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HUMAN T CELL LYMPHOTROPIC VIRUS TYPE I GENOMIC EXPRESSION AND IMPACT ON INTRACELLULAR SIGNALING PATHWAYS DURING NEURODEGENERATIVE DISEASE AND LEUKEMIA

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1. ABSTRACT

HTLV-I has been identified as the etiologic agent of neoplasia within the human peripheral blood T lymphocyte population, and a progressive neurologic disorder based primarily within the central nervous system. We have examined the role of HTLV-I in these two distinctly different clinical syndromes by examining the life cycle of the virus, with emphasis on the regulation of viral gene expression within relevant target cell populations. In particular, we have examined the impact of specific viral gene products, particularly Tax, on cellular metabolic function. Tax is a highly promiscuous and pleiotropic viral oncoprotein, and is the most important factor contributing to the initial stages of viral-mediated transformation of T cells after HTLV-I infection. Tax, which weakly binds to Tax response element 1 (TRE-1) in the viral long terminal repeat (LTR), can dramatically *trans*-activate viral gene expression by interacting with cellular transcription factors, such as activated transcription factors and cyclic AMP response element binding proteins (ATF/CREB), CREB binding protein (CBP/p300), and factors involved with the basic transcription apparatus. At the same time, Tax alters cellular gene expression by directly or indirectly interacting with a variety of cellular transcription factors, cell cycle control elements, and cellular signal transduction molecules ultimately resulting in dysregulated cell proliferation. The mechanisms associated with HTLV-I infection, leading to tropical spastic paraparesis (TSP) are not as clearly resolved. Possible explanations of viral-induced neurologic disease range from central nervous system (CNS) damage caused by direct viral invasion of the CNS to bystander CNS damage caused by the immune response to HTLV-I infection. It is interesting to note that it is very rare for an HTLV-I infected individual to develop both adult T cell leukemia (ATL) and TSP in his/her life time, suggesting that the mechanisms governing development of these two diseases are mutually exclusive.

2. HISTORIC, EPIDEMIOLOGIC, AND CLINICAL PERSPECTIVES

Since equine infectious anemia virus (EIAV) was identified as the first retrovirus in 1904 (1), research concerning the retrovirus family (Retroviridae) has experienced tremendous growth. Retroviral infections have been reported in most vertebrate animals and some invertebrate animals, such as insects and mollusks (2). Traditionally, Retroviridae has been divided into three subfamilies based on pathogenic consequences of infection rather than genomic structures. They are the oncoviruses (Oncovirinae), the slow-growth viruses (Lentivirinae), and the foamy viruses (Spumavirinae) (3). However, since recent nucleotide sequence analyses have demonstrated that this traditional classification does not reflect relationships

at the genomic level, this classification is no longer utilized. The International Committee on the Taxonomy of Viruses (ICTV) has adopted a classification system which divides retroviruses into seven genera: avian-leukosis-sarcoma viruses, mammalian C-type viruses, B-type viruses, D-type viruses, the HTLV-BLV group, lentiviruses, and spumaviruses (2).

The identification of first human retrovirus, human T-cell lymphotropic virus type I (HTLV-I), was reported in 1980 (4). Subsequent serologic studies in 1982 led to the discovery of a second, highly related but distinct virus designated human T-cell lymphotropic virus type II (HTLV-II) (5). Both HTLV-I and HTLV-II belong to the HTLV-BLV Retrovirinae genus, and share about 65% nucleotide sequence similarity. Additional reports have suggested the existence of two additional human T cell lymphotropic viruses, HTLV-IV (6, 7) and HTLV-V (8, 9). The human immunodeficiency virus type 1 (HIV-1), which was originally designated as HTLV-III, is now grouped with the lentivirus family.

In 1977, Takatsuki and coworkers reported studies of a group of patients, all of whom had lymphoid neoplasms, with a well-defined clinical picture and a rather unusual distribution of birthplaces. The neoplastic process observed in this geographically clustered group of patients was referred to as adult T-cell leukemia (ATL) (10, 11). Shortly thereafter, HTLV-I was isolated from T-cell lymphoblastoid cell lines and primary peripheral blood lymphocytes from T-cell leukemia patients in the United States (4), and subsequent epidemiologic, immunologic, genetic, and molecular biologic studies demonstrated for the first time that a human virus, HTLV-I, was the etiologic agent of a human cancer, ATL (4, 12, 13). It is estimated that 1 to 2 million people are infected by HTLV-I in Japan alone, where the virus is endemic (14), and approximately 10 to 20 million people are HTLV-I carriers worldwide (15).

In 1985, Gessain and coworkers (16) found that a group of patients in Martinique with a slowly progressive neurologic disorder, referred to as tropical spastic paraparesis (TSP), had antibodies directed against HTLV-I. One year later, Osame and colleagues (17) reported the existence of HTLV-I-specific antibodies in HTLV-I-associated myelopathy (HAM) patients in a southern region of Japan. Subsequent studies have established that TSP and HAM are identical diseases, and that HTLV-I is the etiologic agent of this disease. The list of diseases associated with HTLV-I infection has been extended in the past several years to include HTLV-I-associated arthropathy (HAAP) (18), HTLV-I uveitis (HAU) (19), cutaneous T-cell lymphoma (CTCL) (20), and several other less characterized diseases (21-23). The molecular mechanisms involved in the HTLV-I-associated etiology of this broad spectrum of diseases in a rather small percentage of HTLV-I-infected patients are not yet clear. Possible explanations range from viral-mediated deregulation of cellular gene expression to tissue damage caused by the host immune response to virus infection.

HTLV-I is usually a sexually or congenitally transmitted virus, although other routes of transmission have been documented, including transmission by contaminated needles shared by intravenous drug abusers (24). Since HTLV-I is strongly cell-associated during invasion of its human host, cell-free virus has been difficult to demonstrate *in vivo*, although such an infection has been achieved recently in an *in vitro* experimental system (25). Routes of HTLV-I infection, all of which likely occur via the passage of infected cells, include (1) vertical transmission from mothers to their children via prenatal, transplacental blood exchange, the birthing process, or postnatal breast feeding (26-28); (2) heterosexual and homosexual transmission (28, 29); (3) transfusion of blood or blood products which contain infected white blood cells, red blood cells, or platelets (30-32); and (4) shared contaminated needles among drug addicts (33).

This review will focus on the role of HTLV-I in the etiology of neoplasia within the human peripheral blood T lymphocyte population, and a progressive neurologic disorder based primarily within the central nervous system. To this end, we will begin to address the role of HTLV-I in these two distinctly different clinical syndromes by examining the life cycle of the virus within relevant target cell populations and subsequently defining the impact of specific viral gene products on cellular metabolic function. This information will then be used as a foundation for discussing the etiological role of HTLV-I in leukemia and neurodegeneration within the immune and nervous systems, respectively.

3. OVERVIEW OF HTLV-I REPLICATION

3.1. Virus structure and life cycle

The mature virion is spherical and enveloped with a diameter of 110 to 140 nm. The host cell-derived viral membrane contains the glycoprotein spikes encoded by the viral *env* gene which encodes two protein components: a 21 kDa transmembrane protein (TM), and a 46 kDa membrane surface glycoprotein (SU). The center of the HTLV-I virion consists of a highly dense, spherical nucleocapsid containing two copies of the 9 kb genomic RNA (which bears all of the characteristics of eukaryotic mRNA), the virus-encoded reverse transcriptase (RT) and integrase (IN) enzymes, $tRNA^{pro}$ (2) which is required as a primer for the initiation of reverse transcription, and the viral protease (PR) enzyme which is responsible for the cleavage of HTLV-I structural proteins.

The life cycle of HTLV-I can be divided into two stages (Figure 1). The first stage includes viral entry, reverse transcription of the viral RNA into DNA, nuclear localization of the proviral DNA, and integration of the proviral DNA into the host cell genome. All these processes are accomplished in the absence of *de novo* viral gene expression by the viral structural proteins and several enzymatic proteins packaged within the virion (34). The second stage uses host cell gene transcription and protein synthesis machinery to complete the processes of viral gene expression and assembly.

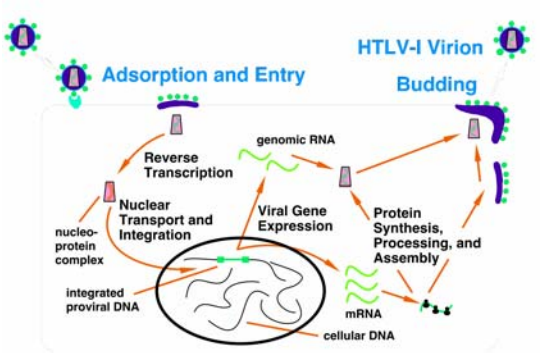

Figure 1. HTLV-I life cycle. Major events in the viral replication cycle include adsorption and entry, reverse transcription, nuclear transport and integration, viral gene expression, and viral protein synthesis, processing, and assembly.

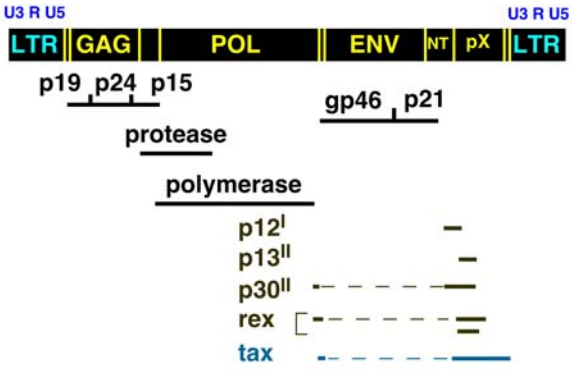

Figure 2. HTLV-I genomic structure. The viral genomic structure and the known viral genes are indicated. The viral mRNAs and the corresponding viral protein products are also shown. Dotted lines represent introns in the viral mRNAs.

Efficient HTLV-I entry into the host cell usually requires direct cell-cell interaction, although successful *in vitro* infections with cell-free virus particles have been documented in several cell lines (35, 36). *In vitro* infection is usually initiated by cocultivation of gamma-irradiated HTLV-I producing cells with target cells, although the infection efficiency is quite low compared to other retroviruses such as HIV-1. Viral attachment and entry into susceptible cells requires a specific cell surface receptor which has not yet been identified but is present on numerous cells, including those of non-human origin (37, 38). Although the majority of cells infected by HTLV-I *in vivo* are $CD4^+$ cells, the CD4 surface molecule has been demonstrated not to be the receptor for HTLV-I (39, 40). Several different approaches have been utilized to identify the receptor for HTLV-I. Sommerfelt and coworkers (41) generated a series of human-mouse somatic cell hybrids and correlated the susceptibility of these hybrids to HTLV-I infection with the presence of a particular human chromosome. In these experiments, all hybrids which were susceptible to HTLV-I infection contained human chromosome 17. Additional studies have localized the gene which encodes the receptor to chromosome 17q. In another approach to identify the cellular receptor for HTLV-I, monoclonal antibodies were used to block HTLV-I infection of susceptible cells. Galvachin and coworkers (40) identified a monoclonal antibody, Mab32-23, which specifically blocks the binding and entry of HTLV-I into activated peripheral blood mononuclear cells (PBMCs). The 32-23 antigen was expressed on the surface of Lq1, a human-mouse somatic hybrid cell line which retains human chromosome 17. Further studies will be necessary to elucidate the nature of this antigen and its corresponding gene.

After entry, reverse transcriptase within the viral capsid initiates the synthesis of the viral DNA by utilizing the single-stranded viral RNA as a template (42). The resultant double-stranded proviral DNA is then transported into the nucleus where the integration of the proviral DNA into the host genome proceeds with the assistance of viral integrase carried within the HTLV-I virion (43). HTLV-I integration appears to take place randomly in the host genome since no specific HTLV-I provirus insertion sites have been identified in most cases of ATL (11).

Following integration, the viral life cycle proceeds into the second stage which includes transcription of viral genes, translation of viral proteins, virion assembly, and virion release. All of these processes require participation of cellular transcription, translation, and transport machinery, as well as the assistance of a number of viral proteins (44). The integrated provirus can be passively spread to daughter cells following host cell division and can remain latent for a prolonged period of time. Following cellular stimulation (the nature of which is ill-defined), the provirus enters an active replication cycle which results in production of progeny virions. *In vitro*, HTLV-I virions are not efficiently released into the cell culture media from the infected target cells; the transfer of viral infectivity is usually accomplished via cell-cell contact (45).

