Seizing of T Cells by Human T-Cell Leukemia/Lymphoma Virus Type 1

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Human T-cell leukemia/lymphoma virus type 1 (HTLV-1) causes neoplastic transformation of human T-cells in a small number of infected individuals several years from infection. Several viral proteins act in concert to increase the responsiveness of T-cells to extracellular stimulation, modulate proapoptotic and antiapoptotic gene signals, enhance T-cell survival, and avoid immune recognition of the infected T-cells. The virus promotes T-cell proliferation by usurping several signaling pathways central to immune T-cell function. Viral proteins modulate the downstream effects of antigen stimulation and receptor–ligand interaction, suggesting that extracellular signals are important for HTLV-1 oncogenesis. Environmental factors such as chronic antigen stimulation are therefore important, as also suggested by epidemiological data. The ability of a given individual to respond to specific antigens is determined genetically. Thus, genetic and environmental factors, together with the virus, contribute to disease development. As in the case of other virus-associated cancers, HTLV-1–induced leukemia/lymphoma can be prevented by avoiding viral infection or by intervention during the asymptomatic phase with approaches able to interrupt the vicious cycle

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of virus-induced proliferation of a subset of T-cells. This review focuses on current knowledge of the mechanisms regulating HTLV-1 replication and the T-cell pathways that are usurped by viral proteins to induce and maintain clonal proliferation of infected T-cells *in vitro*. The relevance of these laboratory findings will be related to clonal T-cell proliferation and adult T-cell leukemia/lymphoma development *in vivo*.

I. INTRODUCTION

The immortalization of T-cells by human T-cell leukemia/lymphoma virus type 1 (HTLV-1) *in vitro* may epitomize early steps of viral oncogenicity and offers an exceedingly interesting model whereby to study the effect of viral genes on transcription factors, cell cycle regulators, growth factors, mediators of cell-to-cell interaction [chemokines, major histocompatibility complex classes I and II (MHC I, MHC II), growth factor receptors, etc.], and sorting and degradation pathways [endoplasmic reticulum (ER)-associated degradation (ERAD), proteasomes, etc.].

Limitations in the *in vitro* experimental models used to study HTLV-1, however, need to be recognized up front. Basic studies on viral entry and the role of viral genes in the regulation of viral replication have been hampered by the inefficiency of cell-free virus infection (De Rossi *et al.*, 1985; Derse *et al.*, 2001; Hoffman *et al.*, 1992; Koralnik *et al.*, 1992b) and by the fact that the identity of the cellular receptor(s) remains unknown (Jones *et al.*, 2002; Manel *et al.*, 2003; Nath *et al.*, 2003).

Although it is possible to immortalize and transform human $CD4^+$ T-cells *in vitro* (Markham *et al.*, 1983; Popovic *et al.*, 1983; Yamamoto *et al.*, 1982), several lines of evidence indicate that the clonal leukemic T-cells found in adult T-cell leukemia/lymphoma (ATLL) invariably fail to grow *in vitro*. Typically, T-cell lines obtained from ATLL patients differ from the leukemic clone, suggesting the *in vitro* outgrowth of infected non-leukemic cells. Indeed, no T-cell line that carries the same chromosomal aberration as the original ATLL cells has been developed.

The effect of ectopic expression of single viral genes on T-cell lines, as well as cells of other lineages, at times appears to be highly context dependent, which may relate to preexisting somatic mutations that may vary among cell lines, suggesting that validation in primary human T-cells may be essential.

The demonstration of the susceptibility of rabbits to HTLV-1 infection (Miyoshi *et al.*, 1985) and the generation of HTLV-1 molecular clones (Derse *et al.*, 1996; Kimata *et al.*, 1994; Nicot *et al.*, 1993; Zhao *et al.*, 1995) infectious in rabbits and nonhuman primates have provided animal models to study the contribution of viral genes to viral replication *in vivo* (Collins *et al.*, 1996; Wei and Fultz, 2002). However, because ATLL does

not develop in any of these animal models, proper validation of the findings obtained in *in vitro* cell systems is not feasible.

Epidemiological, clinical, and laboratory evidence demonstrates that HTLV-1 favors the clonal expansion of infected T-cells *in vivo* (Cavrois *et al*., 1998; Wattel *et al*., 1995), leaving little doubt that HTLV-1 is the cause of ATLL (Murphy *et al*., 1989). Nevertheless, a body of evidence suggests that while restricted viral expression may occur during HTLV-1 infection, the expression of viral genes, including *tax*, may not be required at all in ATLL cells (Franchini *et al*., 1984a; Franchini *et al*., 1984b; Kinoshita *et al*., 1989; Korber *et al*., 1991; Okazaki *et al*., 2001).

Although most of the basic research on HTLV-1 has been on Tax, the viral transactivator (Bex *et al*., 1997; Jeang, 2001; Marriott *et al*., 2002; Yoshida and Suzuki, 1995), studies on other viral proteins also encoded by the 3′ end of the viral genome are emerging (Albrecht and Lairmore, 2002; D'Agostino *et al*., 2002; Gaudray *et al*., 2002; Johnson *et al*., 2001; Nicot *et al*., 2001b, 2003; Zhang *et al*., 2000, 2001).

Tax can immortalize primary T-cells inefficiently, and continuous T-cell receptor (TCR) stimulation and interleukin-2 (IL-2) are often necessary in the first months of culture. This *in vitro* phenomenon may well mimic the *in vivo* situation except that it does not account for the host immune response.

The concerted expression of viral proteins is likely necessary for the maintenance of HTLV-1 infection in the host, as suggested by studies in the rabbit model (Albrecht and Lairmore, 2002). Finally, the low frequency of occurrence of ATLL in infected people suggests the requirement of additional somatic mutations for the development of full-blown disease. In fact, a variety of chromosomal aberrations, such as deletions, translocations, and rearrangements, are found consistently in ATLL cells (Itoyama *et al*., 2001; Kamada *et al*., 1992; Rowley *et al*., 1984).

II. HUMAN T-CELL LEUKEMIA/LYMPHOMA VIRUS TYPE 1 (HTLV-1)

HTLV-1 (Gallo, 1986; Poiesz *et al*., 1980a; Yoshida *et al*., 1982) is a complex retrovirus that infects and induces the *in vitro* growth of human $CD3^+$ T-cells, most often expressing the CD4 molecule (Miyoshi *et al*., 1981). *In vivo*, HTLV-1 is associated epidemiologically with a mature $CD3^+$ $CD4^+$ T-cell type leukemia/lymphoma (Blattner *et al*., 1982; Catovsky *et al*., 1982; Hinuma *et al*., 1981; Robert-Guroff *et al*., 1981), a condition designated as ATLL (Takatsuki *et al*., 1977). HTLV-1 is also associated with a progressive myelopathy designated tropical spastic

paraparesis/HTLV-1–associated myelopathy (TSP/HAM) (Gessain *et al.*, 1985; Osame *et al.*, 1986; Rodgers-Johnson *et al.*, 1985), of probable immune-mediated pathogenesis (Bangham, 2000; Hollsberg and Hafler, 1995; Jacobson *et al.*, 1990; Jacobson, 2002; Osame, 2002).

HTLV-1 is transmitted by breast milk, sexually, and by blood products (Blattner, 1990; Murphy *et al.*, 1989; Yamaguchi and Takatsuki, 1993). It is estimated that 20 million people worldwide may be infected with HTLV-1 (de The and Bomford, 1993) and, of those, approximately 400,000 people (1–2%) will develop leukemia or TSP/HAM in their lifetime (Tajima and Kuroishi, 1985). ATLL cells are of clonal origin and usually carry a single copy of integrated virus (provirus) (Wong-Staal *et al.*, 1983; Yoshida *et al.*, 1984). Clinically, ATLL can be of the smoldering, chronic, or acute type (Takatsuki *et al.*, 1985; Yamaguchi *et al.*, 1983). The current treatments of ATLL fail to induce long-term remission, and even the clinically less aggressive forms of ATLL are fatal.

A. The Viral Genome

HTLV-1 is a single-stranded diploid RNA virus (Seiki *et al.*, 1983), belongs to the family of Deltaretroviruses (Fields *et al.*, 1996), and carries genetic information for structural proteins and enzymes (Gag and Env, reverse transcriptase, protease, integrase) (Bertola *et al.*, 2001; Ha *et al.*, 2002; Heidecker *et al.*, 2002; Kobayashi *et al.*, 1991; Le *et al.*, 2002; Mariani and Beckham, 2003; Muller and Krausslich, 1999; Trentin *et al.*, 1998). The 3′ end of the viral genome was defined previously as the pX region, as its coding capacity was undefined (Seiki *et al.*, 1983). However, that nomenclature is now obsolete, as several viral proteins encoded by open reading frames (ORFs) I–IV, whose expression occurs through alternative splicing of the genomic RNA, have been identified (Fig. 1 and Table I).

Both singly spliced and doubly spliced ORF I mRNAs encode a protein of 12 kDa ($p12^{I}$) (Ciminale *et al.*, 1992; Koralnik *et al.*, 1992a, 1993) that has weak oncogenic potential (Franchini *et al.*, 1993), affects calcium release from cells (Ding *et al.*, 2002), activates the nuclear factor of activated T-cell (NF-AT)-dependent transcription (Albrecht *et al.*, 2002), and affects cell signaling and trafficking of cellular receptors (Johnson *et al.*, 2001; Mulloy *et al.*, 1998a; Nicot *et al.*, 2001b) (Table I). A doubly spliced mRNA from ORF II encodes the HTLV-1 $p30^{II}$ protein (Ciminale *et al.*, 1992; Koralnik *et al.*, 1992a), a negative posttranscriptional regulator of viral expression (Nicot *et al.*, 2003). $p30^{II}$ also affects the transcriptional activity of cAMP-responsive element (CRE) or Tax-responsive element (TRE) promoters (Zhang *et al.*, 2000, 2001).

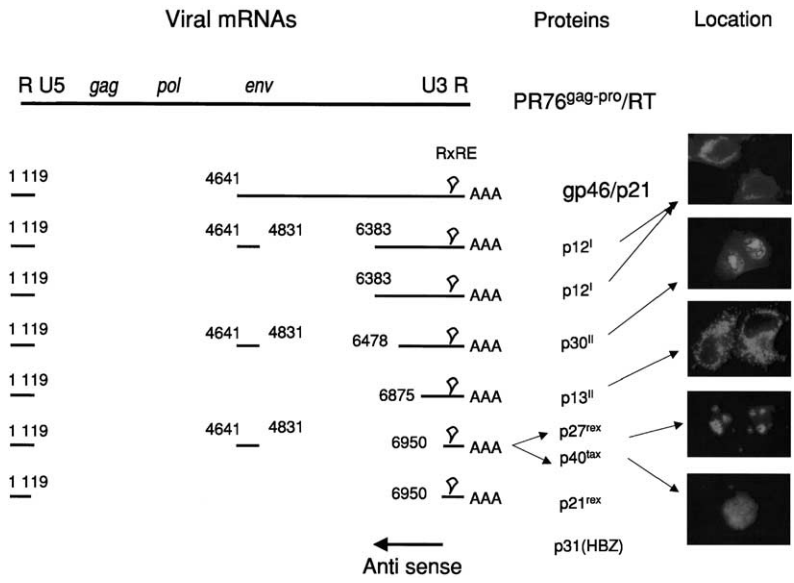

Fig. 1 Diagram of the derivation of singly and doubly spliced mRNAs in the HTLV-1 genomic RNA. Descriptions of the alternatively spliced mRNAs for all these ORFs can be found in the following references: Tax and Rex (Aldovini *et al*., 1986; Seiki *et al*., 1985; Wachsman *et al*., 1985), $p12^{I}$ and $p13^{II}$ (Berneman *et al*., 1992; Koralnik *et al*., 1992a), $p30^{II}$ (Ciminale *et al*., 1992; Koralnik *et al*., 1992a), and $p21^{rex}$ (Furukawa *et al*., 1991; Orita *et al*., 1991).

A protein of 13 kDa ($p13^{II}$) encoded by a singly spliced mRNA from ORF II (Berneman *et al*., 1992; Koralnik *et al*., 1992a, 1993) affects mitochondrial membrane potential (Ciminale *et al*., 1999; D'Agostino *et al*., 2002).

