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KINETICS AND REGULATION OF HTLV-1 GENE EXPRESSION

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ABSTRACT

Human T-Lymphotropic virus type 1 (HTLV-1) is the causative agent of two distinct pathologies, adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells, and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease.

The HTLV-1 expression strategy is characterized by the production of plus- and minus-strand transcripts, alternative splicing and polycistronic translation. This strategy greatly increases the coding potential of the virus, resulting in expression of several regulatory and accessory genes (Tax, Rex, p12, p13, p21rex, p30tof and HBZ) in addition to the structural proteins and virion-associated enzymes common to all retroviruses (Gag, Pro, Pol and Env).

In spite of over 30 years of studies, several key features of the HTLV-1 life cycle and pathogenicity remain obscure. In particular, it is still unclear whether HTLV-1 gene expression is characterized by latency patterns, whether the different viral genes follow distinct kinetics of expression and, if this is the case, which molecular mechanisms control these phenomena.

The work described in the present thesis was aimed at understanding these aspects of HTLV-1 regulation. To this end we optimized a Real Time RT-PCR method using splice-site-specific primers to quantitate the different HTLV-1 transcripts and their kinetics of expression in peripheral blood mononuclear cells (PBMCs) isolated from HTLV-1-infected individuals and in cells transfected with HTLV-1 molecular clones. Results indicated that expression of HTLV-1 mRNAs follows a distinct timing upon reactivation of viral expression, with the mRNA coding for the Tax and Rex regulatory proteins acting as an early "master" transcript preceding expression of the other viral transcripts.

Although it is commonly accepted that Rex acts at a post-transcriptional level controlling the nuclear export and stability of viral mRNAs coding for the virion-associated proteins, the Rex-dependency of tax/rex, p12, p13, p21rex, p30tof and hbz transcripts has not been investigated so far. To test if the kinetics of HTLV-1 gene expression might be dependent on Rex function and to determine the Rex-dependence of individual HTLV-1 mRNAs, we generated a Rex knock-out HTLV-1 molecular clone and analyzed the nucleo-cytoplasmic compartmentalization of the

viral mRNAs. Results demonstrated the strict Rex-dependency of the “two-phase” kinetics and revealed strong nuclear retention of hbz mRNAs, supporting their function as non-coding transcripts. Furthermore our results revealed that the Rex-responsiveness of the different HTLV-1 mRNAs is determined by a novel 72-nucleotides cis-acting regulatory sequence located upstream of exon 3.

Mathematical modelling underscored the importance of a temporal delay between the Tax and Rex functions, which was supported by experimental evidence of a delayed accumulation and longer half-life of Rex compared to Tax.

These data provide evidence for a temporal pattern of HTLV-1 gene expression, reveal major differences in the intracellular compartmentalization of HTLV-1 transcripts and, importantly, provide clues to a long-standing paradox of HTLV-1 regulation, i.e. the different Rex-dependence of viral transcripts in spite of the presence of the Rex-responsive element (RxRE) in the 3' untranslated region of all viral mRNAs.

RIASSUNTO

Il virus T-linfotropico umano di tipo 1 (HTLV-1) è l'agente eziologico di due distinte patologie, la leucemia/linfoma a cellule T dell'adulto (ATLL, adult T-cell leukemia/lymphoma), un'aggressiva neoplasia a carico dei linfociti T CD4+ maturi, e della paraparesi spastica tropicale/mielopatia associata ad HTLV-1 (TSP/HAM, tropical spastic paraparesis/HTLV-1-associated myelopathy), una patologia degenerativa del sistema nervoso centrale.

La strategia di espressione genica di HTLV-1, caratterizzata dalla produzione di trascritti a partire da promotori localizzati sia nel filamento positivo che in quello negativo del genoma virale, da splicing alternativo e da traduzione bicistronica, incrementa notevolmente la capacità codificante di HTLV-1, con la conseguente espressione di numerosi geni regolatori ed accessori (Tax, Rex, p12, p13, p21rex, p30tof e HBZ) in aggiunta alle proteine strutturali e agli enzimi associati al virione, comuni a tutti i retrovirus (Gag, Pro, Pol ed Env).

Nonostante oltre 30 anni di studi, diversi aspetti chiave del ciclo vitale di HTLV-1 e della sua patogenicità rimangono tutt'oggi non noti. In particolare, non è ancora chiaro se l'espressione genica di HTLV-1 sia caratterizzata da stadi di latenza, se i diversi geni virali presentino cinetiche di espressione distinte e quali meccanismi molecolari possano controllare questi fenomeni.

Gli studi descritti nella presente tesi sono stati mirati a comprendere questi aspetti della regolazione genica di HTLV-1. A questo scopo abbiamo sviluppato un protocollo di Real Time RT-PCR associato all'impiego di primer specifici per le diverse giunzioni di splicing al fine di quantificare i diversi trascritti codificati da HTLV-1 e di analizzarne le cinetiche di espressione sia in cellule mononucleate di sangue periferico isolate da individui infettati con HTLV-1, che in cellule trasfettate con cloni molecolari di HTLV-1. I risultati ottenuti indicano che l'espressione degli mRNA codificati da HTLV-1 segue una precisa cinetica dopo riattivazione dell'espressione virale: l'mRNA codificante le proteine regolatrici Tax e Rex agisce come trascritto precoce che precede l'espressione degli altri geni virali.

Sebbene sia comunemente accettato che Rex eserciti la sua funzione a livello post-trascrizionale controllando l'esporto nucleare e la stabilità degli mRNA che codificano le proteine associate al virione, fino ad oggi non è mai stata investigata la

Rex-dipendenza dei trascritti p12, p13, p21rex, p30tof e hbz. Al fine di testare se le cinetiche di espressione genica di HTLV-1 osservate potessero dipendere dalla funzione di Rex e al fine di determinare la Rex-dipendenza dei singoli mRNA virali, abbiamo generato un clone molecolare di HTLV-1 knock-out per Rex e analizzato la compartimentalizzazione nucleo-citoplasmatica dei trascritti virali. I risultati ottenuti hanno dimostrato la stretta Rex-dipendenza delle cinetiche di espressione a "due fasi" ed hanno rivelato una forte ritenzione nucleare degli mRNA codificanti HBZ, supportando la loro funzione come trascritti non codificanti. Inoltre, i risultati ottenuti hanno dimostrato che la responsività a Rex dei differenti mRNA virali potrebbe essere determinata dalla presenza di una sequenza regolatoria di 72 nucleotidi che agisce in cis, localizzata a monte dell'esone 3.

Infine, analisi matematiche hanno sottolineato l'importanza di un ritardo temporale tra le funzioni di Tax e di Rex, supportata dall'evidenza sperimentale di un ritardo nell'accumulo e di un'emivita più prolungata di Rex rispetto a Tax.

I dati ottenuti in questo studio forniscono l'evidenza di una regolazione temporale dell'espressione genica di HTLV-1, rivelano una differente compartimentalizzazione degli mRNA virali e offrono una possibile spiegazione di un paradosso ancora irrisolto della regolazione di HTLV-1, ovvero la differente Rex-dipendenza dei trascritti virali, nonostante la presenza della sequenza responsiva a Rex (RxRE, Rex-responsive element) nella regione 3' non tradotta di tutti i trascritti virali.

1. INTRODUCTION

1.1 Human T-lymphotropic virus type 1: taxonomy, epidemiology and pathogenesis

Human T-Lymphotropic virus type 1 (HTLV-1) was the first human retrovirus to be identified and is the only one with established oncogenic properties (Poiesz et al., 1980; Hinuma et al., 1981).

HTLV-1 belongs to the Retroviridae family, Oncovirinae sub-family, Deltaretrovirus genus, which also includes HTLV-2, -3, -4, simian T-Lymphotropic virus (STLV), and bovine leukemia virus (BLV). STLV and BLV infections are associated with neoplastic diseases, while the pathogenicity of HTLV-2, -3, -4 has not been clearly established (Araujo and Hall, 2004; Feuer and Green, 2005; Mahieux and Gessain, 2009). Deltaretroviruses are referred as "complex" retroviruses. In fact, they present at the 3' end of the genome, the so called "pX region" that encodes the regulatory proteins Tax and Rex and different accessory proteins. Another peculiarity of the Deltaretroviruses is represented by their oncogenic mechanism, which does not involve the expression of a cellular-derived viral oncogene (v-onc), as in the case of acute-transforming retroviruses, or integration in the proximity of a cellular proto-oncogene (c-onc), as in the case of chronic-transforming retroviruses. The mechanism of cell transformation by Deltaretroviruses is instead determined by the ability of Tax protein to de-regulate the expression of a wide range of cellular genes.

HTLV-1 infects about 20 million people worldwide and is endemic in South-Western Japan, Central Africa, the Caribbean Basin, Central and South America and the Melanesian Islands. Sporadic infection occurs in Europe and North America. Transmission of the virus may occur in a "vertical" manner from mother to newborn (e.g. mainly through breastfeeding and in few cases during gestation or peripartum), or "horizontally" through exchange of biological fluids (e.g. sexual contact and parenteral transmission) (Proietti et al., 2005).

HTLV-1 is the causative agent of two distinct pathologies, adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells that is extremely refractory to current therapies (Uchiyama et al., 1977; Tsukasaki et al., 2009), and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease (Gessain et al., 1985; Osame et al., 1986).

In addition, accumulating evidence supports an association between HTLV-1 infection and a number of chronic inflammatory diseases such as uveitis (Mochizuki et al., 1992; Pinheiro, 1995), arthropathy (Murphy et al., 2004b; Yakova et al., 2005) and infective dermatitis (LaGrenade et al., 1990).

The majority of HTLV-1-infected individuals remain asymptomatic throughout life; only 2-5% develop ATLL or TSP/HAM after a latency period of decades or several years, respectively (Kawano et al., 1985). In spite of over 30 years of study, the molecular mechanisms determining ATLL or TSP/HAM have not been yet fully clarified.