3.2. Genes common to HTLV-I and all known retroviruses

The HTLV-I genome contains elements common to many retroviruses, as well as genes unique to HTLV-I. The structural proteins, the virion-associated enzymes, and envelope proteins are encoded by the *gag* (group-specific antigens), *pol*, and *env* genes respectively, which are common to all known retroviruses (Figure 2). After translation into a polyprotein, Gag is eventually cleaved into the 19 kDa matrix (MA), 24 kDa capsid (CA), and 15 kDa nucleocapsid (NC) proteins (45). MA is myristylated at its NH_2-terminal end and interacts with the inner side of lipid membrane (46). CA molecules interact with each other to form a capsid structure, the morphology of which is common to most retroviruses. NC is negatively charged and associates with two copies of the 9 kb viral RNA genome within the capsid structure. HTLV-I protease (PR) is encoded by an open reading frame that spans the 3' end of *gag* to the 5' end of *pol*; translation is achieved by ribosomal frameshifting (47). The catalytic activities of HTLV-I PR are required for the viral life cycle, since PR is

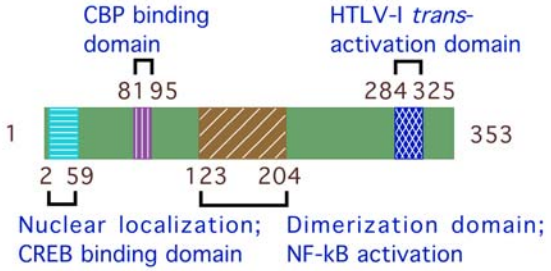

Figure 3. Domains of HTLV-I *trans*-activator Tax. Tax is primarily a nuclear protein and has pleiotropic functional properties involving interaction with multiple cellular transcription factors and signal molecules. The domains responsible for different functions of Tax illustrated in the figure have been determined by several studies (56-58).

responsible for generating mature Gag products (47). HTLV-I *pol* encodes enzymes that perform three distinct functions: Mg^{2+}-dependent reverse transcription, proviral DNA integration, and RNaseH digestion which specifically degrades the RNA in the RNA-DNA duplexes. The *env* gene encodes the viral membrane proteins that have been described previously.

3.3. Genes unique to HTLV-I

The pX region of the HTLV-I genome comprises four ORFs: X-I, X-II, X-III and X-IV (48). Two important viral regulatory proteins, Tax and Rex, are encoded in the distal portion of this region. Both are translated from doubly-spliced subgenomic mRNAs and are essential for the viral life cycle (49). While the 27 kDa Rex protein is primarily encoded by the X-III open reading frame, the 40 kDa Tax protein is mainly encoded by the X-IV reading frame. The initial codons for both Tax and Rex are located in the second exons of their mRNAs. In addition, a 21 kDa protein is encoded by ORF X-III and X-IV by using an internal AUG (50). Since antibodies directed against the C-terminal region of Rex also precipitated the 21 kDa protein, this protein has been referred to as $p21^{Rex}$. Although the function of $p21^{Rex}$ is not yet clear, limited studies have suggested that it may act antagonistically with Rex (51). Tax is a 40 kDa phosphorylated protein and is accumulated mainly in the nuclear matrix region of HTLV-I-infected cells (52, 53). Tax is a viral transcriptional activator and can dramatically increase viral gene transcription through its interaction with the 5' LTR of the proviral genome (54, 55). In addition, Tax can interact with multiple cellular transcription factors and signal molecules to exert pleiotropic functions. The domains responsible for different function of Tax have been determined by several studies (Figure 3) (56-58). Unlike Tax which regulates viral gene transcription directly, Rex (also a nuclear phosphoprotein), modulates viral gene expression at the posttranscriptional level (59). Rex increases the expression of viral genes *gag, pol,* and *env*, and inhibits the synthesis of Tax and Rex by promoting the nuclear export of nonspliced or singly spliced viral mRNAs (60). Rex-mediated nuclear export of nonspliced or singly spliced viral mRNAs requires two specific sequences in the viral genome. The Rex-responsive element (RxRE) maps to the 3' long terminal repeat (LTR) of the virus genome while the *cis*-acting repressive sequence (CRS) is located in the U5 region of the 5' LTR. It has been shown that the binding of Rex to RxRE overcomes the suppressive effect of CRS and favors the cytoplasmic expression of incompletely spliced viral mRNAs (61, 62).

The proximal 655 nucleotides of pX contain ORF X-I and X-II, which can be transcribed into four different mRNAs by alternative splicing (48). pX-ORF I mRNA can be either singly or doubly spliced. However, both species encode only one, highly hydrophobic 12 kDa protein, $p12^{I}$ (63). Although it has been shown that the doubly spliced mRNA pX-rex-ORF I can be translated *in vitro* to generate a 152 amino acid (aa) protein of 27 kDa, *in vivo* translation of pX-rex-ORF I cDNA only produces the 12 kDa protein due to internal initiation (63). In contrast, two protein species are derived from pX-ORF II by two different mRNA splicing events. While the singly spliced pX-ORF II mRNA yields an 87 aa protein of 13 kDa, ($p13^{II}$), the doubly spliced pX-ORF II mRNA encodes a 241 aa protein of 30 kDa, ($p30^{II}$) (63). The functions of $p12^{I}$, $p13^{II}$ and $p30^{II}$ have not been firmly established. Koralnik and coworkers utilized indirect immunofluorescence to examine the cellular localization of these three proteins in transfection assays (63). $p12^{I}$ is found to accumulate in the perinuclear area of cellular endomembranes while $p13^{II}$ and $p30^{II}$ accumulate in the nuclei and nucleoli of transfected cells, respectively. $p12^{I}$ and the E5 oncoprotein of the bovine papillomavirus type 1 (BPV-1) share a significant amount of aa similarity. Both proteins are very hydrophobic and are found in similar cellular compartments. Although $p12^{I}$ alone does not induce focus formation in mouse C127 cells, $p12^{I}$ greatly enhances the capacity of E5 to transform C127 cells in transient cotransfection assays. Furthermore, $p12^{I}$, like E5, can effectively bind to the 16 kDa component of the vacuolar H^{+} ATPase, a cellular target of E5 (64). Therefore, it appears that E5 and $p12^{I}$ evolved convergently to exert at least a subset of similar functions.

4. REGULATION OF HTLV-I GENE EXPRESSION

4.1. HTLV-I LTR and its role in regulating basal viral gene expression

The HTLV-I genome is flanked at each end by a long terminal repeat (LTR), a hallmark of retroviral genomic structure. Each LTR, composed of a U3 (unique 3'), R (repeated), and U5 (unique 5') region (Figure 4), is an integral component of the viral regulatory system and is essential to viral reverse transcription, integration, and transcription. The U3 region is important in regulating proviral gene expression as well as mRNA termination and polyadenylation (65). A salient feature of the HTLV-I LTR is the presence of three imperfect tandem 21-bp repeats in its U3 region which are responsible for Tax-mediated *trans*-activation; these three *cis* acting regulatory elements and intervening sequences have been collectively termed the Tax Responsive Element I (TRE-1). A high degree of sequence homology between these three repeats in LTRs derived from HTLV-I and HTLV-II is suggestive of their functional importance in viral gene expression (45). Each

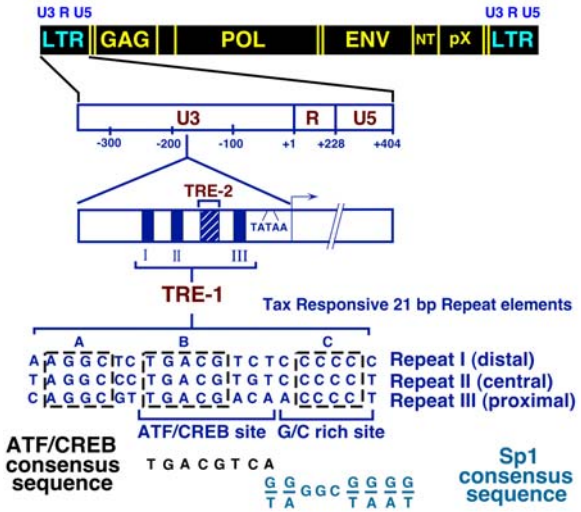

Figure 4. HTLV-I LTR structure. The viral LTRs are located at the both ends of the viral genome. Viral transcription is regulated by the sequence within the U3 region of the 5' LTR. Three 21-bp Tax-responsive elements, which are collectively referred to as Tax-responsive element 1 (TRE-1), are positioned within U3 region of the LTR at positions -251 to -231, -203 to -183, and -103 to -83 relative to the start of transcription. In addition, a second Tax-responsive element 2 (TRE-2) is located between the promoter proximal repeat and the promoter central repeat. The nucleotide sequences of the three 21-bp repeats as well as ATF/CREB and potential Sp1 binding sites are also illustrated.

21-bp repeat contains three completely conserved domains designated A, B, and C from promoter distal end to promoter proximal end. These three domains comprise 13 nucleotides of the 21 bp repeat. Domain B contains the first five of eight bp of the cAMP response element (CRE, TGACGTCA) and is sufficient for the Tax-mediated *trans*-activation in combination with either domain A or domain C (66-68).

The interaction of the HTLV-I LTR with the viral protein Tax and cellular proteins important in the regulation of gene transcription has been under intense investigation. The results from these studies have provided extensive information relevant to understanding the mechanisms involved in regulating viral gene expression. After reverse transcription, the HTLV-I proviral DNA is integrated into the host genome. Cellular transcription factors then bind to the viral LTR and induce the synthesis of a basal level of viral mRNA. Since there is little or no Rex present in the nucleus, the majority of mRNAs are doubly spliced and encode the products of pX region (including Tax and Rex). Tax, in turn, dramatically upregulates viral and cellular gene transcription. Each individual 21-bp repeat is unique with respect to its ability to interact with cellular proteins and Tax. Electrophoretic mobility shift (EMS) analyses performed utilizing oligonucleotides corresponding to each of the three individual repeat elements and nuclear extracts from several HTLV-I target cell lines have resulted in the detection of two DNA-protein complexes formed primarily with the promoter proximal repeat, and other complexes common to each 21-bp repeat (69-73). Antibody supershift and consensus oligonucleotide competition EMS analyses have shown that the DNA-protein complexes common to each 21-bp repeat consist of ATF/CREB family members (CREB, CREM, ATF-1, and ATF-2), whereas DNA-protein complexes unique to the promoter proximal repeat involved Sp1 and Sp3 (69, 72-74). In addition, Fos and Jun derived from U-373 MG glioblastoma cell line or mature monocytic cell line specifically bind the promoter central repeat (75).

The exact nature of the promoter proximal Sp binding sites has not yet been determined. A binding site sequence analysis utilizing TRANSFAC (The Transcription Factor Database) also failed to highlight a definitive Sp binding site within this sequence. This situation is not without precedent since previous studies have demonstrated that Sp1 can recognize and bind to a number of sites which may substantially deviate from the Sp1 consensus core sequence, GGGCGG (TRANSFAC). Because the C domain of the promoter proximal repeat is made up of a GC rich sequence, this portion of the promoter proximal repeat is likely capable of binding Sp1. Another candidate Sp binding sequence (AGGCGT) is located at the 5' end of the promoter proximal repeat and is exactly the same as the upstream Sp1 binding site (site III) in the human immunodeficiency virus type 1 LTR (76). EMS analyses (Yao and Wigdahl, unpublished results) indicated that while mutation in the conserved C domain of the promoter proximal repeat substantially reduced the binding of Sp factors, mutation of the AGGCGT sequence only marginally affected Sp binding to the proximal repeat. However, when both sequences were mutated, the binding of Sp factors to the proximal repeat was almost completely abolished. It has been well-established that the CRE binding site in each 21-bp repeat covers the entire B domain and the adjacent three nucleotides in its 3' end. Our experimental observations indicated that the primary Sp binding site is located in the conserved C domain. The proximal arrangement of binding sites for these two transcription factors may result in a spatial hindrance, which may not allow members from these two transcription factor families to bind to their corresponding sites at the same time. If this hypothesis is correct, mutations that specifically disrupt ATF/CREB binding to the proximal repeat will result in the increased abundance of Sp-DNA complexes, while mutations that specifically disrupt Sp binding to the proximal repeat will lead to increase in ATF/CREB binding. Our EMS analyses (unpublished data) and those of Barnhart (74) have clearly demonstrated competitive binding of Sp1 and CREB to the promoter proximal repeat. Although Sp1 can activate the HTLV-I LTR as well as a truncated promoter construct containing a minimal promoter and a single promoter proximal repeat in the *Drosophila schneider* SL-2 cell line (74), it will be necessary to further address the biological significance of Sp factor binding to the promoter proximal repeat as well as the nature of competitive binding between Sp and ATF/CREB family members to their sites in the promoter proximal repeat.

It has been demonstrated that a number of cellular transcription factors can bind to sequences other than the three 21-bp repeats in the HTLV-I LTR. The region located between the promoter proximal repeat and the promoter central repeat contains two Ets responsive elements, ERR1 and ERR2, which can bind members of Ets proto-oncogene family and mediate Ets1 and Ets2-dependent transcriptional activation (77, 78). Sp1 also can bind to this region to mediate Sp1-dependent activation (79, 80). Recently, Torgeman *et al.* (80) have demonstrated that this Sp1 site is responsible for a 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced, Tax-independent activation of HTLV-I LTR-directed expression. Although TPA exerts most of its biological effect through the protein kinase C (PKC) pathway, PKC is not involved in TPA-mediated Sp1-binding stimulation. Since the Sp1 protein level is not changed in TPA-treated cells, it would appear that a posttranslational modification of Sp1 is responsible for the TPA-mediated effect. A third Sp binding site has been identified in the U5 region in the viral LTR and serves as a repressive element (81, 82).

