calcineurin/NFAT pathway.
1.8.1.5 Putative Role of p12\textsuperscript{I} Variants in HAM/TSP

Proteasome destabilization of viral proteins is thought to be an intracellular defense mechanism against viral infection\textsuperscript{68,309}. Lysine is a known target for covalent binding of ubiquitin\textsuperscript{56,68,309} and the metabolic instability p12\textsuperscript{I} is mediated in part by ubiquitylation at a single lysine residue at position 88 and subsequent proteasomal degradation, as well as by destabilizing residues at its amino terminus\textsuperscript{341}. Interestingly, lysine carrying allele was found only in some TSP-HAM cases irrespective of geographical locations, suggesting that a selective pressure over p12\textsuperscript{I} might occur in the host\textsuperscript{85,341}. It is hypothesized that the reduced stability of p12\textsuperscript{I} in HAM/TSP patients due to this sequence variation may facilitate generation of a viral-specific CTL response, since degradation of p12\textsuperscript{I} would alleviate the reduction of MHC-I molecules at the cell surface\textsuperscript{21}. Interestingly, in a study conducted at the same geographic location, lysine residue at position 88 in p12\textsuperscript{I} was not found in all HAM/TSP patients, but found in an asymptomatic HTLV-1 carrier\textsuperscript{225}. However, since all individuals in this study were born in the same geographic region, it might simply represent a particular HTLV-1 carrier population in which the selective pressure on the p12\textsuperscript{I} sequence would not be occurring. Overall, the significance of natural p12\textsuperscript{I} alleles is unclear, and it is possible that the lysine at position 88 of p12\textsuperscript{I} might have a significant effect on the biological effects of the protein in the host, including giving a possible selective advantage in individuals with a certain MHC-I. Future studies including screening of HTLV-1-infected individuals in other populations may elucidate this further.
1.8.2 pX ORF II p13\textsuperscript{II}

1.8.2.1 Biochemical Characteristics of p13\textsuperscript{II}: Mitochondrial Targeting

Less is known about the function of the smaller protein, p13\textsuperscript{II}, encoded by the 87 carboxy-terminal amino acids of the 241-residue Tof or p30\textsuperscript{II} protein. Initial studies demonstrated p13\textsuperscript{II} localization to the nucleus\textsuperscript{173}, but more-recent reports show mitochondrial localization of the protein\textsuperscript{48,62}, specifically in the inner mitochondrial membrane and cristae\textsuperscript{62}. This localization is mediated by an atypical mitochondrial targeting sequence (MTS) in the N terminus of p13\textsuperscript{II} between amino acids 22-31\textsuperscript{48}(Fig 1.2). In addition, p13\textsuperscript{II} MTS is, at least in part, was able to override the potent NLS of Rev\textsuperscript{66}. While the p13\textsuperscript{II} MTS is also present in p30\textsuperscript{II}, p30\textsuperscript{II} is not localized to the mitochondria, suggesting that the signal has to be near the amino-terminus to direct mitochondrial targeting\textsuperscript{63}. Analysis of the amino acid sequence of p13\textsuperscript{II} reveals no characteristic DNA-binding motifs and previous data show neither DNA binding nor transcriptional activity\textsuperscript{303}.

1.8.2.2 p13\textsuperscript{II} Alteration of Mitochondrial Morphology and Inner Membrane Potential

Functionally, expression of p13\textsuperscript{II} disrupts the mitochondrial inner membrane potential and alters mitochondrial morphology and architecture, leading to apparent mitochondrial swelling and fragmentation of their normal interconnected string-like network, suggesting a role for p13\textsuperscript{II} in induction of apoptosis\textsuperscript{48}. Additionally, it is thought that p13\textsuperscript{II} might possibly cause changes in mitochondrial permeability and/or alter processes like calcium signaling that relies on the mitochondrial network and the ER. Since p13\textsuperscript{II} does not cause substantial cytochrome \textit{c} release\textsuperscript{48} or show any
involvement of the permeability transition pore (PTP) in driven cation fluxes\textsuperscript{62}, the biological significance of p13\textsuperscript{II} mitochondrial localization and disruption of membrane potentials remains unclear. D’Agostino \textit{et al}\textsuperscript{64} consider that p13\textsuperscript{II} might initially induce selective permeability changes causing swelling and subsequent de-energization and irreversible swelling, and suggested that p13\textsuperscript{II} triggers apoptosis. However, thus far, increased levels of apoptosis have never been demonstrated in p13\textsuperscript{II}-expressing cells, leaving open the possibility for other mitochondrion-based functions of this viral protein. Such functions could simply include an increased respiratory activity of mitochondria, which is often accompanied by swelling or Ca\textsuperscript{2+} signaling and Ca\textsuperscript{2+} dependent regulation of the oxidative phosphorylation machinery. Thus, p13\textsuperscript{II} may facilitate later stages of HTLV-1 infection such as assembly and release.

D’Agostino \textit{et al}\textsuperscript{62} demonstrated that amino acids 9–41 of p13\textsuperscript{II} which includes the MTS, folds into an $\alpha$ helix in the context of a membrane-like environment and has specific effects on the permeability of isolated mitochondria to small cations. Furthermore, the presence of the four arginines in the MTS is essential for the increase in mitochondrial ion conductance and \textit{in situ} effects on mitochondrial morphology but not for mitochondrial targeting. However, the observation that the p13\textsuperscript{II} MTS is not cleaved upon import into the mitochondria and that it does not require positively charged residues distinguishes it from most MTS, and suggests that the protein might utilize a mitochondrial import pathway distinct from that described for most MTS, which involves binding to a series of acidic receptors followed by cleavage of the signal.
1.8.2.3 \textit{p13}^{II} \textit{Cellular Protein Interactions}

Hou \textit{et al.},\textsuperscript{126} reported that \textit{p13}^{II} associates with two novel cellular proteins designated \textit{C44} and \textit{C254}. \textit{C254} appears to be rabbit actin binding protein 280 (ABP280) present in the cytoskeleton of many different cell types and functions in the modulation of cell shape and polarity and is essential for migration in melanocytes and other cultured cells. More importantly, ABP-280 is important in the insertion of adhesion molecules into the cell membrane. While the region of ABP-280 that interacts with \textit{p13}^{II} is also involved in interactions with integrin B1, tissue factor, and presenilin, other regions of ABP-280 binds to glycoprotein and the cytoplasmic domain of furin\textsuperscript{126}. \textit{C44} shares homology with archeal adenylate kinases, the eukaryotic homologues of which localize to mitochondria and are involved in energy metabolism. Interestingly, the human homologue of \textit{C44} is expressed in the Jurkat T cell line and proliferating, but not resting, PBMC\textsuperscript{126}. These studies were performed using two molecular clones of HTLV-1, K30p and K34p, which have amino acid differences in \textit{rex}, \textit{p13}^{II}, and \textit{p30}^{II}. Interestingly, only \textit{p13}^{II} K34, but not \textit{p13}^{II} K30 allow the interaction with \textit{C44}. The amino acid sequence of the \textit{p13}^{II} variant used by Ciminale \textit{et al} \textsuperscript{48} was most similar to \textit{p13}^{II} K30, as it had the 25- amino-acid carboxyl tail and that it would not bind \textit{C44}. This might provide clues to the role of specific amino acids within \textit{p13}^{II}, which are critical in binding \textit{C44} and in causing apoptosis. Additionally, \textit{p13}^{II} binds to farnesyl pyrophosphate synthetase (FPPS), an enzyme involved in the mevalonate/squalene pathway, and in the synthesis of farnesyl pyrophosphate, a substrate required for prenylation of Ras\textsuperscript{190}. Interestingly, \textit{G4}, a mitochondrial targeting accessory protein of
BLV also binds to FPPS. Future studies on the functional significance of the interaction between p13\textsuperscript{II} and these cellular proteins will possibly elucidate the role of p13\textsuperscript{II} in altering mitochondrial physiology and thus in HTLV-1 replication and pathogenesis.

Furthermore, Mahana et al.,\textsuperscript{211} reported an increase in Vav phosphorylation in rabbit cells transfected with an HTLV-1 molecular clone that contains two mutations in pX ORF II, resulting in expression of truncated p13\textsuperscript{II} and p30\textsuperscript{II}. Vav is a hematopoietically restricted guanine nucleotide exchange factor for the Rac/Rho family of GTPases and is necessary for T cell activation\textsuperscript{146}. These findings suggest that p13\textsuperscript{II} may play a role in controlling the activation state of Vav, which may relate to viral infectivity and leukemogenesis.

1.8.3 pX ORF II p30\textsuperscript{II}

Earlier studies suggested that ORF II was dispensable for expression of HTLV-1 proteins Tax, Rex, or Env, viral replication and immortalization of primary lymphocytes \textit{in vitro}\textsuperscript{74,302}. In addition, the isolation of a viral clone containing a premature stop codon in pX ORF II, from leukemic cells led to the conclusion that pX ORF II was not necessary for the outgrowth of leukemic clones \textit{in vivo}\textsuperscript{44}. However, possible functional role of pX ORF II during early infection was not ruled out by these initial studies. To specifically test the functional role of pX ORF II in viral replication \textit{in vivo}, our laboratory group inoculated rabbits with lethally irradiated cell lines expressing the wild-type molecular clone of HTLV-1 (ACH) and a clone containing selected mutations in pX
ORF II (ACH.p30/13). While all ACH-inoculated rabbits became infected as early as 2 weeks postinoculation, ACH.p30/13-inoculated animals failed to become infected or maintained low proviral copy numbers in their blood leukocytes. These animals also had weak and transient ex vivo p19 antigen production from their PBMC cultures and anti-HTLV-1 antibody titers declined towards the end of the study. Most strikingly, using quantitative competitive PCR, our laboratory group demonstrated a dramatically reduced (up to 100-fold) viral load in the ACH.p30/13-infected animals. Taken together, these data suggested that pX ORF II is necessary for maintenance of high viral loads in vivo.

1.8.3.1 Biochemical Characteristics of p30II: Features of a Transcription modulator

Several lines of evidence indicate that p30II acts as a transcription factor. Importantly, the protein localizes to the nucleus, specifically the nucleolus of cells transiently transfected with a p30II expression vector. p30II contains a highly conserved bipartite NLS between amino acids 71 to 98, which can be functionally substituted for the NLS of Rex. In addition, p30II contains serine and threonine-rich regions that share distant homology to the activation domain of cellular transcription factors, such as Oct-1/2, Pit-1, and POU-1 (Fig. 1.2). Taken together, these characteristics suggest that p30II has a role in transcription of viral and cellular gene expression.
1.8.3.2 Role of p30II in Modulating Viral and Cellular Gene Expression

Our laboratory group has reported that, when provided in limiting concentrations, p30II expression stimulates HTLV-1 LTR-driven reporter gene activity, even in the presence of Tax, whereas higher concentrations represses LTR and CRE-driven reporter gene activity\(^3\)\(^8\)\(^1\). These activities are analogous to herpes simplex virus type 1 (HSV-1) regulation of its immediate-early (IE) gene promoter. VP16, a potent transcription factor from HSV-1, binds the host cell protein HCF, which facilitates the stable complex formation of the viral protein with Oct-1\(^1\)\(^8\)\(^7,\)\(^3\)\(^6\)\(^6\). The IE gene promoter contains an Oct-1-like motif (TAATGARAT) that is important for IE gene expression, with both positive and negative effects, depending on the context of these cellular transcription factors and VP16\(^3\)\(^6\)\(^6\).

Our laboratory group has also demonstrated that p30II co-localizes with p300 in the nucleus and physically interacts with CBP/p300, at the highly conserved KIX domain, the domain HTLV-1 Tax also interacts with. In addition, p30II is able to disrupt CREB-Tax-CBP/p300 complexes bound to the viral 21-bp TRE repeats\(^3\)\(^8\)\(^0\). Since p30II and Tax interacts with CBP/p300 through the same KIX domain, it is possible that the competitive CBP/p300 binding between p30II and Tax might be the mechanism by which p30II attenuate the formation of these multiprotein complexes and thereby repress transcription on CREB-responsive promoters. Similarly, Tax expression has been demonstrated to interfere with the transcriptional activity of c-Myb and the binding of Tax and c-Myb to KIX domain of CBP was found to be mutually exclusive\(^5\)\(^6,\)\(^5\)\(^3,\)\(^2\)\(^5\)\(^9,\)\(^2\)\(^6\)\(^0\).
The coactivators CBP and p300 mediate transcriptional control of various cellular and viral DNA binding transcription factors. Although these coactivators have divergent functions, they are similar in nucleotide sequence, are evolutionarily conserved, and are commonly referred to as CBP/p300\textsuperscript{30,134}. CBP and p300 are highly related and share many functional properties, however there is evidence that these factors are not interchangeable. Several cellular and viral proteins that interact with either CBP or p300 have been identified, including steroid and retinoid hormone receptors, CREB, c-Jun, c-Myb, Sap-1α, c-Fos, MyoD, p53, Stat-1/2, NF-κB, pp90rsk, TATA-binding protein, TFIIB, HTLV-1 Tax, adenovirus E1A, Kaposi’s sarcoma-associated herpes virus viral interferon regulatory factor protein, and simian virus 40 large T antigen\textsuperscript{30,134,349}. CBP/p300 protein is available only at limiting concentrations within the cell nucleus, causing an environment for competition between coactivators and transcription factors, thus providing an additional layer of regulated gene expression\textsuperscript{98,315,319}. There is evidence of a functional antagonistic relationship between transcription factors, as a result of competition for binding to common regions of CBP/p300\textsuperscript{81,98,134}. Under such a condition of tight competition, relative concentrations of Tax/ p30\textsuperscript{II} at various stages of disease might be a critical factor in determining the levels of viral transcription. It is possible that, at higher concentrations, p30\textsuperscript{II} may support viral persistence by reducing viral expression, and thus reducing immune elimination of HTLV-1 infected cells. Additionally, p30\textsuperscript{II} might also repress cellular genes necessary to maintain viral persistence. However, at low concentrations, p30\textsuperscript{II} enhances TRE/viral over CRE/cellular mediated transcription. p30\textsuperscript{II} has the potential to play a role in promoting viral transcription, cell proliferation, competitively repressing CBP/p300-dependent
cellular gene transcription (e.g., p53-dependent p21WAF1/CIP1 gene activity), and for promoting the spread of the virus in vivo. This is also consistent with previous finding from our laboratory that an infectious HTLV-1 molecular clone failed to maintain viral loads in vivo when p30II and p13II expression was abolished.

Recently, it was reported that p30II modulates LTR mediated transcription, in the context of the entire provirus, by a post-transcriptional mechanism. However, data presented in this thesis further confirm the role of CBP/p300 in the modulation of LTR mediated transcription by p30II, in the context of the provirus. In the presence of increasing concentrations of p300, we were able to rescue the p30II-mediated repression on LTR driven gene transcription, in a dose-dependent manner, irrespective of the presence or absence of the provirus. CBP and p300 bridge transcription factors to relevant promoters, has intrinsic histone acetyltransferase (HAT) activity, and form complexes with proteins such as CBP/p300 binding protein-associated factor, which also exhibits HAT activity. Recently, there is increasing knowledge of the mechanism and functional significance of the interactions between many viral proteins and CBP/p300. In the case of adenovirus oncoprotein E1A, interaction with CBP/p300 is critical for regulation of transcription, suppression of differentiation, and immortalization of cells in culture. The T antigen of SV40 regulates the expression of a group of cellular genes by modifying the HAT activity of CBP/p300 or by bridging the gap between DNA binding transcription factors and components of the general transcription machinery. Identifying the molecular mechanism and functional significance of the interaction between p30II and p300 is very crucial in understanding of the role of p30II in the
pathogenesis and replication of this important human pathogen. Therefore to further understand the molecular mechanism and functional significance of the interaction between p30II and p300, using N terminal and C terminal deletion mutants of p30II, we have identified the motifs within p30II that are critical in binding CBP/p300 and in regulating LTR mediated transcription, in the presence/ absence of the provirus. Herein, we confirm the role of p30II as a regulator viral gene transcription, in association with p300 and identified the amino acid sequence 1-132 to be critical in binding CBP/p300 and amino acids 100-179 of p30II to be critical for its function as a repressor of LTR-mediated transcription, irrespective of the presence or absence of the HTLV-1 provirus. Amino acid domain 100-179 contains important features such as the DNA binding domain, serine and threonine rich residues with homology to Oct-1 and POU family of transcription factors and five lysine residues, four of which represent potential acetylation sites for CBP/p300. Data presented in this thesis define the roles of these four lysine residues in p30II-mediated LTR repression. While CBP/p300-mediated acetylation has become a common theme for regulation of protein function, herein we demonstrate that the intrinsic histone acetyltransferase activity of CBP/p300 is critical in regulation of p30II-mediated LTR repression.

1.8.3.3 HTLV-1 Mediated T Cell Activation /Transformation and the Role of p30II.

Mechanisms of HTLV-1 mediated T cell activation and transformation are not completely understood. Unlike other oncogenic viruses, HTLV-1 does not contain a viral oncogene or integrate at specific sites in the host cell genome, causing activation of
cellular proto-oncogenes \(^{311}\). However, HTLV-1 transforms human primary CD\(^{4+}CD^{8-}\) and immortalizes CD \(^{8+}CD^{4},\) double positive, as well as double negative T cells \(^{54}\) and a multistep oncogenic process is believed to occur during HTLV-1 infection, leading to cell proliferation and subsequent cell transformation. First, TCR engagement results in intracellular calcium increase and Ras/MAPK pathway activation, leading to IL-2 production and IL-2R\(\alpha\) accumulation at cell surface, causing induction of T cells to transit from G0 to G1 phase. Second, IL-2 and IL-2R complex interaction leads to activation of IL-2R/Jak/Stat and Shc/Ras/MAPK pathways, triggering T cell progression from G1 to S phase and eventually inducing cell proliferation. The association between these T cell activation pathways and T cell growth is not fully understood. Based on the studies on the initial stages of HTLV-1-induced T cell activation in ex vivo cultured infected T cell clones from HAM/TSP patients with HAM/TSP, infected T cell clones spontaneously proliferate \textit{in vitro} up to 2 weeks after stimulation in the absence of exogenous IL-2 (involving autocrine IL-2/IL-2R stimulation), while uninfected T cell clones revert to G0 stage\(^{125}\). Tax is thought to increase the expression of IL-2R\(\alpha\), thereby induce this initial activation \(^{128}\). Peripheral blood T cells can be immortalized and subsequently transformed by coculturing with HTLV-1-producing T cells\(^{124,125,235,293}\). \textit{In vitro} immortalization is thought to occur in multiple stages: At \(~4\) weeks after the infection, T cell cultures reach a crisis stage with dramatic decrease in cell number, followed by IL-2 dependent proliferation of cells surviving the crisis characterized by upregulated expression of CD25 as well as MHC-II, polyclonal proviral integration, scarce expression of tax/rex mRNA and transient expression of IL-2. Later, at \(~14\) weeks after infection, oligoclonal proviral integration, abundant expression of tax/rex mRNA
and increased CD25 expression, but not IL-2, are detected\(^\text{158}\). Since transformed cells are resistant to rapamycin treatment, \(^\text{365}\) HTLV-1 infection is believed to circumvent the rapamycin sensitive IL-2/IL-2R pathway. Interestingly, HTLV-1 transformed T cells (but not immortalized T cells) show constitutive activation of IL-2R-associated Jak1/3 and Stat3/5 \(^\text{54,231}\). HTLV-1 Tax and p12\(^\text{\text{I}}\) proteins have been linked to HTLV-1-induced immortalization and transformation. Tax is known to upregulate p21\(^\text{WAF}\) expression, inhibit p53 \(^\text{42}\), inhibit p16\(^\text{INK}\) and thus indirectly activate cyclin D/CDK4/6 \(^\text{330}\), functional disruption of mitotic checkpoint protein MAD1 \(^\text{138}\) while p12 is reported to increase Stat5 activation and modestly enhance proliferation \(^\text{262}\).