ATLL is classified into four clinical forms: acute, chronic, smouldering and lymphoma (Tsukasaki et al., 2009). In some cases the acute phase of ATLL is preceded by peripheral lymphocytosis characterized by poly- or oligoclonal integration of the viral genome. Acute ATLL is characterized by the presence of a dominant proliferating leukemic clone with monoclonal integration of the provirus. These cells possess multi-lobulated nuclei and are called "flower cells". ATLL cells are usually CD3+ CD4+ CD8- CD25+ and frequently accumulate in peripheral blood as well as in lymphoid organs and skin. Furthermore, HTLV-1 infection is accompanied by a high frequency of T-cells expressing the surface marker Forkhead Box P3 (FoxP3) (Chen et al., 2006; Kohno et al., 2005), whose expression is characteristic of regulatory T cells (T_{reg} cells), which play a critical role in suppressing the immune response. However, in ATLL, the FoxP3 positive (FoxP3+) leukemic T cell clones are distinct from the functional T_{reg} population (Abe et al., 2008; Toulza et al., 2009). The increase in FoxP3+ cell frequency in HTLV-1 infection results from the upregulation of the CCL22 chemokine production by HTLV-1-infected cells that express Tax. CCL22 engages the CCR4 receptor expressed on the functional T_{reg} cell population, resulting in an enhancement of the migration and survival of FoxP3+ cells *in vitro*. These FoxP3+ cells may both retard the progression of ATLL and HTLV-1-associated inflammatory diseases and contribute to the immune suppression seen in HTLV-1 infection, especially in ATLL (Toulza et al., 2010).

One of the most common characteristics of ATLL is hypercalcemia, which results from the transcriptional activation of a parathormone-like peptide induced by the

viral protein Tax. The prognosis of acute ATLL is extremely poor with an overall survival of a few months.

TSP/HAM is characterized by a slowly progressive spastic paraparesis, associated with bladder dysfunction and sensory disorders (Rodgers, 1965). Parenchymal and perivascular infiltration of mononuclear cells occurs in the white and gray matter of the spinal cord, resulting in demyelization and fibrosis (Iwasaki, 1990). The presence of infiltrating T-cells in the spinal cord lesions and of Tax-specific cytotoxic T-lymphocytes (CTL) in the cerebrospinal fluid and in the peripheral blood mononuclear cells (PBMCs) suggests that TSP/HAM might have an autoimmune base (Osame, 2002; Verdonck et al., 2007). This hypothesis is consistent with the association between HLA haplotype and the risk of developing TSP/HAM (Barmak et al., 2003).

HTLV-1 infection of PBMCs leads to cell immortalization. After several months of culture it is usually possible to detect a mono- or oligoclonal profile of provirus integration as a result of a process that selects for one or few clones that carry several genetic alterations and acquire the capability to grow in an interleukin-2 (IL-2)-independent manner. These cells usually show a CD3+ CD4+ IL-2R+ (interleukin-2 receptor) phenotype, or, rarely, CD3+ CD8+ IL-2R+ (Green and Chen, 2001). The transformed clones express low levels of viral proteins, suggesting that in this stage the neoplastic phenotype is maintained through a mechanism that is largely independent of viral expression. Silencing of viral gene expression was shown to be due to viral promoter methylation, or accumulation of mutations in the viral tax gene, or deletions of the proviral 5' LTR (Takeda et al., 2004; Miyazaki et al., 2007). The dynamics of infection and immortalization observed *in vitro* recapitulate at least part of the natural history of ATLL *in vivo*. Indeed, ATLL cells also express very little, if any, viral proteins and frequently carry defective proviral copies integrated in the host genome. The propagation and persistence of the infected cells in the host relies on both *de novo* infection of new host cells and, mainly, on "mitotic transmission" of the integrated viral genome to daughter cells (Overbaugh and Bangham, 2001).

1.2 Infection and virus propagation

The HTLV-1 virion consists of a viral core that contains the viral-encoded enzymes (reverse transcriptase, integrase and protease) and the single-stranded diploid RNA

genome surrounded by capsid and matrix proteins. A lipoproteic envelope, composed of a plasma membrane-derived lipid bilayer and the gp21 and gp46 envelope glycoproteins, surrounds the viral core (Figure 1) (Manel et al., 2005; Lairmore and Franchini, 2007). HTLV-1 presents a broad cell tropism *in vitro* (monocytes, microglial cells, epithelial cells, B- and T- lymphocytes), but it is mainly detected in CD4+ T-lymphocytes in ATLL and TSP/HAM patients and in asymptomatic carriers (Manel et al., 2005). Viral spread is mediated through the interaction between the viral envelope protein gp46 and the glucose transporter GLUT-1 (Manel et al., 2003). Consequently, glucose consumption of the infected target cell is inhibited and extracellular milieau acidification is reduced, possibly causing metabolic alterations in the infected cells (Manel et al., 2003; Manel et al., 2005). The gp46-GLUT-1 interaction allows the envelope protein gp21 to mediate cellular membranes fusion with the formation of the “virological synapse”. The virological synapse is an organized contact area whose assembly results from the polarization of the cytoskeleton of the infected cell and the accumulation of HTLV-1 core complexes and the HTLV-1 genome at the cell junction; the virion components are then transferred to the uninfected cell as enveloped particles (Igakura et al., 2003; Majorovits et al., 2008). The microtubule-organizing centre (MTOC) polarization is induced by the engagement of intracellular adhesion molecule-1 (ICAM-1) (Barnard et al., 2005) and activation of the Ras-MEK-ERK pathway (Nejmeddine et al., 2009). Tax is involved in the formation of the virological synapse: it localizes in the contact region between infected and target cells (Nejmeddine et al., 2005) and enhances MTOC formation by stimulating the CREB pathway (Nejmeddine et al., 2005; Nejmeddine et al., 2009). Proteins that mediate antigen recognition and cell adhesion (e.g. hDlg, neuropilin-1, heparan sulphate proteoglycans) also contribute to HTLV-1 binding and entry into the target cell and are part of the "virological synapse" (Pinon et al., 2003; Blot et al., 2004; Ghez et al., 2006).

Another mode of HTLV-1 transmission is mediated through an extracellular biofilm-like structure that stores viral particles, facilitating virus spread after cell-to-cell contact (Pais-Correia et al., 2010). Both the virological synapse and biofilm-mediated transmission are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1 infected subjects and cell-free blood

products are not infectious (Fan et al., 1992; Derse et al., 2001); efficient viral spread instead requires direct cell-to-cell contact.

After virus entry into the target cell, the viral genome is reverse-transcribed by viral reverse transcriptase (RT), producing an RNA-DNA hybrid. The ribonuclease H (RNAse H) component of viral RT degrades the RNA strand, while the DNA strand is used as a template by RT, which also has DNA-polymerase-DNA-dependent activity, to synthesize a complementary DNA strand. The double-stranded DNA circularizes and transfers to the nucleus, where it integrates randomly in the host genome. Integration is mediated by the viral enzyme integrase and by the long terminal repeats (LTRs) located at both ends of the viral genome. Viral genes are then transcribed and translated by the cellular machinery. Virion assembly occurs in the cytoplasm, through the interaction between the viral nucleocapsid and the plasma membrane, and incorporation of two copies of the single stranded RNA genome along with tRNA, RT, protease and integrase.

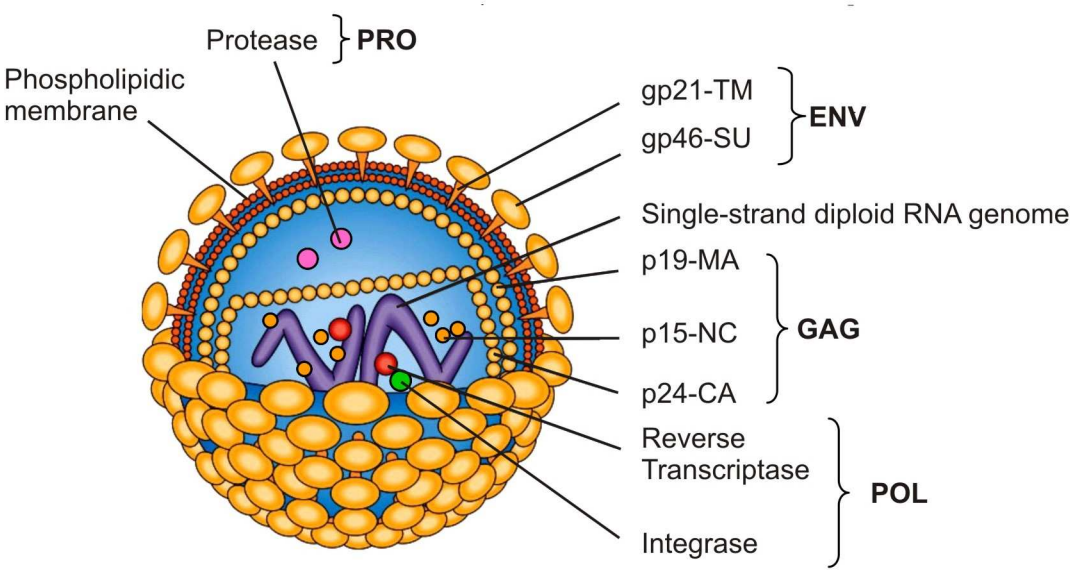

Figure 1. Schematic representation of the HTLV-1 virion (Le Blanc et al., 2001).

1.3 HTLV-I genetic organization

The genome of HTLV-1 reflects the basic structure of the Deltaretrovirus genus (Figure 2): at the 5' and 3' ends are located the LTRs which flank the partially overlapping open reading frames (ORFs) of the gag, pro, pol and env genes that code for enzymes and structural proteins of mature virus particles. The region between the end of the env gene and the 3' LTR is termed the pX region, and contains at least four partially overlapping ORFs, termed x-I through x-IV, coding for regulatory and accessory proteins (Figure 2A). The negative strand of HTLV-1 also contains an ORF located in the pX region (antisense orientation) (Larocca et al., 1989) which codes for the HBZ protein (HTLV-1 bZIP factor) (Figure 2B) (Gaudray et al., 2002).
Expression of the highly condensed HTLV-1 genetic information is achieved through ribosomal frameshifting (which generates a Gag-Pro-Pol polyprotein from the full-length transcript), by alternative splicing and polycistronic translation (which produces distinct mRNAs coding for the env and pX region genes) and through minus-strand transcription which generates at least 2 different transcripts encoding 2 isoforms of the HBZ protein.
The HTLV-1 transcripts can be grouped in 4 major classes (Figure 2A and B):
a) unspliced (US) mRNA, coding for Gag-Pro-Pol, and used as genomic RNA;
b) singly-spliced (SS) mRNAs, coding for the envelope glycoproteins (Env) and for the accessory proteins p21rex, p12 and p13;
c) doubly-spliced (DS) mRNAs, coding for the regulatory proteins p40 (Tax) and p27 (Rex), and for the regulatory/accessory protein p30tof;
d) mRNAs generated from the negative strand, coding for HBZ protein.