4.2. Tax-mediated *trans*-activation of HTLV-I LTR

Tax-mediated *trans*-activation of HTLV-I LTR is dependent on TRE-1 and a sequence located between the promoter central repeat and the promoter proximal repeat called Tax responsive element 2 (TRE-2) (79, 83). The three 21-bp repeat transcriptional enhancers act in a position and direction independent manner. However, it is still controversial whether a single copy of the 21-bp repeat is sufficient to support a Tax-induced enhancer activity. Brady and coworkers constructed a series of CAT reporter mutants which contained a minimal HTLV-I LTR promoter and a number of HTLV-I enhancer constructs in different orientations. These experiments suggested that plasmids containing a single 21-bp repeat were only marginally *trans*-activated by Tax, whereas plasmids containing two 21-bp repeats were *trans*-activated 30-fold in the sense orientation or 16-fold in the antisense orientation (83). Studies by a number of other groups have demonstrated similar results (54, 84-86). Therefore, it is a generally accepted notion that two or more copies of 21-bp repeat sequences are required for significant *trans*-activation by Tax. Nevertheless, Montagne *et al*. cloned a single copy of the promoter proximal repeat upstream of the rabbit beta-globin gene promoter, and determined the promoter activity in the presence or absence of Tax by utilizing a quantitative S1 nuclease protection assay. Under these experimental conditions, the promoter containing one copy of the promoter proximal repeat was strongly stimulated by Tax, and addition of an extra 21-bp repeat only resulted in a moderate increase of the enhancer effect (66).

In order to determine the effect of each individual 21-bp repeat on Tax-mediated *trans*-activation of a minimal HTLV-I promoter, rather than the heterologous promoter construct utilized by Montagne, we have constructed a series of luciferase reporter mutants in which each of three 21-bp repeats was cloned upstream of the HTLV-I minimal promoter. Utilizing transient expression analyses, we have demonstrated that a single copy of the promoter proximal repeat can be *trans*-activated by Tax to about 20% of the level obtained by a full-length HTLV-I LTR (unpublished data). We are currently investigating the functional roles of the promoter distal and the promoter central repeats in Tax-mediated *trans*-activation. At first inspection, these results would appear to contradict those of Brady *et al*. (83). However, the studies performed by Brady and other investigators utilized a CAT reporter system, which is significantly less sensitive than the luciferase reporter system utilized in our studies. In agreement with other investigators utilizing CAT reporter systems, we also have shown that a full-length HTLV-I LTR construct exhibited minimal promoter activity under basal conditions. In contrast, we readily detected promoter activity under basal conditions when a luciferase reporter gene driven by a full-length LTR or a single copy of the promoter proximal repeat construct was utilized.

Although a tremendous amount of knowledge has been accumulated in the past several years, the mechanisms by which the HTLV-I LTR is *trans*-activated by Tax are not yet fully appreciated. Previous *in vitro* DNA footprinting analysis clearly demonstrated protection over each of three 21-bp repeats and other regions of U3. This pattern of protection was unchanged in the presence of Tax (87, 88). Similar results were obtained utilizing EMS analyses (85, 89, 90). Consequently, it has been a long held position that Tax does not bind to the viral DNA but, rather, it exerts its effect by specific interactions with cellular intermediaries (91-93). However, recent evidence suggests that Tax does directly interact with the promoter proximal repeat (94). This strategy is exploited by several other well-characterized viral *trans*-activators such as herpes simplex virus VP16 and adenovirus E1a (95). In 1989, Giam and Xu generated a series of mutants which covered the full-length of the promoter distal repeat and determined whether these mutants were capable of *trans*-activation by Tax (85). These studies demonstrated clearly that mutations located in sequences homologous to the CRE (TGACGTCA) severely diminished *trans*-activation by Tax. Furthermore, the mutations which abolished Tax *trans*-activation were clustered exclusively in the 5' six bases, which indicated the importance of element orientation in *trans*-activation. However, our recent observations have shown that while the 5' six bases of the CRE are important in *trans*-activation, mutation of the last base of the CRE at its 3' end and its 3' adjacent base resulted in a 70% reduction in Tax-mediated transient expression activity when compared to the parental promoter proximal repeat truncation construct. This observation is consistent with the hypothesis that each 21-bp repeat is unique in its ability to bind cellular transcription factors and/or Tax (Yao and Wigdahl, unpublished observations).

A number of genes encoding bZIP DNA binding proteins that specifically interact with the CRE have been identified and cloned (96, 97). The 43 kDa CREB protein of the ATF/CREB family is the prototypical bZIP DNA binding protein. It has a leucine zipper domain in its carboxyl terminus, a *trans*-activation domain in its

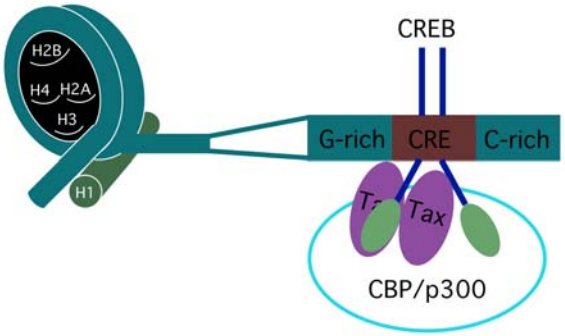

Figure 5. Model of Tax-mediated *trans*-activation of the HTLV-I LTR. A dimer of ATF/CREB transcriptional factors can bind to the CRE site in each 21-bp repeat in the HTLV- LTR. Then, Tax dimerizes and subsequently binds to the basic region of bZIP domain as well as 5' GC-rich DNA sequences flanking the CRE sites. Finally, CBP/p300 is recruited to Tax in a CREB-phosphorylation-independent manner to form a CBP-Tax-CREB-21-bp quaternary complex to upregulate viral gene expression (94, 108, 124).

amino terminus, and a basic DNA binding domain next to the leucine zipper domain. Different members of the ATF/CREB family can bind a CRE as homodimers or heterodimers that form via the leucine zipper domain (98). Nyborg and colleagues have shown that CREB and ATF-2 are the two major T-cell proteins that directly bind to the 21-bp repeats and stimulate HTLV-I transcription *in vitro* (99). Tax *trans*-activates the HTLV-I LTR by enhancing the DNA binding of bZIP proteins, including CREB and ATF-2, to the 21-bp repeats. The Tax-mediated enhancement of DNA binding activity of these proteins appears to be achieved by an increase in bZIP protein dimerization (100, 101). Although CREB and ATF-1 share a high degree of aa sequence homology, Tax effectively interacts with the CREB homodimers or CREB-ATF-1 heterodimers but not with the ATF-1 homodimer (92). It has been puzzling how Tax enhances the DNA binding activity of a number of bZIP proteins that display substantial aa sequence variation. In order to determine the region(s) of the bZIP domain necessary for Tax function, Perini and coworkers (102) utilized EMS analyses to examine the DNA-binding activity of a series of bZIP derivatives. Surprisingly, the results of their experiments demonstrated that the conserved basic region of the bZIP domain is required for increased Tax-dependent DNA binding, which can be abolished by a single change in one of several conserved amino acids. In contrast, no particular sequence in the leucine zipper region was required for Tax function. Furthermore, Tax can selectively alter the DNA binding affinity of several bZIP proteins for the four DNA sites tested. The ability of Tax to increase the DNA binding of bZIP proteins is dependent on both the core binding elements and their flanking sequences. Similar conclusions were also reached by Baranger and coworkers (103). Shenyreva and Munder utilized a modified yeast two hybrid system to further demonstrate that Tax-stimulated transcription requires an unmasked amino terminus of Tax, suggesting that the amino terminal region of Tax is responsible for interaction with CREB (104).

There is a general consensus that Tax-stimulated DNA binding of bZIP proteins is a two-step process involving a direct interaction between Tax and the bZIP basic region. This results in a decreased dissociation constant between the bZIP dimers, and subsequent decrease of the dissociation rate and/or enhancement of the association rate of these dimers with respect to binding DNA sequences (101, 103, 105). However, interaction of Tax with bZIP proteins and formation of a stable ternary Tax-CREB-21-bp repeat complex is not well understood. There are two models to explain Tax-mediated *trans*-activation. Several studies have indicated that it is possible to detect the Tax-bZIP-DNA complex in solution. However, Tax falls off under standard native gel electrophoresis conditions (99, 101, 102, 106). Thus, in the first model, it appears that Tax may function as a molecular chaperone to enhance the dimerization and DNA binding of bZIP proteins and selectively modify their DNA binding specificity (102). On the other hand, studies by Giam and coworkers consistantly demonstrate the presence of the ternary Tax-CREB-21-bp repeat complex in EMS analyses under their experimental conditions (92, 107, 108), and experiments performed by Giebler *et al*. also demonstrated similar results (109). Tie and coworkers used chemical cross-linking, gel filtration chromatography, and a series of Tax mutants with defined functional phenotypes to address the role of Tax in DNA-protein complex formation. These studies indicated that Tax forms a dimer to interact with CREB and this dimerization is essential for Tax to exert its function as a *trans*-activator (108). Jin and Jeang utilized yeast one hybrid and two hybrid systems to map the region(s) in Tax responsible for its dimerization to its zinc finger domain (110). M22 (T130A, L131S), mutant forms of Tax, which have an impaired ability to dimerize and assemble a ternary complex with CREB and the 21-bp repeat, has an impaired *trans*-activation capacity. While Tax mutants M1 and M47 possess similar dimerization capacities in comparison to the wild type Tax, they fail to interact with CREB and the basal transcription factors, respectively (111). Consequently, they are defective in *trans*-activation of TRE-1. Since the M47 mutant still enhances DNA binding of a number of bZIP proteins, it is likely that Tax does not act as a chaperone or a catalytic enzyme to upregulate binding of bZIP proteins to DNA. More likely, Tax upregulates viral gene expression by actively interacting with CREB to participate in assembly of the Tax-CREB-21-bp repeat ternary complex with a stoichiometry of $Tax_2/CREB_2/21\text{-bp repeat}_1$ (108) (Figure 5). Tang and coworkers further demonstrated that Tax-CREB interactions require an intact alpha helix spanning almost the entire CREB basic DNA binding domain. Specifically, amino acid residues Arg^{284}, Met^{291}, and Glu^{299} within the CREB and ATF-1 basic domains are involved in direct contacts with Tax. These three residues are separated by approximately two helix turns and are all positioned on the opposite side of the bZip helix from the conserved DNA-binding residues (112).

4.3. Tax-mediated *trans*-activation of CRE-containing cellular promoters

While Tax can dramatically *trans*-activate the HTLV-I LTR, it can also upregulate expression of several cellular genes, such as interleukin-2, interleukin-2 receptor alpha, and c-*fos*, as well as the HIV-1 LTR (113-116). In addition, Tax has been shown to downregulate the expression of the DNA polymerase beta gene (117). Tax-mediated activation of other cellular and viral promoters requires protein binding sites other than the CRE motif. For instance, Tax activates NF-kappaB resulting in the translocation of NF-kappaB from the cytoplasm into the nucleus to upregulate transcription of the interleukin-2 receptor alpha and HIV-1 LTR (114, 116). The c-*fos* promoter is activated by Tax through a serum response factor binding site (113). In contrast to Tax-mediated *trans*-activation of the HTLV-I LTR, most cellular gene promoters containing CREs have been found to be largely refractile to Tax (90, 105). However, genetic analysis of Tax mutants has suggested that cellular genes whose promoters contain CRE site(s) play an important role in Tax-mediated cell transformation (118). These data suggest that there are two different mechanisms by which Tax activates cellular and HTLV-I CREs, respectively.