A doubly spliced mRNA within the most distal 3′ end of the viral genome encodes two well-studied positive regulators of viral gene expression: the Rex and Tax proteins from ORFs III and IV, respectively (Aldovini *et al*., 1986; Seiki *et al*., 1985; Wachsman *et al*., 1985). The Rex protein regulates viral mRNA export from the nucleus to the cytoplasm by exploiting cellular proteins involved in nuclear cytoplasmic transport (Felber, 1997). ORF III also encodes, by alternative splicing, the $p21^{rex}$ protein (Berneman *et al*., 1992; Orita *et al*., 1991), of unknown function (Table I). Finally, a protein of 31 kDa, HTLV-1 basic region zipper (bZIP) factor (HBZ), is encoded by the 3′ end antisense RNA. HBZ forms heterodimers with CREB-2 and suppresses Tax- and CREB-2–mediated transcription from the TRE within the viral long terminal repeat (LTR) (Gaudray *et al*., 2002; Larocca *et al*., 1989).

Table I Cellular Localization and Targets of HTLV-1 Proteins Described by the 3′ End of the Virus

	mRNA	Protein	Cellular location	Physical interaction with cellular targets	Known functional effects
ORF I	*ORF I* (singly spliced)	$p12^{I}$	ER and Golgi	Vacuolar ATPase (16 kDa)	Unknown
				IL-2R β, IL-2R γ_c	STAT5 transcriptional activation
				Calreticulin	Calcium release
				Calnexin	NF-AT transcriptional activation
				MHC I-Hc	Immune evasion
	Rex ORF I (doubly spliced)		?	?	?
ORF II	*ORF II* (singly spliced)	$p13^{II}$	Nucleus and mitochondria	Farnesyl-pyrophosphate synthesis	Disruption of inner mechanism potential of mitochondria
	Tax ORF II	$p30^{II}$	Nucleolus and nucleus; does not shuttle into and out of nucleus	p300	Modulation of CRE and TRE; viral latency by reducing of Tax and Rex
ORF III	*Tax/Rex* (doubly spliced mRNA)	$p27^{rex}$	Shuttles into and out of nucleus	Nuclear pore	Transport of genomic and singly spliced mRNAs to the cytoplasm
	$p21^{rex}$	$p21^{rex}$	Cytoplasm	?	?
ORF IV	*Tax/Rex*	$p40^{tax}$	Nucleus and cytoplasm	Several cellular targets (see Section II)	Pleiotropic effects (see Section II)
3′ end antisense strand	2.5-kDa antisense RNA at 3′ end of viral genome	p31 (HBZ)	Nucleus	CREB-2	Downregulation of CREB-2–mediated HTLV-1 transcription

Tax increases transcription of the viral promoter, exploiting the CREB/ATF family of transcription factors, and of cellular promoters by usurping the NF-κB, AP-1, and serum responsive factor (SRF) pathways with consequent activation or suppression of a plethora of cytokines and chemokines (Ballard *et al*., 1988; Hiscott *et al*., 2001; Jeang, 2001). Tax also affects cell cycle regulators (Yoshida and Suzuki, 1995), DNA repairs (Marriott *et al*., 2002), and cellular pathways involved in apoptosis.

B. HTLV-1 Open Reading Frames (ORFs) I, II, and III

1. $p12^I$

$p12^I$ is a small hydrophobic protein encoded by both a singly and a doubly spliced mRNA within ORF I. The singly spliced ORF I mRNA for $p12^I$ has been demonstrated in HTLV-1–infected T-cell lines and in *in vitro*–infected macrophages (Koralnik *et al*., 1992b), as well as in *ex vivo* samples from healthy carriers (Ciminale *et al*., 1992; Koralnik *et al*., 1992b, 1993). The doubly spliced ORF I mRNA has also been found mainly in *ex vivo* samples of ATLL (Koralnik *et al*., 1992a).

Importantly, cytotoxic T lymphocytes (CTLs) and serum from HTLV-1–infected individuals or experimentally infected rabbits have been demonstrated to recognize ORF I-derived peptides (Dekaban *et al*., 2000b; Pique *et al*., 2000), providing critical, although indirect, evidence of the synthesis of this protein in infected cells.

$p12^I$ localizes to the ER (Koralnik *et al*., 1993) and Golgi (Ding *et al*., 2001; Johnson *et al*., 2001). While $p12^I$ is not necessary for HTLV-1 replication *in vitro* (Albrecht *et al*., 2000; Derse *et al*., 1997; Robek *et al*., 1998), $p12^I$ contributes to viral infectivity *in vivo* inasmuch as ablation of the acceptor splice site for the singly spliced mRNA is associated with a decreased infectivity in rabbits (Collins *et al*., 1998).

Over the past few years, the understanding of $p12^I$ function has increased significantly. $p12^I$ is a weak oncogene that has amino acid similarities with the bovine papillomavirus type 1 (BPV) E5 protein (Franchini *et al*., 1993) and forms dimers (Trovato *et al*., 1999). Two natural variants of $p12^I$ have been identified, one of which is ubiquitinated at a lysine at position 88 (Fig. 2A), is targeted to the proteasome for degradation, and has a very short half-life (Martins *et al*., 2002; Trovato *et al*., 1999). The amino acid sequence of $p12^I$ is highly conserved among *ex vivo* DNA samples of HTLV-1–infected individuals and contains four proline-rich (PXXP) Src homology 3 (SH3)-binding domains (Franchini, 1995), a motif that has been shown to be important for the interaction of proteins involved in intracellular signaling (Feller *et al*., 1994; Mayer, 2001).

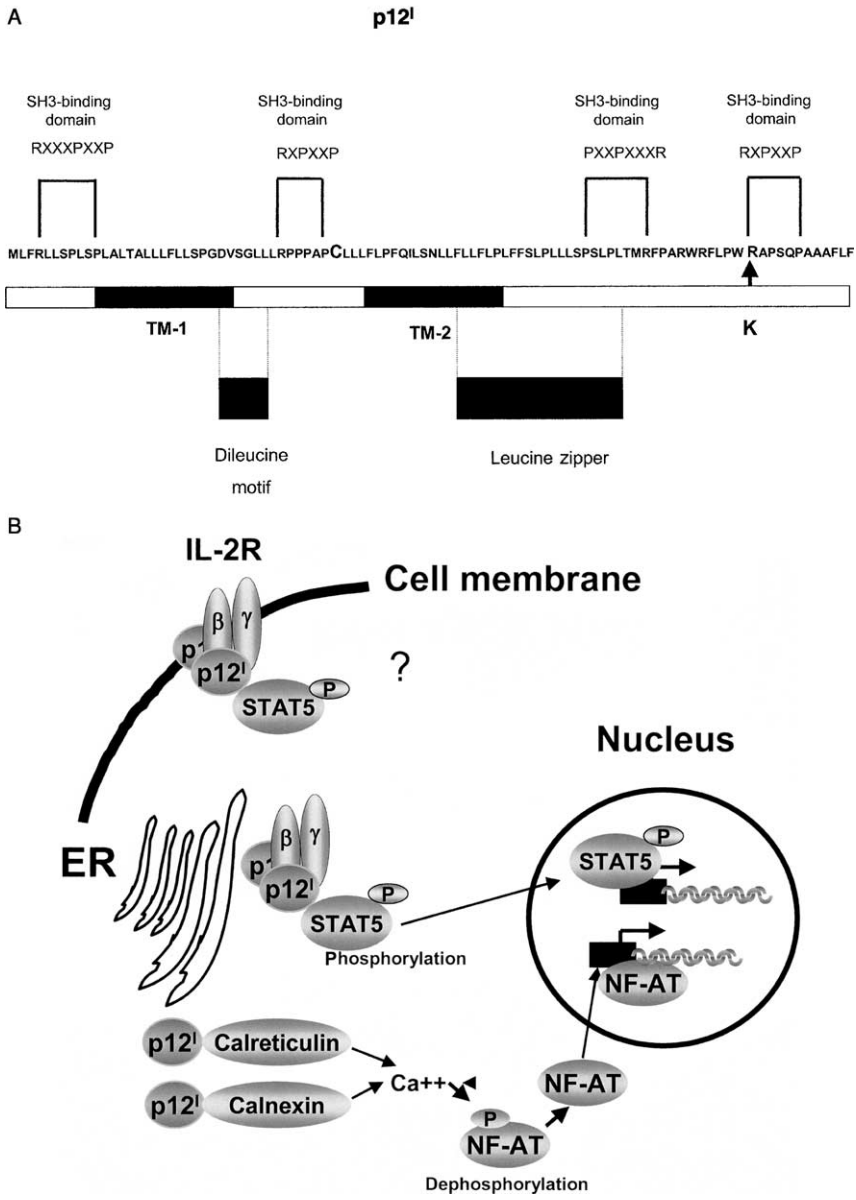

Fig. 2 (*continued*)

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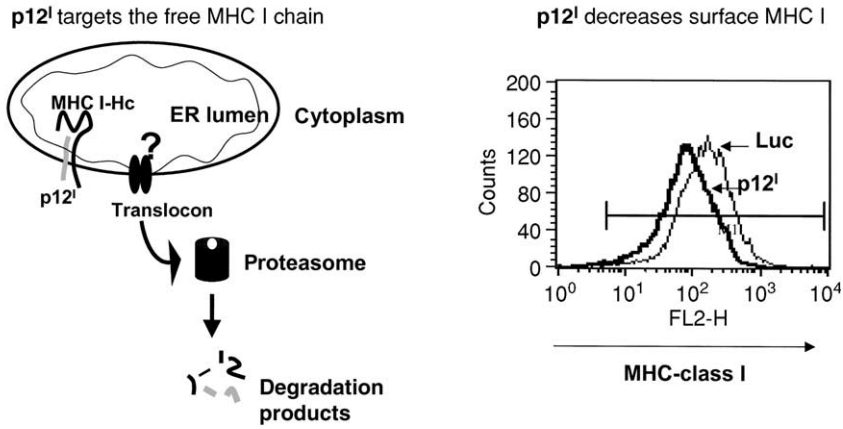

Fig. 2 $p12^I$ cellular targets. (A) $p12^I$ amino acid sequence and domains. (B) The effect of $p12^I$ on T-cell activation. $p12^I$ binds IL-2R β and γ_c chains and activates STAT5 transcriptional activity. $p12^I$ may also modulate T-cell signaling through its interaction with calreticulin and calnexin and the consequent release of intracellular calcium, which in turn may lead to the activation of NF-AT, a transcription regulator. (C, left) $p12^I$ binds and targets MHC I-Hc to the proteasome for degradation. The MHC I-Hc/$p12^I$ complex may be removed from the ER through the translocon and degraded by the proteasome. (Right) Trafficking of MHC I is altered in cells expressing $p12^I$. There was an approximate 50% decrease in MHC I on the surface of Jurkat cells expressing $p12^I$ (thick lines) in comparison to the control (thin lines). Reprinted from Johnson *et al.* (2001).

Indeed, to date, $p12^I$ has been shown to interact with several cellular proteins. $p12^I$ interacts with the 16-kDa subunit of the vacuolar ATPase (Franchini *et al.*, 1993; Koralnik *et al.*, 1995), a complex that is important for the acidification of lysosomes and endosomes (Stevens and Forgac, 1997). The significance of this interaction is at present unknown in the case of HTLV-1 infection. However, in human immunodeficiency virus (HIV) infection, the Nef protein's downregulation of CD4 from the cell surface has been associated with the ability of Nef to interact with the catalytic unit of the vacuolar ATPase, which may connect Nef with the endocytic pathway (Lu *et al.*, 1998b).

The finding that $p12^I$ interacts with IL-2 receptor (IL-2R) β and γ_c chains and affects their surface expression (Mulloy *et al.*, 1996) led to the hypothesis that $p12^I$ may contribute to T-cell proliferation. Indeed, $p12^I$, when expressed in the context of an ectopically reconstituted IL-2R signaling pathway, binds the cytoplasmic domain of IL-2R β involved in the recruitment of Janus-associated kinases (Jak) 1 and 3. As a result of this interaction,

$p12^I$ increases signal transducers and activators of transcription-5 (STAT5) DNA binding (Fig. 2B) and transcriptional activity, and this effect was demonstrated to be dependent on the presence of IL-2R β, γ_c, and Jak3 (Nicot *et al.*, 2001b). Similarly, $p12^I$ increases STAT5 activation in primary T-cells, which acquire a proliferative advantage dependent on TCR signaling. Indeed, the effect of $p12^I$ is observed only following suboptimal treatment of T-cells with low concentrations of αCD3 and αCD28 antibody stimulation together with IL-2 (Nicot *et al.*, 2001b). Collectively, these data suggest that $p12^I$ expression may decrease the threshold of T-cell activation and favor the entry of T-cells into S phase even in conditions of suboptimal antigen stimulation.