Data presented in this thesis establishes the role of p30\(^\text{II}\) as a regulator of cellular gene expression. More importantly, herein we illustrate that p30\(^\text{II}\) activated many key transcription factors involved in T cell signaling/activation, such as NFAT, NF-κB and AP-1, and deregulated the expression of multiple genes involved in T cell activation or cell signaling and in regulation of transcription, translation, apoptosis, cell cycle, cell adhesion as well as immune response. The mechanism of function of p30\(^\text{II}\) is not elucidated; however it is likely involved in T cell activation and subsequent T cell proliferation.
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Figure 1.1. Diagram of HTLV-1 genome and alternatively spliced mRNA and protein species from pX ORFs. Numbers correspond to nucleotide positions of exon splice acceptor and donor sites with respect to the full length HTLV-1 genome. Dotted lines indicate the mRNA while the boxes below the dotted lines indicate coding regions of each protein. Open reading frame from which each protein is produced, is indicated as superscript, on the right of each protein.
Figure 1.2. Diagram of p12\textsuperscript{I}, p30\textsuperscript{II}, and p13\textsuperscript{II} with predicted functional motifs. Abbreviations: TM, transmembrane region; LZip, leucine zipper motif; DxxxLL, dileucine motif; PxxP, SH3 binding motif; K/R, lysine-to-arginine variant at position 88 (arginine at this position increases stability of protein). Boxes below p30\textsuperscript{II} indicate regions sharing sequence homology with the DNA binding domain and homeodomain of Oct-1. Numbers below bars indicate amino acid position numbers.
CHAPTER 2

HUMAN T LYMPHOTROPIC VIRUS TYPE-1 P30\textsuperscript{II} INFLUENCES CELLULAR GENE EXPRESSION AND ENHANCES TRANSCRIPTION ACTIVATION OF T LYMPHOCYTES

INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1), the first characterized human retrovirus, causes adult T cell leukemia/lymphoma (ATL) and is associated with several lymphocyte-mediated disorders such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)\textsuperscript{7}. Mature CD4+ T lymphocytes are the primary targets of HTLV-1 infection\textsuperscript{25}. Although the mechanism by which the virus causes oncogenic transformation of host T lymphocytes is incompletely understood, altered gene expression has been associated with the initiation or progression of ATL \textsuperscript{47}. This complex retrovirus encodes structural and enzymatic gene products, as well as regulatory and accessory proteins from open reading frames (ORF) in the pX region between \textit{env} and the 3’ long terminal repeat (LTR) of the provirus\textsuperscript{19}. The well characterized Rex and Tax proteins are encoded in the ORF III and IV respectively. Rex is a nucleolus-localizing
phosphoprotein, involved in nuclear export of unspliced or singly spliced viral RNA. Tax is a nuclear localizing phosphoprotein that interacts with cellular transcription factors and activates transcription from the viral promoter, Tax-responsive element (TRE) and enhancer elements of various cellular genes associated with host cell proliferation. Emerging evidence has documented the role of pX ORF I and II gene products in the replication of HTLV-1. There are four proteins expressed from these ORFs- p12I, p27I, p13II, and p30II. pX ORFs I and II mRNAs are present in infected cell lines and freshly isolated cells from HTLV-1-infected subjects as well as in ATL and HAM/TSP patients. Antibodies and cytotoxic T cells that recognize recombinant proteins or peptides of the pX ORF I and II proteins are present in HTLV-1 infected patients and asymptomatic carriers.

Using molecular clones of HTLV-1 with selective mutations of ORF I and II, we have tested the requirement of p12I and p13II/p30II in the establishment of infection and maintenance of viral loads in a rabbit model of infection. ORF II protein p30II contains a highly conserved bipartite nuclear localization signal (NLS) and localizes within the nucleus of cells. In addition, p30II contains serine- and threonine-rich regions with distant homology to transcription factors Oct-1 and -2, Pit-1, and POU-M1. p30II also co-localizes with p300 in the nucleus and physically interacts with CREB binding protein (CBP)/p300 and differentially modulates cAMP responsive element (CRE) and TRE mediated transcription. Based on these reports, we hypothesized that p30II functions as a regulator of cellular and viral gene expression to promote HTLV-1 replication.
Gene arrays have primarily been employed to study the changes in gene expression profile of HTLV-1-immortalized and transformed cell lines or in cells from ATL patients and attempts to test the influence of individual HTLV-1 viral proteins on cellular gene expression have been limited to Tax. Herein we used the Affymetrix U133A human gene chip to confirm the role of p30II as a regulator of gene expression and identified several novel and important alterations in gene expression profiles, unique to cell cycle regulation, apoptosis and T cell signaling/activation. In addition, using semi-quantitative RT-PCR, we have confirmed the expression of multiple genes modulated by p30II in primary CD4+ T lymphocytes. Moreover, we have validated the function of p30II in T cell signaling/activation, using reporter assays. This is the first report that demonstrates the role of p30II as an activator of many key transcription factors involved in T cell signaling/activation, such as Nuclear Factor of Activated T cells (NFAT), Nuclear Factor-Kappa B (NF-κB) and Activator Protein-1 (AP-1). Together, our data indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes to promote lymphocyte activation to enhance clonal expansion of infected cells and maintain proviral loads in vivo.
MATERIALS AND METHODS

Lentiviral vectors and other plasmids

The plasmid pWPT-IRES-GFP was generated by cloning the internal ribosome entry site (IRES) sequence from pHR’CMV/Tax1/eGFP (Gerald Feuer, SUNY, Syracuse) into pWPT-GFP plasmid (Didier Trono, University of Geneva). Subsequently, the plasmid pWPT-p30IIHA-IRES-GFP was created by cloning p30II sequence from ACH\textsuperscript{30} with the downstream influenza hemagglutinin (HA1) tag (Fig. 2.1). Sanger sequencing confirmed both the plasmids to have the correct sequence and were in frame. GFP and p30IIHA expression were confirmed by fluorescence activated cell sorting (FACS) analysis (Beckman Coulter, Miami, FL) and western blot respectively. GFP expression from each of the plasmids was confirmed by flow cytometry (Beckman Coulter) and the p30IIHA expression from pWPT-p30IIHA-IRES-GFP plasmid was confirmed by western blot using mouse monoclonal anti- hemagglutinin antibody (1:1000) (Covance, Princeton, NJ) as described previously\textsuperscript{57,58}. The plasmid pME-p30IIHA was created by cloning p30II sequence from ACH with HA1 tag, into pME-18S (G. Franchini, NIH). Other plasmids used include previously reported pRSV-βGal\textsuperscript{58} and AP-1, NF-κB and NFAT-luciferase reporter plasmids\textsuperscript{2}.

Recombinant lentivirus production and infection of Jurkat T lymphocytes and primary CD4+ T lymphocytes

Recombinant lentiviruses were produced by transfecting pHCMV-G, pCMVΔR8.2 and pWPT-p30IIHA-IRES-GFP (sample) or pWPT-IRES-GFP (control) as described
previously\(^1\)\(^8\). Briefly, 293T cells (5 \(\times\) 10\(^6\)) were seeded in a 10-cm dish and transfected the following day with 2 \(\mu\)g of pHCMV-G, 10 \(\mu\)g of pCMV\Delta R8.2 and 10 \(\mu\)g of pWPT-p30\(^\text{II}\)HA-IRES-GFP or pWPT-IRES-GFP using the calcium phosphate method. Supernatant from 10 to 20 dishes was collected at 24, 48 and 72 h post transfection, cleared of cellular debris by centrifugation at 1000 rpm for 10 min at room temp and then filtered through a 0.2 \(\mu\)m filter. The resulted supernatant was then centrifuged at 6,500 g for 16 h at 4\(^\circ\) C. The viral pellet was suspended in cDMEM (DMEM containing 10% FBS and 10% streptomycin and penicillin) overnight at 4\(^\circ\) C and the concentrated virus was aliquoted and stored at –80\(^\circ\) C. To determine the virus titer, serial dilutions of the virus stock were used to spin infect 293T cells and 48 h post infection, eGFP expression and p30\(^\text{II}\) expression was measured by flow cytometry and RT-PCT respectively. Briefly, on the day before infection, 293T cells (1 \(\times\) 10\(^5\)) were seeded in a 6 well plate. The medium was removed the following day and the cells were then incubated with the diluted virus containing 8 \(\mu\)g/ml polybrene (Sigma, St. Louis, MO). Cells were then spin-infected by centrifugation at 2700 rpm for 1 h at 30\(^\circ\) C, supplied with fresh medium and cultured for 48 h. Then cells were treated with trypsin (Invitrogen, Carlsbad, CA), pelleted and resuspended in D-PBS (Invitrogen) for fluorescence activity cell sorting (FACS) analysis on an ELITE ESP flow cytometer (Beckman Coulter). 1 X 10\(^6\) cells were used to perform western blot to detect the expression of p30 \(^\text{II}\) HA. Jurkat T lymphocytes (clone E6.1, American Type Culture Collection) were transduced with recombinant virus at multiplicity of infection of 4 in the presence of 8 \(\mu\)g/ml polybrene (Sigma) and spin-infected at 2700 rpm for 1 h at 25\(^\circ\) C. Primary CD4+ T cells were extracted using dynabead CD4 positive isolation kit (Dynal Biotech, Lake Success, NY).
according to manufacturer’s instructions. Primary CD4+ T cells were stimulated with Phytohemagglutinin (PHA) for 48 hours, transduced with recombinant virus at multiplicity of infection of 20 in the presence of 8 µg/ml polybrene (Sigma) and spin-infected at 2700 rpm for 1 h at 25°C. At 10 days post-transduction, GFP expression of controls and samples were verified to be above 90% by FACS analysis and the presence of p30II mRNA expression in samples (and absence in controls) was verified by RT-PCR (Fig. 2.2).

Probe preparation and microarray analysis

According to the instructions of manufacturers, total cellular RNA was isolated from transduced Jurkat T lymphocytes using RNAqueous (Ambion, Austin, TX). To test the concentration and purity of the RNA samples, absorbance at 260 nm and 280 nm were measured and the 260/280 ratio was calculated using a spectrophotometer (Genequant, Amersham Pharmacia, Piscataway, NJ). The 260/280 ratio of all the RNA samples were between the range of 1.9-2.1. The probe preparation for GeneChip was performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, cDNA was synthesized using genechip T7-Oligo (dT) promoter primer kit (Affymetrix) and superscript double stranded cDNA synthesis kit (Invitrogen), according to the manufacturers instructions. cDNA cleanup was done using Genechip Sample Cleanup module (Affymetrix). In vitro transcription was performed on the cDNA to produce biotin-labeled cRNA with ENZO RNA Transcript labeling kit (Affymetrix), according to the manufacturers instructions. cRNA cleanup was
done using Genechip Sample Cleanup module (Affymetrix). The quality of total RNA and biotin-labeled cRNA of all the samples and controls were checked by calculating the ratio of absorbance at 260 nm and 280 nm (between 1.9 to 2.1) using a spectrophotometer (Genequant) and agarose gel electrophoresis. The labeled cRNA was fragmented to 50–200 nucleotides, and hybridized to U133A arrays (Affymetrix) using GeneChip® Hybridization Oven (Affymetrix). Arrays were washed and stained using GeneChip® Fluidics Station 400 (Affymetrix) and scanned by GeneArray Scanner (Affymetrix).

Quality control criteria evaluations done as part of the basic analysis include (1) The ratios of 3’ signal to 5’ signal of two housekeeping genes, beta-actin and GAPDH were between 0 and 3. (2) The hybridization controls BioB, BioC, BioD, and Cre were all present and in a linear relationship of intensity. (3) The scale factors between arrays did not vary by 3 fold. (4) The background intensity was not significantly higher than expected. (5) The percent of gene present was monitored and found to be not less than the standard 30%. To determine the quantitative RNA level, the average differences representing the perfectly matched minus the mismatched for each gene-specific probe set was calculated. Differential gene expression and comparative analysis was done using Data Mining Tool® (Microarray suite 5) to identify probes with at least 1.5 fold difference in expression between control and p30I and verified for cluster formation by dCHIP software39. The biological and molecular functional grouping of these probes was done using Gene Ontology Mining Tool (Affymetrix)6. The selected probe lists are presented in the supplemental data.
RT-PCR

One μg of RNA was converted to cDNA (Reverse Transcription system, Promega, Madison, WI) as described by the manufacturer. cDNA from 100 ng of total RNA was amplified with AmpliTaq DNA polymerase (Perkin Elmer, Boston, MA), PCR products were separated by agarose gel electrophoresis, normalized to GAPDH and quantified using alpha imager spot densitometry (Alpha Innotech, San Leandro, CA). DNA contamination was tested by performing a control with no reverse transcriptase. The PCR primers for p30II were as follows: TAG CAA ACC GTC AAG CAC AG (forward) and CGA ACA TAG TCC CCC AGA GA (reverse). The PCR primers for CHP were as follows: CCC ACA GTC AAA TCA CTC GCC (forward) and ATG GTC CTG TCT GCG ATG CTG (reverse). The PCR primers for JUN were as follows: CTC TCA GTG CTT CTT ACT ATT AAG CAG (forward) and TTA TCT AGG AAT TGT CAA AGA GAA GATT (reverse). The PCR primers for NFATc were as follows: TTG GGA GAG ACA TGT CCC AGA TT (forward) and TCA TTT CCC CAA AGC TCA AAC A (reverse). The results were expressed as a graph. Statistical analysis was performed using Student’s \( t \) test, \( P < 0.05 \).

Transient transfection and reporter gene assay

Analysis of AP-1, NF-κB, and NFAT transcriptional activity in pME- and pME-p30II-transfected Jurkat T lymphocytes was performed as described previously (25). Briefly, transient transfection of Jurkat T lymphocytes was done by electroporating \( 10^7 \)
cells in cRPMI (RPMI 1640 containing 10% fetal bovine serum (FBS) and 10% streptomycin and penicillin) at 350V and 975 µF using Bio-Rad Gene Pulser II (Bio-Rad, Laboratories, Hercules, CA) with 30 µg of pME-p30 or pME empty plasmid, 10 µg of reporter plasmid (NFAT-Luc, AP-1 Luc or NF-κB Luc), and 1 µg of pRSV-Gal plasmid or 1 ug pWPT-IRES-GFP plasmid. The transfected cells were seeded in six-well plates at a density of 5 X 10^5/ml and were either left untreated or stimulated with 20 ng/ml of phorbol myristate acetate (PMA) (Sigma) or with 2 µM ionomycin (Sigma), or both at 6 h post-transfection, followed by incubation for 18 h prior to lysis for analysis of luciferase activity. Stimulations with anti-CD3 and/or anti-CD28 antibodies (each at 3 µg/ml) (BD Pharmingen, Heidelberg, Germany) were carried out 18 h post-transfection. Following 8 h of stimulation, to measure luciferase activity, the cells were lysed with Cell Culture Lysis Reagent (Promega), and the cell lysates were tested for luciferase activity according to the manufacturer’s protocol. Transfection efficiency was normalized by staining with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) (Sigma) and counting β-Gal expressing cells. Transfection efficiency was also normalized by counting GFP positive cells under the fluorescence microscope. Results were expressed as mean of optimized luciferase activity (luciferase activity / percentage cells stained positive for β-Gal expression) in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student’s t test, P < 0.05.
RESULTS

At 10 days post-transduction, GFP expression of Jurkat T lymphocytes transduced with recombinant lentivirus expressing GFP alone (controls) or p30\textsuperscript{II} and GFP (samples) was confirmed to be > 95%. Presence of expression of p30\textsuperscript{II} mRNA in the sample cells and absence of p30\textsuperscript{II} mRNA expression in control cells were confirmed by RT-PCR. Differential gene expression and comparative analysis was done to identify probes with at least 1.5 fold difference in expression between control and p30\textsuperscript{II} and verified for cluster formation\textsuperscript{39}. We categorized genes deregulated by HTLV-1 p30\textsuperscript{II} into those upregulated or downregulated in expression. We further grouped genes deregulated by p30\textsuperscript{II} based on their functions, such as apoptosis, cell cycle, cell adhesion, transcription / translation factors and T cell activation or cell signaling. In all the categories, p30\textsuperscript{II} appeared to be an overall repressor of cellular gene expression, while selectively increasing the expression of certain key regulatory genes (Table 2.1).

Apoptosis

Based on changes in gene expression in p30\textsuperscript{II} expressing cells (Table 2.1), p30\textsuperscript{II} would be predicted to modulate apoptosis. These include Bcl-2 related/interacting genes such as pro-apoptotic Bcl-2-related protein A1, anti-apoptotic MCL1, cell-death regulator Harakiri, apoptotic protector BNIP1 (downregulated) and pro-apoptotic BIK (upregulated). In addition, p30\textsuperscript{II} expression correlated with downregulation of genes associated with Fas mediated apoptosis pathway such as tumor suppressing subtransferable candidate 3 and TNF receptor superfamily member 25. p30\textsuperscript{II} expression
was also associated with decrease in caspases (2 and 4) and increase in genes associated with the DNA fragmentation pathway (CIDE-B and CIDE-3). In addition, p30\textsuperscript{II} expression correlated with decreased expression of many other apoptosis related genes including CD28, Lck, cyclin B1, Cullin 5, Adenosine A2a receptor, TAF4B and NCK-associated protein 1.

**Cell cycle**

p30\textsuperscript{II} expression altered the expression of multiple genes involved in regulation of different stages of cell cycle. These include checkpoint suppressor 1, cytosolic branched-chain amino acid transaminase 1, histone deacetylase 6, cyclin B1, WEE1 kinase, CDC14A, Lck, JAK2, GAS7, BZAP45, Cullin, Rab6 GTPase activating protein (downregulated) and TERF1, AKAP8, DDX11, MSH2 and JUN (upregulated). Another gene downregulated by p30\textsuperscript{II} expression was MDM2, which, interestingly is overexpressed in certain leukemias\textsuperscript{34} and capable of enhancing the tumorigenic potential of cells by inhibiting p300 / PCAF mediated p53 acetylation\textsuperscript{40}.