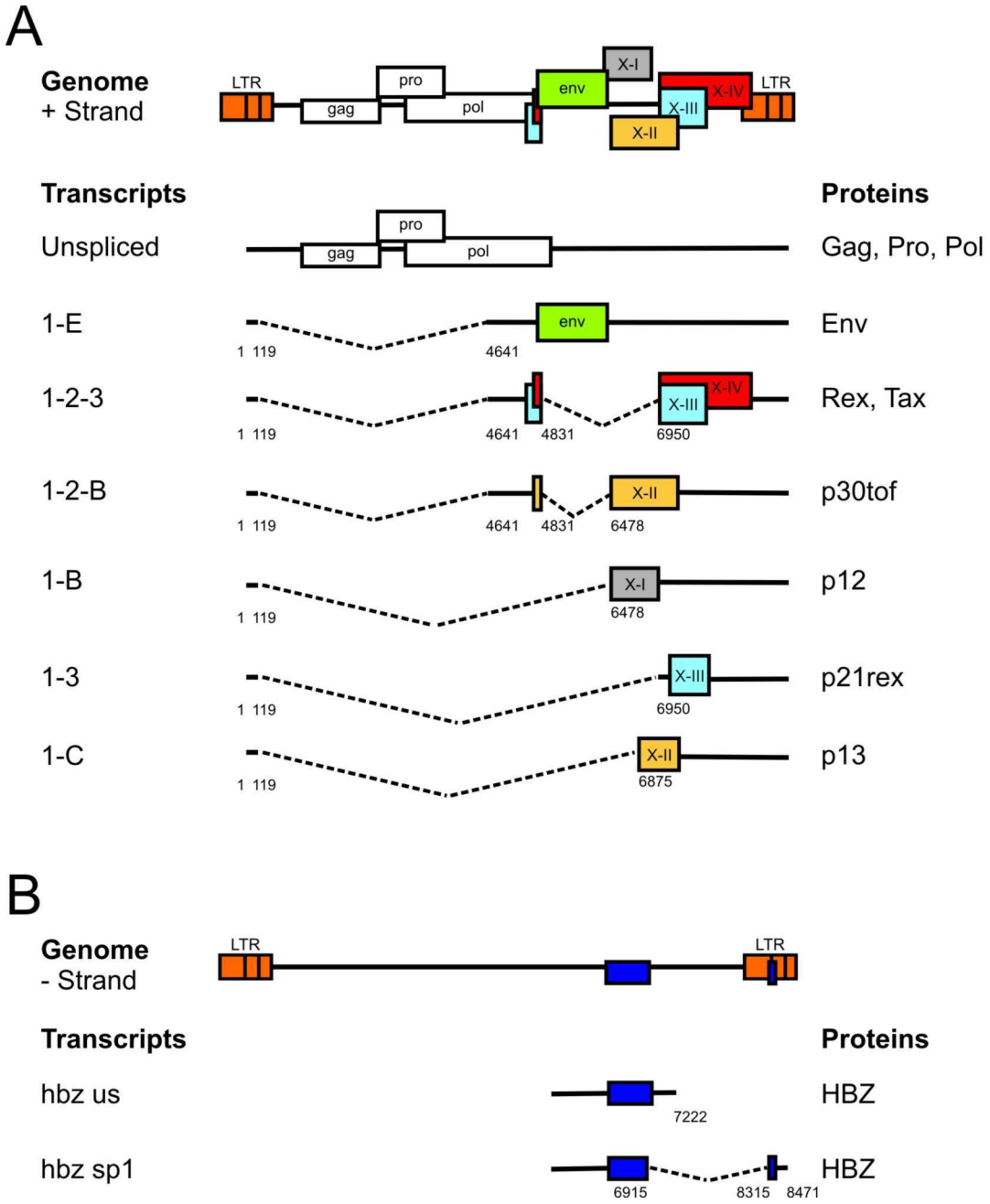

Figure 2. Organization and expression of the HTLV-1 genome. A: Plus-strand ORFs, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates splicing sites used for the generation of the mature mRNAs. Resulting exons are: 1 (1-119), 2 (4641-4831), 3 (6950-8493), B (6478-8493), C (6875-8493) and E (4641-8493). mRNAs are named according to their exonic composition**. B:** For the minus-strand, the ORF, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates the start sites used for the generation of the mature mRNAs. Resulting exons are: hbz us (7222-4834) and hbz sp1 (8471-8315 and 6915-4834).

1.3.1 Expression of structural proteins

The full-length US mRNA (8.6 kb) is packaged into virions as the genomic RNA and is also translated into the structural proteins (Gag) and enzymes (Protease and Polymerase) of mature virus particle (Lairmore and Franchini, 2007). The gag gene codes for the 19 kDa matrix (MA), 24 kDa capsid (CA) and 15 kDa nucleocapsid (NC) structural proteins. The pro gene encodes the viral protease. The 5' portion of the pol gene encodes the reverse transcriptase (RT) protein, which converts the viral single-stranded RNA genome into double stranded DNA through its DNA polymerase and RNaseH activities. Sequences downstream code for Integrase, which is responsible for the integration of the reverse-transcribed viral genome in the host cell genome (Figure 1).

These genes are translated as polyproteic precursors (Gag, Gag-Pro and Gag-Pro-Pol) generated through ribosomal frameshifting at the gag-pro and/or gag-pro-pol junction (Figure 3). The precursors are post-translationally modified by myristylation at the N-terminus, an essential step for their insertion in the internal side of the plasma membrane of the infected cell. After anchoring to the plasma membrane the precursors are cleaved by the viral protease to generate the single mature polypeptides.

The 4.2 kb SS mRNA, transcribed from the env gene, is translated into a 68 kDa precursor which is post-translationally modified by glycosylation and cleavage into two proteins named gp46-SU, localized at the surface of the infected cells and virions and responsible for the binding to the GLUT-1 receptor, and gp21-TM, a transmembrane protein that mediates membrane fusion and formation of the virological synapse.

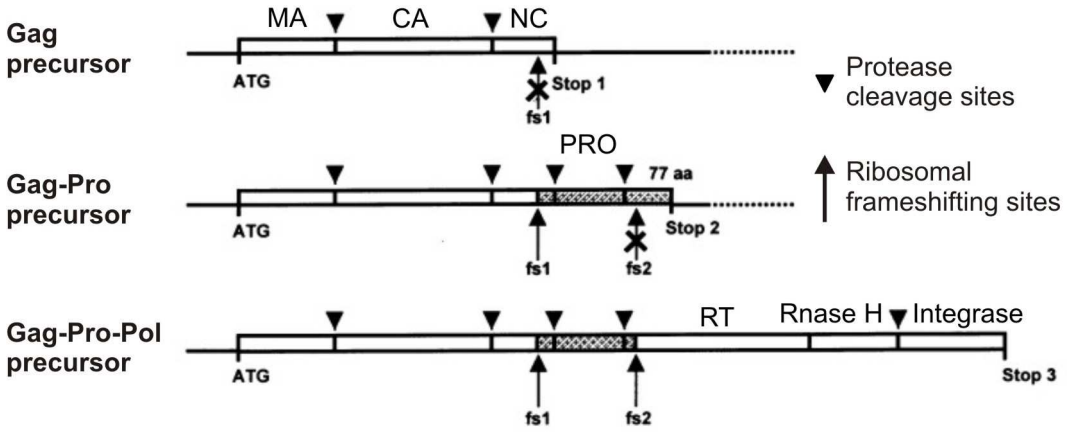

Figure 3. Schematic representation of the Gag-Pro-Pol polyprotein precursors of HTLV-1. Fs1 and fs2: ribosomal frameshifting sites; MA: matrix, CA: capsid; NC: nucleocapsid, PRO: protease, RT: reverse transcriptase (Le Blanc et al., 2001).

1.3.2 Expression of proteins coded in the pX region

The pX region of the HTLV-1 genome contains at least four different partially overlapping open reading frames, termed X-I through X-IV, that code for non structural proteins of HTLV-1. As shown in Figure 2A, expression of the different pX region genes is accessed through alternative splicing and polycistronic translation. All these mRNAs contain exon 1, which is non coding. Singly spliced mRNAs contain exon 1 and different 3' exons, and code for the accessory proteins p13 (mRNA 1-C, translated from a start codon located in the ORF x-II) and p12 (mRNA 1-B, translated from a start codon in the ORF x-I) and for the accessory/regulatory protein p21rex (mRNA 1-3, translated from a start codon located in the ORF x-III) (Ciminale et al., 1992; Koralnik et al., 1992).

Two doubly spliced mRNAs contain exons 1 and 2, and different 3' exons. Exon 2 contains 2 start codons (AUGs). mRNA 1-2-3 is a bicistronic transcript coding for the regulatory proteins Rex and Tax; Rex initiates at the first AUG in exon 2 and continues in the x-III ORF in exon 3, while Tax initiates at the second AUG and continues in the x-IV ORF in exon 3. The 1-2-B mRNA codes for the accessory/regulatory protein p30tof, which is translated from the second start codon of exon 2 and continuing in frame with the x-II ORF in exon B (Ciminale et al., 1992; Koralnik et al., 1992).

1.3.3 Expression of proteins coded by the negative strand of HTLV-1

The negative strand of the HTLV-1 genome contains one ORF located in the pX region (antisense orientation) which generates at least 2 different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). These transcripts code for 2 isoforms of HBZ protein that differ by 7 amino acids at the N-terminus due to the presence of the first exon only in the former transcript. Hbz sp1 has multiple transcriptional initiation sites in the U5 and R regions of the 3' LTR, whereas the hbz us transcript initiates within the tax gene (Figure 2B). Both hbz sp1 and hbz us have TATA-less promoters (Yoshida et al., 2008).

1.4 Functions of the pX region proteins

1.4.1 Tax

Tax is coded by the x-IV ORF and is expressed from a dicistronic doubly-spliced mRNA which contains exon 1, 2 and 3 (mRNA 1-2-3), starting from the second start codon of exon 2 that is in frame with the x-IV ORF (located in the exon 3).