The finding that $p12^I$ also interacts with both calnexin and calreticulin (Fig. 2B), two ER-resident proteins that regulate calcium storage and increase calcium release (Albrecht *et al.*, 2000; Ding *et al.*, 2001), further underscores the importance of $p12^I$ in conferring a proliferative advantage to T-cells (Albrecht *et al.*, 2000; Ding *et al.*, 2001). Antigen stimulation results in an increase in intracellular calcium levels, which signals T-cell activation (Lewis, 2001). Calreticulin is an upstream activator of NF-AT; elevated calcium levels result in the dephosphorylation and translocation of NF-AT to the nucleus, where it binds DNA and modulates gene expression, including the expression of the *IL-2* gene as well as other cytokine genes (Lewis, 2001; Rusnak and Mertz, 2000). By decreasing the threshold of T-cell activation through NF-AT transcriptional activation and increasing the responsiveness of IL-2 by STAT5 activation, $p12^I$ may amplify physiological signaling through the TCR, even in a microenvironment where both antigens and growth factors are limiting (Fig. 2B) and favor the clonal expansion of T-cells.

In addition to increasing T-cell proliferation, $p12^I$ may help establish a chronic viral infection through its interference with the MHC I complex (Johnson *et al.*, 2001; Johnson and Franchini, 2002). Many viruses and bacteria have developed a mechanism(s) to escape the immune response of the host (Tortorella *et al.*, 2000); HTLV-1 causes a chronic infection, suggesting that it may have evolved strategies to protect infected cells from immune recognition. In this regard, $p12^I$ has been shown to bind to the free MHC I heavy chain (MHC I-Hc) in the ER (Fig. 2C, left), where it inhibits its maturation and association with β_2-microglobulin (Johnson *et al.*, 2001). Part of the MHC I-Hc/$p12^I$ complex is removed from the ER and degraded by the proteasome (Johnson *et al.*, 2001), presumably through a pathway that removes misfolded, improperly assembled, or glycosylated proteins from the ER (ERAD) (Fig. 3) (Bonifacino and Klausner, 1994; Plemper and Wolf, 1999). Targeting of the free MHC I-Hc by $p12^I$ represents a novel mechanism of viral interference with MHC I, as other viruses most often target the MHC I-Hc/β_2-microglobulin complex (Furman and Ploegh,

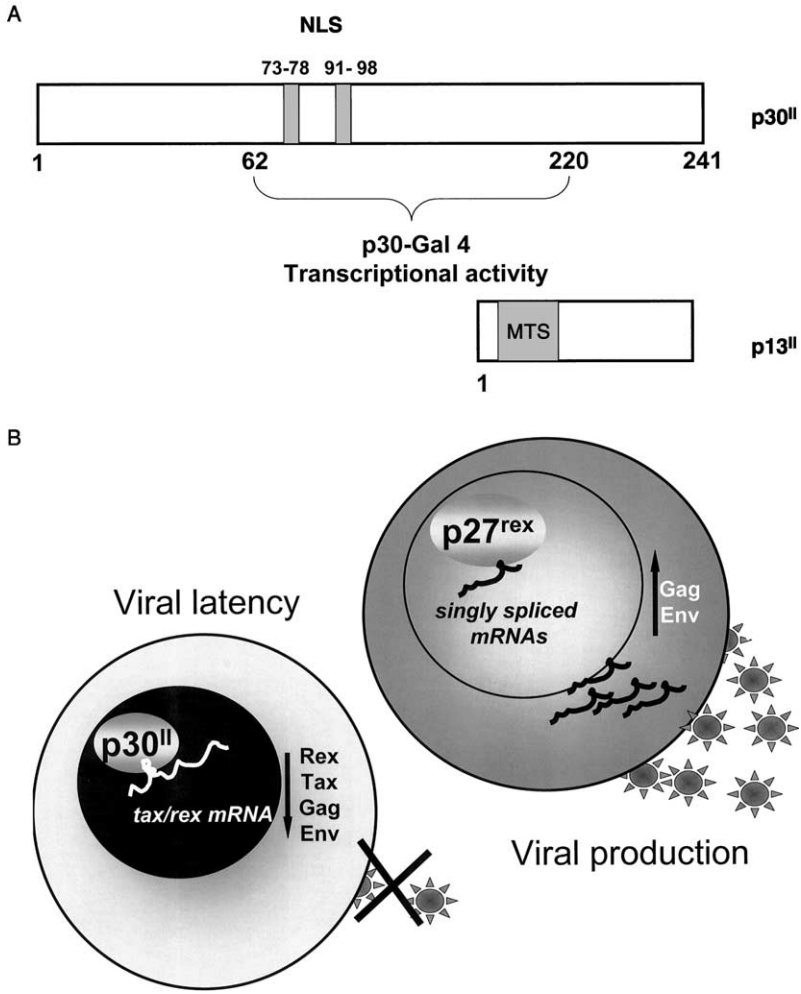

Fig. 3 $p30^{II}$ and $p13^{II}$. (A) Schematic representation of known domain locations in $p30^{II}$ and $p13^{II}$. (B) Role of $p30^{II}$ and Rex proteins, negative and positive regulators, respectively, of viral expression.

2002). $p12^{1}$ induces a reduction of approximately 50% in the levels of MHC I on the surface of T-cells (Johnson *et al.*, 2001) (Fig. 2C, right) and, consequently, this event may translate into decreased MHC I/peptide complexes at the cell surface. Interestingly, in a rat model, it has been demonstrated that a 50% reduction of MHC I expression in cells presenting Tax epitopes resulted in escape from immune recognition (Ohashi

et al., 1999). However, more studies are needed to demonstrate whether the level of the downregulation of MHC I/viral peptide complexes at the cell surface induced by $p12^{I}$ would be sufficient to allow virally infected cells to escape detection by the immune system of the host. In addition, the effect of the two natural variants of $p12^{I}$ on antigen presentation and the mechanism(s) involved in the MHC I-Hc/$p12^{I}$ degradation need to be dissected further.

All together, the effect of $p12^{I}$ on T-cell proliferation and MHC I downregulation may explain the phenotype observed *in vivo* inasmuch as the ablation of $p12^{I}$ in a proviral clone is associated with decreased infectivity in rabbits (Collins *et al.*, 1998). In this scenario, infected T-cells may lose their proliferative advantage and, at the same time, be recognized more efficiently by the immune system of the host, leading to a decrease in virus load (number of cells carrying the provirus). Because the virus load correlates with disease development (Nagai *et al.*, 1998), $p12^{I}$ may play a very important role in the pathogenesis of HTLV-1 infection.

2. $p30^{II}$

A doubly spliced *Tax* ORF II mRNA encodes the $p30^{II}$ protein (Ciminale *et al.*, 1992; Koralnik *et al.*, 1992a) that, when expressed ectopically, localizes to the nucleus and nucleoli of transfected cells (D'Agostino *et al.*, 1997; Koralnik *et al.*, 1993). A singly spliced ORF II mRNA that encodes a protein of 13 kDa ($p13^{II}$) has been found in HTLV-1–infected cells (Koralnik *et al.*, 1992a) and in some samples of *ex vivo* ATLL cells (Berneman *et al.*, 1992). The doubly spliced ORF II mRNA was found independently by Ciminale *et al.* (1992) and Koralnik *et al.* (1992) in HTLV-1–infected cells. This mRNA encodes a protein of 30 kDa, originally designated as either $p30^{II}$ or Tof.

As in the case of ORF I, only indirect evidence of $p30^{II}$ expression in infected cells exists. Cytotoxic T-cells and antibodies to ORF II peptides can be found in HTLV-1–infected individuals (Chen *et al.*, 1997; Pique *et al.*, 2000), and ablation of both $p30^{II}$ and $p13^{II}$ from a molecular clone of HTLV-1 impairs its infectivity in a rabbit model (Bartoe *et al.*, 2000), again providing critical evidence of the expression and importance of these proteins in HTLV-1 infection.

Interestingly, both ORF I and ORF II appear to be dispensable for the infection and immortalization of human T-cells *in vitro* (Albrecht *et al.*, 2000; Derse *et al.*, 1997; Robek *et al.*, 1998). ORF II has the potential to encode the nuclear/nucleolar $p30^{II}$ (Koralnik *et al.*, 1993) and the mitochondrial/nuclear $p13^{II}$ (D'Agostino *et al.*, 2001; Koralnik *et al.*, 1993) proteins, and neither is required for viral replication or immortalization *in vitro* (Bartoe *et al.*, 2000). $p30^{II}$ has been suggested to play a role in the

transcriptional modulation of cellular genes, as it contains serine-rich regions with distant homology to several transcriptional activators: Oct-1, Oct-2, Pit-1, Engrailed, and POU-M1 (Ciminale *et al*., 1992). $p30^{II}$ also carries a bipartite nuclear localization signal (Fig. 3A) (D'Agostino *et al*., 1997).

Enforced expression of $p30^{II}$ alone in nonlymphoid cells had no significant transcriptional effect on the viral LTR in some studies (Ciminale *et al*., 1992; Nicot *et al*., 2003; Roithmann *et al*., 1994) but it did in another (Zhang *et al*., 2000). However, a $p30^{II}$–Gal4 fusion protein has been shown to activate transcription of a Gal4-driven *luciferase* reporter gene, as well as to differentially modulate the activity of HTLV-1 LTR- and CRE-driven reporter genes in nonlymphoid cells (Zhang *et al*., 2000). In this case, it was suggested that this modulation occurs through the interaction of $p30^{II}$ with the transcriptional coactivator, p300.

The coactivators p300 and CREB-binding protein (CBP) act as transcriptional mediators by interacting with various cellular and viral proteins and have intrinsic histone acetyltransferase activity, which is believed to regulate chromatin structure necessary for the initiation of gene transcription (Blobel, 2002).

An epitope-tagged form of $p30^{II}$ ($p30^{II}$-HA1) was found to colocalize with p300 in the nuclei of HeLa cells and bind to CBP/p300, suggesting that p300 may be a cellular target of $p30^{II}$ and may contribute to the transcriptional effects of $p30^{II}$–Gal4 (Zhang *et al*., 2001). $p30^{II}$–Gal4 repression/activation of both TRE- and CRE-driven reporter gene activities (Zhang *et al*., 2001) contrasts with the exquisite specificity of Tax to transactivate viral promoters and may be mediated by the ability of $p30^{II}$ to bind the KIX domain of CBP/p300, as does Tax (Giebler *et al*., 1997; Harrod *et al*., 1998; Kashanchi *et al*., 1998; Kwok *et al*., 1996; Zhang *et al*., 2001), and destabilize the Tax, p300, and CREB complexes *in vitro*. More work will be required to assess whether $p30^{II}$ acts as a transcriptional regulator of viral LTRs in T-cells.

Evidence from Nicot *et al*. (2003) demonstrated that $p30^{II}$ may have a quite different function in the viral life cycle: $p30^{II}$ downregulates viral replication by a novel posttranscriptional mechanism. $p30^{II}$ is localized in the nucleus and nucleoli, does not shuttle into and out of the nucleus, and specifically retains the *tax/rex* mRNA in the nucleus, reducing Tax and Rex protein levels in infected cells. Viral expression is decreased quickly through a posttranscriptional mechanism because the decreased level of Rex impairs the nuclear-cytoplasmic transport of mRNAs for the Gag and Env proteins (Fig. 3B). The decrease in Rex function might be faster than the decrease in Tax function, as the half-life of the Tax protein is 15 h (Hemelaar *et al*., 2001). These data demonstrate that $p30^{II}$ posttranscriptionally silences viral expression and may contribute to viral latency.

One study reported a lack of effect on the expression of Tax or Env following the ectopic expression of $p30^{II}$ (Roithmann *et al.*, 1994). We believe, as also acknowledged by the authors, that this apparent discrepancy may be related to the use of a construct that expressed $p30^{II}$ inefficiently from the doubly spliced mRNA. Interestingly, in that report, forced expression of $p30^{II}$ resulted in decreased expression of Rex and Env even though it was not emphasized by the authors.

3. $p13^{II}$

$p13^{II}$ is encoded by a singly spliced mRNA found in *ex vivo* ATLL samples (Berneman *et al.*, 1992) as well as in infected T-cell lines (Koralnik *et al.*, 1992b). Expressed ectopically, $p13^{II}$ has been shown to localize in the nucleus and mitochondria of transfected cells (Ciminale *et al.*, 1999; D'Agostino *et al.*, 1997, 2001; Koralnik *et al.*, 1993). Indeed, $p13^{II}$ carries a mitochondrial targeting signal (MTS) at its amino terminus (Fig. 3A). $p13^{II}$ expression alters the mitochondrial structure and disrupts the inner membrane potential (Ciminale *et al.*, 1999; D'Agostino *et al.*, 2002). Mitochondria provide much of the energy for the cell as well as have an important impact on the life and death of the cell by releasing cytochrome *c*; thus, $p13^{II}$ is located in a vital cellular organelle (Desagher and Martinou, 2000). It is suggested that the effect of $p13^{II}$ on mitochondria may be important for HTLV-1 replication or pathogenicity, as was demonstrated for African swine fever virus (Rojo *et al.*, 1998). The redistribution of mitochondria to perinuclear viral assembly sites and the subsequent enhancement of mitochondrial respiratory functions by the African swine fever virus may provide energy for viral morphogenesis (Rojo *et al.*, 1998). Studies have demonstrated an interaction of $p13^{II}$ and farnesyl pryophosphate synthetase (FPPS), similar to the bovine leukemia virus (BLV) G4 protein (Lefebvre *et al.*, 2002). FPPS, an enzyme involved in the prenylation of several proteins, including Ras (Rao, 1995), was shown to interact with the BLV G4 protein during cellular transformation (Lefebvre *et al.*, 2002), which raises the possibility that the interaction of $p13^{II}$ and FPPS may play a role in HTLV-1 transformation.