**Cell adhesion**

p30\textsuperscript{II} expression was associated with altered expression of several genes involved in cell adhesion. These include decrease in integrin (integrin \(\beta\)8) immunoglobulin (MADCAM1), a counter-receptor for P-selectin (SELPLG), cadherin (desmocollin 3), protocadherin (PC-LKC) liprin (PF1BP1), CD84 / Ly-9, CD58, CD43 / sialophorin and glycosyl-phosphatidyl-inositol phospholipase D1. Expression of p30\textsuperscript{II} correlated with increase in integrin receptor \(\alpha\)1 subunit and KIT ligand.
Transcription and translation

Expression of p30II was associated with decrease in expression of transcriptional control genes such as TATA-binding protein associated factor 4 (TAF4), two corepressors (Enolase-1 and Chromosome 19 ORF2 protein), a novel specific coactivator for mammalian TEFs, namely TONDU, homeobox genes (mesenchyme homeobox 1, homeobox A1), T-box genes (T-box 21) and proteins containing helix-loop-helix domain, which are known to be critical in cell growth/differentiation and tumorigenesis (neuronal PAS domain protein 2, Myc-associated factor protein, inhibitor of DNA binding-3). Additionally, p30II expression correlated with downregulation of zinc finger proteins (zinc finger protein 36), a group of transcription regulators proposed to be candidates in malignant disorders and coiled coil proteins (JEM-1). p30II was also associated with downregulation of many genes with positive transcriptional effects (including SEC14-like 2, Nurr 1, CITED2 / MRG1, LXR alpha and SMARCA2). Downregulation of HDAC6, a histone deacetylase and nuclear receptor coactivator 3 (CBP interacting protein) with histone acetyltransferase and pCAF/CBP recruiting abilities are particularly interesting, since p30II contains multiple highly conserved lysines, which could play a role in acetylation. Expression of p30II was associated with decrease in GAS 7, which has sequence homology to Oct and POU family of transcription factors. Genes upregulated by p30II also included HTLV enhancer factor, c-Jun, TAF1C, Kruppel-type zinc finger, PQBP1, AF4 and SOX4. Based on this, p30II appears to regulate transcription through various mechanisms and different levels, some of which could be attributed to the interaction between p30II and CBP/p300. Additionally, p30II
expression was associated with decrease in translation initiation factor 2 (IF2) and eukaryotic translation elongation factor 1δ (EEF1D) and increase in eukaryotic translation elongation factor 1α (EEF1A2), a putative oncogene 4

**p30II alters the expression of genes involved in T cell signaling / activation**

Expression of p30II was associated with decrease in mRNA levels of CD28, a co-stimulatory molecule with a distinct role in T lymphocyte activation 10. Cells expressing p30II had upregulated levels of Vav-2 and CD72 and downregulated levels of CD46 and Lck tyrosine kinase, a member of the Src family protein tyrosine kinases that participates in signal transduction pathways initiated by T cell surface receptors such as TCR/CD3, CD2, CD4, CD8, and CD28 43. Additionally, p30II expression correlated with decrease in the level of CHP, an endogenous calcineurin inhibitor, indicating a likely increase in calcineurin activity, which would be predicted to correlate with upregulation of NFAT expression by p30II. Moreover, p30II expression was associated with increased expression of c-Jun and c-Fos, suggesting activation of AP-1 mediated transcription. p30II appeared to downregulate the expression of protein kinase D (PKD), which negatively modulates JNK signaling pathway 26, mediates cross-talk between different signaling systems, and is critical in processes as diverse as cell proliferation, apoptosis, immune cell regulation and tumor cell invasion 52. Interestingly, p30II expression was also associated with decrease in epidermal growth factor (EGF), which stimulates calcium influx 56 and activates ETS domain transcription factor Elk-1 48. In Jurkat T lymphocytes expressing p30II, there were no detectable levels of IKKγ, which is important for NF-κB signaling in response to both
T cell activation signals and Tax\(^{49}\). However p30\(^{II}\) expression increased HPK-1 (Hematopoetic Progenitor Kinase-1), a known NF-\(\kappa\)B activator\(^5\). p30\(^{II}\) expression was associated with decreased Ras GRP2, a guanyl nucleotide exchange factor that increases Ras-GTP, suggesting a decrease in the level of activated Ras (Ras-GTP).

We performed semiquantitative RT-PCR analysis in Jurkat T lymphocytes and primary CD4\(^+\) T lymphocytes to confirm the altered expression of genes involved in T cell activation / signaling pathway (Fig. 2.3A, 2.3B, 2.3C and 2.3D). Overall, HTLV-1 p30\(^{II}\) downregulated the expression of multiple upstream regulators of T cell activation / signaling pathway to enhance NFAT, NF-\(\kappa\)B and AP-1 driven transcription, key factors in T cell proliferation and leukemogenesis.

**HTLV-1 p30\(^{II}\) enhances transcription mediated by NFAT, NF-\(\kappa\)B and AP-1 in Jurkat T lymphocytes.**

Using luciferase reporter assays, we directly tested the ability of p30\(^{II}\) to enhance NFAT, NF-\(\kappa\)B and AP-1 driven transcription. NF-\(\kappa\)B, AP-1, and NFAT luciferase reporter plasmids and p30\(^{II}\) expression plasmid were transiently transfected into Jurkat T lymphocytes, and stimulated with PMA or ionomycin or both, CD3 or CD28 or both. p30\(^{II}\) increased the NFAT driven luciferase reporter gene activity from 2.2 to 10.7 fold depending on co-stimulatory treatment e.g., PMA, ionomycin, CD3, CD28 etc. (Fig. 2.4A), indicating that p30\(^{II}\) effectively enhanced NFAT driven transcription, when stimulated with ionomycin or CD3. HTLV-1 p30\(^{II}\) increased NF-\(\kappa\)B driven luciferase reporter gene activity from 3.1 to 11.4 fold, depending on co-stimulation (Fig. 2.4B),
indicating that p30\textsuperscript{II} is able to effectively enhanced NF-κB driven transcription, significantly even under unstimulated conditions. HTLV-1 p30\textsuperscript{II} modestly increased the AP-1 driven luciferase reporter gene activity from 1.2 to 5.2 fold in the presence of co-stimulator treatments (Fig. 2.4C), indicating that p30\textsuperscript{II} enhanced AP-1 driven transcription, significantly when stimulated with PMA.

**DISCUSSION**

Our study is the most comprehensive analysis of changes in gene expression patterns caused by an individual retrovirus accessory protein. Our approach included methods to strengthen the reliability of our data by (a) use of triplicate samples and appropriate controls (b) use of multiple software for data analysis (c) minimization of nonspecific hybridization and background signals by using Affymetrix chip\textsuperscript{42} (d) use of a well-characterized T lymphocyte system (Jurkat) and (e) verification of microarray data by semiquantitative RT-PCR in Jurkat T lymphocytes and primary CD4\textsuperscript{+} T lymphocytes (f) validation of microarray data by reporter assays, all of which were consistent with our microarray findings. Some of these findings are consistent with previous studies using gene arrays to test HTLV-1-transformed cell lines. For example, HTLV-1 infected cell lines contain low levels of caspase-4 and high levels of JUN\textsuperscript{47} and cyclin B1 levels are low in HTLV-1 leukemic T cells\textsuperscript{28}. Overall, this study not only confirmed that p30\textsuperscript{II} is a regulator of cellular genes, but also identified several potential new functional roles for p30\textsuperscript{II}, in T cell activation or cell signaling and in regulation of apoptosis and cell cycle.
Others have used gene array approaches to study HTLV-1-related changes in gene expression. Harhaj et al. \cite{23} studied the gene expression in HTLV-1 mediated oncogenesis using human cDNA array analysis of normal and HTLV-1 immortalized T cells and found that the expression of a large number of genes involved in apoptosis were deregulated in HTLV-1 immortalized T cells. Subsequently, the same type of cDNA arrays were employed by De La Fuente et al. \cite{15} to study upregulation of a number of transcription factors in HTLV-1-infected cells, including zinc fingers, paired domains, and basic helix-loop-helix (bHLH) proteins. Gene expression profiles of fresh peripheral blood mononuclear cells (PBMC) from acute and chronic ATL patients were used to identify the genes associated with progression of ATL including a T cell differentiated antigen (MAL), a lymphoid specific member of the G-protein-coupled receptor family (EBI-1/CCR7) and a novel human homolog to a subunit (MNLL) of the bovine ubiquinone oxidoreductase complex\cite{31}. Using NIH OncoChip cDNA arrays containing 2304 cancer related cDNA elements, Ng et al. 2001 \cite{44} compared normal and Tax-expressing Jurkat T lymphocytes and identified Tax induced changes in gene expression, associated with apoptosis, cell cycle, DNA repair, signaling factors, immune modulators, cytokines, growth factors, and adhesion molecules. Recently, Affymetrix, GeneChip microarrays containing oligonucleotide hybridization probes representative of ~7000 genes were used to compare the expression profiles of normal activated peripheral blood lymphocytes to HTLV-I-immortalized and transformed cell lines \cite{47}. In this study, by employing a gene chip representing ~33000 genes, we tested the role of p30\textsuperscript{II} on cellular gene expression profile of a larger number of genes.
Expression from the IL-2 promoter requires binding of several transcription factors, including NFAT, AP-1 and NF-κB. NFAT is vital to proliferation of peripheral lymphocytes for HTLV-1 infection while AP-1 is linked to the dysregulated phenotypes of HTLV-1 infected T cells and malignant transformation. Activation of AP-1 occurs through Tax-dependent and independent mechanisms in HTLV-1-infected T cells in vitro and in leukemia cells in vivo. NF-κB is highly activated in many hematopoietic malignancies, HTLV-1 infected T cell lines and in primary ATL cells, even when Tax expression levels are low and due to its anti-apoptotic activity, it is considered to be a key survival factor for several types of cancer. Ours is the first report demonstrating the ability of a retroviral accessory protein to have broad modulating activities on the transcriptional activity of NF-κB, NFAT and AP-1.

Mechanisms employed by p30II to enhance NFAT, AP-1 and NF-κB mediated transcription will require additional research. We have previously reported that another HTLV-1 accessory protein p12I stimulates NFAT mediated transcription, when stimulated with PMA, indicating that p12I acts synergistically with Ras/ MAPK pathway to promote NFAT activation and thus may facilitate host cell activation and establishment of persistent HTLV-1 infection. However p30II enhances NFAT driven transcription significantly when stimulated with ionomycin or CD3, and therefore likely uses a different mechanism than p12I. To modulate NFAT driven transcription and subsequent T cell activation / signaling, it is possible that these two accessory proteins act synergistically. AP-1 is able to interact with transcriptional coactivator CBP/ p300 as well as viral CREs and mediate HTLV-I gene expression. Intriguingly, we have
previously reported that p30\textsuperscript{II} interacts with CBP/p300 at KIX domain of CBP \textsuperscript{11}, influences CRE and TRE mediated transcription \textsuperscript{58} and disrupts CREB-Tax-p300 complexes on TRE probes \textsuperscript{57}. NF-κB (52) and NFAT \textsuperscript{21} are also known to interact with transcriptional coactivator CBP/p300. Therefore it is possible that p30\textsuperscript{II} regulates the transcriptional activity of NFAT, NF-κB and AP-1, at least in part, by its interaction with CBP/p300.

HTLV-1 mediated interference with normal T-cell apoptosis is thought to be a mechanism of tumorigenicity \textsuperscript{25}, but specific mechanisms by which HTLV-1 infection or any particular HTLV-1 gene products influence on T-cell survival are not fully understood \textsuperscript{25}. Similar to the effect of HTLV-1 Tax on apoptosis related genes\textsuperscript{23,44}, we found that p30\textsuperscript{II} also deregulates multiple genes resulting in possible pro-apoptotic and anti-apoptotic effects. Since apoptosis is a well-known mechanism of cellular defense against viral infection, a possible role of p30\textsuperscript{II} in lymphocyte apoptosis might correlate with the requirement of p30\textsuperscript{II} in maintaining proviral loads \textit{in vivo} \textsuperscript{9}. Previous studies indicate that several members of the cell cycle machinery have altered expression in HTLV-1 infected cells \textsuperscript{47}. Several studies examined the aberrations in cell cycle caused by HTLV-1 Tax \textsuperscript{25}; however, not much is known about the role of other HTLV-1 proteins in causing abnormalities in cell cycle.

p30\textsuperscript{II} appears to regulate viral gene expression and modulate immune response. We have previously reported that, p30\textsuperscript{II} activated HTLV-1 LTR at lower concentrations and repressed at higher concentrations. \textsuperscript{58} Interestingly, p30\textsuperscript{II} expression was associated
with downregulation of lck (p56), which suppresses the HTLV-1 promoter \(^{45}\) and upregulate HTLV enhancer factor, which is known to bind to LTR at a region involved in regulation of gene expression by the ets family of transcription factors\(^{38}\). Additionally, p30\(^{II}\) expression was associated with altered expression of cellular genes involved in immune modulation such as CD46, CD43, CD58, IFN\(\gamma\) and CD72.

Overall, this study supports our earlier reports \(^{2,9,57,58}\) and sheds light on the mechanisms by which p30\(^{II}\) functions in HTLV-1 pathogenesis and in leukemogenesis. Many of the effects of p30\(^{II}\) are similar to that of other HTLV-1 proteins like Tax or p12\(^{I}\). It is possible that these proteins act coordinately or synergistically. We postulate that, by modulating the expression of various HTLV-1 proteins, the virus employs selective use of different viral proteins during different stages of the infection. However, since information on the expression profile of HTLV-1 proteins during different stages of the infection is limited, additional studies are required to explore this possibility. Further studies will also be required to verify our data in primary T lymphocytes. Such future studies might provide new directions in the development of therapeutic interventions against HTLV-1 disorders, which are associated with immune mediated mechanisms.

HTLV-1 accessory proteins are functionally homologous to the accessory proteins of bovine leukemia virus (BLV), a deltaretrovirus with conserved organizational structure similar to HTLV-1 that is associated with a naturally occurring lymphoma in cattle \(^{17,29,37}\). BLV-wild type and mutant proviruses that contained deletions in the G4 or R3 genes infected B lymphocytes and permitted the infected cell to resist apoptotic signals \(^{17}\). G4
protein interacts with farnesyl pyrophosphate synthetase (FPPS), an enzyme in the mevalonate/squalene pathway that is critical for synthesis of FPP, a substrate required for prenylation of Ras\textsuperscript{37}. Infectious molecular clones of BLV with mutations in gene regions encoding G4 and R3 were limited in their ability to maintain proviral loads and producing lymphosarcomas in infected sheep\textsuperscript{29}. These reports taken together with our data indicates that complex retroviruses associated with lymphoproliferative diseases rely upon accessory gene products to modify their cellular environment to enhance clonal expansion of the virus genome and thus maintain proviral loads \textit{in vivo}.
REFERENCES


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Table 2.1. Genes modulated by HTLV-1 p30II
*Genes upregulated by p30II, indicating that the signal intensity was either increased by a minimum of 1.5 fold or turned on (not expressed in controls) in at least 2 of the 3 p30II expressing samples. Unmarked genes are downregulated by p30II, indicating that the signal intensity was either decreased by a minimum of 1.5 fold or completely shut down in at least 2 of the 3 p30II expressing samples.
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Figure 2.1. Schematic illustration of lentiviral vectors expressing both p30HA and GFP (sample vector) as bicistronic messages and GFP alone (control vector) from elongation factor 1 alpha promoter. Abbreviations: LTR - Long Terminal Repeats; RRE - Rev Response Element; EF1 α - Elongation Factor 1 alpha promoter; IRES – Internal Ribosome Entry Site; WPRE – Woodchuck Hepatitis Post-transcriptional Regulatory Element.
Figure 2.2. Triplicate p30\textsuperscript{II} samples express GFP and p30\textsuperscript{II} while triplicate controls express only GFP. (A) Flow cytometric analysis illustrating the expression of GFP in Jurkat T cells 10 days post spin-infection with lentiviral vectors. Both sample (expressing p30\textsuperscript{II} and GFP) and control (GFP alone) group contains relatively high and similar levels of GFP. (B) RT-PCR demonstrating the expression of p30\textsuperscript{II} in Jurkat T cells 10 days post spin-infection with lentiviral vectors. Jurkat T cells spin-infected with sample vector express p30\textsuperscript{II} while the control vector spin-infected cells do not express p30\textsuperscript{II}. RT-PCR was performed with triplicate samples and controls. GAPDH was used as a control for the integrity of the message.
Figure 2.3. Semiquantitative RT-PCR of CHP, JUN and NFATc in controls and p30\textsuperscript{II} expressing Jurkat T lymphocytes (A and B) and primary CD4+ T lymphocyte (C and D) samples. PCR products were separated by electrophoresis (A and C), normalized to GAPDH and quantified by densitometry (B and D). Black bars indicate control and grey bars indicate p30\textsuperscript{II} expressing cells. Data points are mean of triplicates. CHP was downregulated while JUN and NFATc was upregulated by p30\textsuperscript{II}. Fold decrease / increase in activity in the presence of p30\textsuperscript{II} are indicated above each bar.
Figure 2.4. p30II activates NFAT, AP-1 and NF-κB transcriptional activity in Jurkat T lymphocytes. Black bars indicate control and grey bars indicate p30II. Data points are mean of triplicate experiments. Fold increase in activity in the presence of p30II is indicated above each bar. p30II increased the NFAT-luc activity from 2.2 to 10.7 fold depending on co-stimulatory treatment e.g., PMA, ionomycin, CD3, CD28 etc. (A), p30II increased NF-κB-luc activity from 3.1 to 11.4 fold (B) and modestly increased the AP-1 driven luciferase reporter gene activity from 1 to 5 fold in the presence of co-stimulator treatments (C).
CHAPTER 3

MOTIFS OF HUMAN T LYMPHOTROPIC VIRUS TYPE 1 P30\textsuperscript{II} CRITICAL FOR ITS INTERACTION WITH P300 AND REGULATION OF LTR TRANSCRIPTIONAL ACTIVITY.

INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T cell leukemia/lymphoma (ATL), and HTLV-1-associated myelopathy/ tropical spastic paraparesis (HAM/TSP) and many immune-mediated disorders\textsuperscript{48,26}. Molecular mechanisms used by the virus to circumvent immune elimination by the host and to facilitate lymphocyte proliferation are not fully understood. The genome of HTLV-1 encodes Gag, Pol, and Env, the common structural and enzymatic proteins characteristic of all retroviruses. Using alternative splicing mechanism and internal initiator codons, this complex retrovirus makes several regulatory and accessory proteins encoded by four open reading frames (ORFs) of the pX region (pX ORF I to IV) between the \textit{env} gene and the 3’ long terminal repeat\textsuperscript{16}. ORF III and IV encode the well characterized Rex and Tax proteins respectively. Rex is a nucleolar-localizing phosphoprotein, involved in
nuclear export of unspliced or singly spliced viral RNA\textsuperscript{21} while Tax is a nuclear-localizing phosphoprotein, which interacts with cellular transcription factors and activates transcription from the viral promoter containing repeats of TRE and enhancer elements of various cellular genes associated with host cell proliferation\textsuperscript{18,34,37,38,47}. pX ORFs I and II also produce alternatively spliced forms of mRNA, which encode four accessory proteins, p12\textsuperscript{I}, p27\textsuperscript{I}, p13\textsuperscript{II}, and p30\textsuperscript{II,17,12,17,28}. Less is known about the role of pX ORF I and ORF II in the replication or pathogenesis of HTLV-1. However, pX ORFs I and II mRNAs are present in infected cell lines and freshly isolated cells from HTLV-1-infected subjects\textsuperscript{28} and in ATL and HAM/TSP patients\textsuperscript{10}. Antibodies\textsuperscript{11,15} and cytotoxic T cells\textsuperscript{36} against recombinant proteins or peptides of the pX ORF I and II proteins are present in HTLV-1 infected patients, and asymptomatic carriers.

We have reported that mutations in the ACH.p30\textsuperscript{II} viral clone that insert an artificial termination codon in the p30\textsuperscript{II} mRNA prevent the virus from obtaining typical proviral levels in the rabbit model of infection\textsuperscript{41}. p30\textsuperscript{II} contains a highly conserved bipartite nuclear localization signal\textsuperscript{14} as well as serine-and-threonine-rich regions with distant homology to transcription factors Oct-1 and -2, Pit-1, and POU-M1\textsuperscript{12}. Taken together, these characteristics suggest that p30\textsuperscript{II} has a role in transcription of viral and cellular gene expression. In addition, a recent report demonstrated that p30\textsuperscript{II} modulates LTR mediated transcription, in the context of the entire provirus, at the post-transcriptional level, by retaining Tax/Rex mRNA in the nucleus\textsuperscript{33}. We have previously reported that, when provided in limiting concentrations, p30\textsuperscript{II} expression stimulated HTLV-1 LTR-driven reporter gene activity, even in the presence of Tax, whereas higher concentrations repressed LTR and CRE-driven reporter gene activity\textsuperscript{50}.