Paca-Uccaralertkun and coworkers (95) performed a series of experiments to determine DNA sequences preferentially bound by the Tax-CREB complex *in vitro*. In their experiments, a number of 47-mer oligonucleotides (containing 15 random bases flanked by restriction site sequences) were incubated with purified CREB and Tax or CREB alone, and the complexes were precipitated by an antibody directed against the COOH-terminal region of Tax or an antibody against CREB. The selected DNAs were cloned and sequenced. After sequencing 34 plasmid clones, two groups of sequences containing a CRE in the middle flanked by a long stretch of G and C residues in the 5' and 3' regions, respectively, were predominantly bound by Tax-CREB. In contrast, CREB alone recognizes only CRE core consensus motifs (GNTGACGT/C) without flanking G- or C-rich sequences. The Tax-CREB selected sequences were very similar to the HTLV-I 21-bp repeats and can be effectively *trans*-activated by Tax. EMS analyses, DNase I footprinting, and transient expression analyses have demonstrated that while G- and C-rich sequences flanking CRE core elements are critical for the formation of the Tax-CREB-DNA ternary complex as well as Tax *trans*-activation, they are not involved in direct contact with the Tax-CREB complex. These results suggest that Tax interacts with CREB resulting in expanded DNA binding specificity of CREB, and forms a multiprotein complex which binds specifically to HTLV-I 21-bp repeats. Similar results have been obtained by Anderson and Dynan (100). Brauweiler and coworkers (105) also performed *in vitro* DNA binding assays and transient expression analyses with the HTLV-I 21-bp repeat sequences as well as a consensus CRE sequence from the human chorionic gonadotropin gene (hCG) promoter. Their observations indicated that Tax can specifically stabilize CREB on the 21-bp repeat but not on the cellular consensus CRE, and this Tax-dependent stabilization is consistent with the *in vivo* Tax *trans*-activation results. In order to determine whether the sequence responsible for both Tax stabilization of CREB binding and Tax-mediated *trans*-activation lies within the 21-bp CRE-like core or within G- and C-rich flanking sequence, two hybrid sequences were generated. One contained the consensus CRE core (TGACGTCA) flanked by sequences from the HTLV-I promoter proximal 21-bp repeat. The second sequence was comprised of the CRE-like core sequence (TGACGACA) derived from the HTLV-I promoter proximal 21-bp repeat flanked by sequences derived from the hCG consensus CRE site. The results from these studies clearly demonstrated that the CRE core, whether consensus or non-consensus, had no effect on Tax-mediated stabilization of CREB binding *in vitro* and Tax-mediated *trans*-activation *in vivo*. In contrast, the sequences adjacent to the CRE core exhibited a striking impact on Tax-dependent CREB binding stability as well as Tax-mediated *trans*-activation. The construct containing the consensus CRE core and the 21-bp repeat flanking sequence displayed an increased DNA binding affinity for CREB in the presence of Tax, and permitted Tax *trans*-activation. These data further support the concept that the sequences that flank the CRE-like core in the 21-bp repeat play a critical role in conferring Tax-mediated *trans*-activation. A similar conclusion was also reached by Yin *et al*. with a CRE site derived from somatostatin gene promoter (119, 120). They further proposed, based on Scatchard analysis, that CREB binds to the somatostatin CRE in a single-step high-affinity binding reaction, whereas CREB complex formation with the 21-bp repeats involves both low- and high-affinity binding reactions (120). Recently Lenzmeier *et al*. utilized high resolution methidiumpropyl-EDTA iron (II) footprinting to demonstrate that Tax widened the CREB footprinting into the GC-rich sequences flanking the viral CRE in the promoter proximal repeat of the HTLV-I LTR. The footprint extension by Tax was specific for the viral CRE since Tax did not exert the similar effect on a cellular CRE. Cross-linking experiments further demonstrated that Tax could be specifically cross-linked to the 5'-flanking sequence of the viral promoter proximal CRE. The cross-linking could be inhibited by chromomycin A_3, a minor-groove DNA binding compound. These recent observations support the concept that it is necessary for Tax to directly bind to the viral 21-bp repeats to exert its *trans*-activation potential (94).

In 1994, several groups reported that CREB was phosphorylated at Ser-133 by protein kinase A (PKA) in response to cellular signaling pathways. Phosphorylation of CREB facilitates binding of a co-factor, CREB-binding protein (CBP), and activates transcription (121, 122). Later, it was demonstrated that adenoviral E1a-associated protein p300 functions as a homologue of CBP (123). In order to compare the ability of Tax to *trans*-activate cellular CREs and HTLV-I CRE-like sequences, Kwok *et al*. generated reporter gene constructs containing either a single copy of the cellular somatostatin CRE or the HTLV-I U3 region. These constructs were transiently transfected into F9 teratocarcinoma cells, which express endogenous CBP but lack endogenous CREB and PKA. Their results indicated that Tax increases CREB-mediated

induction of the cellular CRE only when CREB is phosphorylated, since this Tax-dependent augmentation was abolished when PKA was not present or the consensus phosphorylation site in CREB was mutated. In contrast, Tax dramatically *trans*-activated the reporter gene construct driven by the HTLV-I U3 region even in the absence of PKA. These results suggested that Tax-mediated *trans*-activation of the HTLV-I LTR is independent of CREB phosphorylation. Fluorescence polarization binding assays and avidin-biotin complex assays provided evidence to suggest that when cellular CRE sites were utilized as target sequences, only phosphorylated CREB could recruit CBP. Tax, in turn, interacts with CBP, but not directly with phosphorylated CREB, to augment transcription. In contrast, when an HTLV-I CRE-like site was utilized, Tax promoted the dimerization of both phosphorylated CREB and nonphosphorylated CREB with subsequent binding to target DNA. CBP was recruited to this protein complex by direct contact with Tax, but not with CREB (124). However, assembly of this 21-bp-repeat-CREB-Tax-CBP quaternary complex itself may not be sufficient to initiate transcription. Additional cellular transcriptional factors which interact with the C-terminal *trans*-activation domain of Tax are required for transcriptional activation (56).

Giebler *et al*. further demonstrated that Tax can specifically promote the binding of the KIX domain of CBP to a 21-bp-repeat-CREB complex by up to 4.4 kcal/mol, and the increased binding affinity of the KIX domain is independent of CREB phosphorylation. Tax also increases the binding of the KIX domain to a truncated form of CREB which only contains the 73 amino acid bZIP domain, suggesting that the entire N-terminal CBP interaction domain is not necessary when Tax is present. *In vivo* functional observations were consistent with *in vitro* DNA-protein binding studies, since transfection of the bZIP domain of CREB into F9 cells was sufficient to support Tax-mediated *trans*-activation of the HTLV-I LTR. *In vivo* over-expression of a KIX domain, which does not possess any *trans*-activation activities, partially inhibits Tax-mediated *trans*-activation of the HTLV-I LTR. This indicates that the KIX domain can occupy the CBP binding site on Tax and prevent the interaction between Tax and CREB. Therefore, it seems that 21-bp-repeat-bound CREB only serves as an adapter for Tax to recruit CBP to the viral DNA, and CBP functions as a co-factor in Tax-mediated *trans*-activation of the HTLV-I LTR (109). The minimal region in the KIX domain required for Tax interaction spans amino acid residues 588 to 683, which has a sequence similar to the minimal KIX region essential for strong interaction with phosphorylated CREB. Mutations in KIX can specifically abolish Tax binding while retaining phosphorylated CREB binding, and vice verse, suggesting that Tax and phosphorylated CREB recognize different sets of amino acid residues in the region (125). The region in Tax necessary for binding CBP/p300 has been mapped to a highly protease-sensitive region around amino acid residues 81 to 95 ($_{81}$QRTSKTLKVLTPPIT$_{95}$) (56). It is still not clear how much HTLV-I CRE induction can be attributed to Tax and CBP *in vivo*. However, it is likely that both proteins may contribute to Tax-mediated *trans*-activation of the viral LTR. These data also suggest that the HTLV-I LTR can be *trans*-activated by Tax under basal conditions whereas the activation of cellular CRE-containing promoters requires conditions in which CREB is phosphorylated (124) (Figure 5).

4.4. Cellular RNA polymerase and HTLV-I LTR-directed transcription

Eukaryotic genes are generally classified into three categories, and the genes belonging to each category are transcribed by one of three eukaryotic RNA polymerases. RNA polymerase (Pol) I is responsible for the transcription of class I genes, which make up about 50% of the transcriptional activity in most eukaryotic cells. The primary product of RNA Pol I transcription is ribosomal RNA. All cytoplasmic mRNAs are transcribed by RNA Pol II from class II genes whose promoters usually bear very characteristic sequences. tRNAs and 5S rRNA are products of class III gene transcription, which is mediated by RNA Pol III (126). The HTLV-I LTR is classified as a typical Pol II transcription promoter. The viral LTR contains a TATA box located 30 bp upstream of the transcription initiation site and a number of Pol II transcriptional factor binding sites. The transcripts from the integrated proviral DNA contain a long poly(A) RNA. The HTLV-I LTR can be consistently transcribed *in vitro* by a reconstituted system consisting of TATA-binding protein, TFIIA, recombinant TFIIB, TFIIE, TFIIF, TFIIH, and Pol II (127). However, the presence of an overlapping transcription unit (OTU) within the context of the HTLV-I LTR is still a controversial issue. Piras *et al*. (127) reported that in Hela whole cell extracts, HTLV-I transcription is resistant to alpha-amanitin at concentrations (6 ug/ml) which inhibit the transcription of a well-characterized pol II promoter, the adenovirus major late promoter. Similar to a typical Pol III promoter (such as the adenovirus Ad2 VA-I promoter), HTLV-I transcription was inhibited when a higher concentration of alpha-amanitin (60 ug/ml) was utilized. HeLa whole cell extracts depleted of Pol II by utilizing three different Pol II antibodies could still support transcription driven by the viral LTR, indicating the existence of an OTU in the HTLV-I LTR. HTLV-I OTU transcription generated a correctly initiated transcript as the RNA isolated from an HTLV-I-infected cell line, MT-2. Depletion experiments also demonstrated that TATA-binding protein and TFIIB, but not TFIIC, are required for HTLV-I OTU transcription. Therefore, they proposed that the HTLV-I LTR possesses overlapping promoters: a traditional Pol II promoter and an uncharacterized Pol III promoter requiring an undefined set of transcriptional factors.

In contrast, Lenzmeier and Nyborg (128) investigated the nature of HTLV-I transcription by utilizing a variety of extracts, including HeLa whole cell extracts, treated with RNA Pol inhibitors. Employing *in vitro* run-off transcription assays, investigations have shown that HTLV-I transcription is sensitive to alpha-amanitin in a pattern similar to the adenovirus major late promoter and resistant to the presence of tagetitoxin, an RNA Pol III inhibitor. RNA Pol II is the only Pol that can mediate correct initiation of transcription from the HTLV-I LTR.

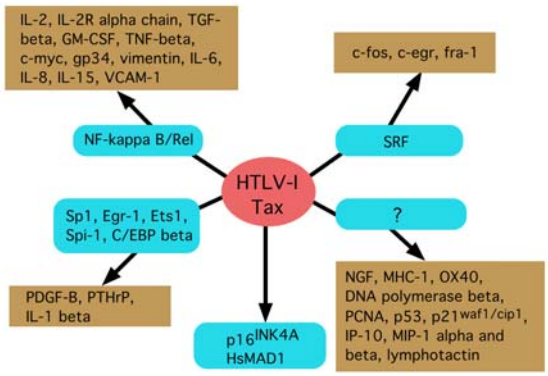

Figure 6. Tax can affect multiple cellular pathways by interacting with numerous cellular molecules (see text for details).

The presence of Tax and exogenous CREB in an *in vitro* transcription system does not change the sensitivity of the HTLV-I LTR to alpha-amanitin, indicating RNA Pol II is also responsible for Tax-mediated *trans*-activation. There are no clear explanations for the obvious discrepancy in observations recorded by the two groups of investigators. However, the viral promoter structure and the presence of a long poly (A) tail in the HTLV-I mRNAs are more consistent with an RNA Pol II-mediated transcription.

5. INTERACTION OF TAX WITH CELLULAR TRANSCRIPTION FACTORS DURING ONCOGENESIS

5.1. Tax modulates cellular gene expression

Tax is highly promiscuous and can *trans*-activate both the HTLV-I LTR and a variety of cellular genes (Figure 6). These genes can be grouped according to the mechanisms by which Tax *trans*-activates their expression. Group 1 genes include cellular IL-2, IL-2R alpha chain, granulocyte macrophage/colony-stimulating factor (GM-CSF), transforming growth factor beta (TGF-beta), tumor necrosis factor-beta (TNF-beta), c-*myc*, vimentin, gp34 (OX40 ligand) (14), IL-6 (129), IL-8 (130), and IL-15 (131), vascular cell adhesion molecule 1 (VCAM-1) (132). Group 2 genes include c-*fos,* c-*egr* (egr-1, -2), and *fra*-1 (14). The genes for c-*sis*/platelet-derived growth factor-B (PDGF-B) (133), parathyroid hormone-related protein (PTHrP) (134), and IL-1beta (135) are included in group 3. Finally, group 4 genes include nerve growth factor (NGF), major histocompatibility complex class I (MHC-I), OX40 (14), proliferating cell nuclear antigen (PCNA) (136), interferon-inducible protein-10 (IP-10), the macrophage inflammatory proteins-1 (MIP-1 alpha, beta), lymphotactin (137), and stromal derived factor-1 (SDF-1/PBSF) (138). The only gene known to be suppressed by Tax is DNA polymerase beta, a cellular enzyme involved in DNA damage repair (117). As discussed above, Tax associates with CREB and recruits CBP to form a ternary complex resulting in dramatic upregulation of HTLV-I LTR-directed transcription. However, the Tax/CREB interaction has a minimal impact on cellular gene transcription (124).