4. Rex and $p21^{REX}$

Two proteins are encoded by ORF III: a 27-kDa protein, Rex ($p27^{rex}$), encoded by the doubly spliced *tax/rex* mRNA, and a smaller singly spliced cytoplasmic protein, $p21^{rex}$ (Berneman *et al.*, 1992; Kiyokawa *et al.*, 1985; Nagashima *et al.*, 1986; Orita *et al.*, 1991). The function of $p21^{rex}$ is unknown. The fact that $p21^{rex}$ mRNA and $p21^{rex}$ proteins were found in several HTLV-1-infected cell lines (Furukawa *et al.*, 1991) and at high

levels in primary uncultured cells from ATLL patients suggests that $p21^{rex}$ may be important in HTLV-1 infection (Berneman *et al*., 1992; Furukawa *et al*., 1991; Kubota *et al*., 1996; Orita *et al*., 1991, 1992).

Rex localizes to the nucleolus of infected cells, shuttles into and out of the nucleus, and plays an important role in the regulation of viral replication (Hidaka *et al*., 1988; Inoue *et al*., 1991; Nagashima *et al*., 1986; Nosaka *et al*., 1989; Ohta *et al*., 1988; Siomi *et al*., 1988; Smith and Greene, 1991). Rex has at least three domains that are important for its functions: an arginine-rich region important for binding to the Rex-responsive element, designated RxRE (Bogerd *et al*., 1991; Bogerd *et al*., 1992; Grassmann *et al*., 1991), a region near the RNA-binding domain that is required for the assembly of multimeric Rex complexes onto the RxRE (Bogerd and Greene, 1993), and a leucine-rich nuclear export signal that interacts with the nuclear export receptor, CRM-1 (Hakata *et al*., 1998; Palmeri and Malim, 1996). Rex also binds to the nucleoporin-like Rev cofactor (Bogerd *et al*., 1995; Fritz *et al*., 1995).

Rex is a site-specific-binding protein whose activity is dependent on its ability to bind to a highly stable stem–loop structure located within the viral LTR, RxRE (Ballaun *et al*., 1991; Bar-Shira *et al*., 1991; Hanly *et al*., 1989; Seiki *et al*., 1988; Unge *et al*., 1991; Yoshida and Seiki, 1987).

Rex is a positive posttranscriptional regulator of viral expression (Hidaka *et al*., 1988) and increases the nuclear export of viral structural genes *env, gag*, and *pol*, necessary for the production of an infectious virus. Rex increases the levels of genomic and singly spliced viral mRNAs in the cytoplasm while decreasing the amount of doubly spliced *tax/rex* mRNAs (Hidaka *et al*., 1988; Inoue *et al*., 1986b). Rex is thought not only to regulate mRNA transport from the nucleus to the cytoplasm (Hanly *et al*., 1989; Inoue *et al*., 1986b), but also to inhibit splicing, increase mRNA stability, and/or enhance the translation of incompletely spliced mRNAs (Grone *et al*., 1996). This function of Rex appears to be regulated by phosphorylation (Adachi *et al*., 1992). Indeed, Rex was shown to contribute to the expression of the *IL-2* gene (McGuire *et al*., 1993) and to stabilize the *IL-2R* α mRNA (Kanamori *et al*., 1990), leading to the speculation that Rex may also contribute to T-cell transformation through an autocrine/paracrine mechanism.

More recently, a role for the Rex protein in the expression of FynB, vascular cell adhesion molecule-1 (VCAM-1), and leukocyte function–associated antigen-3 (LFA-3) has also been proposed (Valentin *et al*., 2001; Weil *et al*., 1999).

Until recently, it has been unclear how the interaction of Rex with the RxRE results in the regulation of some but not other viral mRNAs, as the RxRE is presumably present at the 3′ end of all mRNAs (Fig. 1). However, as discussed in the previous section, HTLV-1 $p30^{II}$ regulates the relative

amount of *tax/rex* mRNA in the cytoplasm (Nicot *et al.*, 2003), and it is possible that $p30^{II}$ also regulates other doubly spliced mRNAs. Thus, Rex and $p30^{II}$ have opposite effects on viral production, Rex posttranscriptionally increases both genomic RNA and structural (Gag, Env) and enzymatic (reverse transcriptase, protease, integrase) mRNA transport, thus increasing virus production. In contrast, $p30^{II}$ decreases virus production by decreasing Rex and Tax function through a posttranscriptional mechanism (Fig. 3).

III. THE HTLV-1 ORF IV

A. Tax: A Transactivator of Viral and Cellular Promoters

Seminal contributions have defined the role of the Tax protein in viral replication and virus–host interaction. Sodroski *et al.* (1984) provided the first evidence that a factor in HTLV-1–infected cells was able to increase transcription of the viral LTR and, later on, this function was ascribed to Tax, a virus-encoded phosphoprotein of 40 kDa (Cann *et al.*, 1985; Felber *et al.*, 1985; Seiki *et al.*, 1985; Sodroski *et al.*, 1985a,b). Collectively, these findings introduced new concepts regarding how complex retroviruses regulate their own transcription/expression and paved the way for a rapid demonstration of a transactivator, the Tat protein, encoded by HIV-1 (Stevenson, 1997).

The dysregulation of IL-2R α expression observed in HTLV-1–infected cells and ATLL (Kronke *et al.*, 1985; Lando *et al.*, 1983) prompted investigation as to whether Tax activates the transcription of IL-2 and the IL-2R (Greene *et al.*, 1986) and led to the discovery of the effect of Tax on the NF-κB pathway (Ballard *et al.*, 1988; Cross *et al.*, 1987; Inoue *et al.*, 1986a; Leung and Nabel, 1988; Maruyama *et al.*, 1987; Ruben *et al.*, 1988; Siekevitz *et al.*, 1987), a central regulatory pathway for the growth and survival of T-cells (Baeuerle and Baltimore, 1996). Evidence that Tax could activate the nuclear oncogene *c-Fos* (Alexandre *et al.*, 1991) led to the identification of SRF as another cellular transcription factor capable of recruiting Tax (Fujii *et al.*, 1992). Finally, the demonstration that Tax binds to both coactivators of transcription, CBP/p300 and p300/CBP-associated factor (PCAF) (Goodman and Smolik, 2000), provided a possible explanation of the effect of Tax on the promoters of other cellular genes, *i.e.*, sequestering the coactivators whose amount is limiting in cells will then affect the expression of an array of cellular genes (Yoshida, 2001). Genetic mapping of Tax activity on these pathways has led to the definition

of specific domains within Tax that affect CREB/ATF, NF-κB, and the coactivator CBP/p300 (Fig. 4A).

1. Tax: A POTENT TRANSCRIPTIONAL ACTIVATOR OF VIRAL LTR

Tax is a 40-kDa phosphoprotein that shuttles into and out of the nucleus (Burton *et al.*, 2000). The ability of Tax to transcriptionally activate the HTLV-1 promoter within the viral LTR has been studied thoroughly and is understood in detail (Fig. 4B). Tax activates transcription of the viral LTR through three imperfect 21-bp repeat elements collectively referred to as the TRE (Brady *et al.*, 1987; Felber *et al.*, 1985; Fujisawa *et al.*, 1985; Seiki *et al.*, 1985). Each TRE contains a core CREB/ATF-binding site (CRE-like) that is flanked by short 5-G– and 3-C–rich sequences that are required for the specificity and activation of each TRE-1 by Tax (Brauweiler *et al.*, 1995; Fujisawa *et al.*, 1989; Jeang *et al.*, 1988; Numata *et al.*, 1991;

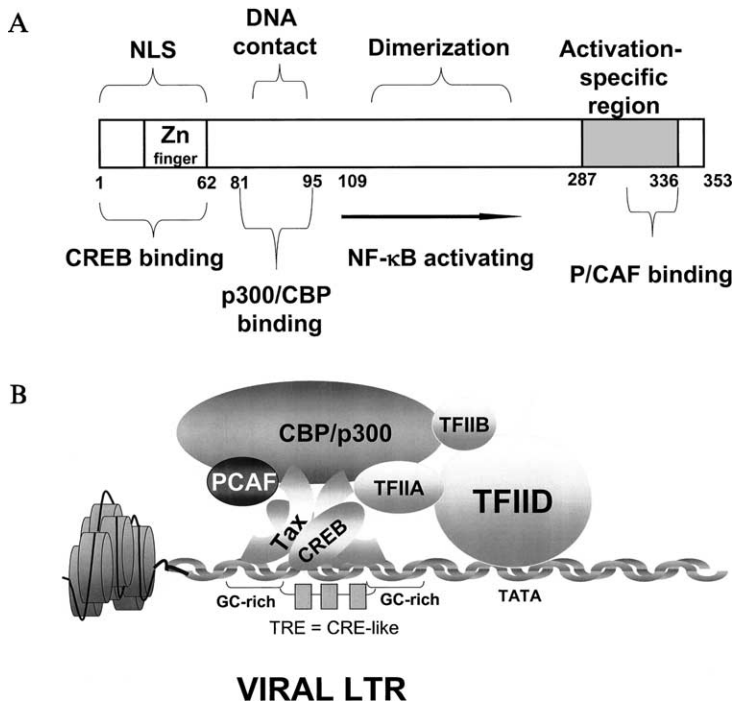

Fig. 4 The Tax protein. (A) Tax domains involved in differentiation, CREB binding, NF-κB activation, and interaction with the p300/CBP and PCAF coactivators. Adapted from Jeang *et al.* (2001). (B) Schematic model of the Tax and CREB transcription complex on the viral LTR.

Paca-Uccaralertkun *et al.*, 1994; Tang *et al.*, 1998). *In vitro*, Tax contacts this G-C–rich DNA-flanking sequence and acts as an anchor to recruit the cellular coactivator CREB to the transcription complex (Kimzey and Dynan, 1998; Lenzmeier *et al.*, 1998), which in turn functions as a dimer and interacts directly through its basic domain-leucine zipper with the CRE-like sequence to activate transcription from the viral LTR (Brauweiler *et al.*, 1995; Goren *et al.*, 1995; Laurance *et al.*, 1997; Paca-Uccaralertkun *et al.*, 1994; Tie *et al.*, 1996; Yin and Gaynor, 1996; Zhao and Giam 1991, 1992). Several studies have suggested that these interactions stimulate the dimerization of bZIP-containing proteins and their DNA-binding activity (Armstrong *et al.*, 1993; Baranger *et al.*, 1995; Perini *et al.*, 1995; Tie *et al.*, 1996; Wagner and Green, 1993; Yin and Gaynor, 1996).

The complexes formed between Tax and CREB family members are high affinity for transactivation of the viral CRE-like sequence but not for the cellular CRE, the natural target of CREB transcription factors (Adya and Giam, 1995; Fujisawa *et al.*, 1989; Suzuki *et al.*, 1993). Unlike CREB-mediated transcription, Tax-mediated transcription is independent of cellular signaling and occurs in the absence of CREB phosphorylation. It has been hypothesized that Tax interacts with minor DNA grooves through a protease-sensitive region spanning amino acid residues 89 to 110 (Connor and Marriott, 2000; Kimzey and Dynan, 1998; Lenzmeier *et al.*, 1998; Lundblad *et al.*, 1998; Tang *et al.*, 1998). This results in appropriate folding of Tax and functional exposure of its carboxy-teminal region, leading to recruitment of the RNA polymerase II transcription machinery complex (Kimzey and Dynan, 1999; Semmes and Jeang, 1995) (Fig. 5).

In addition to CREB, Tax also interacts directly with CREB-2 (Gachon *et al.*, 1998, 2000; Reddy *et al.*, 1997) and with the transcriptional coactivator CBP and its homologue p300 (Giebler *et al.*, 1997; Harrod *et al.*, 1998; Kwok *et al.*, 1996). The acetyl-transferase activity of these factors acetylates histones and unfolds the DNA structure surrounding the promoter region for transcription initiation (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Mutants of Tax unable to interact with CBP/p300 are defective for transactivation of the HTLV LTR. Formation of a Tax/CREB/CBP/p300 complex is not sufficient, and recruitment of PCAF to this complex is required even though the acetyl-transferase activity of PCAF appears to be dispensable (Harrod *et al.*, 2000; Jiang *et al.*, 1999).