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We have shown that p30\textsuperscript{II} is co-localized with p300 in the nucleus and physically interacts with CBP/p300, at the highly conserved KIX domain, the domain HTLV-1 Tax also interacts with. CBP/p300 protein is available only at limiting concentrations within the cell nucleus, causing an environment for competition between coactivators and transcription factors, thus providing an additional layer of regulated gene expression\textsuperscript{35,43}. There is evidence of a functional antagonistic relationship between transcription factors, as a result of competition for binding to common regions of CBP/p300\textsuperscript{5,13,24}. Under such a condition of tight competition, relative concentrations of Tax/ p30\textsuperscript{II} at various stages of disease might be a critical factor in determining the levels of viral transcription. We have previously demonstrated that p30\textsuperscript{II} disrupts CREB-Tax-CBP/p300 complexes bound to the TRE repeats\textsuperscript{49}. At higher concentrations, p30\textsuperscript{II} may support viral persistence by reducing viral expression, thus reducing immune elimination of HTLV-1 infected cells and at low concentrations, enhance TRE/viral over CRE/cellular mediated transcription, thus promote viral transcription and spread of the virus \textit{in vivo}. This is also consistent with our previous results showing that an infectious HTLV-1 molecular clone failed to maintain viral loads \textit{in vivo} when p30\textsuperscript{II} expression was abolished\textsuperscript{41}.

Identifying the molecular mechanism and functional significance of the interaction between p30\textsuperscript{II} and p300 is crucial in understanding the role of p30\textsuperscript{II} in the pathogenesis and replication of HTLV-1. In this study, we have further characterized the nature of this interaction, by identifying the motifs within p30\textsuperscript{II} that are critical in binding CBP/p300 and in regulating LTR mediated transcription. Using N terminal and C terminal deletion mutants of p30\textsuperscript{II}, we have localized the binding site of p300 on p30\textsuperscript{II}.
These serial deletion mutants were employed to identify the domain of p30\textsuperscript{II}, which is important in regulating LTR mediated transcription. Using our serial deletion mutants of p30\textsuperscript{II}, we identified the domain of p30\textsuperscript{II} that is involved in regulating LTR mediated transcription, in the context of the provirus. By dissecting this amino acid domain of p30\textsuperscript{II} (100-179) critical for repressing LTR mediated transcription further, we identified that lysine residue at amino acid position 106 (K3) of HTLV-1 p30\textsuperscript{II} is critical for its repression of TRE-mediated transcription. More importantly, by adding p300, we were able to rescue the p30\textsuperscript{II}-mediated repression on LTR driven gene transcription, irrespective of the presence or absence of the provirus. Our data confirms the role of p30\textsuperscript{II} as a regulator viral gene transcription, in association with p300. This is the first report that dissects p30\textsuperscript{II} and identifies the functional domains important for binding CBP/p300 and in regulating LTR mediated transcription. Furthermore, we found that HTLV-1 p30\textsuperscript{II} represses Tax-mediated LTR transactivation, while HTLV-1 Tax rescues p30\textsuperscript{II}-mediated repression of LTR driven transcription, indicating the ability of these proteins to compete with each other in modulating HTLV-1 LTR transcriptional activity. Taken together, our data indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes, in conjunction with regulatory proteins, to promote cell-to-cell transmission of the virus, clonal expansion of infected cells and maintenance of proviral loads \textit{in vivo}. 
MATERIALS AND METHODS

Cell lines.

293T cells were obtained from G. Franchini (National Cancer Institute, NIH, Bethesda) and Hela-Tat cells were obtained from AIDS Research and Reference Reagent Program, National Institute of Health. 293T and Hela-Tat cells were grown in modified Dulbecco’s eagle medium containing 10% fetal bovine serum and 1% streptomycin and penicillin at 37°C. Cells were split and cultured in six-well plates, 10 cm dishes or chamber slides to 50-60% confluence 16-18 h before transfection.

Plasmids.

The pTRE-Luc plasmid and pRSV-B-Gal have been described previously.\textsuperscript{49,50} pME-p30\textsuperscript{II}HA wildtype and serial deletion mutant plasmids were created by cloning the p30\textsuperscript{II} sequence from HTLV-1 molecular clone, ACH with downstream influenza hemagglutinin (HA1) tag, into pME-18S plasmid (G. Franchini, National Cancer Institute) between 5’ EcoRI and 3’ NotI sites. Site directed mutants were made by substituting lysines in pMEp30\textsuperscript{II}HA with arginine. Fidelity of the plasmids were confirmed by Sanger sequencing and p30\textsuperscript{II} HA protein expression was confirmed by western blot using monoclonal anti-HA antibody (Covance, Berkeley, CA). ACH.30 plasmid has a 24 bp insertion that causes an artificial termination codon in p30\textsuperscript{II40}. pCMV-Tax expresses the HTLV-1 Tax protein and has been described previously.\textsuperscript{32} pCMV-p300 expresses the full-length p300 protein from a CMV I/E promoter (Upstate USA, Inc., Charlottesville, VA).
Cell transfection and reporter gene assay.

For each transfection, 0.3 µg of pTRE-Luc reporter plasmid was cotransfected with pME-p30\textsuperscript{II}HA wildtype or serial deletion / arginine substitution mutant plasmids and 0.4 µg ACH.30 plasmid when specified, using Lipofectamine Plus\textsuperscript{®} (Invitrogen, Carlsbad, CA). When pCMV-p300 plasmid was used, it was also co-transfected. As an internal control for transfection efficiency, 0.1 µg of pRSV-βGal (Invitrogen) was also used in each transfection. pME 18S was used as carrier DNA to equalize DNA concentrations for each transfection. Transfected cells were lysed with 400 µl lysis buffer (Promega, Madison, WI) per well for 25 min. Twenty microliters of each lysate was used to test luciferase reporter gene activity using an Enhanced \textsuperscript{®}Luciferase Assay kit (Promega). To normalize transfection efficiency, cells were washed with PBS, stained with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) (Sigma, St. Louis, MO) and counted βGal expressing cells using 20X objective of light microscope. Co-transfection of pME-p30\textsuperscript{II}HA had no effect on the X-Gal staining (data not shown). Results were expressed as mean of optimized luciferase activity (luciferase activity / percentage cells stained positive for β-Gal expression) in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was done using Student’s t test, P < 0.05.

Western immunoblot assay.

Transiently transfected cells were lysed in RIPA buffer containing PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Cell
lysates were prepared by centrifugation at 14,000 rpm (Beckman, Fullerton, CA) for 20 min at 4°C. Equal amounts of proteins were mixed with Laemmli buffer (62.5 mM Tris; pH 6.8), 2% SDS, 10% glycerol, 0.2% bromophenol blue, 100 mM dithiothreitol (DTT). After boiling for 5 min, samples were electrophoresed through 6 or 10% polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotechnology, Piscataway, NJ) at 100 V for 1 h at 4°C. Membranes were then blocked in Tris-buffered saline containing 5% nonfat milk and 0.1% Tween 20. Proteins were detected with primary monoclonal anti-hemagglutinin (HA) antibody (Covance) followed by an anti-mouse (Cell Signaling Technology, Beverly, MA) immunoglobulin G (IgG)-horseradish peroxidase-conjugated goat antibody. Blots were developed using an enhanced chemiluminescence detection system (Cell Signaling Technology).

Coimmunoprecipitation of p30II with p300.

Sixty percent confluent 293T cells were cotransfected by pME-p30IIHA, pME-p30IIHA serial deletion or arginine substitution mutants and pCMV-p300 using Superfect® (Gibco BRL, Gaithersburg MD). After 48 h, the transfected cells were washed with PBS and resuspended in 400 μl of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg of leupeptin per ml, and 1 μg of aprotinin per ml. Cell suspensions were incubated on ice for 20 min and then lysed by homogenization. The lysates were centrifuged at 14,000 rpm for 20 min at 4°C and incubated overnight at 4°C with 100 ng of a polyclonal HA antibody (Covance). After adding 40 μl of 100% of protein A-agarose slurry, the mixture
was incubated at 4° C for 1 h. The immunoprecipitated complexes were washed twice with 10 volumes of lysis buffer and three times with PBS buffer. The components of the complexes were resolved on 6% SDS-polyacrylamide gels and detected by western immunoblot assay using anti-p300 antibody (Santa cruz biotechnology, Santa cruz, CA). The density of each band was measured and compared to wildtype p30II using a commercial software package (Gel-pro Analyzer software; Media Cybernatic Inc., San Diego, CA). Normal rabbit IgG was used as a negative control in immunoprecipitation assays and equal amounts of input cell extracts were processed to determine the expression of p30II by western immunoblot assay.

**Localization of p30II and co-localization with CBP/p300 by confocal microscopy.**

To detect cellular colocalization of p30II and p300 by immunofluorescence, Hela Tat cells were seeded in two chamber slides (Fisher Scientific) at approximately 50% confluence 18 h prior to transfection. Transfection with 4 µg of pME-p30II-HA wildtype or pME-p30IIHA serial deletion mutants alone or with 2 µg of pCMV-p300 was performed using Superfect (Gibco BRL). At 48 h posttransfection, media were removed and cells were fixed for 15 min using 4% paraformaldehyde. Cells were then incubated with FITC (Fluorescin isothyocyanate) conjugated anti-HA antibody alone (diluted 1:1000; Santa Cruz) or with TRITC (Texas red isothyocyanate) conjugated anti-p300 antibody (diluted 1:1000; Molecular probes) in antibody dilution buffer containing 0.01 M sodium phosphate, 0.5 M NaCl, 0.5% Triton-X 100 and 2% bovine serum albumin overnight at 4° C. The expression of p30II-HA and p300 was evaluated using Zeiss LSM 510 confocal microscope.
RESULTS

HTLV-1 p30II localizes to the nucleus and colocalizes with p300 in the nucleus.

HTLV-1 p30II contains a nuclear localization signal, characteristic of most proteins that function as a transcription factor. Using immunofluorescence and immunoblot methods, previous reports have shown that HTLV-1 p30II was localized in the nucleus of transfected cells. To confirm the subcellular localization of p30II, we performed confocal microscopy on HeLa-tat cells transiently transfected with pMEp30IIHA. Anti-Phalloidin was used to stain the cytokeratin of the cells (Fig 3.1A). Consistent with previous reports, p30II was detected predominantly in the nucleus of our transiently transfected HeLa-tat cells by confocal microscopy (Fig. 3.1B). Based on nuclear localization, as typical of proteins that play a role in transcription, and the sequence homology of p30II with the POU family of transcription factors, we hypothesized that p30II has a role in transcriptional regulation. To test whether p30II colocalizes with p300 in the nucleus, we performed confocal microscopy of Hela-Tat cells transiently cotransfected with pME p30IIHA and pCMV-p300, and verified the subcellular localization of p30II (Fig. 3.1B) and p300 (Fig. 3.1C). Hoechst was used as a nuclear marker (Fig. 3.1D) and we found that p30II-HA colocalized with p300 in the nucleus of cells that expressed both proteins (Fig. 3.1E).
HTLV-1 p30\textsuperscript{II} physically interacts with p300

HTLV-1 p30\textsuperscript{II} contains a bipartite nuclear localization sequence and regions with sequence homology to the DNA binding domain and homeodomain of transcription factors such as Oct-1 (Fig. 3.2A). To test if p30\textsuperscript{II} physically interacts with the cellular transcriptional co-activator p300, we transiently transfected 293T cells with pCMV-p300 and pME p30\textsuperscript{II}HA plasmid encoding full-length p30\textsuperscript{II}, prepared whole-cell extracts and performed co-immunoprecipitation assays. Our data indicated that p300 was immunoprecipitated as a complex with p30\textsuperscript{II} (Fig. 3.2B). In contrast, p300 could not be detected in the complexes precipitated using preimmune serum while p30\textsuperscript{II}HA was detected from the cellular lysate (data not shown).

Amino acids 1-132 of HTLV-1 p30\textsuperscript{II} are critical for its physical interaction with p300

To identify the domain of p30\textsuperscript{II} that is required in its interaction with p300, we performed transient co-transfection of 293T cells with pCMV-p300 and various amino-terminal and carboxyl-terminal serial deletion mutants of p30\textsuperscript{II}, and whole-cell extracts were used for coimmunoprecipitation assays. These mutants of p30\textsuperscript{II} contained serial deletions of both the amino-terminal and carboxyl-terminal regions of p30\textsuperscript{II} (Fig. 3.2A). Each of the p30\textsuperscript{II} mutant proteins was expressed at the expected molecular weight, as indicated by immunoblot analysis (Fig. 3.2B). The subcellular localization characteristics of these mutants were tested by confocal microscopy. Mutants containing amino acids 1-220, 1-179, 1-132 or 71-220 localized to the nucleus while mutants containing amino acids 1-71, 100-179 and 179-241 localized to the cytoplasm (Fig. 3.3). As seen in Fig.
3.2B, we have confirmed that amino acids 1-132 of p30\textsuperscript{II} are critical for binding p300. The p30\textsuperscript{II} mutants 1-132, 1-220 and 1-179 (in the decreasing order) appeared to interact with p300 more intensely compared to the wildtype p30\textsuperscript{II}.

**HTLV-1 p30\textsuperscript{II} modulates LTR driven transcription.**

To test the effect of p30\textsuperscript{II} on LTR mediated transcription, we transiently transfected increasing concentrations of pME p30\textsuperscript{II}HA plasmid with constant amounts of our LTR-luc reporter plasmid and tested for luciferase reporter activity. We found that lower concentrations of the p30\textsuperscript{II} plasmid (below 0.6 µg) consistently activated the HTLV-1 LTR reporter gene activity, but increased amounts (above 0.6 µg) of the plasmid repressed LTR reporter gene activity (Fig. 3.4A). Consistent with our previous report, this data confirms that at low concentrations, p30\textsuperscript{II} has the potential to enhance LTR-mediated transcription while at higher doses, p30\textsuperscript{II} represses the LTR-mediated transcription\textsuperscript{50}.

We tested the ability of p30\textsuperscript{II} to regulate LTR mediated transcription in the presence of the HTLV-1 provirus. By transiently transfecting pME-p30\textsuperscript{II}HA at various doses with ACH.30, provirus deleted for p30\textsuperscript{II} expression, we have found that p30\textsuperscript{II} represses LTR mediated transcription, in a dose responsive fashion (Fig. 3.4B). Interestingly, at concentrations that activated LTR mediated transcription in the absence of ACH.30 (below 0.6 µg), we found that p30\textsuperscript{II} indeed repress LTR mediated transcription if the provirus is present. This difference could be due to the presence of other HTLV-1 proteins in the provirus.
p300 expression reverses the p30\textsuperscript{II}-dependent repression of LTR driven transcription, in the presence or absence of provirus, in a dose-responsive manner.

HTLV-1 Tax is known to activate the LTR. This transactivation function of HTLV-1 Tax is dependent on the co-adaptors CBP/p300. Tax is reported to bind p300 at the KIX domain, the same domain that p30\textsuperscript{II} binds to. Our previous reports suggested that p30\textsuperscript{II} and Tax serve different roles in the regulation of transcription. Based on the hypothesis that p30\textsuperscript{II} represses LTR-driven reporter gene activities as a consequence of competition for limited basal quantities of CBP/p300, we tested if overexpressing p300 rescues p30\textsuperscript{II}-mediated repression of LTR-driven reporter gene expression. By transiently transfecting increasing concentrations of pCMV-p300 with constant concentration of pME-p30\textsuperscript{II}HA plasmid, herein, we demonstrated that p300 expression reverses the p30\textsuperscript{II}-dependent repression of LTR-luciferase reporter gene activity, in the absence of the provirus (Fig. 3.5A). This data confirms that the transcriptional regulatory effect of p30\textsuperscript{II} is p300 dependent.

To test if the p30\textsuperscript{II} mediated repression of the LTR-mediated transcription is p300 dependent in the context of the provirus, we transiently transfected increasing amounts of pCMV-p300 plasmid with constant amounts of pME-p30\textsuperscript{II}HA plasmid and ACH.30, the provirus deleted for p30\textsuperscript{II} expression. Interestingly, even in the presence of the provirus, increasing doses of p300 reversed the ability of p30\textsuperscript{II} to repress the LTR mediated transcription, in a dose responsive fashion (Fig. 3.5B). Taken together, this data further confirm that the transcriptional regulatory function of p30\textsuperscript{II} on the LTR is p300 dependent, in the presence of the provirus as well.
HTLV-1 p30\textsuperscript{II} represses Tax-mediated LTR transactivation and HTLV-1 Tax rescues p30\textsuperscript{II}-mediated repression of LTR driven transcription

Since HTLV-1 p30\textsuperscript{II} and Tax bind CBP/p300 at the KIX domain and since the transcriptional regulatory functions of p30\textsuperscript{II} and Tax on the LTR are p300 dependent, we hypothesized that HTLV-1 Tax and p30\textsuperscript{II} might competitively influence the transcriptional regulatory function of each other on the HTLV-1 LTR. To test the effect of p30\textsuperscript{II} on Tax-mediated LTR transactivation, we transiently co-transfected increasing concentrations of pME p30\textsuperscript{II}HA plasmid with constant concentration of LTR-luc reporter plasmid, as well as pCMV-Tax plasmid and tested the luc reporter activity. We found that p30\textsuperscript{II} consistently repressed the Tax transactivation of the HTLV-1 LTR reporter gene activity, in a dose dependent manner (Fig. 3.6A). Conversely, to test the effect of HTLV-1 Tax on p30\textsuperscript{II}-mediated repression of the LTR transcriptional activity, we transiently co-transfected increasing concentrations of pCMV-Tax plasmid along with constant concentration of LTR-luc reporter plasmid as well as pME p30\textsuperscript{II}HA plasmid and tested the luc reporter activity. We found that Tax consistently rescued the p30\textsuperscript{II}-mediated repression of the LTR reporter gene activity, in a dose dependent manner (Fig. 3.6B).

HTLV-1 p30\textsuperscript{II} contains multiple domains critical for its repression of LTR driven transcription

To identify the structural domain of p30\textsuperscript{II} that is critical for repression of LTR driven transcription, 293T cells were transiently co-transfected with a series of carboxyl-terminal and amino-terminal truncation mutants of p30\textsuperscript{II} and LTR-luciferase reporter
plasmid, and was tested for their ability to reduce the LTR-luciferase activity. The luciferase activity elicited by LTR-luciferase construct, in the presence of the full-length p30\textsuperscript{II} was compared to luciferase activity in the presence of each of the serial deletion mutants (Fig. 3.7A). Luciferase activity elicited by the serially deleted mutants 1-220, 1-179, 1-132 and 1-71 were higher than that by the full-length p30\textsuperscript{II} (in the decreasing order of luciferase activity), while in the presence of mutants 179-241, 100-179 and 71-220 (in the decreasing order of luciferase activity), the luciferase activity was less than that in the presence of the wildtype p30\textsuperscript{II}. This data shows that the region between 71-220 is able to repress the LTR-luc reporter, even more than the wildtype p30\textsuperscript{II}, indicating that these amino acid sequences are critical for the repression of LTR driven transcription by p30\textsuperscript{II}. However, mutant containing amino acid sequences 1-220 appeared to elicit the highest LTR driven reporter activity, which suggests the existence of a domain that inhibits the transcriptional repressive function of p30\textsuperscript{II} in this mutant, which is likely amino acids 1-71. This is also consistent with the higher luciferase activity elicited by the mutant containing only amino acids 1-71, compared to the wildtype p30\textsuperscript{II}. However, it appears that amino acids 1-71 do not inhibit the repressive function of the wildtype p30\textsuperscript{II}. A comparison of 1-220 mutant p30\textsuperscript{II} and the wildtype p30\textsuperscript{II} raises the possibility of another critical domain at amino acids 220-241, which helps the wildtype overcome the inhibitory role by amino acids 1-71. This is also consistent with the increased ability of the mutant containing 179-241 to repress LTR mediated transcription, compared to the wildtype. However, we cannot rule out the possibility of a change in the physical conformation or other properties of the mutant proteins, causing the observed differences in their transcriptional activity. These data indicate that the amino acid sequence from 71-
220 of p30\textsuperscript{II} is important for its function as a repressor of LTR-mediated transcription. The mutant containing amino acid sequence 100-179 was also able to repress LTR-mediated transcription at levels similar to that of 71-220. The differences in activity between these mutant was not statistically significant (p < 0.15), and therefore, the amino acids 100-179 appears to be the structural motif within p30\textsuperscript{II}, most critical for its repression of LTR mediated reporter activity.