Tax is a 353-amino acid, 40-kDa, mainly nuclear, phosphoprotein that transcriptionally controls the expression of viral genes and of a large number of cellular genes. Different functional domains have been mapped in the Tax sequence. The hydrophobic N-terminal domain contains the nuclear localization signal (NLS) that overlaps with a zinc finger region that is crucial for Tax's interaction with the CRE-binding/activating transcription factor (CREB/ATF) and the serum responsive factor (SRF). The central region of Tax encompasses a kinase-inducible domain (KID) that mediates the interaction with the kinase-inducible exchange (KIX) domain of the transcriptional co-activators CREB binding protein (CBP) and p300, and the dimerization domain. The C-terminal region contains a domain involved in the interaction of Tax with the transcriptional co-activator p300/CBP-associated factor (P/CAF). Tax was initially described as an activator of LTR-directed transcription (Felber et al., 1985). Three imperfectly conserved 21 base-pair (bp) repeat sequences called Tax responsive elements (TRE) located in the U3 region of the LTR are necessary and sufficient to confer Tax responsiveness (Brady et al., 1987). The TRE element contains an octamer motif TGACG(T/A)(C/G)(T/A) that is flanked by a G stretch and a C stretch at the 5' and 3' sides, respectively (Jeang et al.,

1988). This octamer shares homology with the consensus cAMP-responsive element (CRE) (5'-TGACGTCA-3'). Nevertheless, Tax exhibits poor affinity for DNA and does not bind directly to the TRE element (Giam and Xu, 1989) but interacts with members of the CREB/ATF family: CREB, CREM, ATF1, ATF2, ATF3, ATF4 (CREB2) and X-box-binding protein 1 (XBP1). These proteins share a common cluster of basic residues allowing DNA binding and a leucine zipper (b-Zip) domain involved in homo- and heterodimerization. Dimer formation modulates their DNA binding specificity and transcriptional activity. Tax promotes formation of a Tax-CREB/ATF-TRE ternary complex by interacting with the b-Zip domain of CREB/ATF factors. Tax enhances the dimerization of CREB/ATF factors, increasing their affinity for the viral CRE (Perini et al., 1995; Wagner and Green, 1993; Anderson and Dynan, 1994; Yin and Gaynor, 1996) and further stabilizes the ternary complex through direct contact of the GC-rich flanking sequences (Kimzey and Dynan, 1998; Lundblad et al., 1998). Tax then recruits coactivators (CBP/p300 and P/CAF) to facilitate transcriptional initiation. The ability of Tax to dimerize is required for efficient ternary complex formation and for optimal transactivation (Jin and Jeang, 1997; Tie et al., 1996). Normally, CREB-CBP/p300 interaction is controlled by CREB phosphorylation in response to different signal transduction pathways. In contrast, Tax, by directly interacting with the co-activator, physically links CREB and CBP/p300, making CREB phosphorylation dispensable. In this way, viral genes transcription becomes independent from cellular signals.

Tax also binds to transducers of regulated CREB activity (TORCs), a family of co-activator of CREB. Tax interacts with the three members of this family (TORC1, TORC2 and TORC3) (Koga et al., 2004; Siu et al., 2006) and TORCs cooperate with Tax to activate the LTR in a CREB and p300-dependent manner. Thus, TORCs are thought to associate with the Tax ternary complex and participate in transcriptional activation.

In addition to activating the viral LTR promoter, Tax stimulates transcription of a large number of cellular genes through interactions with the cellular transcription factors CREB/ATF, NF-κB and SRF.

Tax activates a variety of cellular genes through its interactions with CREB/ATF proteins, for example those encoding interleukin 17 (IL-17) and c-fos (Dodon et al., 2004; Alexandre and Verrier, 1991). Conversely, Tax also represses expression of

cyclin A, p53 and c-myb by targeting CREB/ATF factors (Kibler and Jeang, 2001; Mulloy et al., 1998; Nicot et al.,2000).

The NF-κB/Rel family of transcription factors play a central role in Tax-mediated cell transformation. In mammals, the NF-κB family is composed of five structurally related members, RelA, RelB (p65), c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100), which form dimeric complexes that transactivate or repress target genes bearing a NF-κB-responsive elements in their promoters or enhancers (Siebenlist et al., 1994; Perkins 2007). p105 and p100 are precursor proteins that are cleaved in the mature p50 and p52 proteins, respectively. These factors share a common Rel-homology domain (RHD) mediating their dimerization, DNA binding and nuclear localization. These factors can be activated by a series of stimuli such as antigens or cytokines. In non-activated cells, NF-κB dimers are trapped in the cytoplasm by inhibitory proteins called IκBs such as p105, p100, IκBα, IκBβ and IκBγ (C-terminal region of p105), that mask the nuclear localization signal of NF-κB factors through physical interaction (Siebenlist et al., 1994; Perkins 2007). NF-κB activation involves phosphorylation of IκB inhibitors by the IκB kinase (IKK), which contains 2 catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO (NF-κB essential modulator), which triggers their ubiquitination and subsequent proteasomal degradation, resulting in nuclear translocation of NF-κB dimers (Perkins 2007; Karin and Ben Neriah, 2000). Tax associates with RelA, c-Rel, p50 and p52 after their translocation in the nucleus (Suzuki et al., 1993; Murakami et al., 1995; Suzuki et al., 1994) but also directly recruits RelA from the cytoplasm (Lamsoul et al., 2005; Azran et al., 2005). After interaction with these NF-κB factors, Tax increases their dimerization which is essential for binding to target promoters (Suzuki et al., 1993; Suzuki et al., 1994; Petropoulos et al., 1996). When the complex is bound to the promoter, Tax recruits the CBP/p300 and PCAF co-activators (Bex et al., 1998; Bex and Gaynor, 1998), leading to transcriptional activation. This interactions of Tax with NF-κB transcription factors only explains part of Tax1-mediated NF-κB activation since the completion of this process also requires cytoplamic events. In the canonical pathway, Tax associates with the IKKγ/NEMO subunit (Harhaj and Sun, 1999; Jin et al., 1999) as well as with activating upstream kinases such as MAPK/ERK kinase kinase 1 (MEKK1) and TGF-β activating kinase 1 (TAK1) (Yin et al., 1998; Wu and Sun, 2007). Tax thus connects activated kinases to the IKK

complex and forces the phosphorylation of IKKα and IKKβ leading to degradation of IκBα and IκBβ (Harhaj and Sun, 1999; Jin et al., 1999). In addition, Tax binds directly to the IKKα and IKKβ subunits and activates their kinase activity independently of the upstream kinases (Chu et al., 1998). A third level of Tax interference with the NF-κB pathway is its direct binding to IκBs and their degradation independently of IKK phosphorylation (Hirai et al., 1994; Suzuki et al., 1995). Tax further interacts with two subunits of the 20S proteasome, favors anchorage of p105 and accelerates its proteolysis (Rousset et al., 1996). Tax thus leads to IκB degradation at multiple levels, thereby allowing nuclear translocation of NF-κB independently of external stimuli.

Tax has been shown to increase the expression of the transcriptional factors AP-1 (activator protein -1) a homo- or heterodimeric complex of Fos (c-Fos, FosB, Fra1 and Fra2) and Jun (c-Jun, JunB and JunD) (Fujii et al., 1991; Fujii et al., 2000). Fos and Jun are under the transcriptional control of the serum responsive factor (SRF) in response to various stimuli such as cytokines, growth factors, stress signals and oncogenes. SRF binds to the SRF responsive element (SRE) located in the Fos/Jun promoters, which contain two binding sites: a CarG box (CC(A/T)6GG) and an upstream Ets box (GGA(A/T)). Once SRF occupies the CArG box, the ternary complex factor (TCF) establishes protein interactions with SRF and subsequently binds the upstream Ets site. This complex then recruits the co-activators P/CAF and CBP/p300 to activate transcription. Tax activates transcription of promoters under the control of SRE motifs (Alexandre and Verrier, 1991; Fujii et al., 1991; Alexandre et al., 1991) without direct binding to the DNA but through interactions with transcription factors associated with the SRF pathway. This interaction results in increased binding of SRF to the SRE (Dittmer et al., 1997). Once the complexes are stabilized, Tax recruits the coactivators CBP/p300 and P/CAF and mediates transactivation (Shuh and Derse, 2000).

Furthermore Tax is able to interact with proteins stimulating the G1-S phase transition of the cell cycle through different mechanisms including transcriptional activation or repression, post-translational modifications and protein-protein interactions (Jeang et al., 2004; Marriott and Semmes, 2005). Tax interacts with cyclins-D1, -D2 and -D3 as well as with cyclin-dependent kinases (CDKs) 4 and 6 but not with CDK1 or CDK2 (Haller et al., 2002; Haller et al., 2000; Fraedrich et al.,

2005; Neuveut et al., 1998). Through these interactions, Tax stabilizes the cyclin D2/CDK4 complex and enhances its kinase activity, leading to hyperphosphorylation of retinoblastoma protein (Rb). Tax also associates with the CDK inhibitors (CDKI) p15INK4b and p16INK4a and counteracts their inhibitory activity against CDK4 (Suzuki et al., 1996; Suzuki and Yoshida, 1997; Low et al., 1997; Suzuki et al., 1999). Finally, Tax binds to Rb and enhances its proteosomal degradation (Kehn et al., 2005).

Tax also inactivates the p53 tumor suppressor, thus impinging on the major pathway controlling genome integrity and favouring the emergence of a large spectrum of molecular alterations in infected cells (Tabakin-Fix et al., 2005).

Tax shows a strong anti-apoptotic activity through NF-κB activation (Kawakami et al., 1999), transcriptional activation of anti-apoptotic factors Bcl-XL, Bfl1 and HIAP-1 (Tsukahara et al., 1999; Nicot et al., 2000; De La Fuente et al., 2003) and downregulation of the pro-apoptotic protein Bax (Brauweiler et al., 1997).

Tax targets multiple components of the DNA damage repair pathway and promotes DNA abnormalities. Moreover Tax subverts mechanisms monitoring chromosomal segregation during mitosis; in fact, one of the hallmarks of Tax-expressing cells is chromosomal instability and aneuploidy (Marriott et al., 2002). Tax interacts with different proteins involved in centrosome amplification and in the mitotic spindle assembly checkpoint (SAC). For example, Tax interacts with the anaphase promoting complex (APC), which controls the metaphase-anaphase transition and correct execution of mitosis. APC directs the ubiquitination and proteosomal degradation of cyclin B1 and Pds1p/securin, key regulators of mitosis. Both securin and cyclin B act by inhibiting separase, a protease that destroys the connections linking sister chromatids. In normal cells chromosomes start to segregate only after they have been attached to the kinetocore and are subjected to mechanical tension (Nasmyth, 2005). In Tax-positive cells the decrease in cyclin B and securin starts in S phase, before the cell enters mitosis. This results in incorrect activation of separase and unequal chromosomal separation between cells (Liu et al., 2005).

Tax was also reported to modulate the transforming growth facor β (TGFβ) pathway and the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.