Importantly, *in vivo* genomic footprinting of the viral LTR using cell extracts of HTLV-1–infected cells has confirmed and extended the aforementioned results obtained *in vitro* (Datta *et al.*, 2000, 2001). In addition, chromatin immunoprecipitation studies using extracts of HTLV-1–infected cells (Lemasson *et al.*, 2002; Lu *et al.*, 1998a) have indeed demonstrated the presence of CREB, CREB-2, ATF-1, ATF-2, *c-Fos*, c-Jun, and both

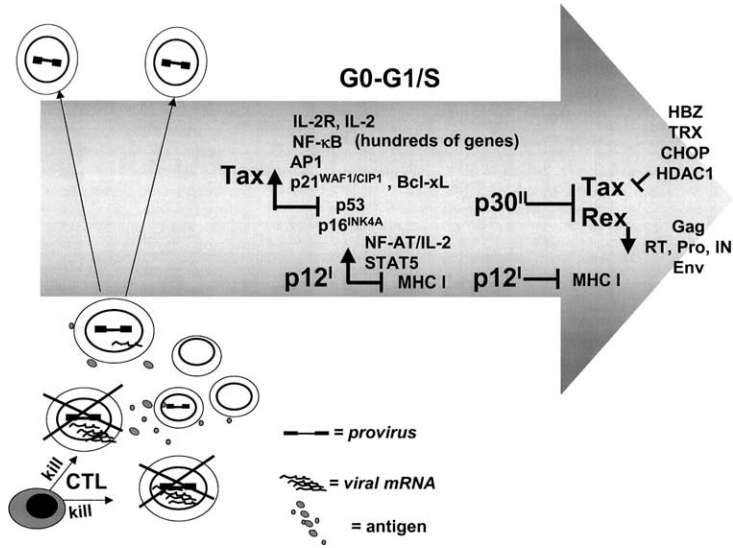

Fig. 5 Model of HTLV-1 propagation *in vivo*. This model summarizes hypothetical mechanisms based on laboratory findings on how HTLV-1 proteins promote the transition of T-cells from a resting to a proliferative state, T-cell survival, and T-cell evasion from immune recognition.

p300- and CBP-binding proteins on the DNA of the HTLV-1 LTR. Interestingly, the histone deacetylases (HDAC1, HDAC2, and HDAC3) were also found associated with the complex, consistent with the demonstration of a physical and functional interaction of Tax with HDAC1 *in vitro* and *in vivo* (Ego *et al.*, 2002).

2. Tax INITIATES AND MAINTAINS CONSTITUTIVE ACTIVATION OF THE NF-κB PATHWAY

Tax acts on several levels to initiate and maintain constitutive activation of the NF-κB pathway (Hiscott *et al.*, 2001) (Fig. 4). The NF-κB pathway is a central regulator of genes involved in cell growth, host immune response, and programmed cell death (Baeuerle and Baltimore, 1996; Barkett and Gilmore, 1999; Ghosh *et al.*, 1998; Rayet and Gelinas, 1999). Activation of NF-κB is rapid, occurs in the absence of *de novo* protein synthesis, and results in the transcriptional activation of over 100 genes. Members of the NF-κB family of transcription factors use a conserved Rel homology domain to form homo- and heterodimers and bind the κB GGGRNNYYCC DNA motif on cellular genes (Rayet and Gelinas, 1999). In unstimulated cells, NF-κB factors are sequestered in the cytoplasm through interaction

with inhibitory partners IκB (IκBα, IκBβ, IκBε, p100, p105, and Bcl-3). IκBα and IκBβ are prominent in the retention of RelA heterodimers in the cytoplasm (Fig. 2). Upon cellular activation by mitogens, cytokines, or stress, IκBα and IκBβ are rapidly phosphorylated and targeted for ubiquitination and proteasomal degradation. Consequently, unmasked nuclear localization signals of Rel heterodimers allow their translocation to the nucleus and NF-κB–dependent transcription.

Tax has been shown to interact directly with different members of the NF-κB family (Beraud *et al.*, 1994; Hirai *et al.*, 1992; Kanno *et al.*, 1994; Murakami *et al.*, 1995; Suzuki *et al.*, 1993, 1995). The quest for the kinase(s) involved in IκB phosphorylation led to the discovery of a high molecular mass complex of 700 to 900 kDa with specific kinase activity (Chen *et al.*, 1996; Lee *et al.*, 1997). Analysis of that complex revealed the presence of IκB kinases, IκκαIκκα and IκκβIκκβ (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997; Regnier *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEKK1) (Lee and Koretzky, 1998), and NF-κB–inducing kinase (NIK) (Nakano *et al.*, 1998). Studies suggest that MEKK1 and NIK are upstream kinases of IκκIκκ and that IκκαIκκα is a preferred substrate for NIK, whereas IκκβIκκβ is a preferred substrate for MEKK1 (Ling *et al.*, 1998; Yin *et al.*, 1998). Subsequently, a noncatalytic regulatory subunit, IκκγIκκγ/NF-κB essential modulator (NEMO), was also found in the complex (Rothwarf *et al.*, 1998; Yamaoka *et al.*, 1998; Yin *et al.*, 1998).

In Tax-expressing cells, constitutive nuclear expression of NF-κB (Chu *et al.*, 1998; Yin *et al.*, 1998) is found together with phosphorylation and degradation of both IκBα and IκBβ (McKinsey *et al.*, 1996). Although Tax is predominantly a nuclear protein (Bex *et al.*, 1997), a fraction of Tax responsible for NF-κB activation resides in the cytoplasm (Li and Gaynor, 1999; Nicot *et al.*, 1998). Evidence that the IκκIκκ complex was a target of Tax-mediated phosphorylation of IκB originally came from the observation that a fraction of Tax coprecipitates with IκκαIκκα- and IκκβIκκβ-containing complexes (Chu *et al.*, 1998; Geleziunas *et al.*, 1998; Uhlik *et al.*, 1998) and that kinase-defective mutants of IκκαIκκα effectively block Tax-mediated NF-κB activation (Geleziunas *et al.*, 1998; Yin *et al.*, 1998). However, it was subsequently demonstrated that Tax is only recruited to the complex through direct binding to IκκγIκκγ/NEMO (Chu *et al.*, 1999; Harhaj and Sun, 1999; Xiao and Sun, 2000).

The mechanism by which the Tax–IκκγIκκγ interaction stimulates IκκαIκκα and IκκβIκκβ kinase activity remains unclear. A possible interpretation is that IκκγIκκγ function results in conformational changes and molecular adaptations and helps the formation of Tax/IκκIκκ complexes (Jin *et al.*, 1999). Binding to and recruitment by Tax of IκκγIκκγ to the IκκIκκ complex is, however,

not sufficient to signal activation. Dominant-negative mutants of NIK effectively block Tax-mediated NF-κB activation (Geleziunas *et al.*, 1998; Uhlik *et al.*, 1998), suggesting that NIK-mediated I$\kappa\kappa\alpha$ phosphorylation is required. The interaction of Tax with MEKK1 was also found to be important, as Tax mutants unable to activate NF-κB do not interact with MEKK1 and a dominant-negative form of MEKK1 blocks Tax-mediated NF-κB activation (Yin *et al.*, 1998). These results suggested that MEKK1-mediated I$\kappa\kappa\beta$ phosphorylation is also required.

3. Tax ACTIVATION OF OTHER CELLULAR TRANSCRIPTION FACTORS

Tax is recruited by SRF to specific cellular promoters of immediate early nuclear oncogenes such as *c-Fos*, *Fos-related antigen-1* (*Fra-1*), *early growth response gene-1* (*Egr-1*), and *Egr-2* (Alexandre *et al.*, 1991; Fujii *et al.*, 1992, 1994; Herdegen and Leah, 1998; Suzuki *et al.*, 1993; Tsuchiya *et al.*, 1993). Tax interacts directly with a region encompassing amino acid residues 422 to 435 of SRF (Fujii *et al.*, 1995), and binding of the complex to specific CarG box motifs allows some restriction in the subset of promoters that are regulated through this pathway (Fujii *et al.*, 1994). Recruitment of CBP/p300 is required for transcriptional activation of the SRF pathway by Tax (Shuh and Derse, 2000).

Another less studied aspect of Tax is its stimulation of AP-1 transcriptional activity, which is very important for T-cells, as AP-1 is one of several transcription factors involved in activation of the IL-2 promoter (Armstrong *et al.*, 1993; Jin and Howe, 1997; Mori *et al.*, 2000). The demonstration of constitutive activation of AP-1 in HTLV-1–infected cells and in ATLL suggests the importance of this pathway in T-cell growth (Mori *et al.*, 2000). Interestingly, in ATLL cells, activation of this pathway appears to be Tax independent (Mori *et al.*, 2000).

4. Tax AS A TRANSREPRESSOR OF TRANSCRIPTION

The first evidence that Tax can act as a transcriptional repressor through basic helix–loop–helix factors was obtained while studying the *β-polymerase* gene promoter (Jeang *et al.*, 1990; Uittenbogaard *et al.*, 1994). Subsequently, it was shown that Tax also represses the *Lck* and *p53* promoters through a similar mechanism (Lemasson *et al.*, 1997; Uittenbogaard *et al.*, 1995). More recently, the $p18^{INK\text{-}1c}$ promoter was also found to be repressed by Tax through the E-box motif present in the promoter region. The hypothesized mechanism is a direct competition by Tax for E-box–binding protein E47 recruitment of CBP/p300 (Suzuki *et al.*, 1995,

1999b). Finally, the *Bax*, *c-myb*, and *B-myb* promoters have been demonstrated to be repressed by Tax (Brauweiler *et al.*, 1997; Nicot *et al.*, 2000a,c). In the case of *c-myb* and *B-myb* promoters, however, this effect was due to Tax's inhibition of c-Myb transactivation of its own promoter through activation of NF-κB and displacement of CBP/p300 by RelA on the c-Myb promoter (Nicot *et al.*, 2001a). In addition, Tax has also been shown to repress the expression of transforming growth factor (TGF)-β1 (Arnulf *et al.*, 2002; Lee *et al.*, 2002; Mori *et al.*, 2001c). This may have important implications in HTLV-1 pathogenesis because TGF-β is a potent inhibitor of T-cell proliferation and cytotoxicity. The mechanism by which Tax exerts its action is still unclear and it has been proposed that Tax may compete for coactivator CBP/p300 recruitment, prevent Smad heterocomplex nuclear translocation, or interfere with Smad DNA-binding activity.

5. TRANSCRIPTIONAL ACTIVATION OF GROWTH FACTORS AND GROWTH-FACTOR RECEPTORS BY Tax

Because Tax usurps the NF-κB pathway, which is central in immune regulation, several studies have investigated the effect of Tax on the expression of interleukins as well as the expression of their receptor chains. The hypothesis being investigated was whether a specific paracrine–autocrine mechanism(s) could be the basis of the spontaneous T-cell proliferation observed in HTLV-1–infected individuals or in ATLL cells.

An autocrine mechanism may be key in the growth of some HTLV-1–infected T-cells *in vitro*, and the specific cytokine receptor may vary from cell line to cell line. Indeed, Tax has been shown to transcriptionally activate the promoters of IL-2 and the IL-2R α chain through the NF-κB pathway (Ballard *et al.*, 1988; Cross *et al.*, 1987; Inoue *et al.*, 1986a; Leung and Nabel, 1988; Maruyama *et al.*, 1987; Ruben *et al.*, 1988; Siekevitz *et al.*, 1987). Similarly, transcription of IL-15 and the IL-15R α chain is increased by Tax through NF-κB (Azimi *et al.*, 1998, 2001; Mariner *et al.*, 2001) and, in the chronically infected HUT102 cell line, the expression of both ligand and receptor are upregulated, supporting the notion of an autocrine growth mechanism. However, as discussed in Section VI, the notion of a possible autocrine mechanism of growth has been called into question by the finding that inhibitors of Jak3 and STAT5 activation did not interfere with the growth of these T-cells (Kirken *et al.*, 2000).

Tax also activates IL-1 α, IL-4, IL-6, IL-8, and IL-10 through the NF-κB pathway (Li-Weber *et al.*, 2001; Mori and Prager, 1996, 1998; Mori *et al.*, 1998; Yamashita *et al.*, 1994), and the number of cytokines and receptors activated transcriptionally by Tax is likely to increase over time.