To determine if 100-179 itself is the structural domain that is critical for repressing LTR-mediated transcription, in the presence of the provirus, we performed transient transfection of 293T cells with ACH.30, LTR-luc and the carboxyl-terminal and amino-terminal truncation mutants of p30\textsuperscript{II} described above and measured the luciferase activity. The luciferase activity elicited by LTR-luciferase construct, in the presence of the full length / wildtype p30\textsuperscript{II}, in the context of the provirus was then compared to luciferase activity in the presence of each of the serial deletion mutants (Fig. 3.7B). Similar to the findings in the absence of the provirus, luciferase activity elicited by the serially deleted mutants 1-179, 1-220, 1-71 and 1-132 were higher than that by the full-length p30\textsuperscript{II} (in the decreasing order of luciferase activity), and in the presence of mutants 100-179, the luciferase activity was less than that in the presence of the wildtype p30\textsuperscript{II}. Taken together, these data indicate that the amino acid sequence 100-179 of p30\textsuperscript{II} is critical in repression of LTR-mediated transcription in the presence or absence of the HTLV-1 provirus.
Lysine 106 (K3) of HTLV-1 p30\(^{II}\) is critical for its repression of LTR driven transcription.

HTLV-1 p30\(^{II}\) contains 6 lysine residues (K1-K6) (Table 3.1). Interestingly, 5 of these are within the critical domain comprised of amino acids 100-179. Importantly, four (K2-K5) of the lysine residues within this domain are part of a consensus acetylation sequence (G/SK motif),\(^6\) representing potential acetylation sites for CBP/p300. To determine the role of these lysine residues in p30\(^{II}\)-mediated repression of LTR driven transcription, 293T cells were transiently co-transfected with the arginine substitution mutants for each lysine residue of p30\(^{II}\) and LTR-luciferase reporter plasmid, and was tested for their ability to reduce the LTR-luciferase activity. The luciferase activity elicited by LTR-luciferase construct, in the presence of the full-length p30\(^{II}\) was compared to luciferase activity in the presence of each of the arginine substitution mutants (Fig. 3.8A). Luciferase activity elicited by the arginine substitution mutant K3 (amino acid 106) was higher than that by the full-length p30\(^{II}\), pME empty plasmid, arginine substitution mutant K2 (amino acid 103), arginine substitution mutant K4 (amino acid 123) and arginine substitution mutant K5 (amino acid 143) (in the decreasing order of luciferase activity), indicating a loss of repression function with substitution of lysine at amino acid position 106. This data shows that the lysine residue at amino acid position 106 is critical for repressing the LTR-luc reporter.

To determine the role of these lysine residues in p30\(^{II}\)-mediated repression of LTR driven transcription, in the presence of the provirus, we performed transient transfection of 293T cells with ACH.30, LTR-luc and the arginine substitution mutants of p30\(^{II}\)
described above and measured the luciferase activity. The luciferase activity elicited by LTR-luciferase construct, in the presence of the full length / wildtype p30^{II}, in the context of the provirus was then compared to luciferase activity in the presence of each of the arginine substitution mutants (Fig. 3.8B). Similar to the findings in the absence of the provirus, luciferase activity elicited by the arginine substitution mutant K3 (amino acid 106) was higher than that by the full-length p30^{II}, pME empty plasmid, arginine substitution mutant K2 (amino acid 103), arginine substitution mutant K4 (amino acid 123) and arginine substitution mutant K5 (amino acid 143) (in the decreasing order of luciferase activity), indicating a loss of repression function with substitution of lysine at amino acid position 106. This data confirms that the lysine residue at amino acid position 106 is critical for repressing the LTR-luc reporter in the presence of the provirus as well. Taken together, these data indicate that the lysine residue at amino acid position 106 within p30^{II} is important for its function as a repressor of LTR-mediated transcription in the presence or absence of the HTLV-1 provirus.

**DISCUSSION**

Our data is the first to dissect p30^{II} and demonstrate the functional motifs of p30^{II} that are critical in binding p300 and in repressing LTR mediated transcription. Identification of these motifs is important for defining the molecular mechanism of p30^{II}-mediated repression of LTR-driven transcription. This information is vital in gaining more insight into HTLV-1 gene expression and pathogenesis. We found that the motif
critical for binding p300 is amino acid sequence 1-132 while the motif that plays a major role in repressing LTR-mediated transcription is amino acid sequence 100-179, which contains the entire DNA binding region, part of the homeodomain with homology to Oct-1 and POU family of transcription factors as well as 5 lysine residues, four of which are part of a G/SK consensus acetylation sequence representing potential acetylation sites for CBP/p300.

The coactivators CREB binding protein (CBP) and p300 mediate transcriptional control of various cellular and viral DNA binding transcription factors. Although these coactivators have divergent functions, they are similar in nucleotide sequence, are evolutionarily conserved, and are commonly referred to as CBP/p300. CBP and p300 are highly related and share many functional properties, however there is evidence that these factors are not interchangeable. Several cellular and viral proteins that interact with either CBP or p300 have been identified, including steroid and retinoid hormone receptors, CREB, c-Jun, c-Myb, Sap-1a, c-Fos, MyoD, p53, Stat-1/2, NF-κB, pp90rsk, TATA-binding protein, TFIIB, HTLV-1 Tax, adenovirus E1A, Kaposi’s sarcoma-associated herpes virus viral interferon regulatory factor protein, and simian virus 40 large T antigen.

CBP and p300 bridge transcription factors to relevant promoters, has intrinsic histone acetyltransferase (HAT) activity, and form complexes with proteins such as CBP/p300 binding protein-associated factor, which also exhibits HAT activity. Recently, there is increasing knowledge of the mechanism and functional significance of
the interactions between many viral proteins and CBP/p300. In the case of adenovirus oncoprotein E1A, interaction with CBP/p300 is critical for regulation of transcription, suppression of differentiation, and immortalization of cells in culture$^{1,3,31}$. Simian virus T antigen regulates the expression of a group of cellular genes by modifying the HAT activity of CBP/p300 or by bridging the gap between DNA binding transcription factors and components of the general transcription machinery$^{4,19}$. Identifying the molecular mechanism and functional significance of the interaction between p30$^{II}$ and p300 is very crucial in understanding of the role of p30$^{II}$ in the pathogenesis and replication of this important human pathogen.

Previously, using deletion mutants of CBP/p300 in GST pull-down assays, we localized the binding site of CBP/p300 for p30$^{II}$ to a highly conserved KIX region. Interestingly, KIX domain is the binding site of CBP/p300 for HTLV-1 Tax protein as well. In addition, p30$^{II}$ was able to disrupt CREB-Tax-CBP/p300 complexes bound to the viral 21-bp TRE repeats$^{49}$. These results indicate that p30$^{II}$ might act in contrast to the role of HTLV-1 Tax. The role of CBP and p300 coactivators in HTLV-1 gene expression has been the focus of many previous reports. HTLV-1 Tax, a transactivator of LTR mediated transcription, is critical in the activation of the HTLV-1 viral genes through its interaction with the p300 and CBP coactivators$^8$.

Although CBP and p300 mediate the activities of various transcription factors, many earlier reports prove that it is present in the cell only at limiting concentrations. Even small reductions in the concentrations of these coactivator are damaging in many
instances, like the human Rubinstein-Taybi syndrome where loss of a single CBP allele causes developmental defects. In experiments where transcription factors are overexpressed, it is possible that the capacity of the endogenous CBP/p300 is overridden and the effects due to competition may be more obvious. Many such studies have demonstrated the competition between different molecules for CBP/p300. Even though some of these involve two mutually antagonistic transcription factors with the same shared binding site on CBP/p300, competition does not necessarily require having the same binding site. In circumstances where CBP/p300 levels are limiting, there could be selective preference of one over the other, causing an exclusion of one of the proteins. In our study, we found that HTLV-1 p30II and Tax appear to compete with each other in modulating the transcriptional activity from the LTR. Since HTLV-1 p30II and Tax interacts with CBP/p300 through the same KIX domain, we believe that this is through competitive CBP/p300 binding between p30II and Tax. Similar mechanism involving Tax and CBP/p300 has been previously reported. The binding of Tax and c-Myb to KIX domain of CBP was found to be mutually exclusive and Tax expression was shown to interfere with transcriptional activity of c-Myb.

An environment of coactivator competition between transcription factors provides an additional layer of regulated gene expression. The relative amounts of Tax and p30II may be critical in determining which protein binds to CBP/p300 and the consequent regulation of LTR mediated transcription of viral genes from the HTLV-1 viral promoter. When its levels are low, p30II also enhance transcription from the viral promoter. Under such circumstances, p30II and Tax might act synergistically. However, at higher
concentrations, p30\textsuperscript{II} antagonizes the Tax transactivation of the HTLV-1 promoter. There are similar reports on positive and negative effects by the same protein in other viruses, such as herpes simplex virus type 1 (HSV-1), in which the interaction between cellular and viral transcription factors play a critical role in the regulation of the immediate-early (IE) gene promoter. VP16, a potent transcription factor from HSV-1, binds the host cell protein HCF, which facilitates the stable complex formation of the viral protein with Oct-1\textsuperscript{46}. The IE gene promoter contains an Oct-1-like motif (TAATGARAT) that is important for IE gene expression, with both positive and negative effects, depending on the context of these cellular transcription factors and VP16\textsuperscript{44}.

CBP and p300 bridge transcription factors to relevant promoters and through its intrinsic histone acetyl transferase (HAT) activity, acetylates lysine residues at the amino terminus of histones, which results in modification of chromatin structure to allow access of the transcriptional machinery. CBP/p300 is known to form complexes with proteins such as CBP/p300 binding protein-associated factor, which also exhibits HAT activity\textsuperscript{20}. p300 also directly acetylate transcription factors such as p53, GATA-1 and c-myb and thus augment their sequence-specific DNA-binding activity\textsuperscript{42}. Simian virus T antigen regulates the expression of a group of cellular genes by modifying the HAT activity of CBP/p300 or by bridging the gap between DNA binding transcription factors and components of the general transcription machinery\textsuperscript{4,19}. c-myb oncprotein of the avian retrovirus group is known to be acetylated by CBP/p300 at its multiple lysine residues\textsuperscript{13}. This is considered to be the mechanism by which CBP/p300 regulates the functions of c-myb. p30\textsuperscript{II} contains six highly conserved lysine residues, 5 of which are within the amino
acid region 100-179, four of which are part of the consensus acetylation sequence (G/SK motif)\(^6\) and thus represent potential acetylation sites for CBP/p300 (Table. 3.1). We found that the lysine residue at amino acid position 106 (K3) of HTLV-1 p30\(^\text{II}\) is critical for its repression of LTR driven transcription in the presence or absence of the provirus. Based on this, it is possible that the intrinsic HAT activity of CBP/p300 is utilized to acetylate and potentially modulate the transcriptional regulatory function of p30\(^\text{II}\).

Recently, it was reported that p30\(^\text{II}\) modulates LTR mediated transcription, in the context of the entire provirus, by a post-transcriptional mechanism.\(^3^3\) Previous studies from our laboratory demonstrated that HTLV-1 p30\(^\text{II}\) directly interacts with CBP/p300 to modulate gene transcription.\(^4^9,5^0\) Data presented in this report further confirms the role of CBP/p300 in the modulation of LTR mediated transcription by p30\(^\text{II}\), in the context of the provirus. In the presence of increasing concentrations of p300, there was a dose-dependent rescue of LTR driven gene transcription, irrespective of the presence or absence of the provirus. Although, we cannot rule out the possibility of a post-transcriptional mechanism by HTLV-1 p30\(^\text{II}\), this report presents convincing evidence for a p300 dependent transcriptional regulatory function. Therefore, HTLV-1 p30\(^\text{II}\) appears to be a multi-functional protein with transcriptional and post-transcriptional role in regulating gene expression.

As of yet, there is not much information regarding the relative levels of various HTLV-1 proteins during various stages of the infection. It is possible that differences in expression levels of various viral proteins, leading to differences in transcriptional
regulation as described above might be the mechanism by which HTLV-1 infection/disease progresses through various stages\textsuperscript{39}. This is likely to be in synergy with differential regulation of cellular gene regulation by Tax and/or p30\textsuperscript{II} and thus changing the immune responses in accordance with different stages of progression of HTLV-1 infection and disease. Our current data can be supported by recent findings from our laboratory where an infectious HTLV-1 molecular clone failed to maintain viral loads \textit{in vivo} when p30\textsuperscript{II} expression was abolished\textsuperscript{41}. Taken together, our data indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes to promote cell-to-cell transmission of the virus, clonal expansion of infected cells and maintenance of proviral loads \textit{in vivo}.
REFERENCES


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Lysine residue and amino acid position within p30\textsuperscript{II} | Percentage conservation
--- | ---
K1 (50) | 87.5
K2 (103) | 66.7
K3 (106) | 87.5
K4 (123) | 87.5
K5 (143) | 91.7
K6 (152) | 91.7

Table 3.1. Lysine residues in p30\textsuperscript{II} are conserved among various HTLV isolates. Amino acid position of each lysine is indicated in parenthesis.
Figure 3.1. p30II localizes to the nucleus and colocalizes with p300 in the nucleus of transiently transfected HeLa-Tat cells. Hela Tat cells were seeded in two chamber slides (Fisher Scientific) at approximately 50% confluence 18 h prior to transfection, and co-transfected with 4 µg of pME-p30II-HA and 2 µg of pCMV-p300 using Superfect (Gibco BRL). At 48 h post-transfection, media were removed and cells were fixed for 15 min using 4% paraformaldehyde. Cells were then incubated with FITC conjugated anti-HA antibody (diluted 1:1000; Santa Cruz) and TRITC conjugated anti-p300 antibody (diluted 1:1000; Molecular probes) overnight at 4° C. Panel A represents the cytokerin staining of the cells using anti-Phalloidin while Panel D represents the nuclear staining of the cell using Hoechst. The expression of p30II-HA (Panel B) and p300 (Panel C) were evaluated using Zeiss LSM 510 confocal microscope (40X) and the images were overlayed using Adobe photoshop (Panel E). These results are representative of a minimum of triplicate experiments.
Figure 3.2. Amino acids 1-132 of HTLV-1 p30\textsuperscript{II} are critical for its physical interaction with p300. (A) Schematic diagram of the known/putative functional domains of p30\textsuperscript{II}. Schematic diagram of the carboxyl terminal and amino terminal deletion mutants of p30\textsuperscript{II} is indicated. Bipartite NLS indicates the nuclear localization sequence. Regions with sequence homology to the DNA binding domain and homeodomain of Oct-1 are indicated. The numbers on both sides of p30\textsuperscript{II} indicate length of the peptide as number of amino acids. Letters within the boxes indicate amino acids in p30\textsuperscript{II} and Oct-1. (B) Top panel represents the immunoprecipitation of p300 with p30\textsuperscript{II} and serial deletion mutants of p30\textsuperscript{II}. Physical binding between various deletion mutants of p30\textsuperscript{II} and p300 was tested by expressing pCMV-p300 and pME-p30\textsuperscript{II}HA wildtype or serial deletion mutant plasmids in 293T cells, p300 pull-down from the cellular lysates using polyclonal anti-HA antibody, followed western immunoblot assay with anti-p300 antibody. Below each line, the binding is indicated, as density of each band measured by Gel-pro Analyzer software (middle panel). The results above are representative of three experiments. The protein expression of the carboxyl terminal and amino terminal deletion mutants of p30\textsuperscript{II} was tested using monoclonal anti-HA antibody (lower panel).
Figure 3.3. Subcellular localization characteristics of various p30\textsuperscript{II} serial deletion mutants p30\textsuperscript{II} in transiently transfected HeLa-Tat cells. Hela Tat cells were seeded in two chamber slides (Fisher Scientific) at approximately 50 % confluence 18 h prior to transfection, and co-transfected with 4 µg of each pME-p30\textsuperscript{II}-HA serial deletion mutant using Superfect (Gibco BRL). At 48 h post-transfection, media were removed and cells were fixed for 15 min using 4 % paraformaldehyde. Cells were then incubated with FITC conjugated anti-HA antibody (diluted 1:1000; Santa Cruz) overnight at 4\textdegree C. Cytokeratin staining of the cells was performed using Anti-Phalloidin. Amino acid domains present in each mutant is indicated. The expression of p30\textsuperscript{II}-HA was evaluated using Zeiss LSM 510 confocal microscope (40X). These results represent a minimum of triplicate experiments.
Figure 3.4. HTLV-1 p30\textsuperscript{II} differentially modulates LTR-mediated transcription in the absence or the presence of the provirus. (A) In the absence of the provirus, p30\textsuperscript{II} enhances LTR mediated transcription at low concentrations (upto 0.6 \(\mu\)g) and represses LTR mediated transcription at higher doses (above 0.6 \(\mu\)g) (B) In the presence of full-length provirus, p30\textsuperscript{II} represses LTR mediated transcription in a dose responsive fashion. 293T cells were transiently cotransfected with 0.3 \(\mu\)g of pLTR-luciferase reporter plasmid, increasing amounts of pME-p30\textsuperscript{II} HA, +/- 0.4 \(\mu\)g of ACH.30 proviral plasmid and the luciferase activity was measured. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student’s \(t\) test. * indicates P < 0.05.
Figure 3.5 p300 expression reverses the p30II-dependent repression of LTR driven transcription, in the absence or the presence of the provirus. (A) In the absence of the provirus, p300 expression reverses the p30II-dependent repression of LTR driven transcription, in a dose-responsive manner. (B) p300 reverses the p30II-dependent repression of LTR driven transcription, in the presence of the provirus, in a dose-responsive manner. 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30II-HA, increasing quantities of pCMV-p300, +/- 0.4 µg of ACH.30 proviral plasmid and the luciferase activity was measured. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student’s t test. * indicates P < 0.05.
Figure 3.6 HTLV-1 p30II and Tax appear to compete with each other in modulating the LTR mediated transcription. (A) p30II represses Tax-mediated LTR transactivation in a dose-dependent manner. (B) Tax rescues p30II-mediated repression of LTR driven transcription in a dose-dependent fashion. 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid along with either 0.4 µg of pCMV-Tax plasmid and increasing quantities of pME-p30II HA (Fig. 6A) or 1.2 µg of pME-p30II HA plasmid and increasing quantities of pCMV-Tax (Fig. 6B). pME 18S (Fig. 6A) or a plasmid containing CMV promoter (Fig. 6B) was used as carrier DNA to equalize DNA concentrations for each transfection. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student’s t test. * indicates P < 0.05.
Figure 3.7. Amino acids 100-179 of p30II is critical for its repression of LTR driven transcription, in the absence or the presence of the provirus. (A) In the absence of the provirus, amino acids 100-179 of p30II are critical for its repression of LTR driven transcription. (B) In the presence of the provirus, amino acids 100-179 of p30II are critical for its repression of LTR driven transcription. 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30II-HA wildtype or serial deletion mutants, +/- 0.4 µg of ACH.30 proviral plasmid and the luciferase activity was measured. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student’s t test. * indicates P < 0.05.
Figure 3.8. Lysine 106 (K3) of p30\textsuperscript{II} is critical for its repression of LTR driven transcription in the absence or the presence of the provirus. (A) In the absence of the provirus, lysine 106 (K3) of p30\textsuperscript{II} is critical for its repression of LTR driven transcription. (B) In the presence of the provirus, lysine 106 (K3) of p30\textsuperscript{II} is critical for its repression of LTR driven transcription. 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30\textsuperscript{II}-HA wildtype or site directed mutants, +/- 0.4 µg of ACH.30 proviral plasmid and the luciferase activity was measured. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student’s t test. * indicates P < 0.05.
CHAPTER 4