Experimental evidence accumulated so far underscores the role of Tax as an essential effector of HTLV-1 mediated tumorigenicity. Tax expression induces an immune response since it is the major target of cytotoxic T lymphocytes (CTLs). To escape from CTLs, ATLL cells frequently lose the expression of Tax by several mechanisms (loss of the viral promoter, nonsense or missense mutation of the tax gene, epigenetic change by hypermethylation). Thus, it is speculated that Tax plays an important role in the persistent proliferation of HTLV-1-infected cells during the carrier state, with the mutator phenotype conferred by Tax promoting accumulation of genetic and epigenetic changes that finally lead to Tax-independent proliferation and escape from the host immune system following silencing of Tax (Yasunaga and Matsuoka, 2007).

1.4.2 Rex

Rex is coded by the x-III ORF and it is expressed from a dicistronic doubly-spliced mRNA which contains exon 1, 2 and 3 (mRNA 1-2-3), starting from the first start codon of exon 2 that is in frame with the x-III ORF (located in the exon 3).

Cellular RNA processing leads to rapid splicing of intronic sequences and to the destruction of incompletely spliced transcripts, which contrasts with the need for unspliced and partially spliced mRNAs required for HTLV-1 replication. HTLV-1 and other related virus (e.g. HIV) have evolved a molecular mechanism in which viral regulatory proteins (Rex for HTLV-1 and Rev for HIV-1) interfere with the cellular splicing and nuclear export machinery, leading to cytoplasmic accumulation of the unspliced genomic RNA encoding Gag, Pro, Pol, and the singly spliced RNA encoding Env (Inoue et al., 1986; Inoue et al., 1987; Hidaka et al., 1988; Seiki et al., 1988; Inoue et al., 1991).

Rex is a 189-amino acid, 27 kDa nuclear/nucleolar phosphoprotein that is able to shuttle between the nucleus and the cytoplasm (Palmeri and Malim, 1996; Narayan et al., 2003), allowing the nucleo-cytoplasmic export of incompletely spliced viral RNA, controlling in this way viral gene expression at the post-transcriptional level. This function of Rex is mediated through direct interaction with a 254-nucleotide stem-loop cis-acting RNA element termed the Rex-responsive element (RxRE) (Grone et al., 1994), present in the U3/R region of the 3' LTR of all HTLV-1 transcripts. Nucleotides critical for Rex binding in vitro have been mapped to a discrete 12-nucleotide RNA sequence that is predicted to form a stem-bulge-stem

structure (Ahmed at al., 1990; Bogerd et al., 1991; Bogerd et al., 1992). Rex's interaction with its RxRE is inhibited by the cellular protein, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Dodon et al., 2002).

Different functional domains have been mapped in the Rex protein. The N-terminal arginin-rich region (amino acids 1-19) serves as nuclear localization signal (NLS) (Siomi et al., 1998; Nosaka et al., 1989) and as RNA binding domain (RBD) which mediates Rex binding to the RxRE in the viral RNAs (Grassman et al., 1991; Bogerd et al., 1992). A leucine-rich sequence located near the middle of the protein (amino acids 79-99) functions as activation domain (AD) (Weichselbraun et al, 1992b) and contains the nuclear export signal (NES) (Palmeri and Malim, 1996; Kim et al., 1996). The NES interacts with the protein chromosome region maintenance interacting protein 1 (CRM1/exporting 1) and allows export of the Rex-viral mRNA complex from the nucleus to the cytoplasm (Borgerd et al., 1995). CRM1 belongs to the importin-β family, whose members act as RNA transporters between nuclear and cytoplasmic compartments (Borgerd et al., 1998). Mutations of the four leucine residues within the Rex-activation domain demonstrate that they are critical for nuclear export of mRNA (Kim et al., 1996; Palmeri and Malim, 1996). The two NES flanking regions (amino acids 57-66 and 106-124 respectively) (Bogerd et al., 1995) constitute the Rex multimerization domain (Bogerd and Greene, 1993) and are required for the assembly of Rex into multimeric structures upon binding to the RxRE. The formation of Rex multimers on the RxRE is critical for the nuclear export of viral mRNA since mutations of Rex that failed to form multimers act as dominant-negative mutants (Bogerd et Greene, 1993). In addition to functioning as a Rex exporter, CRM1 serves as an inducing factor for Rex multimerization on viral mRNA by aiding in complex formation (Englmeier et al., 2001; Hakata et al., 1998). Residues 411 and 414 of CRM1 are critical for Rex multimerization, a region distinct from the one involved in the export of Rex. The translation-initiation factor eIF-5A may also play a part in the formation of the Rex homo-oligomers (Katahira et al., 1995). Collectively, the requirement for multiple bound copies of Rex on the RxRE is believed to protect the mRNA from being spliced or sequestered in the nucleus.

Figure 4 summarizes the Rex-mediated nucleocytoplasmic export of incompletely spliced viral mRNAs. After Rex-RxRE binding and Rex multimerization, CRM1 is recruited into the complex. CRM1 uses guanosine diphosphate/guanosine

triphosphate (GDP/GTP) guanine nucleotide exchange of the GTPase Ran to function. CRM1 binds to RanGTP, along with the Rex-mRNA complex, and translocates this complex across the nuclear pore by interacting with phenylalanine-glycine rich nucleoporins. In the cytoplasm RanGTP is then converted to RanGDP and is released from the Rex-mRNA complex. The regulator of chromosome condensation 1 (RCC1) catalyzes the conversion of GTP to GDP (Bischoff et al., 1991). Other proteins affecting Rex function include Ran Binding Protein 3 (RanBP3), a scaffold protein that stabilizes the RanGTP-CRM1-Rex-mRNA complex in the nucleus (Hakata et al., 2003; Englmeier et al., 2001), and SRC-associated in mitosis 68 (Sam 68), which is able to increment tRex-mRNA binding in a CRM1-independent fashion (Reddy et al., 1999; Reddy et al., 2000).

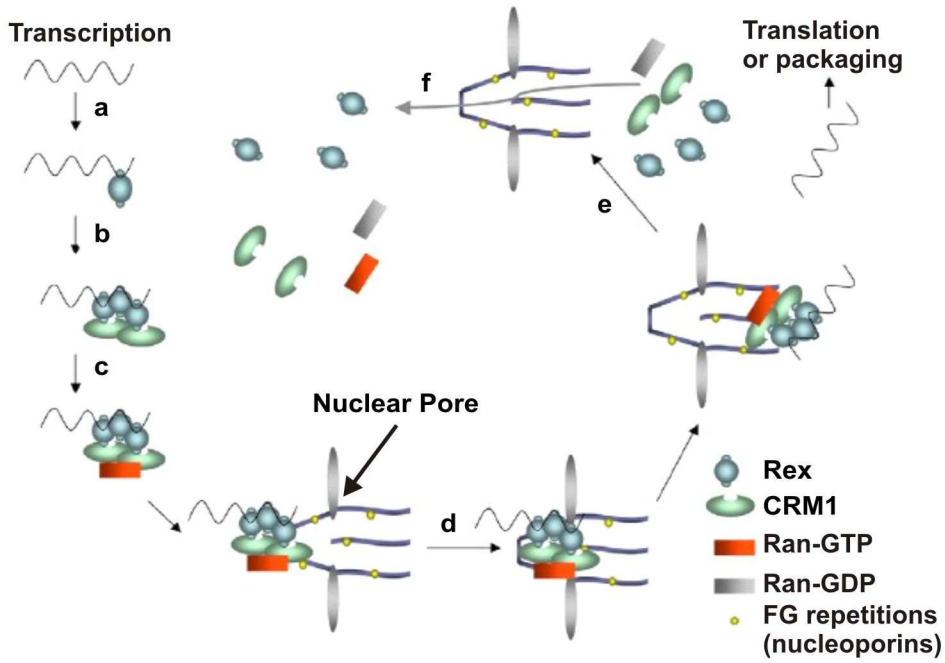

Figure 4. Rex-mediated nucleocytoplasmic export of viral mRNAs. Rex binding to the RxRE sequence in the viral mRNA after transcription (a). Rex multimerization (b) and viral mRNA/Rex/CRM1/RanGTP complex formation (c). Complex translocation from the nucleus to the cytoplasm is mediated by the interactions with phenylalanine-glycine rich nucleoporins (d). Conversion of RanGTP to RanGDP in the cytoplasm with subsequent complex dissociation and viral mRNA release (e). The other components of the complex return into nucleus to start another cycle (taken from Younis and Green, 2005).

In addition to its role in RNA trafficking, Rex has been shown to inhibit splicing *in vitro*, during the initial phase of spliceosome formation (Bakker et al., 1996), and to increase mRNA stability in the nucleus (Grone et al., 1996).

Experiments performed with protein kinase inhibitors indicated that Rex function is modulated by phosphorylation on serine/threonine residues (Adachi et al., 1990; Kesic et al., 2006), a property that highlights the importance of cellular factors in the multi-level regulation of HTLV gene expression (Younis and Green, 2005).

Furthermore Rex has been shown to affect cellular gene expression. Rex was able to augment Tax-mediated upregulation of IL-2 and stabilize the IL-2 receptor-α chain (IL-2Rα) mRNA, thereby prolonging its half-life independent of nuclear-cytoplasmic transport (McGuire et al., 1993; Kanamori et al., 1990). In this manner, Rex may contribute to cellular transformation of HTLV-1 infected cells. Rex has also been shown to increase FynB (p59fyn) expression, possibly by affecting FynB splicing (Weil et al., 1999). FynB is a src family protein-tyrosine kinase that regulates T-cell receptor stimulation (Cooke et al., 1991). Cotransfection experiments in Jurkat T-cells demonstrated that Rex could augment Tax's ability to increase expression of vascular cell adhesion molecule-1 (VCAM-1) and lymphocyte function-associated antigen-3 (LFA-3) (Valentin et al., 2001). Both VCAM-1 and LFA-3 are important proteins in T-cell adhesion, aiding in proliferation of uninfected cells and virus spread.

Although Rex is not required for cellular immortalization *in vitro*, it is necessary for infectivity and viral persistence *in vivo* (Ye et al., 2003), since expression of the US and SS viral RNAs encoding structural proteins is necessary for the assembly of virions. The fact that these mRNAs depend on Rex for expression suggests that the Rex-RxRE interaction may function as a molecular switch controlling the transition between productive and latent phases of HTLV-1 infection.

Recently Rex has been shown to act as RNA silencing suppressor protein. The interaction of Rex with Dicer, a member of the RNase III family of nucleases that converts the double-stranded shRNA (short hairpin RNA) to the single-stranded siRNA (small interfering RNA) in the cytoplasm, inhibits Dicer activity and thereby reduces the efficiency of the conversion of shRNA to siRNA (Abe et al., 2010).