The promoters of group 1 genes contain NF-kappaB binding sites, and these promoters are *trans*-activated by Tax through both direct interaction between Tax and NF-kappaB/Rel and Tax-mediated translocation of NF-kappaB/Rel from cytoplasm to nucleus (114, 129-131, 139-148). NF-kappaB was first discovered as a p50 and p65 heterodimer, and these two subunits were subsequently classified as two members of the Rel/NF-kappaB family (149). The Rel/NF-kappaB family is comprised of NF-kappaB1 p50, p65 (RelA), c-Rel, v-Rel, RelB, NF-kappaB2 p52, and *Drosophila dorsal* and *dif*. The members of the NF-kappaB family share a conserved Rel homology domain of about 300 amino acid that is crucial for their DNA binding and dimerization. Members in this family can interact with each other to form either homodimers or heterodimers, and specifically regulate a wide variety of gene promoters that contain similar yet distinct NF-kappaB binding sites. Under unstimulated conditions, most NF-kappaB proteins are associated with the inhibitor, IkappaB, and these complexes are located in the cytoplasm as inactive forms (147, 150). IkappaB proteins are also part of a family that includes IkappaB-alpha, -beta, -gamma, p105 (a precursor of NF-kappaB1 p50), p100 (a precursor of NF-kappaB2 p52), Bcl-3, and *Drosophila cactus* and *relish*. All IkappaB molecules contain ankyrin-like repeats that are required for binding to NF-kappaB. Upon binding to NF-kappaB, IkappaB covers the nuclear localization signal (NLS) of NF-kappaB proteins to prevent their nuclear translocation. A number of compounds, as well as bacterial and viral agents, such as PMA, TNF-alpha, and HTLV-I Tax, can induce the dissociation of IkappaB from the NF-kappaB/IkappaB complex, resulting in a rapid degradation of IkappaB and the translocation of NF-kappaB from cytoplasm to nucleus (151). The degradation of IkappaB alpha is followed by its rapid resynthesis since the promoter of IkappaB alpha contains an NF-kappaB binding site. Newly synthesized IkappaB alpha binds to NF-kappaB and blocks the nuclear translocation of NF-kappaB. This feedback-loop control mechanism ensures that activation of NF-kappaB is a transient and tightly regulated process (Figure 7).

In HTLV-I-infected cells, however, NF-kappaB is constitutively activated (139). This is, at least in part, because activation of NF-kappaB through IkappaB beta is persistent and is only induced by certain inducers, including Tax (152). IkappaB alpha degradation is mediated by proteasomes, and triggered by the phosphorylation of IkappaB alpha at serine 32 and serine 36 and subsequent ubiquitination at lysine 21 or lysine 22 (153). However, the degradation of IkappaB beta requires stronger signals than that of IkappaB alpha. In T cells, the CD28 signal is required for the degradation of IkappaB beta. McKinsey *et al*. (152) have provided evidence to demonstrate that Tax-induced IkappaB beta is also degraded through the ubiquitin-proteasome pathway. Nevertheless, NF-kappaB selectively *trans*-activates IkappaB alpha gene expression, resulting in the chronic lack of cytoplasmic IkappaB beta in the presence of Tax.

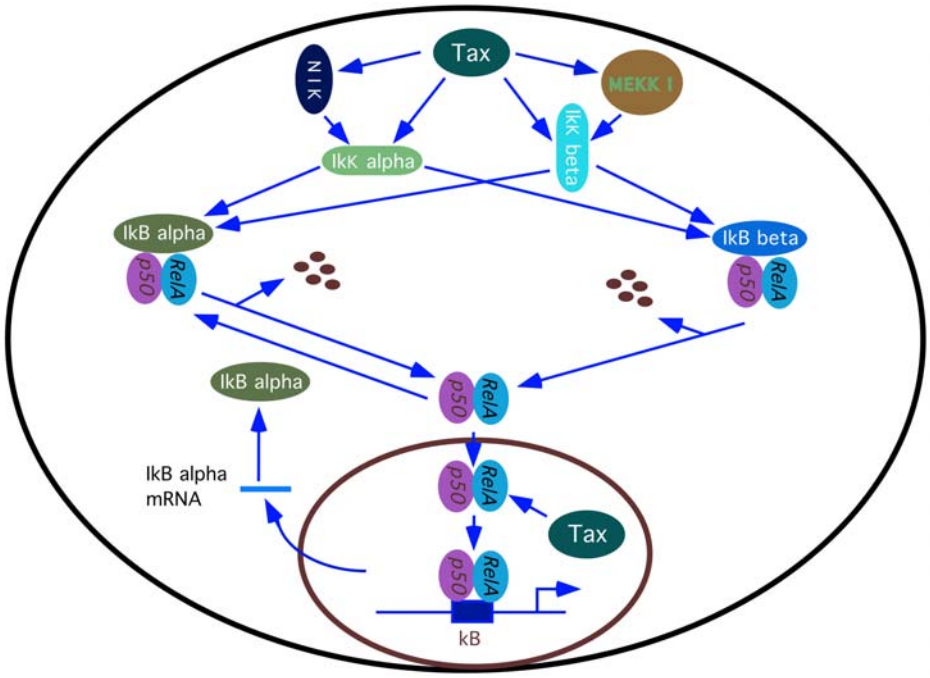

Figure 7. The mechanisms of Tax-mediated activation of NF-kappaB. Promoters containing NF-kappaB binding sites are *trans*-activated by Tax through both direct interaction between Tax and NF-kappaB/Rel and Tax-mediated translocation of NF-kappaB/Rel from cytoplasm to nucleus (see text for details).

Transient transfection of Tax into Jurkat cells activates cellular protein kinases, Ikappa kinase (IKK) alpha and IKK beta, which in turn phosphorylate IkappaB alpha. Furthermore, this Tax-mediated IkappaB alpha phosphorylation also requires another cellular protein kinase, NIK (NF-kappaB inducing kinase) (154). The physical interaction of NIK and IKKs has been detected in cotransfected 293 cells leading to IKK activation. Therefore, it appears that NIK is located upstream of the IKKs and is involved in regulation of IKK activities (155-157). On the other hand, Yin *et al*. reported recently that Tax also physically interacts with the N terminus of MEKK1, a protein kinase which is a component of an IKK complex, resulting in MEKK1 activation. Specifically, Tax expression stimulates the activity of IKK beta leading to the phosphorylation and subsequent degradation of IkappaB alpha (158). It is not clear at this moment what causes this obvious discrepancy. It may result from the different experimental systems utilized in these studies (154).

Alternatively, it has been demonstrated that Tax physically interacts with p100, p105 and IkappaB-gamma through their ankyrin motifs and releases p50/RelA NF-kappaB complexes from their inhibitors in the cytoplasm. This results in the nuclear translocation of p50/RelA and transcriptional activation, a mechanism which is independent of IkappaB phosphorylation and degradation (151, 159, 160). Finally, Tax has been shown to colocalize with NF-kappaB p50 and p65 subunits in nuclear bodies. These Tax-containing nuclear bodies also contain splicing factors Sm and SC-35, transcription components including the largest subunit of RNA Pol II, cyclin-dependent kinase CDK8, and specific transcripts from promoters bearing NF-kappaB binding sites that can be *trans*-activated by Tax (161-163). Furthermore, Tax directly interacts with NF-kappaB1 p50 (164), NF-kappaB2 p52 (165, 166), NF-kappaB p65 and c-Rel (146) through their Rel homology domains and binds to the NF-kappaB binding site. However, Tax does not affect the amount of DNA/NF-kappaB complexes. Transient transfection assays with F9 cells, an undifferentiated embryonic carcinoma cell line which lacks the factors required for Tax-mediated transcriptional activation through the NF-kappaB pathway, have demonstrated that co-transfection of either NF-kappaB p65 or c-Rel with a luciferase gene driven by a promoter containing NF-kappaB binding sites resulted in substantially increased luciferase activity. Co-transfection of Tax with either p65 or c-Rel resulted in an additional 6-8 fold increase in luciferase activity. Tax mutants that did not bind to either NF-kappaB p65 or c-Rel directly failed to display synergistic activity. Thus, Tax acts cooperatively with NF-kappaB p65 or c-Rel to augment the expression of the promoters containing NF-kappaB binding sites (146). Therefore, Tax can modulate NF-kappaB through a number of distinct processes leading to activation of gene expression. The effect of NF-kappaB activation by Tax on T cell transformation will be discussed later.

Tax enhances gene expression of group 2 genes via direct interaction with serum response factor (SRF) and promoters containing a serum reponsive element (SRE) (167, 168). The SRE motif of the human c-*fos* promoter is a dyad symmetry element (DSE) composed of a CArG box

sequence that constitutively binds a dimer of SRF even without mitogenic stimulation (169). The 5' end of the CArG box is recognized by $p62^{TCF}$ (ternary complex factor), which interacts with SRF to form a ternary complex and further enhances the DNA binding affinity of SRF. *In vivo*, the *trans*-activation activity of a mutated Tax can be rescued by the acidic activation domain of VP16 fused to SRF, indicating that Tax and SRF interact functionally with each other. Therefore, when Tax is present, the transcription of the promoters containing a CArG site can be activated without mitogenic signals (167).

Recently, several groups of investigators demonstrated that Tax can *trans*-activate group 3 genes c-*sis*/PDGF-beta, PTHrP P2, and pro-interleukin-1 beta promoters through either zinc finger transcriptional factors or members of the Ets family of transcription factors (133-135). The B-chain/c-*sis* of platelet-derived growth factor (PDGF), the cellular homologue of the viral *sis* oncogene (v-sis), has been suggested to play an important role in the process of transformation (170, 171). Biologically active PDGF is either a homo- or heterodimer of two polypeptides, A and B (172). The transcription of the c-*sis* proto-oncogene is tightly controlled in normal T cells, but is greatly enhanced in HTLV-I-infected T cells (133, 173). Trejo *et al*. previously demonstrated that a region within the c-*sis*/PDGF-beta promoter (-64 to -45) is required for *trans*-activation by Tax and was designated Tax-responsive element 1. EMS analyses indicated that Sp family members (Sp1 and Sp3) as well as a member of the immediate early response gene family (NGFI-A/Egr-1) are the major transcription factors that bind to this region and mediate Tax-responsiveness (174). Tax can substantially increase *in vitro* RNA synthesis from a construct containing the -257 to +74 region of the c-*sis*/PDGF-beta promoter. CCACCC and GNGNGGGNG sequences are crucial for Tax-mediated *trans*-activation, since mutation in this sequence dramatically reduces the effect of Tax on the promoter. The mechanism of Tax-augmented transcription relies on the capability of Tax to significantly increase the DNA binding affinity of both Sp1 and Egr-1 to their cognate sites within Tax-responsive element 1, forming a ternary complex of Sp1 or Egr-1, Tax and DNA. *In vitro* co-immunoprecipitation analyses utilizing both purified proteins and whole cell extracts have provided additional evidence that Tax indeed directly interacts with Sp1 and Egr-1. Physically mapping the domain of Tax responsible for the interaction with these two transcription factors will provide critical tools to further dissect this pathway.

PTHrP is considered as the causative agent of humoral hypercalcemia that is one of the major complications of ATL (134). Recently, PTHrP has been suggested to be involved in regulating proliferation and apoptosis in normal and malignant cells (175, 176). The PTHrP promoter contains binding sites between -73 and -53 for transcription factors Ets1 and Sp1. Ets1 is a member of the Ets transcription factor family that is characterized by the presence of an approximately 85 aa conserved domain responsible for binding to a purine-rich core sequence. Ets factors are usually weak transcriptional activators and often associate with transcription factors of unrelated families to activate gene expression (177). It has been shown that Tax acts synergistically with Ets1 to *trans*-activate the PTHrP P2 promoter in HTLV-I-infected cells. In the yeast two-hybrid system, Tax was shown to interact with Ets1, and this interaction was essential for its synergistic effect since a Tax mutant which prevented the Tax/Ets1 interaction abolished the cooperative effect on *trans*-activation of the PTHrP P2 promoter. *In vitro* coimmunoprecipitation assays demonstrated that Tax was capable of enhancing the binding between Ets1 and Sp1, and formed a ternary complex with these two transcription factors. Furthermore, Ets1-dependent Tax-mediated *trans*-activation of the PTHrP P2 promoter relied on the adjacent Sp1 site since mutation of this Sp1 site dramatically reduced Tax/Ets-1 *trans*-activation of the PTHrP P2 promoter (134).

Although HTLV-I primarily infects $CD4^+$ T cells, HTLV-I has also been found in B lymphocytes (178), endothelial cells (179), monocyte/macrophage cells (180), and fibroblasts (181). Subsequent to activation, cells of the monocyte/macrophage lineage start to express IL-1 beta, an important inflammatory and immunoregulatory cytokine, from the normally silent human proIL-1 beta gene. Dhib-Jalbut *et al*. have demonstrated that HTLV-I Tax upregulates IL-1 beta expression in both human primary microglia and peripheral blood macrophages, which might play an important role in HTLV-I-associated diseases such as HAM/TSP (182). Tax-mediated *trans*-activation of the proIL-1 beta gene requires binding sites for Spi-1 (promoter sequence –131 to +12), a member of the Ets transcription family, and NF-IL6 (CCAAT/enhancer binding protein beta, C/EBP beta), a member of the basic region-leucine zipper (bZIP) family. Tax physically interacts with both Spi-1 and NF-IL6 *in vitro*, and increases the binding of both to the proIL-1 beta gene promoter (135). Again, Tax *trans*-activates the proIL-1 beta gene promoter through protein-protein interaction with two transcription factors.

The mechanisms by which Tax induces the group 4 gene expression and suppresses DNA polymerase beta gene expression are not clear. Due to the promiscuous nature and the extremely pleiotropic function of Tax, more cellular genes whose expression patterns can be changed by Tax will likely be discovered.