HISTONE ACETYLTRANSFERASE (HAT) ACTIVITY OF P300 MODULATES HUMAN T LYMPHOTROPIC VIRUS TYPE 1 P30II-MEDIATED REPRESSION OF LTR TRANSCRIPTIONAL ACTIVITY

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of an aggressive and often fatal CD4+ T cell malignancy, known as adult T-cell leukemia (ATL), a chronic neurodegenerative disease, called HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) and is also implicated in various immune-mediated disorders. The virus infection persists in 15-20 million people worldwide, but is a particular problem in endemic regions of the Caribbean, Japan, Africa, South America, and among at-risk populations within the United States. HTLV-1 is a complex retrovirus, which encodes the typical gag, pol and env gene products, and unique regulatory and accessory genes encoded in four open reading frames (pX ORF I-IV) between the env gene and the 3’ long terminal repeat (LTR). Four accessory proteins designated p12I, p27I, p13II and p30II are expressed from these highly conserved ORFs.
Using molecular clones of HTLV-1 with selective mutations of pX ORFs I and II, our laboratory was the first to identify functional roles of p12\textsuperscript{I}, p13\textsuperscript{II}/p30\textsuperscript{II} for establishment of infection in the rabbit model and for infection of resting T-cells\textsuperscript{7}.

p30\textsuperscript{II}, also known as Tof, is a 241 amino acid protein that localizes in the nucleolus and nucleus of transiently transfected cells. The viral protein has a bipartite nuclear localization signal (NLS) located between amino acids 73-98, which contains two arginine-rich stretches separated by a 12 amino acid spacer and serine/threonine-rich regions. This region shares distant homology to the activation domain of the POU family of transcription factors. We have previously demonstrated that p30\textsuperscript{II} functions as a transcriptional regulator and differentially modulates cAMP responsive element (CRE) and Tax-responsive element (TRE)-mediated transcription through its interaction with CBP/p300\textsuperscript{52}. At low concentrations, p30\textsuperscript{II} activated the transcription from the HTLV-1 LTR. However, at higher concentrations, p30\textsuperscript{II} repressed HTLV-1 LTR driven transcription\textsuperscript{52}. In addition, p30\textsuperscript{II} regulates CRE and TRE-mediated transcription in the presence or absence of Tax\textsuperscript{52}. We have also reported that p30\textsuperscript{II} interacts with cellular transcriptional regulator, CBP/p300, through the KIX domain, similar to the transactivating protein, Tax, encoded in pX ORF-III\textsuperscript{51}. More importantly, p30\textsuperscript{II} inhibits the assembly of Tax-p300-CREB multiprotein complexes on the TRE promoter\textsuperscript{51}. Recently, using serial deletion mutants of p30\textsuperscript{II}, we localized the domain of p30\textsuperscript{II} that is critical for its binding to CBP/p300 and in mediating its transcriptional activity. In addition, we also demonstrated that p30\textsuperscript{II}-mediated LTR repression can be rescued by p300 \textsuperscript{51}(Michael et al, 2004, submitted). Recently, Nicot et al \textsuperscript{34} reported a post-
transcriptional role of HTLV-1 p30\textsuperscript{II} in modulating LTR mediated transcription. Therefore, p30\textsuperscript{II} appears to be a multi-functional protein with transcriptional and post-transcriptional role in regulating viral gene expression.

Recent studies have provided insight into the mechanism and functional significance of the interactions between many viral proteins and CBP/p300. CBP and p300 bridge transcription factors to relevant promoters and through their intrinsic histone acetyl transferase (HAT) activity, acetylates lysine residues at the amino terminus of histones, which results in modification of chromatin structure to allow access of the transcriptional machinery. p300 also directly acetylates transcription factors such as p53 and GATA-1 and thus augment their sequence-specific DNA-binding activity\textsuperscript{43}. Additionally, CBP/p300 is known to form complexes with proteins such as p300/CBP-associated factor (P/CAF), which also exhibits HAT activity\textsuperscript{17}. HTLV-1 Tax also associates with the LTR through its interaction with CREB. These Tax-CREB promoter complexes act as a high-affinity binding site to recruit multifunctional cellular coactivators CBP, p300 and P/CAF to activate the expression of viral genes from the viral promoter\textsuperscript{28}.

Identifying the molecular mechanism and functional significance of the interaction between p30\textsuperscript{II} and p300 is crucial in understanding the pathogenesis and replication of HTLV-1. p30\textsuperscript{II} contains six highly conserved lysine residues, four of which are part of the consensus acetylation sequence (G/SK motif)\textsuperscript{6}, and thus represent potential acetylation sites for CBP/p300. We have previously demonstrated that lysine 106 (K3) of
p30II is critical in LTR repression (Michael et al, 2004, manuscript submitted). Based on this, we hypothesized that the intrinsic HAT activity of CBP/p300 is utilized to acetylate and potentially modulate the transcriptional regulatory function of p30II. Herein, we have characterized the interaction between p30II and p300 and demonstrate that p300 HAT mutants do not rescue p30II-mediated LTR repression as much as wild type p300. In addition, we also show that p30II is acetylated and that the deacetylation by histone deacetylase-1 (HDAC-1) enhances p30II-mediated HTLV-1 LTR repression. Moreover, inhibition of deacetylation by trichostatin A decreases p30II-mediated HTLV-1 LTR repression. This is the first report to demonstrate that the HAT activity of p300 is crucial in modulating HTLV-1 gene expression from the LTR by p30II. Overall, our study confirms the role of p30II as a regulator of viral gene transcription, in association with p300. Taken together, our data indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes to modulate viral gene expression, in context with cellular-mediated acetylation. Thus, HTLV-1 regulates its gene expression in concert with cellular transcriptional regulators to avoid immune elimination from the host, and facilitate clonal expansion of infected cells and maintenance of proviral loads in vivo.
MATERIALS AND METHODS

Cell lines.

293T cells were obtained from G. Franchini (National Cancer Institute, NIH, Bethesda) and grown in modified Dulbecco’s eagle medium containing 10% fetal bovine serum and 1% streptomycin and penicillin at 37°C. Cells were split and cultured in six-well plates or 10 cm dishes to 50-60 % confluence 18 h before transfection.

Plasmids.

The pTRE-Luc plasmid and pRSV-B-Gal have been described previously. pME-p30\textsuperscript{II}HA plasmid contains p30\textsuperscript{II} sequence from HTLV-1 molecular clone, ACH upstream of influenza hemagglutinin (HA1). Site directed mutants K2, K3, K4 and K5 harbor single arginine substitution of lysines in pMEp30\textsuperscript{II}HA at amino acid position 103, 106, 123 or 143 respectively. Fidelity of these plasmids were confirmed by Sanger sequencing and p30\textsuperscript{II} HA protein expression was confirmed by western immunoblot assay using monoclonal anti-HA antibody (Covance, Berkeley, CA ). ACH.30 plasmid has a 24 bp insertion that causes an artificial termination codon in p30\textsuperscript{II40}. pCMV-p300 expresses the full-length p300 protein from a CMV I/E promoter (Upstate USA Inc., Charlottesville, VA). HDAC-1 plasmid codes for HDAC-1 and was a kind gift from S. Schreiber, Harvard University, Cambridge, MA. Wild type and HAT mutants of P/CAF have been described previously.
Cell transfection and reporter gene assay.

For each transfection, 0.3 µg of pTRE-Luc reporter plasmid was cotransfected with pME-p30HA plasmid and 0.4 µg ACH.30 plasmid when specified, using Lipofectamine Plus® (Invitrogen, Carlsbad, CA). When pCMV-p300 wild type or mutant plasmids were used, it was also co-transfected at quantities specified. When HDAC-1 plasmid was used, it was also co-transfected at 0.0, 0.5, 1.0 or 1.5 µg concentrations. When specified, 0.0, 0.6, 1.2 or 2.4 µg of wild type P/Caf plasmid was also co-transfected. When HAT mutant P/Caf plasmid was used, 2.4 µg of this plasmid was also co-transfected. As described previously12, TSA (Sigma, St. Louis, MO) was added to the culture medium at 100 nm concentration, at 24 h posttransfection. As an internal control for transfection efficiency, 0.1 µg of pRSV-βGal (Invitrogen) was also used in each transfection. pME 18S was used as carrier DNA to equalize DNA concentrations for each transfection. Transfected cells were lysed with 400 µl lysis buffer (Promega, Madison, WI) per well for 25 min. Twenty microliters of each lysate was used to test luciferase reporter gene activity using an Enhanced Luciferase Assay kit® (Promega). To normalize the transfection efficiency, cells were washed with PBS, stained with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) (Sigma) and βGal expressing cells were counted using the microscope. Co-transfection of pME-p30HA had no effect on the X-Gal staining (data not shown). Results were expressed as mean of optimized luciferase activity (luciferase activity / percentage cells stained positive for β-Gal expression) in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was done using Student’s t test, P < 0.05.
**Immunoprecipitation of p30II with anti-acetyl lysine antibody.**

Sixty percent confluent 293T cells were cotransfected with 15 µg of either pME-p30IIHA alone or with 10 µg pCMV-p300 wild type or mutant plasmids using Superfect (Gibco BRL). After 48 h, the transfected cells were washed with PBS and resuspended in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 µg of leupeptin per ml, and 1 µg of aprotinin per ml. Cell suspensions were incubated on ice for 20 min and the lysates were centrifuged at 14,000 rpm for 20 min at 4°C and 1.5 mg of the lysate was incubated for 2 hours at 4°C with 100 ng of anti-acetyl lysine antibody (Upstate USA Inc). After adding 70 µl of 100% of protein A/G agarose slurry (Pierce, Rockford, IL), the mixture was incubated overnight at 4°C. The immunoprecipitated complexes were washed twice with 10 volumes of lysis buffer. The components of the complexes were resolved on 15% SDS-polyacrylamide gels and detected by western immunoblot assay using monoclonal HA antibody (Covance).
RESULTS

Histone acetyltransferase (HAT) activity of p300 modulates p30\textsuperscript{II}-dependent repression of LTR driven transcription

HTLV-1 Tax mediated transactivation of the viral LTR is dependent on transcriptional co-adaptors CBP/p300. Tax is reported to bind p300 at the KIX domain, the same domain that p30\textsuperscript{II} binds\textsuperscript{51}. Our previous reports suggested that p30\textsuperscript{II} and Tax serve different roles in the regulation of transcription. Recently, we also reported that overexpressing p300 rescues p30\textsuperscript{II}-mediated repression of LTR-driven reporter gene expression, indicating the possibility that p30\textsuperscript{II} represses LTR-driven reporter gene activities as a consequence of competition for limited basal quantities of CBP/p300. Our findings that the lysine residue at amino acid position 106 (K3) of p30\textsuperscript{II} is critical for repressing TRE-mediated transcription, suggested a potential role of acetylation in p30\textsuperscript{II}-mediated LTR repression (Michael et al, 2004, manuscript submitted). To test the role of p300 HAT domain on p30\textsuperscript{II}-dependent repression of LTR driven transcription, we transiently co-transfected pME-p30\textsuperscript{II}HA and an LTR-luciferase reporter plasmid along with either wild type or HAT mutant of p300 plasmids. In contrast to the wild type p300, p300 HAT mutants only partially rescued the p30\textsuperscript{II}-dependent repression of LTR-luciferase reporter gene activity (Fig. 4.1A).

We then tested the role of histone acetyltransferase activity mediated by p300 plasmids on p30\textsuperscript{II}-dependent repression of LTR driven transcription, in the presence of the HTLV-1 provirus. We transiently co-transfected pME-p30\textsuperscript{II}HA, LTR-luc reporter
plasmid and ACH.30, a provirus deleted for p30II expression, along with either wild type or HAT mutants of pCMV-p300, and tested the luciferase reporter activity. Interestingly, p300 HAT mutant plasmids failed to fully rescue p30II-dependent repression of LTR-luciferase reporter gene activity, compared to the wildtype p300 plasmid, in the presence of the HTLV-1 provirus as well (Fig. 4.1B). These data confirm that the HTLV-1 LTR transcriptional regulatory effect of p30II is dependent on the HAT activity of p300.

Deacetylation by HDAC-1 enhances p30II-dependent repression of HTLV-1 LTR driven transcription

Histone acetyl transferases and histone deacetylases (HDACs) form multiprotein complexes with transcriptional factors and are known to regulate their transcriptional activity. In addition, HDAC-1 is known to physically and functionally interact with HTLV-1 Tax and repress the LTR transactivation function of Tax. To test if deacetylation by HDAC-1 has a similar role on p30II-dependent repression of LTR mediated transcription, we transiently co-transfected increasing concentrations of HDAC-1 plasmid with constant concentration of pME-p30IIHA plasmid and LTR-luciferase reporter plasmid and tested the luciferase reporter activity. Our data indicated that HDAC-1 expression enhanced p30II-dependent repression of LTR-luciferase reporter gene activity, in a dose-dependent manner (Fig. 4.2A).

We then tested the role of deacetylation on p30II-dependent repression of LTR mediated transcription, in the presence of the HTLV-1 provirus, by transiently co-transfecting increasing concentrations of the HDAC-1 plasmid with constant
concentration of pME-p30^{II}HA plasmid, LTR-luciferase reporter plasmid and ACH.30, a provirus deleted for p30^{II} expression. Our data indicated that addition of HDAC-1 enhanced p30^{II}-dependent repression of LTR-luciferase reporter gene activity, in a dose-dependent manner, in the presence of the provirus (Fig. 4.2B). These data further support our findings that the HTLV-1 LTR transcriptional modulatory function of p30^{II} is dependent on deacetylation by HDAC-1.

**Acetylation by trichostatin A (TSA) decreases p30^{II}-dependent repression of HTLV-1 LTR driven transcription**

Reversible modification of the core histones is crucial in the regulation of gene expression. While histone acetylation is typically associated with nucleosomal decondensation necessary for transcriptional activation, histone deacetylation is associated with nucleosomal condensation and subsequent transcriptional repression. Repression of Tax transactivation of HTLV-1 LTR by HDAC-1 can be restored when treated with an HDAC inhibitor, namely, trichostatin A. Similarly, we tested the effect of inhibition of deacetylation on p30^{II}-dependent repression of LTR mediated transcription. To determine the most effective concentration of TSA, we transiently co-transfected 293T cells with 1.0 µg of HDAC-1 plasmid, 1.2 µg of pME-p30^{II}HA plasmid and 0.3 µg of LTR-luciferase reporter plasmid, and added either 0.0, 100, 200 or 400 nM TSA at 24 h post-transfection. We then tested the luciferase reporter activity at 48 h post-transfection and compared the difference in luciferase activity in the presence of various concentrations of TSA and found that 100nM TSA had the highest effect in decreasing
p30\textsuperscript{II}-dependent LTR repression (Fig. 4.3). Next, to determine the effect of TSA at different concentrations of HDAC-1, we transiently co-transfected 293T cells with increasing concentrations of a HDAC-1 plasmid with constant concentration of pME-p30\textsuperscript{II}HA plasmid and LTR-luciferase reporter plasmid, and added 100 nM TSA at 24 h post-transfection. We then tested the luciferase reporter activity at 48 h post-transfection and compared the difference in luciferase activity in the presence or absence of TSA. Our data demonstrated that TSA, an inhibitor of deacetylation decreases p30\textsuperscript{II}-dependent repression of LTR-luciferase reporter gene activity, at various doses of HDAC-1, in the absence of the provirus (Fig. 4.4A).

We then tested the role of inhibition of deacetylation on p30\textsuperscript{II}-dependent repression of LTR mediated transcription, in the presence of the HTLV-1 provirus, by transiently co-transfecting increasing concentrations of HDAC-1 plasmid with constant concentration of pME-p30\textsuperscript{II}HA plasmid, LTR-luciferase reporter plasmid and ACH.30, a provirus deleted for p30\textsuperscript{II} expression and by adding 100 nM TSA at 24 h post-transfection. We then tested the luciferase reporter activity at 48 h post-transfection and compared the difference in luciferase activity in the presence or absence of TSA. Interestingly, addition of TSA decreased p30\textsuperscript{II}-dependent repression of LTR-luciferase reporter gene activity, at various concentrations of HDAC-1, in the presence of the provirus as well (Fig. 4.4B). These data confirm that the HTLV-1 LTR transcriptional regulatory effect of p30\textsuperscript{II} is dependent on deacetylation by HDAC-1 and inhibition of deacetylation by TSA.
P/CAF does not rescue p30\textsuperscript{II}-dependent repression of HTLV-1 LTR driven transcription

P/CAF is an important coactivator that mediates transcription through HAT activity\textsuperscript{43}. P/CAF acetylates various nonhistone transcription-related proteins, such as the chromatin proteins HMG17 and HMG I(Y), activators p53, MyoD, human immunodeficiency virus (HIV) Tat, and general transcription factors TFIIE and TFIIF\textsuperscript{43}. In addition, P/CAF is recruited to the Tax responsive element sites on the HTLV-1 LTR, through direct interaction with Tax and enhance Tax-mediated HTLV-1 transcription, in a HAT-independent manner\textsuperscript{21}. We therefore tested if P/CAF has a role on p30\textsuperscript{II}-dependent repression of HTLV-1 LTR driven transcription. We transiently co-transfected increasing concentrations of P/CAF plasmid with constant concentration of pME-p30\textsuperscript{II}HA plasmid and LTR-luc reporter plasmid and tested the luciferase reporter activity. Our data indicated that, unlike p300, P/CAF does not rescue p30\textsuperscript{II}-dependent repression of HTLV-1 LTR driven transcription (Fig. 4.5). In addition, to test if the HAT activity of P/CAF influences p30\textsuperscript{II}-dependent repression of HTLV-1 LTR driven transcription, we transiently co-transfected pME-p30\textsuperscript{II}HA plasmid and LTR-luc reporter plasmid along with either wild type P/CAF plasmid or P/CAF HAT mutant plasmid, and tested the luc reporter activity. We found that the P/CAF HAT mutant did not significantly alter p30\textsuperscript{II}-dependent repression of HTLV-1 LTR driven transcription, compared to wild type P/CAF (Fig. 4.5). These data confirm that the HTLV-1 LTR transcriptional regulatory effect of p30\textsuperscript{II} is p300-dependent and not P/CAF-dependent.
HTLV-1 p30\textsuperscript{II} is acetylated by transcriptional co-activator p300

To test if HTLV-1 p30\textsuperscript{II} is acetylated by transcriptional co-activator p300, we transiently transfected 293T cells with pMEp30\textsuperscript{II}HA alone or in the presence of either the wild type p300 or p300 HAT mutants, and whole-cell extracts made at 48 h post-transfection were used for immunoprecipitation assays. We used anti-acetyl lysine antibody pull down and subsequent probing with monoclonal HA antibody. Demonstration of acetylation was performed with a weak, but consistent band, presumed to be acetylation. We detected the presence of a 27 kDa protein in the immunoprecipitated samples containing p30\textsuperscript{II}, which appeared more intense when expressed in the presence of exogenous wild type p300 (Fig. 4.6). Moreover, in the presence of p300 HAT mutants, we did not see an increase in the presence of the 27 kDa band, suggesting that the HAT activity of p300 is critical in the acetylation of p30\textsuperscript{II} (Fig. 4.6). Based on this data, it appears that HTLV-1 p30\textsuperscript{II} is acetylated by the transcriptional co-activator p300. Moreover, we tested four arginine substitution mutants, carrying a mutation in the lysine residues, and found that there was a decrease in the intensity of the 27 kDa band in the K3 mutant, compared to wildtype or K2 or K4 or K5 mutants of p30\textsuperscript{II} (Fig. 4.6). Interestingly, we have previously demonstrated that K3 mutant that contains an arginine substitution of the lysine at amino acid 106 of p30\textsuperscript{II} is critical for its repression of LTR driven transcription (Michael et al, 2004, manuscript submitted). Our data indicated that HTLV-1 p30\textsuperscript{II} is acetylated at lysine 106 (K3) by the transcriptional co-activator p300. Follow up experiments are being performed (a) using nuclear lysates (b) at different time points and (c) using an \textit{in vitro} transcription/translation system.
DISCUSSION

Our data is the first to demonstrate that the histone acetyl transferase activity of p300 is critical for modulation of HTLV-1 LTR mediated transcription by p30\textsuperscript{II}. These findings confirm previous reports from our laboratory on the transcriptional regulatory function of HTLV-1 p30\textsuperscript{II}\textsuperscript{51,52}. We have previously reported that the amino acid domain 100-179 of p30\textsuperscript{II} is critical for repression of HTLV-1 LTR, in the presence or absence of the provirus. Amino acids 100-179 of HTLV-1 p30\textsuperscript{II} contains 5 lysine residues, four of which are part of a G/SK consensus acetylation sequence\textsuperscript{6} representing potential acetylation sites for CBP/p300, and we have reported that the lysine residue at amino acid position 106 (K3) of HTLV-1 p30\textsuperscript{II} is critical for p30\textsuperscript{II}-mediated repression of LTR driven transcription in the presence or absence of the provirus (Michael et al, 2004, manuscript submitted).