1.4.3 p21rex

p21rex is coded by the X-III ORF and it is expressed from a singly-spliced mRNA which contains exon 1 and 3 (mRNA 1-3). p21rex is a truncated isoform of Rex lacking the N-terminal arginine-rich domain of the full-length protein. It was hypothesized that it might act as a repressor of full-length Rex, thereby inhibiting the expression of transcripts coding for structural proteins, enzymes and accessory proteins (Ciminale et al., 1995; Heger et al., 1999) and playing a role as a latency-inducing factor in the HTLV-1 life cycle.

1.4.4 p30tof

p30tof is coded by the x-II ORF and it is expressed from a doubly-spliced mRNA which contains exon 1, 2 and B (mRNA 1-2-B), starting from the second start codon of exon 2 that is in frame with the x-II ORF (located in the exon B).

p30tof is a 241-amino acid nucleolar-nuclear non-shuttling protein (Ciminale et al., 1992; D'Agostino et al., 1997). Two arginine-rich domains were identified as nucleolar retention signal (NoRS) (amino acids 73-78) and NLS (amino acids 91-98) respectively (D'Agostino et al., 1997; Ghorbel et al., 2006). Two additional NLS were identified in the N-terminal and C-terminal regions of the protein (Ghorbel et al., 2006).

An HTLV-1 molecular clone containing a mutation in the x-II ORF is still able to produce infective virions and immortalize human T-lymphocytes (Robek et al., 1998), but shows a drastic reduction in *in vivo* infectivity in animal models (Bartoe et al., 2000).

p30tof functions at the post-transcriptional level by inhibiting the nuclear export of the tax/rex mRNA; this effect results in a global inhibition of viral gene expression, suggesting that p30tof might act as a latency factor (Nicot et al., 2004). Additional studies have shed light on the mechanism used by the virus to discriminate between viral mRNAs that are p30tof responsive (tax/rex) and those that are Rex responsive (gag/pro/pol and env). p30tof specifically forms complexes with Rex, with complex formation increased by the presence of viral mRNAs (Sinha-Datta et al., 2007). In particular, p30tof was found to interact with the RNA-binding domain of Rex and thereby prevent Rex from interacting with the RxRE. p30tof efficiently interacts with Rex only when it is bound to RNA. Since p30tof could interact with tax/rex but not

gag/pro/pol or env mRNAs, these data explain that p30tof is able to prevent tax/rex nuclear export but has little or no effect on other viral mRNAs (Sinha-Datta et al., 2007). Although it is critical for HTLV-I to reduce its expression to evade immune detection and clearance, complete latency would not benefit the virus, which in early stages needs some Tax expression to alter cell cycle checkpoints, alter DNA repair, and extend the lifespan of infected cells, thus facilitating transformation. In agreement with such a model, studies show that Rex permits export of residual p30tof-bound tax/rex mRNA allowing a low level of virus expression (Sinha-Datta et al., 2007). Rex may oppose p30tof function by recruiting a limiting cellular factor and inducing a conformational change in p30tof or in the local RNA structure that releases p30tof from its p30 responsive element (RE) and allows export of some tax/rex mRNA to the cytoplasm.

In addition to its post-transcriptional functions, p30tof affects transcrption from promoters with cellular CRE and viral TRE sequences by interacting with the co-activator CBP/p300 (Zhang et al., 2000; Zhang et al., 2001). p30tof represses the transcription from the CRE sequence in a dose-dependent manner. On the other hand, when expressed at low levels, p30tof activates the transcription from HTLV-I LTR; however, at high levels, p30tof represses LTR transcrption. p30tof interacts with the kinase-inducible exchange domain (KIX) of p300/CBP, also known to bind to CREB and Tax. Furthermore, p30tof can disrupt the assembly of the CREB–Tax–p300/CBP complex on TRE sequence. Taken together, these data suggest that p30tof might decrease transcription of the viral genome, thereby facilitating viral latency.

p30tof also affects the expression of a number of cellular genes, at the transcriptional and post-transcriptional level, including adhesion molecules and genes involved in T-cell activation and apotosis (Michael et al., 2004; Taylor et al., 2009).

p30tof interacts with PU.1 inhibiting its DNA binding and transcription activity, resulting in the down-regulation of Toll-like receptor 4 (TLR4) expression from the cell surface (Datta et al., 2006). In addition, since PU.1 autoregulates expression from its own promoter, decreased PU.1 transcriptional activity effectively reduces endogenous PU.1 expression. Moreover, p30tof-mediated inhibition of GSK3-b potently suppresses the production of pro-inflammatory cytokines (MCP-1, TNF-a, and IL-8), while concurrently augmenting production of the anti-inflammatory

cytokine IL-10 following stimulation of TLR4 in human macrophages (Datta et al., 2006).

Finally p30tof recruits the co-activator Tat-interacting protein 60 (TIP60) and promotes the formation of the Myc/TIP60 transcription complex on the Myc-response E-box element and transactivate transcription (Awasthi et al., 2005). Due to the importance of Myc as a proto-oncogene, p30tof may contribute to the transformation of the HTLV-1-infected cell. In fact, p30tof has been shown to modulate expression of cellular genes involved in the cell cycles and apoptosis (Michael et al., 2004; Taylor et al., 2009; Datta et al., 2007). Recent studies have shown that p30tof expression results in alteration of the cell cycle events that would promote early viral spread and T cell survival (Datta et al., 2007).

1.4.5 p13

p13 is coded by the x-II ORF and is expressed from a singly spliced mRNA which contain exon 1 and C (mRNA 1-C) (Ciminale et al., 1992; Koralnik et al., 1992).

p13 is a 87-amino acid protein that corresponds to the C-terminal portion of p30tof (Koralnik et al., 1992) and accumulates in mitochondria (Ciminale et al., 1999).

Studies of a p13-knockout virus showed that although the protein is dispensable for viral replication in cultered cells (Derse et al., 1997; Robek et al., 1998), it is required for establishing a persistent infection in a rabbit experimental model (Hiraragi et al., 2006).

Immunoelectron microscopy and fractionation experiments demonstrated that p13 is an integral membrane protein and accumulates mainly in the inner mitochondrial membrane. p13 induces specific alterations in mitochondrial morphology, resulting in isolated clusters of round-shaped, swollen mitochondria (Ciminale et al., 1999; D'Agostino et al., 2002; D'Agostino et al., 2005).

Functional studies of p13 revealed that it inhibits proliferation of HeLa cells and Jurkat T cells and sensitizes Jurkat T cells to apoptosis triggered by ceramide and Fas ligand (Silic-Benussi et al., 2004; Hiraragi et al., 2005). p13 also interferes with the ability of HeLa cells and Ras/Myc-transformed primary fibroblasts to grow as tumors in nude mice, suggesting that it may exert tumor-suppressor-like activity (Silic-Benussi et al., 2004).

Functional mapping studies demonstrated that p13 contains a mitochondrial targeting signal (MTS) spanning amino acids 21-30 (Ciminale et al., 1999) that contains 4 arginines in the positions 22, 25, 29 and 30. The MTS of p13 (LRVWRLCTRR) is predicted to fold in an α-helix, with the 4 arginines forming a positively charged face that imparts amphipathic properties to this region (D'Agostino et al., 2002). Biophysical and biochemical analyses of a synthetic peptide spanning residues 9-41 ($p13_{[9-41]}$) confirmed that this region folds into an amphipathic α-helix upon exposure to membrane-mimetic solutions (D'Agostino et al., 2002). Although p13 mutants carrying substitutions of the arginines with glutamines, prolines, or alanines and leucines retain mitochondrial targeting, they produce little or no mitochondrial fragmentation/swelling, indicating that the arginines of the amphipathic α-helical domain are essential for these effects (D'Agostino et al., 2002).

In vitro assays carried out using isolated rat liver mitochondria and purified full-length synthetic p13 demonstrated that the peptide triggers an inward K^+ current that induces depolarization and activation of the electron transport chain; these changes are accompanied by increased mitochondrial reactive oxygen species (ROS) production, which along with membrane depolarization, lowers the opening threshold of the permeability transition pore (PTP) (Silic-Benussi et al., 2009).

The effects of p13 on inner membrane potential ($\Delta\psi$) and ROS production are also observed in living cells (Biasiotto et al., 2010; Silic-Benussi et al., 2010). Studies of the influence of p13 on ROS indicate that the protein may have a distinct impact on cell survival and proliferation depending on the cell's inherent ROS levels, with activation predominating in normal resting T-cells and death-promoting effects in transformed cells (Silic-Benussi et al., 2010). In the context of the HTLV-1 propagation strategy, p13 would provide a mechanism to increase the pool of "normal" infected cells while promoting the elimination of cells acquiring a transformed phenotype, thus favouring lifelong persistence of the virus in the host.

1.4.6 p12

p12 is coded by the x-I ORF and it is expressed from an singly-spliced mRNA which contains exon 1 and B (mRNA 1-B). p12 localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus (Koralnik et al., 1993; Ding et al., 2001; Johnson et

al., 2001). While this protein is not required for HTLV-1 replication *in vitro*, it plays a key role in the stabilization of a productive viral infection *in vivo* (Albrecht et al., 2000; Collins et al., 1996; Derse et al., 1997; Robek et al., 1998).

p12 is a higly hydrophobic protein, whose amino acid sequence contains four proline-rich Src homology 3 (SH3) domains that mediate interactions with proteins involved in cell signal transduction, two trans-membrane leucine zipper motifs that mediate targeting of the protein to the ER and other endomembrane compartments (Koralnik et al., 1993; Ding et al., 2001; Johnson et al., 2001) and a calcineurin-binding motif (Kim et al., 2003).

p12 interacts with the β and γ_c chains of the interleukin-2 receptor (IL-2R), resulting in reduced surface expression (Mulloy et al., 1996). Furthermore, p12 binds to the cytoplasmic domain of the IL-2R β chain, which is involved in the recruitment of the Janus-associated kinases 1 and 3 (Jak1 and Jak3). This interaction determines an increase in the transcriptional activity of the signal transducers and activators of transcription-5 (STAT-5), providing a proliferative advantage to T cells (Nicot et al., 2001).

p12 was also shown to sequester free MHC class I heavy chains (MHC-I-Hc), preventing their binding to β2-microglobulin. p12-bound MHC-I molecules are translocated in the cytosol where they are degraded by the proteasome. The overall result of this interaction is a decrease in functional MHC-I on the surface of HTLV-1 infected cells that would thus escape from CTL recognition and clearance by the immune system (Johnson et al., 2001). Furthermore, p12 causes a reduction in the expression of ICAM-1 and ICAM-2, which mediate adhesion of natural killer (NK) cells to the infected cells, resulting in the protection of HTLV-1-infected primary CD4+ T cells from NK cell-mediated cytotoxicity (Banerjee et al., 2007). These effects are particularly relevant in the context of HTLV-1 infection, which is able to induce a strong humoral and cellular immunitary response (Bangham, 2003).