IV. THE EFFECT OF HTLV-1 ON CELL CYCLE REGULATORS

HTLV-1 infection disrupts the physiological mechanisms of T-cell growth and induces DNA damage (Chieco-Bianchi *et al.*, 1988) and indefinite growth of $CD4^+$ T-cells (Markham *et al.*, 1983; Miyoshi *et al.*, 1981; Popovic *et al.*, 1983). Early observations that p53 is stabilized in the absence of genetic mutation in most HTLV-1–infected T-cells (Reid *et al.*, 1993; Yamato *et al.*, 1993) prompted further studies on cell cycle regulation in HTLV-1 infection. In 1996, Cereseto and colleagues demonstrated that stabilization of p53 was associated with its transcriptional impairment and a failure of HTLV-1–infected cells to arrest in G1 following DNA damage. Despite functional impairment of p53, the p53-responsive gene $p21^{WAF1/CIP1}$ was upregulated transcriptionally in infected T-cells. Importantly, in these cells, Tax expression was associated with an increased expression of $p21^{WAF1/CIP1}$ (Akagi *et al.*, 1996; Cereseto *et al.*, 1996). Thus, efforts have focused on the effect of HTLV-1 and/or Tax on regulators of G0/G1/S and G2/M transition and DNA repair.

A. Tax and p53

High levels of p53 have been reported in HTLV-1–infected cells as well as in Tax-expressing cells in the absence of genetic mutation in the *p53* gene (Reid *et al.*, 1993; Yamato *et al.*, 1993). Despite high levels of nuclear p53 in HTLV-1–infected cells, its transcriptional activity was found to be impaired in response to ionizing radiation, and irradiated cells failed to arrest in G1 (Akagi *et al.*, 1996; Cereseto *et al.*, 1996). Indeed, viral infection is associated with stimulation of the G1- to-S-phase transition (Schmitt *et al.*, 1998). Ectopic expression of Tax and a p53-*luciferase* reporter construct in nonlymphoid cells (Mulloy *et al.*, 1998a) or lymphoid cells (Pise-Masison *et al.*, 1998a) indeed demonstrated that Tax impaired p53 transcriptional activity as well as its ability to induce apoptosis. Similar observations were reported in stable T-cell lines expressing only Tax (Akagi *et al.*, 1997b).

The molecular basis for p53 transcription impairment by Tax is understood in part (Pise-Masison *et al.*, 1998b). The cellular pathways involved in p53 inactivation by Tax appear to be context dependent (Pise-Masison *et al.*, 2001). Tax activation of the CREB/ATF pathway appears to be more important than NF-κB in some studies (Mulloy *et al.*, 1998a; Van *et al.*, 2001), whereas Tax-mediated NF-κB activation appears to be more important in others (Chaudhry *et al.*, 2002; Pise-Masison *et al.*, 2000, 2001).

Indeed, Tax was unable to repress p53 in p65 knockout cells (Pise-Masison *et al.*, 2000), supporting a role for the NF-κB pathway in p53 functional impairment in murine fibroblasts (Pise-Masison *et al.*, 2000). Tax does not appear to bind p53, alter the nuclear location of p53 (Mulloy *et al.*, 1998a; Pise-Masison *et al.*, 1998a; Yamato *et al.*, 1993), or affect its ability to bind DNA in gel-shift mobility assays (Mulloy *et al.*, 1998a; Pise-Masison *et al.*, 2000). Tax may enhance, through the NF-κB pathway, the activity of a kinase that phosphorylates p53 on serine residues 15 and 392, which in turn may impair its p53 interaction with the transcription factor IID (TFIID) (Pise-Masison *et al.*, 2000).

Another hypothesis proposed for p53 inactivation favors a direct competition between Tax and p53 for the recruitment of coactivators CBP/p300, perhaps in cells where CBP/p300 is limiting in amount (Ariumi *et al.*, 2000; Livengood *et al.*, 2002; Pise-Masison *et al.*, 2001; Suzuki *et al.*, 1999b; Van Orden *et al.*, 1999). Studies also indicate that Tax represses p53 homologues p73α and p73β, possibly through competition for CBP/p300 recruitment (Kaida *et al.*, 2000; Lemasson and Nyborg, 2001). It is worth noting that while competition for CBP/p300 between Tax and p53 may occur *in vitro* or in transient transfection assays where the proteins are overexpressed, chromatin immunoprecipitation assays suggest that CBP/p300 is present at sufficient quantities in cells to bind to both viral and cellular promoters in HTLV-1–infected cells (Lu *et al.*, 2002). Indirect mechanisms of p53 inactivation/modulation by Tax have also been proposed because of the interaction of Tax with the hTid-1 (Cheng *et al.*, 2001), a human homologue of the *Drosophila* tumor-suppressor protein Tid56 that interacts with the human papillomavirus type 16 (HPV-16) E7 oncoprotein (Schilling *et al.*, 1998).

B. Tax and G1/S Transition

Accumulating evidence suggests that Tax interferes with the G1/S transition. Tax decreases the length of the G1 phase while leaving unaltered the time of the S phase in undamaged murine fibroblasts (Lemoine and Marriott, 2001).

Cell cycle progression is controlled by sequential activation and inactivation of cyclin-dependent protein kinases (CDK), which in turn are regulated by the stoichiometry of inhibitory proteins that bind to the cyclin/CDK complexes. Dysregulation of the cyclin/CDK complexes may lead to cell division prior to repair of eventual DNA damage, with consequent accumulation of genetic defects in the daughter cells. The cyclin D–CDK4/6 complexes are the first to be activated in early G1 phase and associate with D-type cyclins (D1, D2, and D3). In resting cells, inhibitors of

the cyclin D–CDK4/6 complexes maintain the retinoblastoma (Rb) tumor-suppressor protein family (Rb, p107, and p130) in a hypophosphorylated state bound tightly to the E2F transcription factor. Activation of cyclin D–CDK4/6 complexes leads to the hyperphosphorylation of Rb and the release of E2F followed by E2F-mediated transcription and progression to late G1 (Hinds and Weinberg, 1994; Nevins, 1992). Cyclin D–CDK4/6 complexes become active when the stoichiometry of the specific inhibitors p15, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4}$ bound to them decreases (Morgan, 1995; Peter and Herskowitz, 1994; Sherr and Roberts, 1995). Once Rb has been hyperphosphorylated and E2F released, the entry of cells into late G1 phase requires the activation of cyclin E–CDK2 complexes to bypass the restriction point and enter S phase (Harper *et al*., 1993). The activity of cyclin E–CDK2 complexes is under the control of cyclin-dependent kinase inhibitors (CKI) known as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$.

Importantly, in HTLV-1–infected T-cells, $p27^{KIP1}$ is limiting and constitutive activation of cyclin E–CDK2 complexes has been observed (Cereseto *et al*., 1999b). The expression of $p21^{WAF1/CIP1}$ is regulated through p53-dependent and independent pathways and its upregulation is associated with G1 arrest in most cell types (El-Diery *et al*., 1993; Harper *et al*., 1993). Similarly, high amounts of $p27^{KIP1}$ suppress cell proliferation.

Tax appears to act at various steps in G1/S transition. Tax binds and inactivates $p16^{INK4A}$ and transrepresses the expression of $p18^{INK4C}$ (Haller *et al*., 2000; Low *et al*., 1997; Suzuki *et al*., 1996; Suzuki *et al*., 1999a). In addition, increased levels of *cyclin D2* have been reported in HTLV-1–infected cell lines (Akagi *et al*., 1996; Mori *et al*., 2002; Santiago *et al*., 1999) and Tax has been shown to activate the *cyclin D2* promoter directly (Huang *et al*., 2001). Furthermore, studies indicate that Tax inhibits the TGF-β signaling pathway (Arnulf *et al*., 2002; Lee *et al*., 2002; Mori *et al*., 2001a), which activates the transcription of p15 and $p16^{INK4A}$ through Smad3 and Smad4 heterodimer formation, adding an additional level of Tax control to the G1/S progression. Interestingly, however, the activation of E2F-mediated transcription can be induced by Tax in a $p16^{INK4A}$-negative T-cell line, suggesting that Tax may act at additional levels (Lemasson *et al*., 1998).

$p21^{WAF1/CIP1}$ has been reported to act as a proliferative factor in T-cells (Macleod *et al*., 1995; Zhang *et al*., 1994) and indeed, in HTLV-1–infected cells, $p21^{WAF1/CIP1}$ is upregulated, despite functional inactivation of the tumor-suppressor p53 (see previous section), and the high level of $p21^{WAF1/CIP1}$ is not associated with cell cycle arrest (Akagi *et al*., 1996; Cereseto *et al*., 1996). In Tax-expressing cells, $p21^{WAF1/CIP1}$ is associated with cyclin E–CDK2 (Cereseto *et al*., 1999b), as well as cyclin A–CDK2 (de La Fuente *et al*., 2000a). A possible interpretation of these findings is that the cyclin-dependent kinases associated with $p21^{WAF1/CIP1}$ in T-cells

are not inhibited by increasing amounts of this protein, and indeed the complexes may be stabilized and kinase activity further stimulated. An increase in G1/S transition that correlated with increased G1 CDK activity, despite a high level of $p21^{WAF1/CIP1}$, was also observed in the Tax-transduced human T-cell line Kit225 (Iwanaga *et al.*, 2001).

C. Tax and DNA Repair

Tax's promotion of G1/S transition may prevent cells from pausing and repairing DNA. Ectopically expressed Tax transrepresses the *β-polymerase* gene (Jeang *et al.*, 1990), an important cellular DNA repair enzyme. This finding has led to the speculation that Tax may do so also in HTLV-1–infected cells, thereby inhibiting *β-polymerase* expression and subsequent activity in base excision repair (BER). More direct evidence, however, exists on the effect of Tax on nucleotide excision repair (NER). Tax inhibits NER in rat fibroblasts exposed to ultraviolet radiation (Kao and Marriott, 1999); importantly, this inhibition is rescued by functional p53 (Kao *et al.*, 2000b). Because Tax increases proliferating cell nuclear antigen (PCNA) expression (Lemoine *et al.*, 2000), it has been hypothesized (Marriott *et al.*, 2002) that PCNA may be in excess and sequester $p21^{WAF1/CIP1}$, thereby rendering $p21^{WAF1/CIP1}$ limiting. In turn, this would result in a failure to block DNA replication (Li *et al.*, 1994) with a consequent increased rate of nucleotide misincorporation (Mozzherin *et al.*, 1996). However, while this hypothesis may be applicable to fibroblasts, this mechanistic interpretation may not apply to HTLV-1–infected T-cells, as they express high levels of $p21^{WAF1/CIP1}$ (Akagi *et al.*, 1996; Cereseto *et al.*, 1996, 1999b; de La Fuente *et al.*, 2000b).

Tax has also been shown to bind topoisomerase I and inhibit its catalytic activity (Suzuki *et al.*, 2000; Yoshida and Suzuki, 2000). In fibroblasts, enhanced mutation frequency of the cellular genome and increased gene amplification have been directly related to Tax expression (Lemoine and Marriott, 2002; Miyake *et al.*, 1999).

D. Tax and G2/M

Early morphological observations revealed that, as a consequence of HTLV-1 infection, T-cells accumulate lobulated nuclei (Poiesz *et al.*, 1980b). Indeed, in blood smears of ATLL patients, the flower-like cells with multilobulated nuclei are pathognomonic for ATLL (Uchiyama *et al.*, 1977; Yamaguchi and Takatsuki, 1993). This morphological alteration, coupled with the limited frequency of mitosis found in ATLL cells, suggests

that HTLV-1 may interfere with cell division. In HTLV-1–infected T-cell lines, the G2/M checkpoint appears to be functional, as the infected T-cells arrest in G2/M following ionic irradiation (Cereseto *et al*., 1996). Indeed, ectopic expression of Tax is associated with G2/M arrest and accumulation of polymorphic nuclei (Fu *et al*., 2002).

Tax interaction with the human orthologue (Hardwick and Murray, 1995) of mitotic arrest deficiency protein 1 (MAD1), designated Tax-binding protein 181 (TXBP181) (Jin *et al*., 1998), has been linked to Tax-induced multinuclei–micronuclei formation, observed earlier by several investigators (Majone *et al*., 1993; Saggioro *et al*., 1994; Semmes *et al*., 1996). However, this interpretation is now open to question (Campbell *et al*., 2001) because a sequencing error in the original report of TXBP181 resulted in the preparation and use of an antibody against a peptide fragment not present in human MAD1 (hsMAD1). Thus, hsMAD 1 is an 83-kDa, not a 150-kDa, protein located in kinetochores during mitosis and associated with nuclear pores during interphase, and not with interphase centrosomes, as reported for TXBP181 (Jin *et al*., 1998). The mechanism responsible for Tax-induced micronuclei formation, as well as the role of MAD1 in this process, therefore requires further investigation.