5.2. Cell growth and transformation of HTLV-I-infected T cells

HTLV-I infection results in transformation of human primary $CD4^+$, $CD8^-$, DR^+, and $CD25^+$ T cells *in vitro* and *in vivo* (15). As mentioned previously, ATL develops in 1 out of 1,000-2,000 HTLV-I-infected carriers after about a 20 to 30 year latency period, suggesting that multiple steps are required for the development of full-blown disease. Statistical analysis of the relationship between age and occurrence of ATL in 357 cases suggested that HTLV-I-infected T cells require the completion of five independent events prior to the development of ATL (183). Primary T cell cultures established from HTLV-I-infected

individuals usually display an activated, IL-2-dependent, immortalized phenotype rather than an IL-2-independent, transformed phenotype (184). However, if these cells are repeatedly cocultured with normal uninfected adult or umbilical cord T cells *in vitro*, transformed T-cell lines eventually emerge (185-187), suggesting the importance of clonal selection.

A number of retroviruses can transform their host cells by utilizing one of three mechanisms. The first mechanism, referred to as insertional mutagenesis, involves insertion of proviral DNA adjacent to a cellular oncogene leading to dysregulated expression of the oncogene. One example of this type of transformation is avian leukosis virus (ALV)-induced lymphomas in which c-myc gene expression is upregulated (188). In addition, a recent report has indicated that an HTLV-I infected T cell line, HUT 102, produces a significant amount of IL-15, a potent and normally poorly expressed T cell growth-promoting cytokine. The increased level of IL-15 expression resulted from fusion of the HTLV-I R region in the 5' LTR to the IL-15 gene and subsequent upregulated gene expression (189). However, this mechanism cannot be used to explain the general transformation process induced by HTLV-I since no specific HTLV-I provirus insertion sites have been identified in most cases of ATL (11).

The second mechanism involves the integration of proviral DNA-encoded oncogene into the host genome, resulting in the expression of the oncogene. One example is the *src* gene carried by Rous sarcoma virus (RSV) (190). So far, no comparable oncogene sequence has been identified in the HTLV-I genome. Thus, this mechanism is unlikely to be operative in HTLV-I-induced transformation.

In the third mechanism, retroviral gene products act in *trans* to regulate both viral and cellular gene transcription and translation. Based on overwhelming data, Tax is considered the primary viral gene product involved in leukemogenesis (191). In fact, Tax has been shown to transform established rodent fibroblast cell lines. These transformed cells are able to generate tumors in nude mice (192). Although expression of Tax alone in rat embryo cells did not change the morphology of the cells, transfection of Tax with activated Ras in the rat embryo cells gave rise to transformed cells that were tumorgenic in nude mice (193). Transduction of the pX gene into human cord blood lymphocytes and thymus T cells utilizing a viral vector resulted in immortalization that was still IL-2 dependent (194).

The mechanisms whereby Tax transforms cells and the specific domains of Tax that are necessary for the transformation process are not yet clear. Smith and Greene (57) generated a series of Tax mutants which spaned the entire Tax aa sequence and tested their *trans*-activation capacity through either the NF-kappaB or ATF/CREB pathways. Two mutants were particularly interesting. Tax M22, which retained more than 50% of its *trans*-activation function through the ATF/CREB pathway in comparison to the wild type Tax, failed to *trans*-activate the HIV-1 LTR which contains NF-kappaB sites. In contrast, Tax M47, which does not *trans*-activate the HTLV-I LTR (which contains three ATF/CREB sites), *trans*-activated the HIV-1 LTR through the NF-kappaB pathway to levels comparable to the wild type Tax. By utilizing these mutants, they were able to dissect the Tax-mediated transformation into two pathways. Rat fibroblasts (Rat2) that stably expressed either wild type Tax or Tax M22 displayed marked morphological changes and anchorage-independent growth with a high tumorgenic capacity in nude mice. In contrast, Rat2 cells that stably expressed Tax M47 did not manifest any changes in morphology and growth patterns. These results suggested that Tax-induced transformation is likely mediated by the ATF/CREB pathway (118).

In contrast, Yamaoka *et al*. (195) utilized a different set of Tax mutants that functionally segregated ATF/CREB and NF-kappaB *trans*-activation pathways to investigate their transformation abilities. Their results indicated that Tax mutants deficient in their ability to induce NF-kappaB failed to transform another established rat fibroblasts (Rat1), Rat2, and NRK cells. A mutant that lacked the ability to *trans*-activate the HTLV-I LTR displayed a wild type Tax transformation capacity. Furthermore, stable coexpression of the NF-kappaB precursor (p100), a member of the IkappaB family, with wild type Tax inhibited Tax-mediated NF-kappaB activation as well as transformation. Therefore, Yamaoka *et al.* concluded that activation of the NF-kappaB pathway by Tax plays an important role in Tax-induced cell transformation (118). To date, there is no clear explanation for these diametrically opposed results.

However, there are several other reports which support the view that activation of NF-kappaB is crucial for transformation of cells by Tax. Tax mutants which failed to *trans*-activate the bovine leukemia virus (BLV) LTR were fully capable of transforming rat embryo fibroblasts in cooperation with the H-*ras* oncogene (196). Kitajima *et al*. (197) demonstrated that the growth of an HTLV-I-transformed T cell line as well as Tax-transformed fibroblasts derived from Tax-transgenic mice was inhibited by introducing antisense oligonucleotides specifically targeted against RelA to block NF-kappaB expression. These results indicated that active NF-kappaB is required for maintaining the malignant cells. Recently, Matsumoto *et al*. (198) generated another set of Tax mutants which segregated the ATF/CREB, NF-kappaB and SRF pathways. Expression of these mutants in Rat-1 cells alone or in rat embryo fibroblasts (REF) with activated *ras* resulted in distinctly different outcomes. Tax mutants that can activate the NF-kappaB pathway but are deficient in activation of the ATF/CREB and SRF pathways transformed Rat-1 cells. Therefore, active NF-kappaB appears essential for transformation of Rat-1 cells, a conclusion consistent with that of Yamaoka (195). In contrast, Tax mutants that activated the CArG box transformed REF cells to a level comparable to wild type Tax, whereas Tax mutants which failed to *trans*-activate the CArG box were unable to transform REF cells. Consequently, Tax-mediated activation of the CArG box pathway appears necessary for

the transformation of REF cells by Tax. However, a role for the ATF/CREB pathway in Tax-mediated transformation in REF cells cannot be ruled out, since the Tax mutants used in these experiments were not able to separate the ATF/CREB pathway from the CArG box pathway. Thus, it appears that at least two distinct routes are utilized by Tax to transform rat fibroblasts. The mechanisms governing the different requirements for Tax transformation of these two cell types are not yet clear. One possible explanation is the difference in cell backgrounds (Rat-1 is an immortalized cell line, whereas REF cells represent a primary cell population). It is possible that one or more events occurred in Rat-1 cells during the immortalization process which permitted the cells to bypass the requirement for the CArG pathway. Another possible explanation may involve the presence of an active *ras* in REF transformation experiments. *ras* activates NF-kappaB and may replace the requirement for Tax-mediated NF-kappaB activation (198).

Several recent reports have indicated that Tax can also *trans*-activate selected cellular promoters through direct interaction with Sp1, NGFI-A/Egr-1, Ets1, Spi-1, and NF-IL6 (133-135). These discoveries provide several new directions of investigation relevant to the process of Tax-induced cell transformation. As discussed before, Trejo *et al*. demonstrated that Tax interacts with the zinc finger transcription factors Sp1 and NGFI-A/Egr-1 to *trans*-activate the c-*sis*/PDGF-beta promoter. PDGF is a potent growth hormone and chemoattractant for cells of mesenchymal origin involved in tissue damage repair and early development (173, 199). The *sis*/PDGF-beta gene, a cellular homologue of the simian sarcoma virus oncogene v-*sis* (170, 171) that encodes the B chain of PDGF, is not normally expressed in lymphocytes, but is upregulated in HTLV-I-infected T cells. PDGF, which is found in either homodimeric or heterodimeric combinations of A and B chains, binds to two kinds of receptors on the cell surface (172). The alpha receptor binds to both A and B chains with a high affinity, while the beta receptor only binds to the B chain (200). Under normal conditions, lymphocytes do not express PDGF receptor on their cell surfaces, but HTLV-I-infected T cells contain a substantial amount of PDGF-beta receptor mRNA and protein that can be immunoprecipitated with antibodies against PDGF receptor (201). These results suggest that HTLV-I infection results in the dysregulated expression of both PDGF and its receptor. The abnormally expressed PDGF and its receptor, in turn, comprise an autocrine loop by which T cells undergo malignant transformation. Indeed, the involvement of PDGF in transformation has been documented. Introduction of a cDNA clone of c-*sis* (derived from HUT-102, a cell line derived from a cutaneous T cell lymphoma and infected with HTLV-I) into mouse 3T3 cells, which express both types of PDGF receptors, resulted in transformation (202).

As previously discussed, HTLV-I infection induces the expression of a variety of genes, including different cytokines and their receptors. Among these, the most intriguing and well studied molecules are IL-2 and its receptor (IL-2R), which are both important in leukemogenesis. IL-2 and IL-2R constitute a primary system by which mature peripheral T cells are able to grow and proliferate (203, 204). IL-2R is composed of three subunits: alpha, beta, and gamma. Resting T cells only express beta and gamma subunits, which make up a low affinity IL-2R. Subsequent to T cell activation, the alpha subunit is expressed and joins the beta and gamma subunits to form a high affinity receptor (IL-2 alpha) for IL-2 (205). IL-2 exerts its effect on T cells by binding to its receptors and causing phosphorylation and activation of beta and gamma subunit-associated kinases, Janus kinase 1 and 3 (JAK1 and JAK3), and signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5). This activating signal cascade eventually drives T cells from G0/G1 to S phase and completion of the cell cycle (206, 207). One salient feature of leukemic cells derived from ATL patients and T cells immortalized by HTLV-I *in vitro* is the constitutive expression of IL-2R alpha (186, 208, 209). This leads to the hypothesis that constitutive expression of the high affinity IL-2R is crucial for the growth of HTLV-I-transformed T cells.

HTLV-I Tax activates a wide variety of genes which may result in the early stages of leukemogenesis and it is the only viral factor required to immortalize primary T cells *in vitro* (210, 211). However, in newly isolated leukemic cells, the expression of Tax and other viral proteins is rarely detected, suggesting that a distinct, Tax-independent pathway is utilized in the latter stages of leukemogenesis (212, 213). Although there is evidence to suggest that the IL-2/IL-2R autocrine loop is the mechanism driving the leukemogenesis process in cells cultured *in vitro* after isolation from ATL patients (214, 215), most newly isolated leukemic cells from the majority of ATL patients neither produce IL-2, nor respond to IL-2 (213, 216, 217). These observations challenge the hypothesis that IL-2R alpha is important in maintaining leukemic cell growth.

In order to directly test this hypothesis, Richardson *et al*. (218) generated an endoplasmic reticulum-targeted single-chain antibody to specifically block the cell surface expression of IL-2R alpha. Expression of this intracellular antibody in two IL-2-independent HTLV-I-transformed cell lines effectively reduced IL-2R alpha expression on the cell surface to levels undetectable by flow cytometric analysis. However, the growth rate of the cell surface IL-2R alpha-depleted cells was identical to the parental cells, indicating that expression of IL-2R alpha was dispensable for *in vitro* growth of the HTLV-I-transformed cell line. The results from this experiment do not rule out the possibility that expression of IL-2R alpha is necessary for IL-2-dependent cell growth during the early stages of leukemogenesis or immortalization. There are several reports suggesting that the transition from IL-2-dependent to IL-2-independent cell growth happens at a relatively late stage of leukemogenesis (218).

Recently, results from several investigations have indicated that JAK1, JAK3, STAT3, and STAT5, which are

positioned downstream of the IL-2 signal transduction pathway, are constitutively activated in some HTLV-I-transformed cell lines *in vitro*, and the acquisition of constitutive phosphorylation of JAKs and STATs correlates with the loss of IL-2 dependency in HTLV-I-infected cord blood lymphocytes (219, 220). To determine if this *in vitro* model reflects viral leukemogenesis *in vivo*, Takemoto *et al*. (221) determined the status of JAK/STAT phosphorylation and the DNA binding activity of STAT in cell extracts of uncultured leukemic cells from 12 ATL patients. Constitutive DNA binding activity of one or more STAT proteins was detected in malignant T cells from 8 of 12 ATL patients, and a direct correlation between the activation of JAK3, STAT-1, STAT-3, and STAT-5 and cell-cycle progression was shown with leukemic cells derived from four patients examined. The mechanism underlying constitutive activation of JAK/STAT proteins caused by HTLV-I infection will require further clarification.

IL-2 induced tyrosine phosphorylation of IL-2 receptor beta chain (IL-2R beta), JAK1, JAK3, and STAT proteins is dephosphorylated in normal cells. Recently, Migone *et al*. (222) demonstrated that this dephosphorylation is mediated by tyrosine phosphatase 1 (SHP1) containing a *src* homology 2 (SH2) domain. Upon IL-2 stimulation, IL-2R beta and associated signal molecules are phosphorylated, which in turn recruits SHP1 to the IL-2 receptor complex, resulting in dephosphorylation of IL-2R beta, JAK1, and JAK3. However, SHP-1 expression is significantly diminished or undetectable in several IL-2-independent HTLV-I-infected cell lines that possess the constitutive activity of JAK/STAT proteins. Furthermore, they found that down-regulation of SHP-1 expression is correlated with the loss of IL-2-dependency in HTLV-I-infected T cells.