Furthermore, p12 interacts with calreticulin and calnexin (Ding et al., 2001), two ER-resident proteins that regulate Ca^{2+} storage and release, and diminishes calcium available for release from the ER stores and currently increasing cytosolic Ca^{2+} level, suggesting a p12-mediated Ca^{2+} leakage from the ER (Ding et al., 2002). Moreover, p12 induces nuclear factor of activated T-cells (NFAT) activation (Albrecht et al., 2002), by interacting with calcineurin, a Ca^{2+}-responsive protein phosphatase that

controls NFAT activity (Kim et al., 2003). Taken together these effects decrease the threshold for T-cell activation (Nicot et al., 2005).

The modulation of Ca^{2+} homeostasis and signalling induced by p12 are in line with a recent study demonstrating alterations in Ca^{2+} homeostasis and gene expression in HTLV-1 infected cells (Akl et al., 2007). The modulation of Ca^{2+} signalling is a shared mechanism for different viruses to facilitate their infection (Chami et al., 2006; Zhou et al., 2009): HIV-1 Nef and HCV core are some examples of virally encoded proteins controlling Ca^{2+} levels, Ca^{2+}-dependent transcription, and infectivity (Bergqvist et al., 2003; Manninen and Saksela, 2002).

1.4.7 Hbz

The negative strand of the HTLV-1 genome contains one ORF located in the pX region (antisense orientation) which generates at least 2 different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). The difference between hbz sp1 and hbz us is the presence of the first exon in the former transcript. Hbz sp1 has multiple transcriptional initiation sites in the U5 and R regions of the 3' LTR, whereas the hbz us gene initiates within the tax gene (Figure 2B). Both hbz sp1 and hbz us have TATA-less promoters (Yoshida et al., 2008). It has been reported that the basal transcription factor Sp1 is critical for many TATA-less promoters (Boam et al., 1995; Liu and Cowell, 2000). Consistent with this, the transcription of hbz sp1 is dependent on Sp1 (Yoshida et al., 2008). The TRE sequence in the 3' LTR also functions to enhance transcription of the hbz anti-sense transcripts (Yoshida et al., 2008; Landry et al., 2009). However, the enhancing activity for anti-sense transcription is relatively weak when compared with sense transcription. This is consistent with the finding that transcription of the hbz gene is relatively constant in ATLL cases regardless of the expression levels of Tax (Saito et al., 2009).

Hbz sp1 is translated into a protein of 206 amino acids, while hbz us produces a protein of 209 amino acids. The two HBZ isoforms differ by 7 amino acids in the N-terminus (Murata et al., 2006). The HBZ SP1 protein is more abundant and has a longer half-life than HBZ US isoform (Yoshida et al., 2008), and the hbz sp1 mRNA is expressed at higher levels compared to the hbz us mRNA (Usui et al., 2008). The HBZ protein contains an N-terminal transcriptional activation domain (AD), a central

domain (CD) and a C-terminal basic ZIP domain (bZIP) (Gaudray et al., 2002). It localizes in the nucleus with a speckled pattern and contains three regions associated with its nuclear localization: two regions rich in basic amino acids and a DNA binding domain (Hivin et al., 2005). HBZ is not necessary for viral replication or immortalization *in vitro*, but increases infectivity and viral persistence *in vivo* (Arnold et al., 2006).

HBZ interacts with a number of transcription factors, including CREB-2, p300/CBP, Jun family members, and NF-κB (Matsuoka and Green, 2009). Through interactions mediated by its bZIP domain, HBZ abolishes the ability of CREB-2 to bind to the TRE in the HTLV-1 LTR, resulting in the suppression of transcription from the 5' LTR by Tax (Gaudray et al., 2002). HBZ interacts with CBP/p300 via LXXLL-like motifs in its N-terminal region, leading to suppression of viral transcription by inhibiting the recruitment of CBP/p300 to the HTLV-1 promoter (Clerc et al., 2008).

HBZ's bZIP domain also mediates formation of heterodimers with several AP-1 transcriptional family members, such as c-Jun, JunB, and JunD, and modulates their activity (Basbous et al., 2003; Thebault et al., 2004). Binding of HBZ to JunB and c-Jun decreases their DNA binding activity by preventing their interaction with Fos, leading to repression of the AP-1 complex (Matsumoto et al., 2005). Additional AP-1 transcriptional repression is explained by HBZ-mediated reduction in c-Jun stability via the proteasome-dependent pathway (Matsumoto et al., 2005) and sequestration of JunB by HBZ within nuclear bodies (Hivin et al., 2007). In contrast to JunB and c-Jun, the interaction of HBZ with Jun-D stimulates its transcriptional activity (Thebault et al., 2004), and results in the activation of JunD-dependent cellular genes including human telomerase reverse transcriptase (hTERT) (Kuhlmann et al., 2007).

HBZ has been shown to inhibit the activation of the classical NF-κB pathway by two different mechanisms: by inhibiting the DNA binding of the NF-κB subunit p65 and by increasing the expression of PDLIM2, the E3 ubiquitin ligase of p65, leading to enhanced ubiquitination and degradation of p65 (Zhao et al., 2009).

HBZ expression is associated with proliferation of ATLL cells *in vivo* and *in vitro* (Satou et al., 2006; Arnold et al., 2008). Mutational analyses of the hbz gene showed that hbz mRNA, rather than HBZ protein, has a growth-promoting effect on T-cells (Satou et al., 2006) possibly by up-regulating the transcription of the E2F1 gene and its downstream targets.

An intriguing aspect of ATLL pathogenesis is represented by the fact that Tax expression is not detected in about 60% of leukemia cases despite its proven central role in leukemogenesis; i.e. it immortalizes T-lymphocytes *in vitro* and induces cancer in transgenic animals (Takeda et al., 2004). Three mechanisms for inactivating Tax expression have been described: 1) genetic changes (nonsense mutation, deletion, and insertion) of the *tax* gene (Takeda et al., 2004; Furukawa et al., 2001), 2) deletion of the 5' LTR (Tamiya et al., 1996; Miyazaki et al., 2007) and 3) DNA methylation of the 5' LTR (Koiwa et al., 2002; Taniguchi et al., 2005). One possible scenario is that since Tax is the major target of cytotoxic T-lymphocytes (CTL) *in vivo (*Kannagi et al., 1991), these mechanisms to disrupt or decrease Tax expression facilitate the escape of ATLL cells from host CTL. Interestingly, analyses of HTLV-1 proviruses in ATLL cells revealed an intact hbz gene and lack of deletion or methylation of the 3' LTR (Fan et al., 2010; Yoshida et al., 2008), suggesting that hbz gene expression plays a critical role in the development of this disease.

1.5 Regulation of HTLV-1 gene expression: open questions and paradoxes

Although considerable knowledge about HTLV-1 gene expression regulation has been gained up to now, important questions still remain open and intriguing paradoxes unsolved.

Early studies established that HTLV-1 gene expression is controlled by two key regulatory circuits (Figure 5): a positive transcriptional regulatory feedback loop provided by the viral trans-activator Tax that drives transcription of the viral genome (Felber et al., 1985), and a negative post-transcriptional regulatory feedback loop provided by Rex that, by binding to the Rex-responsive element (RxRE) present at the 3' end of HTLV-1 transcripts, enhances the nuclear export and expression of a subset of mRNAs coding for the virion-associated proteins Gag-Pol and Env (Inoue et al., 1986; Inoue et al., 1987; Hidaka et al., 1988; Seiki et al., 1988; Inoue et al., 1991).

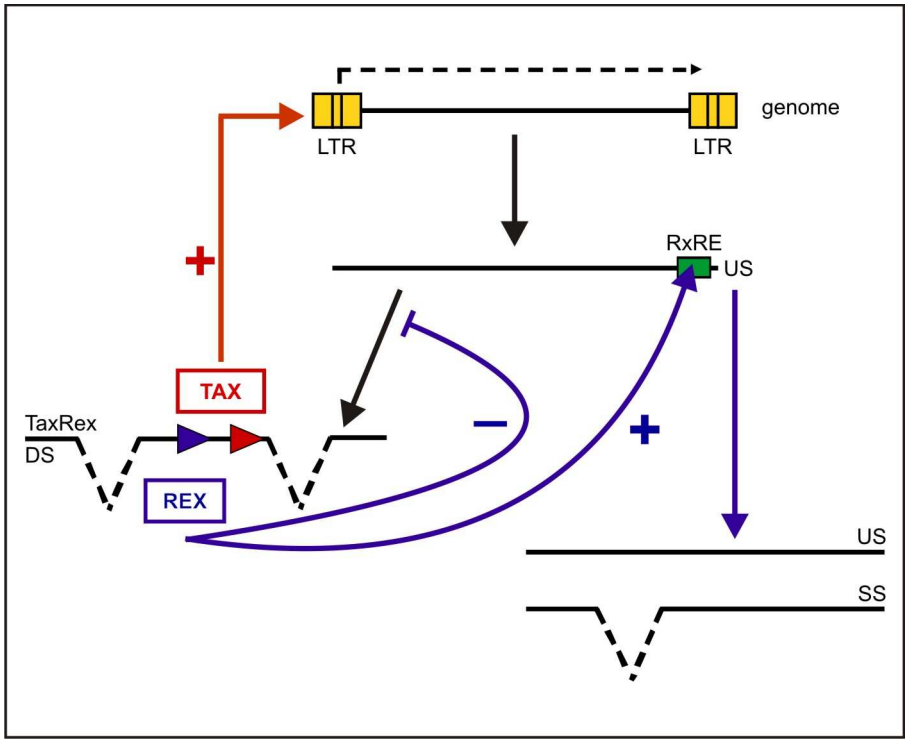

Fig. 5. Feedback regulatory loops controlling HTLV-1 gene expression.
Tax drives transcription of the viral genome and Rex mediates a post-transcriptional regulatory loop that enhances the nuclear export and expression of Rex-dependent mRNAs.

More recently it has become apparent that the HTLV-1 expression strategy is more complex and is characterized by production of positive- and negative-strand transcripts, alternative splicing and polycistronic translation (Lairmore and Franchini, 2007). This strategy greatly increases the coding potential of the virus resulting in expression of several regulatory and accessory genes (Tax, Rex, p12, p13, p21rex, p30tof and HBZ) in addition to the structural proteins and virion-associated enzymes common to all retroviruses (Gag, Pro, Pol and Env).