E. Tax and Apoptosis

Unbalanced activation of signal transduction pathways, inhibition of cell cycle checkpoints, and accumulation of genetic defects are generally strong signals for a commitment to apoptosis. Current understanding is that Tax possesses both pro- and antiapoptotic activities and that cellular fate is dictated by the dominant activity that could be influenced by the cell type, the time and levels of Tax expression, accumulation of cellular mutations, and microenvironment. While transient expression of Tax invariably results in apoptotic cell death (Cereseto *et al*., 1999a; Chlichlia *et al*., 1997; Hall *et al*., 1998; Kao *et al*., 2000a, 2001; Los *et al*., 1998; Nicot and Harrod, 2000; Yamada *et al*., 1994), HTLV-1–transformed cell lines that express sizeable amounts of Tax are usually resistant to most apoptotic stimuli. This phenomenon may be a consequence of selection of cells that have acquired constitutive expression of antiapoptotic genes (Arai *et al*., 1998; Kasai *et al*., 2002; Kishi *et al*., 1997; Mori *et al*., 2001a; Nicot *et al*., 1997; Ruckes *et al*., 2001; Tsukahara *et al*., 1999; Yamada *et al*., 1999).

Notably, Tax has been shown to increase expression of the antiapoptotic Bcl-xL in T-cells, ATLL patient samples, and murine cells through a mechanism that is in part involved in the activation of NF-κB and direct transactivation of the *Bcl-xL* promoter (Mori *et al*., 2001a; Nicot *et al*., 2000a). Conversely, molecular mechanisms by which Tax triggers apoptosis

have been identified as the sequestration of coactivators CBP/p300 in association with a permanent NF-κB activation. Accordingly, mutants of Tax defective in CBP/p300 binding or NF-κB–mediated activation (Fig. 4) have reduced apoptotic activity in nonlymphoid cells, and directed nuclear expression of the minimal coactivator-binding peptide of Tax causes apoptosis (Nicot and Harrod, 2000).

In activated T-cells, Tax-induced apoptosis associated with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) expression is mediated by the NF-κB pathway, whereas Fas ligand (FasL) upregulation by Tax involves the NF-AT pathway (Rivera-Walsh *et al.*, 2001; Rivera *et al.*, 1998). More recent evidence (Chaudhry *et al.*, 2002) also demonstrates that Tax induces apoptosis in activated Jurkat T-cells and that this effect is inhibited by the addition of a Fas–IgG fusion protein that blocks FasL, whose expression is increased by Tax (Chaudhry *et al.*, 2002; Mulloy *et al.*, 1998a; Rivera *et al.*, 1998). Interestingly, the report of Chaudhry and colleagues (2002) also demonstrated that Tax-induced apoptosis was independent of p53-induced apoptosis and that the proapoptotic effect of Tax and p53 in T-cells was abolished when the two proteins were expressed together. Tax may also inhibit apoptosis by inducing antiapoptotic chemokines, such as the I-309 ligand of the CCR8 receptor (Ruckes *et al.*, 2001).

F. Tax and the Proteasome

Proteasomes are constituted of catalytic proteinase complexes that degrade misfolded cellular proteins. Proteasome degradation is also used to quickly eliminate the activity of proteins whose expression needs to be tightly regulated through the cell cycle or other molecules involved in signaling. Additionally, proteolytic processing of proteins for presentation by the MHC I complex occurs through the proteasome (Rock *et al.*, 1994). Tax associated with the MC9 (α3) and HsN3 (β7) subunits of the proteasome when these subunits were coexpressed with the viral transactivator (Beraud and Greene, 1996; Petropoulos and Hiscott, 1998; Rousset *et al.*, 1996). More recently, an association of Tax with intact proteasomes and enhanced proteasome proteolytic activity on prototype peptides was demonstrated in Tax-expressing cells (Hemelaar *et al.*, 2001). Interestingly, despite the enhancement of proteolytic activity, Tax was not degraded rapidly and displayed a long half-life (15 h). Because the activity of Tax was observed in nuclear proteasomes, it was proposed that IκBα is tethered by Tax to the proteasome and is degraded and depleted in the nucleus with a consequent release and activation of NF-κB (Hemelaar *et al.*, 2001). Indeed, the use of proteasome inhibitors has been proposed as a therapeutic approach for

ATLL because of its constitutive activation in ATLL (Tan and Waldmann, 2002) (see ATLL section).

Because Tax is immunodominant, others have investigated whether the increased proteolytic activity of proteasomes induced by Tax may be related to the immunodominance of Tax. However, both the long half-life of Tax and experiments in mice indicate that the immunodominance of Tax may not be determined by its interaction with proteasomes (Lomas *et al*., 2002).

V. OVERALL HTLV-1– OR Tax-INDUCED TRANSCRIPTION PROFILES IN T-CELLS BY DNA MICROARRAY OR SUBTRACTION–HYBRIDIZATION TECHNIQUES

Because Tax affects several cellular transcription factors, one would expect that viral infection causes upregulation and downregulation of several cellular genes. Few studies have been published thus far on the genome-wide expression of T-cells infected by HTLV-1 using DNA microarray technology or subtraction–hybridization techniques.

The atlas human cDNA expression array has been used to compare the expression pattern of peripheral blood mononuclear cells (PBMC) infected or not with the virus (Harhaj *et al*., 1999) and of the HTLV-1–transformed T-cell line C8166 versus the uninfected CEM cell line (de La Fuente *et al*., 2000a). The Affymetrix GeneChip microarray representative of more than 7000 genes has been used for normal PBMCs and five HTLV-1–infected T-cell lines (Pise-Masison *et al*., 2002). A single study using NIH Oncochip cDNA arrays has been performed on the Jurkat cell line (JPX-9) that can be induced to express higher levels of Tax (Ng *et al*., 2001). Normal activated postmitotic PBMC cDNA has been used in subtraction–hybridization studies with cDNA from cultured ATLL cells from a patient (Ruckes *et al*., 2001). Some of these studies report upregulation of cellular genes and others report both upregulation and downregulation. Because of the complexity of data and of the differences in chip composition, a full definition of genes that are affected by viral infection remains incomplete. However, a consensus on the expression of some cellular genes emerges (Table II). Enhanced expression of cell cycle/antiapoptotic genes includes *cyclin B1*, $p21^{WAF1/CIP1}$, , and antiapoptotic *Bcl-xL* genes, confirming prior biological/biochemical findings (Akagi *et al*., 1996; Cereseto *et al*., 1996; Mori *et al*., 2001a; Nicot *et al*., 2000a). In contrast, *caspase-8* was consistently downregulated (Table II). Among the interleukins and their receptors, upregulation of

Table II Microarray Analysis of Cellular Gene Transcripts in HTLV-1–Infected or Tax-Expressing T-Cells

Gene	HTLV-1[a] PBMCs, immortalized (Atlas)	HTLV-1 cell lines						
		C8166[b] PBMCs	CEM C8166[c] (Atlas)	HUT102[b] PBMCs	Bes[b] PBMCs	Champ[b] PBMCs	ACH.WT[b] PBMCs	Jurkat/Tax/ Jurkat[d]
p21$^{WAFI/CIPI}$	↑	↑	↑	↑	↑	↑	↑	↑
cyclin A	↑							
cyclin D3	↑							
cyclin D2	↑		↑					↑
cyclin B1	↑	↑	↑	↑	↑	↑	↑	
caspase-8	↓	↓		↓	↓	↓	↓	
Bcl-xL		↑	↑		↑	↑	↑	
IL-2R α		↑	↑	↑	↑	↑	↑	
IL-2								
IL-15				↑	↑	↑	↑	
IL-15R α				↑	↑	↑	↑	↑

[a]From Harhaj *et al.* (1999).
[b]From Pise-Masison *et al.* (2002).
[c]From de La Fuente *et al.* (2000a). This paper reported only upregulated genes.
[d]This paper reported mainly upregulated genes.

IL-2R α but not IL-2 was also consistently found, consistent with previous findings (see Section VI). In contrast, IL-15 α appears to be upregulated in some but not other HTLV-1–infected T-cell lines and PBMCs. Similarly, IL-15 is not upregulated in all cell lines, and IL-15 expression does not appear to be induced by Tax in Jurkat cells (Table II).

VI. HTLV-1 PROTEINS INVOLVED IN IN VITRO ONCOGENICITY

IL-2 maintains the *in vitro* growth of mitogen-activated human T-cells for a definite time (2 months) (Morgan *et al*., 1976). The terms *immortalization* and *transformation* of T-cells have been used commonly in the field and refer to ligand (IL-2)-dependent or -independent indefinite growth of HTLV-1–infected T-cells, respectively.

The mechanisms of indefinite T-cell growth acquired in the course of viral infection *in vitro* likely differ among HTLV-1–infected T-cell lines and result from selection of the most fit cells after several years of *in vitro* propagation. Thus, these cell line "models" may epitomize events that occur during the multistep oncogenic process that ultimately leads to ATLL (Tsukasaki *et al*., 2001).

Biochemically, the distinction between HTLV-1–immortalized and transformed T-cells has been associated in most, but not all, infected T-cells with constitutive activation of the Jak/STAT signaling pathways (Migone *et al*., 1995; Mulloy *et al*., 1998b; Xu *et al*., 1997), as well as with a reduction of the level of Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) (Migone *et al*., 1998). However, Kirken *et al*. (2000) demonstrated that the inhibitor of Jak3, AG-490, does indeed decrease Jak3 and STAT5 phosphorylation without affecting growth of the HTLV-1–infected T-cell lines MT-2 and HUT102, suggesting that the activation of the downstream effector for both the IL-2 and the IL-15 signaling pathways may be functionally redundant for the proliferation of HTLV-1–infected T-cells. These data are therefore at odds with the hypothesis of autocrine mechanisms of cell growth as proposed for IL-2–independent cell lines as well as with the hypothesis that HTLV-1–infected T-cell lines, such as the HUT102 T-cell line, may grow because of their expression of both IL-15 and the IL-15R α since the IL-15R complex also signals through STAT5 (Azimi *et al*., 1998; Mariner *et al*., 2001). It is possible that cytokines other than IL-2 and IL-15 contribute to the growth of some HTLV-1–infected T-cell lines and, should this be correct, the concept of ligand independence may not be viable at all.

Immortalization of T-cells may be a model that better mimics the clonal expansion of T-cells *in vivo* with the caveat that *in vivo* viral expression is likely contained because of host immune surveillance.

Immortalization of primary human T-cells (indefinite growth) in the presence of IL-2 can be induced by infection with various HTLV-1 isolates or by Tax expression alone (Grassmann *et al.*, 1989; Iwanaga *et al.*, 1999; Tanaka *et al.*, 1991). However, in some cases, continuous stimulation of the TCR is also required (Tanaka *et al.*, 1991). This difference may be explained by the notion that, when HTLV-1 isolates are used, other HTLV-1 antigens may provide the continuous TCR stimulation necessary to maintain T-cell responsiveness to exogenous IL-2.

ORFs I and II do not appear to be necessary for T-cell immortalization, although the presence of ORF I augments viral infectivity (Albrecht *et al.*, 2000; Derse *et al.*, 1997; Robek *et al.*, 1998), whereas Tax is likely very important. The oncogenic properties of Tax may be related not only to its direct effect on cell cycle checkpoint inhibitors, but also to its ability to activate transcription of a wide range of cellular genes involved in cellular growth/survival through stimulation of the CREB or NF-κB transcriptional pathways (Kelly *et al.*, 1992). In an attempt to define whether Tax-mediated CREB- or NF-κB–dependent transcription was responsible for transformation, several laboratories have used Tax mutants to activate one or the other pathway (Fig. 4A).

Immortalization of T-cells using molecular clones in which the wild-type *tax* gene was replaced with mutants demonstrated the importance of NF-κB (Robek and Ratner, 1999). However, a Tax mutant unable to activate NF-κB retained its ability to immortalize T-cells when expressed in the context of a herpesvirus saimiri (HVS) vector (Rosin *et al.*, 1998). This discrepancy may be explained by the level of Tax expression, the presence of other HTLV-1 genes in the former study, or the conditions of T-cell growth, or it may relate to differences among mutated sites.

In nonlymphoid cells, the term *transformation* usually refers to a loss of contact inhibition in cells that nevertheless remain serum dependent. Because of the genetic differences among the Tax cDNAs used and the different cell types used in different laboratories, it is difficult at this time to relate with certainty which of the specific domains of Tax are necessary. In fact, these experiments have yielded conflicting results (Table III) and, while some investigators found Tax-mediated CREB activation critical, others reported just the opposite and found Tax-mediated NF-κB activation essential (Akagi *et al.*, 1997a; Coscoy *et al.*, 1998; Kitajima *et al.*, 1992; Matsumoto *et al.*, 1997; Yamaoka *et al.*, 1996). Tax was shown to cooperate with the *ras* protooncogene in the transformation of primary rodent fibroblasts (Pozzatti *et al.*, 1990), but Tax is insufficient to transform various fibroblast cell lines (Tanaka *et al.*, 1990; Yamaoka *et al.*, 1996).