Interestingly, the down-regulated expression of SHP-1 is not a phenomenon unique to HTLV-I-transformed T cells. Similar down-regulation has also been documented in Burkitts' lymphomas and germinal center B lymphocytes (223). These observations suggest that the constitutively activated IL-2 signal pathway (resulting either from the IL-2/IL-2R alpha autocrine loop or from the HTLV-I-associated constitutive phosphorylation of JAK/STAT proteins) plays a key role in T cell transformation, and SHP-1 acts as a negative regulator of the IL-2 signal transduction pathway. There is a large gap between the IL-2R signal pathway mediated by activated JAK/STAT and cell cycle control. The mechanisms underlying connection of these two processes are unknown. It is noteworthy that the IL-2R signal pathway can also be mediated by SHC/RAS/MSPK proteins (224, 225); their roles in T cell transformation induced by HTLV-I infection will require continued investigation.

A number of important cell cycle regulatory proteins have been shown to be deleted, mutated or inactivated in some HTLV-I-transformed cells. Hatta *et al*. (226) reported that leukemic cells from 10 of 37 ATL patients lost p15 and/or $p16^{INK4A}$, which are inhibitors of cyclin-dependent kinase 4 (Cdk4) and Cdk6, respectively. During G1/S phase transition, Cdk4 and Cdk6 couple with cyclin D to form enzymatically active complexes which, in turn, phosphorylate retinoblastoma (Rb) protein. Hyperphosphorylated Rb releases E2F that promotes transcription of a variety of cellular genes which drive cells into S phase (227, 228). Tax can directly interact with $p16^{INK4A}$ to prevent the formation of a $p16^{INK4A}$/Cdk4 complex *in vitro*, resulting in Cdk4 activation (229). Recently, Schmitt *et al*. (230) generated a tetracycline repressor-based Tax expression system to investigate Tax stimulation of T cells. In this system, Tax expression was driven by a promoter that was suppressed by the presence of tetracycline. Primary human cord blood T cells transduced by a Tax expression vector displayed abnormal proliferation similar to HTLV-I-infected lymphocytes without the presence of tetracycline. After tetracycline treatment, T cells stopped growing and were arrested in the G1 phase. Re-expression of Tax in the cells resulted in entry of the arrested cells into S phase. This Tax-dependent cell cycle progression correlates well with Tax-mediated upregulation of Cdk4 and Cdk6 activities. In the absence of Tax, the activities of Cdk4 and Cdk6 were substantially diminished, but the expression of Cdk4 and Cdk6 was not changed, suggesting that Tax influences Cdk4 and Cdk6 activities at the post-transcriptional level. Consistently, a loss of control of Cdk4 and Cdk6 activity has been observed in transformed and tumor cells (231, 232).

p53, a tumor suppressor protein, is integrally involved with cell cycle regulation. Loss of p53 function by either missense mutation or interaction with viral transforming proteins such as SV40 large T antigen (233), adenovirus E1b (234), or human papillomavirus E6 proteins (235), strongly correlates with the occurrence of cell transformation. Several studies have demonstrated that p53 was mutated or deleted in only 30% of HTLV-I-infected cell lines and cells from ATL patients (236, 237). Surprisingly, the half life of p53 in HTLV-I-transformed human T cells significantly increased rather than decreased, and the p53 gene did not contain any mutations (238). Further studies indicated that stabilized p53 in HTLV-I-transformed cells was incapable of *trans*-activating a p53-responsive reporter plasmid in transfection assays and failed to mediate apoptosis induced by gamma-irradiation. Therefore, the p53 in HTLV-I-transformed cells appears to be nonfunctional (239, 240). Pise-Masison *et al*. further demonstrated that Tax was responsible for the observed stabilization and inactivation of p53, since Tax inhibited p53-mediated *trans*-activation by more than 10-fold in cotransfection assays, and this inhibition was not dependent on a specific DNA sequence. Since Tax does not physically bind to p53, Tax might inhibit p53 function through a novel posttranslational modification (240). Recent results support this hypothesis. It has been shown that when Tax is present, p53 is hyperphosphorylated at Ser15 and Ser392. Phosphorylation of Ser15 alone prevents p53 from binding to TFIID, while p53 can still bind to DNA in a sequence specific manner. While both Ser15 and Ser37 are

phosphorylated, p53-TFIID interaction is restored but p53-MDM2 binding is blocked (241).

$p21^{waf1/cip1}$(wild-type p53 activated fragment 1/cycling dependent kinases interacting protein 1), another cell cycle control protein, limits the transition from G1 to S (227, 228) by forming quaternary complexes with cyclins, Cdks, and proliferating cell nuclear antigen (242). Although the expression of $p21^{waf1/cip1}$ is induced by p53 in normal cells, the expression level in HTLV-I-infected cell lines is elevated despite the presence of nonfunctional p53. The constitutive expression of $p21^{waf1/cip1}$ has also been observed in Tax-1, a human T cell line stably expressing Tax. Further studies demonstrated that Tax is able to *trans*-activate the promoter of the $p21^{waf1/cip1}$ gene, resulting in p53 independent expression of $p21^{waf1/cip1}$ (239). At first glance, high level expression of the cell cycle control protein $p21^{waf1/cip1}$ in T cell lines stably expressing Tax is somewhat perplexing. However, further studies suggested that the $p21^{waf1/cip1}$ protein, at low stoichiometric amounts, serves as an assembly factor for enzymatically active cyclin/cdk complex formation. At higher stoichiometric amounts, the $p21^{waf1/cip1}$ protein transforms this complex from active to an inactive form (243). It is possible that the expression level of $p21^{waf1/cip1}$ induced by Tax in HTLV-I-infected cells may be within the concentration range that constitutively promotes assembly and activation of cyclin/cdk complexes (239).

The oncogenicity of Tax has been studied extensively in Tax transgenic mice. Although the Tax transgenic mice generated in different models displayed various phenotypes, no published data have indicated that transgenic mice carrying the Tax gene alone developed $CD4^+$ T cell malignancy, a phenomenon observed in human ATL. Transgenic mice carrying a Tax gene driven by the HTLV-I LTR usually developed mesenchymal tumors and neurofibromatosis (244, 245). Transgenic mice whose Tax gene expression was regulated by the human granzyme B promoter (GzmB Pr), a mature T lymphocyte specific promoter, developed $CD4^-CD8^-$ large granular lymphocytic (LGL) leukemia with elevated cytokine expression (246, 247). In contrast, Tax under the control of the IgG promoter did not result in any pathologic phenotypes in transgenic mice (248). These obvious discrepancies are most likely due to the use of different promoters, which may lead to various levels of Tax gene expression in different cell types.

6. HTLV-I AND ADULT T-CELL LEUKEMIA (ATL)

According to one epidemiological study published in 1997, there were about 9.3 million cases of cancer reported worldwide (249). Approximately 15% of these cases can be attributed to various infectious agents, such as hepatitis B virus, hepatitis C virus, human papillomaviruses, Epstein-Barr virus, HTLV-I, Helicobacter pylori, schistosomes, and liver flukes. It is estimated that HTLV-I is responsible for about 1% of all leukemia (249). Only a small segment of the population infected by HTLV-I (1 in 1,000-2,000 seropositive individuals per year) will develop ATL in their lifetime with a 20 to 30 year latency period (250). The likelihood of developing any symptoms related to HTLV-I infection is between 5% and 10% during the lifetime of an individual (251). The average age of ATL manifestation is 55, and men are 40% more likely to suffer from ATL than women (250, 252). Currently, ATL is divided into four subcategories (smoldering, chronic, acute, and lymphoma) according to numerous clinical and laboratory features (including the percentage of abnormal T cells in the peripheral blood), lactic acid dehydrogenase (LDH) and calcium blood levels, and malignant tumors in various organs (252, 253).

Smoldering type ATL makes up about 5% of all ATLs. Patients display mild symptoms and a prolonged clinical course with a low level of leukemic cells in the peripheral blood. Skin lesions caused by infiltration of leukemic cells are usually present while lymph node abnormalities are minimal (254). About 20% of HTLV-I-infected patients with clinical symptoms fall into chronic ATL category. This form of the disease is very similar to the smoldering type of ATL except that there is additional involvement of visceral organs, resulting in lymphadenopathy, hepatosplenomegaly, and marginally increased leukemia cells (253). Smoldering and/or chronic types of ATL may represent either distinct disease entities or transitional states leading to more malignant acute ATL.

Acute ATL represents about 55% of all ATL forms and is characterized by rapid disease progression with systemic lymphadenopathy, hypercalcemia due to a high rate of bone turnover mediated by elevated osteoclast activity, lytic bone lesions, hepatosplenomegaly, skin abnormalities, high serum levels of LDH, and various cytokines released from cancer cells. Clinical symptoms of acute ATL are fever, cough, malaise, dyspnea, thirst, drowsiness, and lymph node enlargement (10, 11, 252). About 20% of ATL cases are of the lymphoma type ATL. Patients with lymphoma type ATL present with enlargement of lymph nodes but with no leukemic cells detected in the peripheral blood. ATL patients are immunocompromised and subject to opportunistic infections by viruses, bacteria, fungi, and protozoa (252). ATL is a fatal disease with median survival time from the onset of the disease ranging from 24.3 months for chronic ATL, 10.2 months for lymphoma ATL, to 6.2 months for acute ATL (252).

There is no definitive T cell phenotype associated with ATL, although most cells display a deeply lobulated nucleus. The T cell markers on these cells are also heterogeneous and typically include $CD2^+$, $CD3^+$, $CD5^+$, $CD7^-$, $CD4^+$, and $CD8^-$ markers (255). The $CD8^+$ marker is rarely detected on leukemic cells of ATL patients (256). Patients with this disease are usually characterized by clonal expansion of mature peripheral blood T cells, each harboring a single copy or multiple copies of HTLV-I sequences. Ohshima *et al*. utilized an inverse polymerase chain reaction (IPCR), a more sensitive method than Southern blot hybridization analysis, to study the status of

clonality of HTLV-I-infected cells in asymptomatic HTLV-I carriers. Data derived from peripheral blood mononuclear cells (PBMC) obtained from 16 asymptomatic carriers indicated that about 44% of carriers have already shown either mono- or oligoclonal HTLV-I integration in their PBMCs. However, further step(s) are required to fulfill the malignant process (257).

Recent studies have demonstrated that there is a correlation between the copy number of HTLV-I sequences per cell and ATL clinical manifestation. Patients with multiple HTLV-I insertions in a single cell have severe clinical symptoms involving leukemic cell infiltration into unusual organs such as the uvea and retina, whereas patients with a single copy of HTLV-I sequence per cell display a mild clinical course with skin lesions (258). Moreover, it seems that HTLV-I integration patterns also affect ATL pathogenesis. Tsukasaki *et al*. utilized pX and *gag-pol* probes to investigate HTLV-I proviral integration patterns in PBMCs of 68 ATL patients. They detected defective provirus integration patterns that lack *gag-pol* sequences in 20 patients (29.4%). Thirty four patients (50.6%) were found to have a single, complete proviral genome, and the remaining 14 patients (20.6%) exhibited multiple proviral integration sites in PBMC preparations. Interestingly, different integration patterns correlated with different disease prognoses. The median survival time for patients with one, two or three proviral integration patterns was 6.8, 24.4, and 33.3 months, respectively (259).

7. HTLV-I AND TROPICAL SPASTIC PARAPARESIS (TSP)

In addition to ATL, HTLV-I has also been demonstrated to be the etiologic agent of TSP (16, 17). Patients with TSP usually display spasticity of lower extremities, weakness of lower extremity muscle, disturbed superficial sensory capabilities, and dysfunction of the urinary bladder (260). Seven out of every ten thousand HTLV-I proviral carriers develop TSP. TSP has a relatively shorter latency in comparison to ATL, ranging from months to decades (261). HTLV-I seropositive women are almost three times more likely to develop TSP than men, and the average age of occurrence of disease is 43 years (14). It has been observed that TSP patients usually carry a much higher viral burden when compared to asymptomatic carriers. By utilizing PCR, Kubota and coworkers determined that the HTLV-I proviral DNA load was between 2 and 20 copies per 100 PBL from TSP patients as compared to 0.04-8 copies per 100 PBL from asymptomatic carriers (262). Similar results have been obtained by a number of other investigators (263-265). The higher proviral DNA load in TSP patients appeared to be caused by increased viral replication (263). However, these early results were generated from relatively small sample numbers utilizing less accurate conventional PCR methodology. Therefore, no firm correlation has been established between HTLV-I proviral DNA load and the risk of developing TSP in the asymptomatic HTLV-I-infected carrier population.