CREB binding protein (CBP) and p300 are highly related and evolutionarily conserved transcriptional coactivators with similar nucleotide sequence, but divergent functions. CBP and p300 are not interchangeable, however they share many functional properties\textsuperscript{45}, such as mediating the transcriptional control of various cellular and viral DNA binding transcription factors\textsuperscript{9,16}. There are several cellular and viral proteins that interact with either CBP or p300, including steroid and retinoid hormone receptors, CREB, c-Jun, c-Myb, Sap-1a, c-Fos, MyoD, p53, Stat-1/2, NF-κB, pp90rsk, TATA-binding protein, TFIIB\textsuperscript{4,16,19,20}, HTLV-1 Tax, adenovirus E1A, Kaposi’s sarcoma-associated herpes virus (KSHV) viral interferon regulatory factor protein (vIRF), simian
virus 40 large T antigen, HIV-1 Tat, small delta antigen of hepatitis delta virus and Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA3C) and EBNA2, and herpes simplex virion protein-16 (VP16) \(^{1-3,10,23,27,29,44,46}\). Additionally, proteins such as papillomavirus E 2 protein employs novel mechanism of transcriptional activation, by interacting with cellular protein, AMF-1 to form complexes with p300\(^{36}\).

Acetylation at lysine residues within nucleosomal histones is known to neutralize the basic charge of lysine and thus loosen the integrity of nucleosomes. CBP and p300 bridge transcription factors to relevant promoters and through its intrinsic histone acetyl transferase (HAT) activity, acetylates lysine residues at the amino terminus of histones, which results in modification of chromatin structure to allow access of the transcriptional machinery. Recently, the mechanism and functional significance of the interactions between many viral proteins and CBP/p300 are increasingly understood. For example, interaction between the adenovirus oncoprotein E1A and CBP/p300 is critical for E1A-mediated regulation of transcription, suppression of differentiation, and immortalization of cells in culture\(^1,3,32\). Modification of the HAT activity of CBP/p300 or the bridging of the gap between DNA binding transcription factors and components of the general transcription machinery is the mechanism by which simian virus large T antigen regulates the expression of a group of cellular genes\(^4,16\). Acetylation of c-myb oncoprotein of the avian retrovirus group by CBP/p300 at multiple lysine residues is considered to be the mechanism by which CBP/p300 regulates the functions of c-myb\(^10\). Acetylation of hepatitis \(\delta\) virus small \(\delta\) antigen by p300, at lysine 72 is thought to influence its cellular localization and viral RNA synthesis\(^33\). HIV Tat is acetylated by p300 at lysine 50 and 51 in the TAR RNA binding domain, which promotes the dissociation of Tat from TAR
RNA during early transcription elongation. Acetylation of Tat is also thought to facilitate dissociation of the Tat cofactor cyclinT1 from TAR RNA, serving to transfer Tat onto the elongating RNA polymerase II. Overall, acetylation of Tat by p300/CBP is important for its transcriptional activation of the HIV promoter. EBNA3C, which is essential for EBV-dependent immortalization of human primary B lymphocytes, interacts with p300 and acetylation is thought to be critical in its function. In addition, EBV nuclear protein protein EBNA2 and herpes simplex VP16 acidic domains utilize the intrinsic HAT or scaffolding properties of p300 to activate transcription. Interestingly, KSHV vIRF interacts with and inhibits p300 HAT activity, resulting in drastic reduction of nucleosomal histone acetylation and alteration of chromatin structure and thus help the virus to circumvent the host antiviral immune response and to induce a global alteration of cellular gene expression.

An array of transcriptional activators, coactivators, and histone deacetylases are known to participate in the regulation of HTLV-1 transcription in infected T lymphocytes. Chromatin immunoprecipitation analysis investigating factor binding and histone modification at the integrated HTLV-1 provirus in infected T lymphocytes, demonstrated the presence of Tax, CBP, p300, histone deacetylases and a variety of ATF/CREB and AP-1 family members such as CREB, CREB-2, ATF-1, ATF-2, c-Fos, and c-Jun, at the HTLV-1 promoter. CBP stimulates Tax-mediated HTLV-1 LTR transcription initiation and reinitiation from a naked DNA template in vitro while p300 and P/CAF act as coactivators for Tax-dependent HTLV-1 LTR transcription. Tax directly interacts with both P/CAF and p300/CBP in a multi-histone acetyltransferase/
activator-enhancer complex. Histone H3 and H4 acetylation occurs at 3 regions within the proviral genome, and histone H4 acetylation on the HTLV-1 promoter is known to increase upon inhibition of histone deacetylases, leading to increase in viral RNA. Histone acetylation by CBP/p300 is critical in activation of HTLV-1 transcription from chromatin templates in vitro. Furthermore, it is necessary that the coactivator HAT activity has to be tethered to the template by Tax and CREB, since a p300 mutant that fails to interact with Tax was unable to facilitate transcription or acetylate histones. p300 mediates HTLV-1 LTR expression by targeted nucleosomal acetylation of histones H3 and H4, leading to RNAP II and TFIID recruitment to the chromatin template. CREB-2, also known as ATF-4, is known to activate the HTLV-1 promoter, in association with Tax. CREB-2 is acetylated by CBP/p300, but not by P/CAF, however CREB-2 acetylation does not affect the Tax transactivation of HTLV-1 LTR.

CBP and p300 mediate the activities of various transcription factors, however they are present only at limiting concentrations in cells. Even small reductions in the concentrations of these coactivator are damaging in many instances, such as the human Rubinstein-Taybi syndrome where loss of a single CBP allele causes developmental defects. Our study demonstrates that deacetylation by HDAC-1 enhances p30II-dependent repression of HTLV-1 LTR driven transcription while inhibition of deacetylation by TSA decreases p30II-dependent repression of HTLV-1 LTR driven transcription. Therefore, it appears that p30II acts as an activator of the LTR when acetylated and as a repressor when deacetylated. Since the level of p300 is limited in cells, we believe that the endogenous amount of p300 is sufficient to acetylate p30II when
provided at lower concentrations, but not at higher concentrations. This is consistent with our earlier reports that at lower concentrations, p30II acts as an activator of HTLV-1 LTR mediated transcription, and as a repressor of LTR mediated transcription at higher concentrations52 (Michael et al, 2004, manuscript submitted).

CBP/p300 is known to form complexes with proteins such as P/CAF, which is important for transcription as a coactivator and due to its ability to activate selective promoters via intrinsic HAT activity8,26,38,39,17. P/CAF interacts with p300 and CBP at the same site as adenoviral oncoprotein E1A in a competitive manner, and acetylates either free histones or nucleosomes49, primarily on lysine-14 of histone H3, and more weakly on lysine-8 of histone H441. P/CAF is important for transcription and is required as a HAT and coactivator43. Importantly, P/CAF has the ability to acetylate various nonhistone transcription-related proteins, such as the chromatin proteins HMG17 and HMG I(Y), activators p53, MyoD and general transcription factors TFIIE and TFIIF43. In addition, P/CAF acetylates HIV-1 Tat at Lys28 in the activation domain, and enhances Tat binding to the Tat-associated kinase, CDK9/P-TEFb24. P/CAF also interacts with and acetylates adenoviral E1 B protein, and this is known to interfere with the physical interaction between P/CAF and p53, preventing its activation and thus contributing to viral transformation30. P/CAF interacts with and acetylates polyomavirus large T antigen, thus stimulate DNA replication when tethered near the polyomavirus origin47. Besides, interaction between human papillomavirus E7 oncoprotein and the acetyl transferase domain of P/CAF, result in reduction of its acetyltransferase activity and thereby, alteration of cellular gene expression and growth5. In EBV-infected cells that do not
express the EBV encoded oncoproteins EBNA2 or LMP1, p300 expression enhances the
ability of EBNA2 to up-regulate LMP1 expression and through its intrinsic HAT activity,
P/CAF can further potentiate this p300 effect\(^4^6\). Interestingly, P/CAF is recruited to the
Tax responsive element sites on the HTLV-1 LTR, through direct interaction with Tax
and enhance Tax-mediated HTLV-1 transcription, in a HAT-independent manner\(^2^1\).
However, based on our findings, HTLV-1 p30\(^{\text{II}}\)-mediated repression of the LTR mediated
transcription appears to be dependent on p300, but independent of P/CAF.

Recently, it was reported that p30\(^{\text{II}}\) modulates LTR mediated transcription, in the
context of the entire provirus, by a post-transcriptional mechanism\(^3^4\). However, we have
previously reported that p300 rescues LTR driven gene transcription, in a dose-dependent
manner, irrespective of the presence or absence of the provirus. This report provides
additional evidence and further elucidates the mechanism of p300-dependent LTR-
mediated transcriptional modulation by HTLV-1 p30\(^{\text{II}}\). Therefore, HTLV-1 p30\(^{\text{II}}\) appears
to be a multi-functional protein with transcriptional and post-transcriptional role in
regulating gene expression.

Using deletion mutants of CBP/p300 in GST pull-down assays, previously, we
localized the binding site of CBP/p300 for p30\(^{\text{II}}\) to a highly conserved KIX region\(^5^1\).
Interestingly, KIX domain is the binding site of CBP/p300 for HTLV-1 Tax protein as
well\(^4^8\). In addition, p30\(^{\text{II}}\) was able to disrupt CREB-Tax-CBP/p300 complexes bound to
the viral 21-bp TRE repeats\(^5^1\). In circumstances where CBP/p300 levels are limiting,
proteins are known to compete for binding CBP/p300 and there could be selective
preference of one over the other, causing an exclusion of one of the proteins\(^1^0\). We have
previously reported that HTLV-1 p30\textsuperscript{II} and Tax compete with each other in modulating the transcriptional activity from the LTR, possibly through competitive CBP/p300 binding between p30\textsuperscript{II} and Tax \textsuperscript{52}(Michael \textit{et al}, 2004, manuscript submitted). Such an environment of coactivator competition between transcription factors provides an additional layer of regulated gene expression. Overall, we believe that the relative amounts of Tax and p30\textsuperscript{II} may be critical in determining which protein binds to CBP/p300 and the consequent regulation of LTR mediated transcription of viral genes from the HTLV-1 viral promoter. At low concentrations, p30\textsuperscript{II} and Tax might act synergistically to enhance transcription from the viral promoter, but at higher concentrations, we believe that p30\textsuperscript{II} might antagonize the Tax transactivation of the HTLV-1 promoter (Fig. 4.7). Information on the relative levels of various HTLV-1 proteins during various stages of the infection is lacking and it is possible that differences in expression levels of various viral proteins, leading to differences in transcriptional regulation might be the mechanism by which HTLV-1 infection/disease progresses though various stages. This is likely to be in synergy with differential regulation of cellular gene regulation by Tax and/or p30\textsuperscript{II} and thus changing the immune responses in accordance with different stages of progression of HTLV-1 infection and disease. Our present data can be supported by recent findings from our laboratory where an infectious HTLV-1 molecular clone failed to maintain viral loads \textit{in vivo} when p30\textsuperscript{II} expression was abolished\textsuperscript{42}. Overall, our data indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes to regulate viral gene expression from the LTR, avoid immune elimination by the host and facilitate clonal expansion of infected cells and maintenance of proviral loads \textit{in vivo}. 

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Figure 4.1. p300 HAT mutants only partially rescue p30\textsuperscript{II}-dependent repression of LTR driven transcription in the absence of the provirus (A) or the presence of the provirus (B). 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30\textsuperscript{II} HA, 2.4 µg of either the wild type p300 plasmid or one of the p300 HAT mutant plasmids, M3 or M7, +/- 0.4 µg of ACH.30 proviral plasmid and the luciferase activity was measured. A plasmid containing CMV promoter was used as carrier DNA to equalize DNA concentrations for each transfection. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed, comparing to the values obtained in the presence of p30\textsuperscript{II} and CMV empty plasmid, using Student’s \( t \) test. * indicates \( P < 0.05 \).
Figure 4.2. Addition of HDAC-1 enhances p30II-dependent repression of HTLV-1 LTR driven transcription in the absence of the provirus (A) or the presence of the provirus (B). 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30II HA, 0.0, 0.5, 1.0 or 1.5 µg HDAC-1 plasmid and +/- 0.4 µg of ACH.30 proviral plasmid and the luciferase activity was measured. A plasmid containing the same promoter as the HDAC-1 plasmid was used as carrier DNA to equalize DNA concentrations for each transfection. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed, comparing to the values obtained in the presence of p30II and no HDAC-1 plasmid, using Student’s t test. * indicates P < 0.05.
Figure 4.3. Addition of 100 nM Trichostatin A (TSA) causes a decrease in the enhancement of p30\textsuperscript{II}-dependent repression of HTLV-1 LTR driven transcription by HDAC-1. 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30\textsuperscript{II} HA and 1.0 µg HDAC-1 plasmid. At 24 h post-transfection, 100 nM, 200 nM or 400 nM TSA (Sigma) was added to the culture medium, as described previously\textsuperscript{12} and the luciferase activity was measured. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed, comparing to the values obtained in the absence of TSA, using Student’s \textit{t} test. * indicates P < 0.05. In the presence of 200 nM TSA, P = 0.23. In the presence of 400 nM TSA, P = 0.34
Figure 4.4. Addition of Trichostatin A causes a decrease in the enhancement of p30II-dependent repression of HTLV-1 LTR driven transcription by HDAC-1 in the absence (A) or the presence of the provirus (B). 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30II HA, 0.0, 0.5, 1.0 or 1.5 µg HDAC-1 plasmid and +/- 0.4 µg of ACH.30 proviral plasmid. A plasmid containing the same promoter as the HDAC-1 plasmid was used as carrier DNA to equalize DNA concentrations for each transfection. At 24 h post-transfection, 100 nM TSA (Sigma) was added to the culture medium, when specified, as described previously\textsuperscript{12} and the luciferase activity was measured. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed, comparing between values obtained in the presence or absence of TSA, at the same concentration of HDAC-1, using Student’s $t$ test. \* indicates $P < 0.05$. 
Figure 4.5. Expression of P/CAF does not rescue p30II-dependent repression of HTLV-1 LTR driven transcription. 293T cells were transiently cotransfected with 0.3 µg of pLTR-luciferase reporter plasmid, 1.2 µg pME-p30II HA and 0.0, 0.6, 1.2 or 2.4 µg wildtype P/CAF or 2.4 µg P/CAF HAT mutant plasmid, and the luciferase activity was measured. A plasmid containing the same promoter as the P/CAF plasmids (CMV promoter) was used as carrier DNA to equalize DNA concentrations for each transfection. Results represent the mean of optimized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed, comparing to the values obtained in the presence of p30II alone, using Student’s t test. Statistical significance was also performed, to compare between P/CAF wildtype and HAT mutant, using Student’s t test. * indicates P < 0.05.
Figure 4.6. HTLV-1 p30II appears to be acetylated by the transcriptional co-activator p300 at lysine 106 (K3). (A) p30II appears to be acetylated by the transcriptional co-activator p300. Demonstration of acetylation was performed with a weak, but consistent band, presumed to be acetylation. 293T cells were transiently cotransfected with 15 µg of pME-p30II HA wildtype alone or in the presence of 10 µg of either the wildtype p300 expression plasmid or p300 HAT mutant. Cell lysates were pulled down using anti-acetyl lysine antibody (Upstate USA Inc.) and subsequently immunoprecipitated with monoclonal anti-HA antibody (Covance). Follow up experiments are being performed (a) using nuclear lysates (b) at different time points and (c) using an in vitro transcription/translation system (B). p30II appears to be acetylated at lysine 106 (K3). Demonstration of acetylation was performed with a weak, but consistent band, presumed to be acetylation. 293T cells were transiently cotransfected with 15 µg of pME-p30II HA wildtype or arginine substitution mutants K2, K3, K4 or K5. Cell lysates were pulled down using anti-acetyl lysine antibody (Upstate USA Inc.) and subsequently immunoprecipitated with monoclonal anti-HA antibody (Covance). K2, K3, K4 and K5 represent single amino acid mutations in p30II, where arginine is substituted for lysine at amino acid position 103, 106, 123 or 143 respectively.
Figure 4.7. Schematic model diagram of the potential mechanism by which HTLV-1 p30II modulates LTR-driven transcription. Tax and p30II compete with each other in modulating the LTR-driven transcription, possibly through competitive CBP/p300 binding, since both the viral proteins bind CBP/p300 through the KIX domain. At low concentrations, p30II and Tax might act synergistically to enhance transcription from the viral promoter, but at higher concentrations, we believe that p30II antagonizes Tax transactivation of the LTR, by sequestering p300, and thus disrupting CREB-Tax-CBP/p300 complexes at the TRE repeats on the viral promoter.
CHAPTER 5

SYNOPSIS AND FUTURE DIRECTIONS

HTLV-1 is able to evade immune elimination by the host and facilitate lymphocyte proliferation, the molecular mechanisms of which are still not completely understood. Distinct features of HTLV-1 pathogenesis include HTLV-1 mediated T cell activation and transformation, which has been extensively investigated with specific focus on the role of HTLV-1 Tax\textsuperscript{12,14}. Tax, is the viral transcriptional transactivator protein, which is considered to be the major viral oncoprotein with critical role in HTLV-1 mediated T cell activation and lymphocyte transformation\textsuperscript{12,14}. Animal model studies and \textit{in vitro} transformation assays have established the oncogenic potential of Tax\textsuperscript{12,20,30}. Although Tax appears to be responsible for multiple events necessary for HTLV-1-mediated lymphocyte immortalization, it is not certain whether Tax aids the virus to establish persistent infection. Recent studies from our laboratory, using molecular clones of HTLV-1 with selective mutations of ORF I and II, demonstrated that the expression of the accessory proteins encoded by pX ORFs I and II, p12\textsuperscript{I} and p13\textsuperscript{II}/p30\textsuperscript{II} are critical for the efficient HTLV-1 infection and maintenance of viral loads \textit{in vivo} in a rabbit model of infection, although potentially dispensable for viral replication under activation conditions \textit{in vitro}\textsuperscript{1,4,34}. Additionally, previous reports from our laboratory\textsuperscript{42,43} and data
presented in this thesis demonstrate that HTLV-1 pX ORF II encoding protein, p30\textsuperscript{II} regulates viral gene expression from HTLV-1 LTR and may thus be critical for the virus to evade immune recognition and thereby facilitate the establishment of persistent infection \textit{in vivo}. In addition, data presented in this thesis demonstrate that p30\textsuperscript{II} plays an important role in regulating cellular gene expression and activates many key transcription factors involved in T cell activation, such as NFAT, NFκB and AP-1. These results not only add to our understanding of the role of HTLV-1 p30\textsuperscript{II} in HTLV-1 pathogenesis, but also shed light on the potential mechanism by which p30\textsuperscript{II} modulates HTLV-1 viral gene expression and cellular gene expression in T lymphocytes. Taken together, data presented herein, indicates that HTLV-1, a complex retrovirus associated with lymphoproliferative disorders, uses accessory genes products such as p30\textsuperscript{II} to promote lymphocyte activation to enhance clonal expansion of infected cells and maintain proviral loads \textit{in vivo}.