Table III Tax Domains Relevant for "Transformation" in Different Cell Types

Tax mutant	Cell type	Vector	Cellular pathway
M22, M47	Human PBMC	HTLV molecular clone	NF-κB (Robek and Ratner, 1999)
M22, M47	Mouse T cell	Cytomegalovirus (CMV) promoter driven	NF-κB (Iwanaga *et al*., 1999)
S258A	Human PBMC	Herpesvirus	CREB/ATF (Rosin *et al*., 1998)
M47, G148V	Rat fibroblast	CMV promoter driven	NF-κB (Yamaoka *et al*., 1996)
M22, M47	Rat fibroblast	CMV promoter driven	CREB/ATF (Smith and Greene, 1991)
M22, G148V	Rat fibroblast	CMV promoter driven	NF-κB (Liu *et al*., 2001)

Tax's activation of the phospho-inositol 3 kinase pathway (PI3K) seems required for the transformation of rat fibroblasts (Liu *et al.*, 2001) and, interestingly, inhibitors of PI3K but not other inhibitors of G1/S cell cycle progression have been demonstrated to be able to arrest HTLV-1–infected cells in G1 following ionic irradiation (Cereseto *et al.*, 1999b).

VII. IN VIVO EXPANSION OF T-CELL CLONES AND VIRAL LATENCY

Most of the T-cells in the blood of adults are resting memory T-cells (in the G0 phase of the cell cycle) generated by prior antigen exposure. Following successive antigen encounters, these immune cells become committed to proliferation within hours (Sallusto *et al.*, 1999).

HTLV-1 is found in memory T-cells of both $CD4^+$ (mainly) and $CD8^+$ phenotypes (Nagai *et al.*, 2001a; Richardson *et al.*, 1990). Polyclonal integration of the virus is observed in infected individuals even though oligoclonal expansion of some T-cells is often observed, suggesting on one hand an ongoing *de novo* infection of T-cells and on the other selective expansion over time of specific T-cell clones carrying the provirus. Cell-free infection of T-cells is probably a rare event. HTLV-1 may be able to infect cycling cells, as the dissolution of the nuclear membranes that occurs before cell division may be necessary for integration of the viral DNA in the cellular genome. Clonal expansion of cells carrying the provirus likely occurs following antigenic stimulation.

There is a recognized discrepancy between the number of cells carrying the provirus and those expressing viral mRNA, as demonstrated by studies in New World nonhuman primates (Kazanji *et al.*, 2000) whereby *tax* mRNA could not be amplified from blood T-cells, despite the presence of provirus. Similar results have been reported in HTLV-1–infected individuals in whom a high virus load was nevertheless associated with few cells expressing viral mRNA by *in situ* hybridization (Gessain *et al.*, 1991). Thus, the provirus is likely dormant in most provirus-carrying cells and may become transcriptionally active only after antigen stimulation. Indeed, a high frequency of T-cell clonal expansion has been associated with chronic antigenic stimulation in carriers of *Strongyloides stercoralis* (Gabet *et al.*, 2000). A higher frequency of leukemia has been reported in individuals carrying this parasite (Cavrois *et al.*, 1998; Hayashi *et al.*, 1997).

One could predict that following antigen exposure in the microenvironment, only a minority of provirus-positive cells are stimulated and express viral proteins. That is not to say that given the right antigenic stimulation, a cell carrying the provirus would necessarily express viral proteins. Cellular

factors may influence expression of the provirus, independent of extracellular signals. Extensive methylation of proviral DNA (Clarke *et al*., 1984) of the 5′ viral LTRs (Datta *et al*., 2001) and, to a lesser degree, of the 3′ LTR has been reported (Koiwa *et al*., 2002), and this finding has been related to the silencing of viral gene expression (Datta *et al*., 2001).

In order to survive the vigorous $CD4^+$ (Goon *et al*., 2002; Hanon *et al*., 2000b) and $CD8^+$ (Bangham, 2000; Jacobson *et al*., 1988; Nagai *et al*., 2001b) T-cell immune response of the host, some infected T-cells must be able to conceal viral expression. It is likely that some of the infected T-cells that express high levels of viral RNA and proteins will succumb to virus-specific $CD8^+$ CTLs (Asquith *et al*., 2000). Given the evolutionary success of HTLV-1, the virus must have evolved functions to vigorously counteract immune recognition rather than leaving it to chance, such as integration site, proviral, or LTR methylation, and infection and functional impairment of $CD8^+$ T-cells (Hanon *et al*., 2000a,c; Kubota *et al*., 2003; Lim *et al*., 2000). This may be particularly important because, in provirus-carrying activated T-cells that progress through the S phase of the cell cycle, viral expression will be increased by the IL-2–induced CREB/ATF expression, which, together with Tax, will activate viral expression (Feuerstein *et al*., 1996).

We favor the hypothesis that infected T-cells stimulated with low levels of antigen (or with low affinity for the antigen) may be able to produce viral proteins in amounts sufficient to undergo cell cycle progression and cell division and avoid recognition by the immune system. Even though most infected cells expressing virus may die, those that survive would have additional opportunities to divide.

The following model could be hypothesized (Fig. 5). The activation of IL-2 (IL-15) and IL-2R α (IL-15R α) chain receptors is central for T-cell division. Indeed, both of these ligand–receptor pairs are HTLV-1 targets. Tax activates $p21^{WAF1/CIP1}$ expression, usurps NF-κB (Akagi *et al*., 1996; Cereseto *et al*., 1996; Hiscott *et al*., 2001), inhibits p53-induced apoptosis (Chaudhry *et al*., 2002; Mulloy *et al*., 1998a), and promotes entry into the cell cycle. Tax activates cyclin D–CDK4 kinase activity by blocking CDK4 inhibitors (Low *et al*., 1997; Suzuki *et al*., 1999a; Yoshida, 1999) and accelerates G1/S transition (Lemoine and Marriott, 2001). Tax activates AP-1, the IL-2 (IL-15) and IL-2R α (IL-15R α) promoters for S-phase transition, and Bcl-xL to promote survival. At the same time, $p12^I$, by increasing NF-AT activity (Zhang *et al*., 2000, 2001), increases IL-2 production and augments 10-fold the responsiveness of T-cells to IL-2 (Nicot *et al*., 2001b). During S phase of the cell cycle, however, Tax expression may be decreased by the ensuing expression of $p30^{II}$, leading to a temporary viral latency. The viral HBZ, as well as cellular proteins such as thioredoxin (Trx), CCAAT/enhancer-binding protein (C/EBP), homologous proteins

(*e.g.*, CHOP), and HDAC1, may also block Tax transcriptional activity (Ego *et al.*, 2002; Sasada *et al.*, 2002). Decreased viral expression, coupled with the $p12^{I}$-induced decrease of MHC I on the cell surface, may help some of the infected T-cells avoid immune recognition and undergo mitosis.

This model implies a cell cycle–dependent appearance in the cytoplasm of doubly or singly spliced mRNAs, tightly controlled expression of viral and cellular genes by transcriptional, posttranscriptional, and, likely, post-translational mechanisms, and simultaneous usurpation by viral proteins of pathways essential for the survival and growth of T-cells. Experiments in the years to come will assess the plausibility of this model.

VIII. ADULT T-CELL LEUKEMIA/LYMPHOMA

Tax expression during repeated T-cell division and clonal expansion by shortening of the G1/S progression interferes with DNA repair and may favor the genetic instability of and accumulation of genetic defects in clonally expanded T-cells (Kamada *et al.*, 1992; Mortreux *et al.*, 2001; Tsukasaki, 2002). There is a continuous immunological pressure exerted by the host immune response, as demonstrated, for example, by the appearance of CTL escape variants in Tax (Niewiesk *et al.*, 1994), which may result in the selection of cells that carry deletions in the proviral DNA (Furukawa *et al.*, 2001; Morozov *et al.*, 2000; Ohshima *et al.*, 1991; Okazaki *et al.*, 2001; Tamiya *et al.*, 1996). These cells may not be able to express viral proteins but may have acquired genetic lesions that recapitulate the effect of Tax on NF-κB, AP-1, cell cycle checkpoint inhibitors, and apoptosis.

Indeed, both NF-κB and AP-1 transcription factors are constitutively active in ATLL cells (Mori *et al.*, 2000). $p16^{INK4A}$ and $p15^{INK4B}$ are often deleted or methylated in ATLL cells and this circumstance is associated with progression to ATLL (Drexler, 1998; Fujiwara *et al.*, 1999; Hatta *et al.*, 1995; Hatta and Koeffler, 2002; Hoffman *et al.*, 1992; Nosaka *et al.*, 2000; Pombo-de-Oliveria *et al.*, 2002; Trovato, *et al.*, 2000). Similarly, alteration of the *Rb* gene has also been reported in ATLL (Hatta *et al.*, 1997). The *p53* gene is mutated in approximately 30% of ATLL cases (Sakashita *et al.*, 1992) and is stabilized and functionally impaired in ATLL cells (Takemoto *et al.*, 2000). A significant mutation/deletion of *hBUB1* or *hBUBR1*, components of the mitotic checkpoint in budding yeast, was also observed in 40% of ATLL cases (Ohshima *et al.*, 2000). Genes that regulate the survival of cells, such as *Bcl-xL*, *survivin*, an inhibitor of the apoptosis protein (IAP) family member, and cyclooxygenase-2 (COX-2), are elevated in *ex vivo* samples from ATLL patients (Mori *et al.*, 2001b,d; Nicot *et al.*, 2000a) as is the expression of $p21^{WAF1/CIP1}$, even in the absence

of demonstrable Tax expression. Constitutive activation of some of the STAT proteins has been associated with active DNA synthesis (S phase) and G2/M transition in ATLL cells (Takemoto *et al*., 1997).

Thus, in the late stage of infection, the acquisition of stable somatic mutations may render the function of viral proteins redundant, favoring the selection of deleted/mutated provirus to decrease the risk of elimination by the immune system. One of these cell clones will outgrow the others and cause leukemia/lymphoma in the host.

IX. SUMMARY

HTLV-1 infects cycling memory T-cells, which in turn can revert to a resting state. The ability of HTLV-1 to seize control of T-cell replication appears to depend on antigen stimulation. Antigen exposure awakens the dormant provirus in specific immune cells. Because T-cells depend on the interaction of IL-2 and IL-2R for growth, HTLV-1 has evolved several strategies to arrogate these pathways. The stoichiometry, as well as the catalytic activity of Tax, appears to be very important during T-cell entry into the cell cycle. $p12^{I}$ may function as an adaptor that integrates activation and proliferation signals. Rex and $p30^{II}$ tightly regulate viral expression to avoid immune recognition together with $p12^{I}$ that affects MHC I trafficking to the cell surface. Expression of viral genes seizes temporary control of T-cell growth/survival, and a larger pool of T-cells carrying the provirus is established. Some of these T-cells will succumb to the physiological contraction of T-cell number with decreasing antigen levels. Some cells will succumb to the immune system's defenses and some, likely a small fraction, will survive and become quiescent again. During this phase, a cell-free virus may be produced, which may explain the maintenance of a vigorous virus-specific immune response.

The degree of T-cell clonal expansion during the life of an HTLV-1–infected individual depends on the frequency of exposure to the specific antigens and the ability of the virus to preserve the life of the cell carrying its genome. Several factors thus contribute to the development of ATLL: (1) the presence of virus, genetic, and environmental factors and (2) the immunological competence of the individual.

Pharmacological intervention in ATLL has the same shortcomings as other forms of hematopoietic-malignancy therapy, as viral proteins may not be needed to maintain the leukemic phenotype. While ATLL cannot be cured, it is a preventable disease. Prevention of HTLV-1 infection is an obvious approach. Strategies such as screening of blood or blood products and preventing breast-feeding have been quite successful in decreasing

HTLV-1 infection in developed countries. However, transmission has not been eliminated. In endemic developing countries, these approaches may not be feasible.

Several vaccine modalities have been tested in animal models (Dekaban *et al.*, 2000a) and those studies suggest that it may be possible to develop an effective vaccine for HTLV-1 given its genetic stability. Unfortunately, the relatively low frequency of disease and marketing considerations have discouraged companies from pursuing the development of an HTLV-1 vaccine. Understanding of the steps that lead to ATLL in humans necessitates large prospective studies. Further identification of the functional role of proteins in the replication and latency of the virus in experimental models of infection may lead to a better definition of viral targets during the asymptomatic phase of infection.

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