Recently, Nagai *et al.* (266) utilized PCR with a dual-labeled fluorogenic probe to examine the correlation between proviral DNA load and incidence of TSP in 202 TSP patients and 243 asymptomatic HTLV-I-infected carriers. The average HTLV-I proviral DNA copy number per $1x10^4$ PBMC was 798 in TSP patients, 120 in HTLV-I-infected asymptomatic carriers, and 496 in HTLV-I-infected asymptomatic carriers who are family members of TSP patients. PBMCs from female TSP patients harbor a significantly higher amount of provirus than those from their male counterparts. The study strongly suggested that genetic factors play a role in the process leading to a high proviral DNA load *in vivo*. The HTLV-I proviral DNA load in PBMCs is directly correlated to the extent of CNS neurological damage of TSP patients and may be used as an indicator to predict the development observed in TSP among HTLV-I-infected asymptomatic carriers.

The mechanisms associated with HTLV-I infection and the subsequent development of TSP are not yet clear. At least four hypotheses have been proposed to provide possible explanations for the observed neurological damage. In the first hypothesis, it is proposed that HTLV-I can directly invade specific CNS cell population such as neurons and glial cells, to cause CNS damage. Several groups of investigators have detected HTLV-I-infected cells in the CNS of TSP patients. However, it is still controversial as to whether these HTLV-I-infected cells are of nervous system origin or represent a subset of T cells infiltrating into the CNS from the periphery. For example, Lehky *et al*. utilized *in situ* hybridization to demonstrate the presence of cells containing HTLV-I RNA in spinal cord and cerebellar sections of three TSP patients. Histological analysis demonstrated that at least some of these infected cells were astrocytes (267). In contrast, Hara and coworkers failed to detect HTLV-I proviral DNA in preserved spinal cord sections from TSP patients utilizing a similar technique (268).

Evidence suggests that the LTR sequences of some retroviruses play a role in tissue- and cell type-specificity and may also be involved in determining the course of disease associated with infection (269). When transgenic mice were generated utilizing the LTRs from either CNS- or T cell-tropic HIV strains, expression of the reporter gene within the nervous system was detected only in mice transgenic for LTRs derived from the CNS of infected individuals with neurologic damage (270). Similarly, transgenic mice containing a beta galactosidase transgene driven by an HTLV-I LTR isolated from a patient with TSP expressed the reporter gene primarily within the CNS (271). Use of EMS analyses have resulted in the detection of a unique DNA-protein complex when the promoter central 21 bp repeat was reacted with nuclear extracts derived from either the U-373 MG glioblastoma cell line or the THP-1 mature monocytic cell line. Additional studies have demonstrated that this DNA-protein complex is comprised of the AP-1 components, Fos and Jun (75). *In vitro* transient transfection assays have demonstrated that U-373 MG glioblastoma cells could

support basal and Tax-mediated *trans*-activation driven by the HTLV-I LTR (71).

In the second hypothesis, it has been proposed that $CD8^+$ T cells mediate CNS damage (272). Similar to ATL, $CD4^+$ T cells are the major cell population infected by HTLV-I in TSP patients, and they usually demonstrate clonal rearrangement of the T-cell receptor (273). TSP patients exhibit a state of immune activation including both B and T cells. Matsui and Kuroda recently compared the CNS immunological status of 19 TSP patients and 6 asymptomatic HTLV-I-infected carriers. Their results revealed that B cells as well as $CD4^+$ and $CD8^+$ T cells exhibited an activated phenotype in patients with a short history of TSP (less than 5 or 6 years), whereas the patients with a longer history of TSP (more than 10 years) only exhibited elevated cytotoxicity of $CD8^+$ T cells. These results suggest that the relative role of humoral and cellular immunity may vary during the progression of TSP (274). A number of investigators have also demonstrated the presence of activated $CD8^+$ cells in spinal cord specimens of TSP patients (275-277). Based on these observations, they hypothesized that $CD4^+$ T cells are activated subsequent to HTLV-I infection. Subsequently, they infiltrate the CNS and transfer infectivity to the constituent cells of the CNS. Cytotoxic $CD8^+$ T cells recognize viral epitopes on HTLV-I-infected cells in the CNS, resulting in cytotoxic demyelination.

So far, the presence of high levels of $CD8^+$ T cells directed specifically against the HTLV-I Tax protein has been demonstrated in all TSP patients (278-281). In contrast, HTLV-I-specific $CD8^+$ T cells from PBL were not detected in two ATL patients in one study (282). The HTLV-I Tax-specific CTL from TSP patients are class I, HLA-A2 allele restricted and recognize a 9 amino acid peptide spanning Tax 11-19 (LLFGYPVYV) (272, 283, 284). The peptide derived from Tax 11-19 has extremely high affinity for the HLA A2 complex and can bind to the HLA-A2 molecule at femtomole levels to induce HTLV-I Tax 11-19 specific CTL-mediated lysis. Direct evidence linking HTLV-I-specific CTL to CNS damage in TSP patients has been derived from a spinal cord biopsy from a TSP patient. Magnetic resonance imaging of the spinal cord of the patient revealed a number of lesions. Almost all of the T cells present in the lesions were $CD8^+$ (272).

The third hypothesis proposes that an autoimmune response mediates HTLV-I- associated CNS damage. Investigators who support this hypothesis believe it is possible that HTLV-I infection activates peripheral cytotoxic T cells or T helper cells which then migrate to the CNS and attack cross-reactive autoantigens presented on target cells or induce an inflammatory reaction (a phenomenon similar to one that occurs in experimental autoimmune encephalomyelitis).

In the fourth hypothesis, it is proposed that CNS dysfunction observed in TSP patients is the result of bystander damage to specific CNS cell populations via indirect mechanisms. HTLV-I infection not only activates target cell gene transcription but also induces the expression of cell surface molecules and the secretion of various cytokines. A number of studies utilized RT-PCR, northern blotting and *in situ* hybridization have demonstrated that steady state levels of mRNAs of TNF alpha, GM-CSF, IFN gamma, IL-1 alpha, IL-6 were elevated in PBMCs of TSP patients (285, 286). TNF alpha can be detected in CSF of TSP patients (287). VCAM-1 gene expression was also elevated in TSP patients in comparison to HTLV-I-infected asymptomatic carriers (132, 286, 288). The elevated levels of cytokine expression were due to the presence of Tax (132, 288) (Figure 6). Immunocytochemical staining of spinal cord samples from autopsied TSP patients utilizing a number of antibodies directed against several cytokines have demonstrated that TNF-alpha, IFN-gamma, and IL-1 beta were expressed in infiltrated macrophages, microglia, and astrocytes in inflammatory lesions (289). Therefore, it appeared that both astrocytes and microglia were involved in the inflammatory process (289). The inflammatory responses induced by cytokines have been well documented. For example, TNF-alpha that is produced by activated T cells and macrophages (290) has been suggested to be one of the etiologic agents of the inflammatory state associated with the progressive neurologic disease observed in HIV-1-infected individuals (291, 292), with multiple sclerosis (293), and in 50% of patients with Guillain-Barré syndrome (294). Additionally, TNF alpha is toxic to oligodendrocytes and causes demyelination *in vitro* (295).

Tax protein is not only a potent *trans*-activator intracellularly, but also an extracellular cytokine, regulating cell proliferation and gene expression in uninfected cells (296-299). Tax is secreted from HTLV-I-infected and -transformed cells such as C81, MT4, and PX1 (296, 298) and can be taken up by cells. Lindholm *et al.* (299) demonstrated that pre-B lymphocytes could take up extracellular Tax. This event was linked to NF-kappaB translocation from cytoplasm to nucleus. The mechanisms governing Tax secretion from HTLV-I-infected and -transformed cells as well as Tax taken up by cells need further examination. In order to determine the possible role of Tax in the development of TSP, Cowan and coworkers demonstrated that synthesis of TNF alpha in NT2-N cells, postmitotic cells that phenotypically and functionally resemble mature primary human neurons, was induced by extracellular soluble Tax in a dose-dependent manner as determined by RT-PCR and ELISA. Prolonged treatment with Tax resulted in Tax-dependent cell death (300). Similarly, Dhib-Jalbut *et al.* demonstrated that extracellular Tax also induced TNF-alpha and IL-6 expression in primary adult human microglia and peripheral blood macrophages (182). Therefore, circulating extracellular Tax in CNS, which either diffuses into the CNS from peripheral blood or secreted by $CD4^+$ T cells, infiltrating the CNS can upregulate inflammatory cytokine expression resulting in neural degeneration in TSP patients in absence of detectable amount of the HTLV-I virus (269).

A decade ago, a new group of cytokines referred to as chemokines, and their receptors were discovered.

Recently, these molecules have been implicated in the development of TSP. Chemokines are basic, low molecular weight proteins (8-12 kDa), and have been classified into four groups, CXC, CX_3C, CC and C, based on the number and spacing between their two conserved cysteine residues in their N-termini. CXC subfamily is comprised of IL-8, IP-10, melanoma growth stimulatory activity (MGSA), and SDF-1. CC subfamily includes MIP-1 alpha and beta, RANTES, monocyte chemotactic protein-1 (MCP-1). Fractalkine, which is chemotactic for T cells and monocytes, and lymphotactin, which is chemotactic for lymphocytes, belong to CX_3C and C subfamilies, respectively (301). All chemokine receptors are seven transmembrane proteins that are linked to guanine nucleotide binding proteins (G-proteins) (302). Currently, 15 different chemokine receptors have been identified (303). Binding of chemokines to their receptors triggers conformational changes within integrin molecules resulting in increased binding affinity of integrins to the cell adhesion molecules, ICAM and VCAM (304). This is probably one of the mechanisms by which lymphocytes infiltrate into different tissues.

A number of studies have demonstrated that HTLV-I infection of T cells results in expression of numerous chemokines and cell adhesion molecules whose level of expression is normally under tight control (130, 132, 137, 138, 305-307). Normally, IL-8 is not constitutively expressed. However, its expression can be induced by a number of molecules including TNF-alpha, IL-1, endotoxin, lectins, and phorbol ester in a variety of cell types including endothelial, epithelial, synovial and T cells, fibroblasts, and some tumor cells (305). IL-8 was orginally thought to primarily attract and activate neutrophils. Later, it was found that IL-8 exerts more chemotactic effect on T cells than neutrophils. Therefore, IL-8 is an important chemokine for regulating the migration and distribution of lymphocytes and neutrophils *in vivo* (308). In HTLV-I-infected T cell lines and newly isolated ATL cells, IL-8 is constitutively expressed (130, 309). Transient transfection with Tax expression vector resulted in IL-8 expression in Jurkat cells. Results from deletion and mutation analyses of IL-8 promoter have demonstrated that NF-kappaB and AP-1 sites were responsible for Tax-mediated *trans*-activation of the IL-8 promoter (305). Constitutive expression of IL-8 by HTLV-I-infection cells may result in abnormal T cell infiltration and accumulation in infected lesions. In addition, HTLV-I-infected T cells also express RANTES, SDF, MIP-1alpha, and MIP-1 beta. Expression of Tax is sufficient to induce the expression of these genes (137, 138).

As discussed previously, the presence of high levels of $CD8^+$ T cells directed specifically against the HTLV-I Tax protein in both peripheral blood and in CSF has been demonstrated in all TSP patients (278-281). Not only can these CTLs attack HTLV-I-infected cells resulting in direct cell damage, but also can secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase, a large family of Zn^{2+} endopeptidases that can degrade matrix proteins and a variety of cell surface molecules (310). They include IFN-gamma, TNF-alpha, IL-16, MIP-1 alpha, MIP-1 beta, and matrix metalloproteinase-9 (311). Therefore, it seems that the same mechanisms regulating normal inflammatory responses *in vivo* are also involved in CNS tissue damage in HTLV-I-infected patients.

8. CONCLUSION

The past twenty years of research have resulted in the accumulation of a wealth of knowledge about HTLV-I and its pathogenesis. Human global activities provide HTLV-I with unprecedented opportunities to spread worldwide. Consistent with this notion, an extensive epidemiological study carried out in Europe recently indicated that at least 2 out of 1,000 women in antenatal clinics in France and the United Kingdom were HTLV-I carriers (312). Although routine screening for HTLV-I-positive blood donors by detection of viral structure protein antibodies has been performed in the United States since 1988 (313), recent studies demonstrated that this method substantially underestimated the prevalence of HTLV-I infection in the general population in the United States since patients with mycosis fungoides harbor HTLV-I Tax-related sequences in their genomes without antibodies to the virus. Therefore, development of safer and more reliable blood donor screening methods including both molecular and serological tests should be given high priority (314).

The T cell transformation caused by HTLV-I infection is a long and complex process involving multiple players. Among these, Tax is the most important factor contributing to the viral-mediated initial stage of transformation. The highly promiscuous and pleiotropic nature of Tax presents a formidable challenge to scientists working on elucidating the mechanisms of its function at molecular, cellular, and organism levels. It is evident that Tax dysregulates cell regulation by mediating abnormal cellular gene expression, and by interacting with cell cycle control elements as well as cell signal transduction molecules. Basic studies concerning gene transcriptional regulation and cell cycle control will be crucial to improve our understanding of HTLV-I pathogenesis. In addition, studies of other oncogenic viruses such as SV40 virus, human papilloma virus, and adenovirus will greatly expedite our understanding of these processes since most of these viruses utilize similar strategies to transform their host cells. The ultimate goal is to fully understand HTLV-I pathogenesis at the molecular level to facilitate the design of effective therapeutic treatments or preventive strategies.

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