5.1 Role of p30\textsuperscript{II} in T cell signaling / activation and transformation

Gene array data presented in this thesis shed light on the possible role of p30\textsuperscript{II} in T cell signaling / activation. Expression of p30\textsuperscript{II} was associated with decrease in mRNA levels of CD28, a co-stimulatory molecule with a distinct role in T lymphocyte activation. Besides, p30\textsuperscript{II} expression appeared to upregulate Vav-2 and CD72 while downregulating CD46 and Lck tyrosine kinase, a member of the Src family protein tyrosine kinases that participates in signal transduction pathways initiated by T cell surface receptors such as TCR/CD3, CD2, CD4, CD8, and CD28. Additionally, p30\textsuperscript{II} expression correlated with decrease in the level of CHP, an endogenous calcineurin inhibitor, indicating a likely increase in calcineurin activity, which would be predicted to
correlate with upregulation of NFAT expression by p30II. Moreover, p30II expression was associated with increased expression of c-Jun and c-Fos, suggesting activation of AP-1 mediated transcription. In our gene array studies, p30II expression was associated with decreased expression of protein kinase D (PKD), which negatively modulates JNK signaling pathway, mediates cross-talk between different signaling systems, and is critical in processes as diverse as cell proliferation, apoptosis, immune cell regulation and tumor cell invasion. Interestingly, p30II expression was also associated with decrease in epidermal growth factor (EGF), which stimulates calcium influx and activates ETS domain transcription factor Elk-1. In Jurkat T lymphocytes expressing p30II, there were no detectable levels of IKKγ, which is important for NF-κB signaling in response to both T cell activation signals and Tax. However, p30II expression increased HPK-1 (Hematopoietic Progenitor Kinase-1), a known NF-κB activator. p30II expression was associated with decreased Ras GRP2, a guanyl nucleotide exchange factor that increases Ras-GTP, suggesting a decrease in the level of activated Ras (Ras-GTP). In addition, using luciferase reporter assays, we found that p30II enhanced NFAT, NF-κB and AP-1 driven transcription. Therefore, it is possible that p30II increases IL-2 production, cause T cell activation and lymphocyte transformation.

5.2 Identify the mechanism by which p30II activates NF-κB, NFAT and AP-1 mediated transcription

Herein, we demonstrate that p30II enhanced NFAT, NF-κB and AP-1 driven transcription. p30II increased the NFAT driven luciferase reporter gene activity by 2.2, 4.5, 7.7 and 10.7 fold, when left unstimulated, stimulated with PMA or ionomycin alone
or both respectively and 5.2, 2.4 and 5 fold in the presence of CD3 or CD28 alone or both respectively, indicating that p30\textsuperscript{II} effectively enhanced NFAT driven transcription, when stimulated with ionomycin or CD3. HTLV-1 p30\textsuperscript{II} increased the AP-1 driven luciferase reporter gene activity by 2.6, 5.2, 1.3, 4.9 fold, when left unstimulated, stimulated with PMA or ionomycin alone or both respectively and only 1.2, 1 and 1 fold in the presence of CD3 or CD28 alone or both respectively, indicating that p30\textsuperscript{II} effectively enhanced AP-1 driven transcription, significantly when stimulated with PMA. HTLV-1 p30\textsuperscript{II} increased the NF-κB driven luciferase reporter gene activity by 11.4, 3.7, 3.1 and 3.8 fold, when left unstimulated, stimulated with PMA or ionomycin alone or both respectively and 5.6, 6 and 3.6 fold in the presence of CD3 or CD28 alone or both respectively, indicating that p30\textsuperscript{II} is able to effectively enhance NF-κB driven transcription, significantly even under unstimulated conditions.

Binding of transcription factors such as, NFAT, AP-1 and NF-κB is required for the expression from the IL-2 promoter. While NFAT is vital to proliferation of peripheral lymphocytes for HTLV-1 infection \cite{38}, AP-1 is linked to the dysregulated phenotypes of HTLV-1 infected T cells \cite{11} and malignant transformation \cite{28}. Activation of AP-1 occurs through Tax-dependent and independent mechanisms in HTLV-1-infected T cells \textit{in vitro} and in leukemia cells \textit{in vivo} \cite{11}. NF-κB is highly activated in many hematopoietic malignancies, HTLV-1 infected T cell lines and in primary ATL cells, even when Tax expression levels are low \cite{28} and due to its anti-apoptotic activity, it is considered to be a key survival factor for several types of cancer.
In parallel, HIV-1 accessory proteins Nef and Vpr have been implicated in regulation of cellular gene expression during HIV-1 infection. Vpr-induced modest increase in NFAT, AP-1, NF-κB and LTR-dependent transcription is dependent on its ability to cause G2 arrest. While Nef stimulates calcium signaling to activate NFAT, Vpr potentiates Nef-induced activation of NFAT-dependent transcription by functioning farther downstream. Previous studies from our laboratory have indicated that p12I mediated NFAT activation is dependent on cytosolic calcium, similar to HIV-1 Nef. Similar to HIV-1 Nef and Vpr, it is possible that p12I and p30II act synergistically, to modulate NFAT driven transcription and subsequent T cell activation / signaling.

Data presented in this thesis demonstrates the first HTLV-1 accessory protein with broad modulating activities on the transcriptional activity of NF-κB, NFAT and AP-1. However, additional research is crucial in elucidating the mechanisms employed by p30II to enhance NFAT, AP-1 and NF-κB mediated transcription. It will also be valuable to determine the functional domains and specific amino acids within p30II, critical for the activation of NF-κB, NFAT and AP-1 mediated transcription.

5.3 Determine if p30II has an effect on IL-2 production and lymphocyte proliferation

Although T lymphocyte proliferation is highly regulated, HTLV-1 has developed numerous mechanisms to destabilize this control and induce T lymphocyte proliferation in the absence of appropriate signals. High levels of IL-2 are necessary to promote the progression of resting T cells through cell cycle, leading to T lymphocyte
proliferation\textsuperscript{16,17}. Most of the HTLV-1 infected T lymphocytes require IL-2 to proliferate during early stages of the infection, although the T lymphocyte proliferation becomes IL-2 independent later in the infection \textsuperscript{39}. The role of Tax on the IL-2 pathway and lymphocyte proliferation has been extensively investigated\textsuperscript{12}. In addition, previous studies from our laboratory showed that another HTLV-1 protein, namely, p12\textsuperscript{I} enhances IL-2 production in T lymphocytes\textsuperscript{9}. Data presented in this thesis demonstrates that p30\textsuperscript{II} enhanced NFAT, NF-κB and AP-1 driven transcription. Based on this, we hypothesize that p30\textsuperscript{II} has a major effect on IL-2 production and lymphocyte proliferation.

5.4 Does p30\textsuperscript{II} affect cell cycle progression in T lymphocytes?

Dysregulation of the cell cycle is a key concept in HTLV-1 leukemogenesis and the ability of Tax to dysregulate the cell-cycle machinery to regulate DNA replication and cell division has been a focus of a multitude of investigations. Interestingly, as described in this thesis, we found that p30\textsuperscript{II} expression altered the expression of multiple genes involved in the regulation of different stages of cell cycle. These include checkpoint suppressor 1, cytosolic branched-chain amino acid transaminase 1, histone deacetylase 6, cyclin B1, WEE1 kinase, CDC14A, Lck, JAK2, GAS7, BZAP45, Cullin, Rab6 GTPase activating protein (downregulated) and TERF1, AKAP8, DDX11, MSH2 and JUN (upregulated). Another gene downregulated by p30\textsuperscript{II} expression was MDM2, which, interestingly is overexpressed in certain leukemias\textsuperscript{24} and capable of enhancing the tumorigenic potential of cells by inhibiting p300 / PCAF mediated p53 acetylation\textsuperscript{27}. 
In parallel, HIV-1 Vpr is known to cause G2/M cell cycle arrest, characterized by low levels of Cyclin B1, by directly binding and inhibiting the in vitro activity of a phosphatase, Cdc25C13. In addition, the cell cycle regulatory kinase, WEE-1, is also depleted following prolonged G2 arrest induced by Vpr40. Interestingly, overexpression of WEE-1 suppresses Vpr-mediated apoptosis and is thought to regulate Vpr and γ irradiation-mediated apoptosis and possibly serve as a general regulator linking the cell cycle to some pathways of apoptosis40. Our gene array findings that p30II expression was associated with decreased expression of cyclin B1 and WEE-1, indicate another functional similarity between p30II and Vpr. Based on the clues available from our gene array study, future studies are essential not only to verify the findings, but also to test the functional and biological significance of p30II in HTLV-1 pathogenesis.

5.5 Does p30II affect cell-to-cell adhesion between T lymphocytes?

Lymphocytes naturally infected with HTLV-1 produce virtually no cell-free infectious HTLV-1 particles and cell-to-cell contact is necessary for efficient transmission of the virus between cells and between individuals7,10. However, until recently, there was little known about the mechanism of cell-to-cell spread of HTLV-1. Recently, Igakura et al19 demonstrated that cell contact rapidly induces polarization of the cytoskeleton of the HTLV-1 infected cell to the cell-cell junction. The molecules that mediate this process are not elucidated, and HTLV-1 Env protein is thought to be involved in this, because it is the only HTLV-1 protein expressed intact on the outside of the virus infected cell. Nonetheless, HTLV-1 is known to upregulate the expression levels of certain adhesion molecules such as integrins32,36, which would increase the chances of cell to cell adhesion.
Gene array data presented in this thesis shed light on the possible mechanisms by which p30\textsuperscript{II} functions in HTLV-1 pathogenesis and in leukemogenesis, including in cell-to-cell adhesion between T lymphocytes. Based on our findings, p30\textsuperscript{II} expression appeared to alter the expression of several genes involved in cell adhesion. These include decrease in integrin (integrin $\beta_8$) immunoglobulin (MADCAM1), a counter-receptor for P-selectin (SELPLG), cadherin (desmocollin 3), protocadherin (PC-LKC) liprin (PPF1BP1), CD84 / Ly-9, CD58, CD43 / sialophorin and glycosyl-phosphatidyl-inositol phospholipase D1. Expression of p30\textsuperscript{II} correlated with increase in integrin receptor $\alpha_1$ subunit and KIT ligand. Additional studies are essential to confirm these findings and to test their functional and biological significance in HTLV-1 pathogenesis.

5.6 Determine the significance of critical functional domains and specific amino acids within p30\textsuperscript{II}, \textit{in vivo} in rabbit model of infection

Data presented in this thesis demonstrates that amino acids 100-179 of p30\textsuperscript{II} are critical in regulating LTR mediated transcription, irrespective of the presence or absence of the provirus, in our LTR-luciferase reporter assays. We have also identified the domain of p30\textsuperscript{II}, which is critical in the interaction between p30\textsuperscript{II} and Tax. By dissecting the amino acid domain (100-179) of p30\textsuperscript{II} critical for repressing LTR mediated transcription further, we demonstrated that the lysine residue at amino acid position 106 (K3) of HTLV-1 p30\textsuperscript{II} is critical for its repression of TRE-mediated transcription. \textit{In vivo} experiments using infectious molecular clones with mutations in the motifs or specific amino acids, critical for the functions \textit{in vitro} are necessary to determine the biological significance of these findings. Therefore, the findings from the deletion and site directed
mutational analysis, presented in this thesis, are valuable in designing experiments to reintroduce these mutations into infectious molecular clones to test their functional significance in vivo.

5.7 Does HTLV-1 p30II form complexes at the HTLV-1 promoter, to modulate LTR mediated transcription?

An array of transcriptional activators, coactivators, and histone deacetylases are known to participate in the regulation of HTLV-1 transcription in infected T cells. Chromatin immunoprecipitation analysis investigating factor binding and histone modification at the integrated HTLV-1 provirus in infected T cells demonstrated the presence of Tax, CBP, p300, histone deacetylases and a variety of ATF/CREB and AP-1 family members such as CREB, CREB-2, ATF-1, ATF-2, c-Fos, and c-Jun, at the HTLV-1 promoter. CBP stimulates Tax-mediated HTLV-1 LTR transcription initiation and reinitiation from a naked DNA template in vitro while p300 and pCAF act as coactivators for Tax-dependent HTLV-1 LTR transcription. Tax directly interacts with both pCAF and p300/CBP in a multi-histone acetyltransferase/activator-enhancer complex and histone acetylation by CBP/p300 is critical in the activation of HTLV-1 transcription from chromatin templates in vitro. p300 mediates HTLV-1 LTR expression by targeted nucleosomal acetylation of histones H3 and H4, leading to RNAP II and TFIID recruitment to the chromatin template.

Our laboratory has previously demonstrated that p30II binds p300 at the KIX domain, which is the same domain that HTLV-1 Tax also binds to. Data presented in this thesis demonstrates that p30II and Tax compete to modulate the HTLV-1 LTR
mediated transcription. In addition, our data suggests that p30\textsuperscript{II} is acetylated and that the deacetylation by Histone Deacetylase-1 (HDAC-1) enhances p30\textsuperscript{II}-mediated HTLV-1 LTR repression while inhibition of deacetylation by trichostatin A decreases p30\textsuperscript{II}-mediated HTLV-1 LTR repression. Herein, we also demonstrate that the HAT domain of p300 is crucial in modulating HTLV-1 gene expression from the LTR by p30\textsuperscript{II}. It will be important to identify if p30\textsuperscript{II} interacts with proteins other than CBP/p300, present at the HTLV-1 promoter, and thus form complexes at the HTLV-1 promoter, to modulate the LTR mediated transcription.

5.8 Identify the functional domains and specific amino acids within p30\textsuperscript{II}, which are critical in nuclear retention of tax/rex mRNA and protein-protein interactions

Recently, Nicot \textit{et al.}\textsuperscript{31} reported that p30\textsuperscript{II} modulates LTR mediated transcription, in the context of the entire provirus, by a post-transcriptional mechanism, involving the nuclear retention of tax/rex mRNA. Identifying the functional domains and specific amino acids within p30\textsuperscript{II}, which are critical in its ability to retain tax/rex mRNA in the nucleus, will be of use in elucidating the mechanism of function of p30\textsuperscript{II} further. Ongoing studies in our laboratory are aimed at identifying various proteins that interact with p30\textsuperscript{II}. To elucidate the functional role of p30\textsuperscript{II} in HTLV-1 pathogenesis, it will be useful to identify the functional domains and specific amino acids within p30\textsuperscript{II}, which are critical in such protein-protein interactions. Various serial deletion and site directed mutants of p30\textsuperscript{II} described in this thesis, can be valuable tools in such investigations.
5.9 Temporal expression pattern and interaction between HTLV-1 regulatory and accessory proteins during various stages of the HTLV-1 infection

Based on recent reports from our own laboratory and data presented in this thesis, molecular functions for pX ORF I-encoded p12\textsuperscript{I} and ORF II encoded p30\textsuperscript{II} appears to be associated with HTLV-1-induced T cell activation, particularly in NFAT, NF-κB and AP-1 pathways\textsuperscript{2,8,9,23}. While the mechanism of p30\textsuperscript{II} function awaits additional research, p12\textsuperscript{I} function has been established to be calcium-dependent\textsuperscript{8}, independent of Tax and possibly occurs before Tax is expressed during a natural infection. However, highly activated T cells mediated by expression of p12\textsuperscript{I} and/or p30\textsuperscript{II} likely allow HTLV-1 provirus to integrate into host cell genome and permit the early viral infection. Since Tax is also able to cause T cell activation, it appears to be redundant for HTLV-1 to use multiple proteins to activate T lymphocytes. It is possible that these proteins act coordinately or synergistically. By modulating the expression of various HTLV-1 proteins, the virus might employ selective use of different viral proteins during different stages of the infection. Particularly, it will be important to test if p12\textsuperscript{I} and p30\textsuperscript{II} act synergistically or compete with each other, in modulating NFAT mediated transcription. Similar studies to elucidate the interaction of p30\textsuperscript{II} and Tax in modulating NF-κB mediated transcription may also be valuable in further understanding HTLV-1 pathogenesis.

In addition, previous results from our laboratory demonstrated that HTLV-1 Tax and p30\textsuperscript{II} both bind cellular transcriptional co-activator CBP/p300 at the KIX domain\textsuperscript{42}. Recent studies from our laboratory indicated that p30\textsuperscript{II} activate the HTLV-1 LTR
mediated transcription at lower concentrations, and repress the LTR mediated transcription at higher concentrations\textsuperscript{43}. Based on the data presented herein, HTLV-1 Tax and p30\textsuperscript{II} appears to compete with each other in modulating the HTLV-1 LTR mediated transcription. When its levels are low, p30\textsuperscript{II} also enhances transcription from the viral promoter. Under such circumstances, p30\textsuperscript{II} and Tax might act synergistically. However, at higher concentrations, p30\textsuperscript{II} antagonizes the Tax transactivation of the HTLV-1 promoter. In addition, the data presented in this thesis indicate that p30\textsuperscript{II} modulates the LTR mediated transcription in a p300 dependent manner. The amount of p300 is limited in cells and such an environment of coactivator competition between transcription factors is known to provide an additional layer of regulated gene expression\textsuperscript{6}. Interestingly, recent unpublished findings from our laboratory indicate that HTLV-1 p12\textsuperscript{l} increases the levels of p300. Therefore, it appears that the relative amounts of Tax, p30\textsuperscript{II} and p12\textsuperscript{l} may be critical in modulating LTR mediated transcription of viral genes from the HTLV-1 viral promoter. It is possible that differences in expression levels of various viral proteins leading to differences in transcriptional regulation, might be the mechanism by which HTLV-1 infection/ disease progresses though various stages. This is likely to be in synergy with differential regulation of cellular gene regulation by Tax, p30\textsuperscript{II} and p12\textsuperscript{l}, and thus changing the immune responses in accordance with different stages of progression of HTLV-1 infection and disease. However, since information on the expression profile of HTLV-1 proteins during different stages of the infection is limited, additional studies designed to test the temporal expression patterns of HTLV-1 regulatory and accessory proteins during viral infection are required to explore this possibility.


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