An implication of the feedback regulation of HTLV-1 gene expression mediated by Tax and Rex is that viral transcripts might be expressed with a distinct timing during the course of the viral life cycle, with a switch from early (Rex-independent) to late (Rex-dependent) genes. Nevertheless, this switch in HTLV-1 expression has proven difficult to demonstrate experimentally even in recent studies carried out *in vitro* systems based on transfection of an HTLV-1 molecular clone, in PBMCs infected with HTLV-1 in cell culture and in a rabbit animal model of HTLV infection (Li et al., 2009).

Furthermore, although it is commonly accepted that Rex acts at a post-transcriptional level controlling the nuclear export and stability of viral mRNAs coding for the virion-associated proteins Gag-Pol and Env, the Rex-dependency of all the individual alternatively spliced HTLV-1 transcripts has not been investigated so far. Extrapolating from results obtained on the tax/rex, env and gag transcripts (Hidaka et al., 1988), current models of HTLV-1 regulation predict that multiply-spliced mRNAs such as p30tof should be Rex-independent, while unspliced and singly-spliced mRNAs (p12, p13, p21rex) should be Rex-dependent. Hbz transcripts, which are transcribed from the minus strand, do not contain the RxRE sequence and thus are predicted to be Rex-independent.

Furthermore, a long-standing paradox of HTLV-1 gene expression regulation is represented by the fact that, even if only a subset of HTLV-1 mRNAs are Rex-dependent, the RxRE sequence is present in the 3' LTR of all the positive strand viral mRNAs, suggesting that the Rex-dependence of a transcript might be determined by distinct(s) cis-acting regulatory sequence(s) that would be present on some transcript and absent on others as a result of alternative splicing.

Another intriguing paradox is represented by the fact that the positive and negative regulatory feedback loops that control HTLV-1 gene expression are mediated by two proteins, Tax and Rex respectively, encoded from the same bicistronic doubly spliced mRNA. Simultaneous action of the two proteins would cause the positive and negative regulatory loops to work against each other, resulting in an inhibition of viral gene expression. In this view, a mechanism able to delay Rex function with respect to that of Tax seems to be necessary for allowing viral mRNA expression.

2. AIMS OF THE STUDY

In spite of over 30 years of study, several key features of the HTLV-1 life cycle and pathogenicity remain obscure. In particular, it is still unclear whether HTLV-1 gene expression is characterized by latency patterns, whether the different viral genes follow distinct kinetics of expression, and which molecular mechanisms control these processes.

The work described in the present thesis was aimed at understanding these aspects of HTLV-1 regulation. To this end we optimized a Real Time RT-PCR method using splice-site-specific primers to quantitate the different HTLV-1 transcripts and their kinetics of expression in peripheral blood mononuclear cells (PBMCs) isolated from HTLV-1-infected individuals and in cells transfected with HTLV-1 molecular clones. To analyze the Rex-dependence of individual HTLV-1 mRNAs and to test if the kinetics of HTLV-1 gene expression might be dependent on Rex function, we generated a Rex knock-out HTLV-1 molecular clone. Furthermore we investigated the intracellular compartmentalization of individual HTLV-1 transcripts, in the presence and in absence of Rex, and analyzed the turnover of Tax and Rex, the main regulatory proteins of HTLV-1.

3. MATERIALS AND METHODS

3.1 Cell culture

The HeLa-derived cell line HLtat, which constitutively expresses the human immunodeficiency virus type 1 (HIV-1) Tat protein (Schwartz et al., 1990) was maintained in DMEM (Dulbecco modified Eagle medium; Sigma-Aldrich) supplemented with 10% FCS (fetal calf serum; Invitrogen), 100 units/mL penicillin, and 20 units/mL streptomycin.

The HTLV-1-infected cell line C91PL (Popovic et al., 1983) was maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 2 mM L-glutamine (GIBCO) and penicillin/streptomycin.

3.2 Plasmids

The Rex and Tax proteins were expressed from the full-length tax/rex mRNA, using plasmid pBS1-2-3, which contains the viral 5' LTR and exons 1, 2 and 3 (including the 3'LTR) of the infectious HTLV-1 molecular clone CS-HTLV-1 (Derse et al., 1995), cloned into pBluescript (Stratagene).

Plasmid ACH, which contains the HTLV-1 proviral genome, has been previously described (Kimata et al., 1994). Plasmid ACH-Rex knock-out (ACH-Rex-KO), lacks the Rex AUG and was derived from the HTLV-1 molecular clone ACH by digestion with SphI followed by removal of 3' overhangs (including the Rex initiation codon) with T4 DNA polymerase and religation.

3.3 Transfections

To analyze Tax and Rex protein expression kinetics, HLtat cells were seeded in 35-mm cell culture dishes at 1.5 x 10^5 cells/dish and transfected one day later with 1 µg of pBS1-2-3; in alternative 3 x 10^5 cells were seeded in 60-mm cell culture dishes and transfected one day later with 1 µg of ACH plasmid.

For Real-Time RT-PCR analysis HLtat cells were plated in 100-mm cell culture dishes at 6 x 10^5 cells/dish and transfected one day later with 2 µg of ACH or ACH-Rex KO. Cultures were harvested at 0, 16, 24 and 48 hours post-transfection to quantify viral transcripts by Real Time RT-PCR.

Transfections were carried out by using the FuGENE 6 Transfection Reagent (Roche) or GeneJuice (Novagen) following the manufacturer's recommendations.

3.4 Analysis of Tax and Rex expression by flow cytometry

To analyze the kinetics of Tax and Rex protein expression from plasmid pBS1-2-3 or ACH, HLtat cells were harvested at 8, 16, 32 and 48 hours after transfection. Cells were detached with trypsin, rinsed in phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde-PBS for 20 minutes, washed in PBS, permeabilized in 0.2% Triton-PBS for 10 minutes, washed again, blocked with 3% BSA (bovine serum albumin)-PBS for 30 minutes and, after washing, incubated for 1 hour with mouse anti-Tax monoclonal antibody (Lee et al., 1989) (1:100, 1.5% BSA-PBS) and rabbit anti-Rex polyclonal antibody (Bhat et al., 1993) (1:500, in 1.5% BSA-PBS). After washing with PBS, cells were incubated for 1 hour with Alexa 633-conjugated goat anti-mouse and Alexa 488-conjugated chicken anti-rabbit antibodies (Molecular Probes) diluted 1:1000 in 1.5% BSA-PBS. After washing the cells, Tax and Rex protein expression was detected by flow cytometry using a FACSCalibur apparatus (Becton Dickinson Coulter, BD Biosciences) equipped with 633-nm Helium-Neon and 488-nm Argon lasers. Alexa 633 and Alexa 488 fluorescent signals were analyzed using the FL4 (661±16 nm) and the FL1 (530±30 nm) detection lines, respectively. For each sample 30,000 (pBS1-2-3 plasmid) or 100,000 (ACH) gated events were examined. Data were analyzed using the Summit v4.3 software (Dako).

3.5 Analysis of Tax and Rex degradation rates by immunoblotting

To analyze the degradation rates of the Tax and Rex proteins, HLtat cells transfected with pBS1-2-3 or ACH were treated with 10 μM cycloheximide 24 hours after transfection and harvested in "disruption buffer" (Paris kit, Ambion) at 3, 8, 24 and 32 hours following cycloheximide treatment.

Lysates were subjected to SDS-PAGE and electrotransferred to Hybond-C Extra nitrocellulose membrane (GE Healthcare). Blots were blocked for 1 h in 2% milk (Roche)-0.05% Tween 20-PBS, washed with 0.05% Tween 20-PBS and incubated for 2 hours with mouse anti-Tax monoclonal antibody (1:500), rabbit anti-Rex polyclonal antibody (1:5000) or mouse anti-α-Tubulin monoclonal antibody (1:2000; Sigma-Aldrich) in 3% BSA-0.05% Tween-PBS followed by 1.5 hrs' incubation with

horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Pierce) diluted 1:5000 in 2% milk-0.05% Tween-PBS. Blots were developed using chemiluminescence reagents (Supersignal, Pierce) and immunoreactive bands were visualized and quantified using a BioRad ChemiDoc XRS imager. Data were normalized by dividing Tax and Rex signals by the tubulin signal. Protein half-life was estimated by fitting a linear decay model to the data, assuming a constant degradation rate.

3.6 Patients

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral venous blood samples donated by patients with a clinical diagnosis of ATLL or TSP/HAM attending the clinic at the National Centre for Human Retrovirology, Imperial College Healthcare NHS Trust, St. Mary's Hospital or King's College Hospital, London, UK.

CD8 (CD8+) positive T-cells were depleted from the samples using anti-CD8 paramagnetic beads (Miltenyi Biotec) according to the manufacturer's instructions (Hanon et al., 2000). After CD8+ T-cell depletion, remaining PBMC sub-populations were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM/L glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin and harvested in TRIZol (Invitrogen) at 2, 4, 8, 24 and 48 hours for RNA extraction and viral transcript quantification.

3.7 Real Time RT-PCR

RNA was extracted from primary cells using TRIZol and from cell lines using the Paris Kit (Ambion) to separate cells into nuclear and cytoplasmic fractions. The quality and the quantity of RNA were determined using the Agilent 2100 Bioanalyzer. RNA from patient samples was reverse-transcribed using AMV-reverse transcriptase (Finnzymes) and random hexamers (plus-strand transcripts) or the specific hbz-primer AS2: 5'-TCTTCCTCCAAGGATAATAGCCCGTCCA-3'. Total, cytoplasmic and nuclear RNA from cultured cells (1 µg) was treated with DNAase (Invitrogen) and reverse-transcribed using SuperScriptII reverse transcriptase (Invitrogen) and random hexamers (plus-strand transcripts), or the specific hbz-AS2 primer.

Table 1 lists the sequences of the primers and probes and concentrations used to detect alternatively spliced HTLV-1 transcripts. Probes were 5′ end-labeled with FAM and 3′ end-labeled with TAMRA. As an internal control, GAPDH mRNA or 18S rRNA were analyzed in parallel by using the Endogenous Control Human GAPDH kit or Ribosomal RNA Control Reagents (Applied Biosystems). PCR reactions were performed with an ABI Prism 7900 HT Sequence Detection System by using 5 μl of each diluted RT sample (10 ng/μl) and 20 μl of diluted Taqman Universal PCR Master Mix (Applied Biosystems) and primers and probes at the concentrations listed in the table; each reaction was performed in duplicate. The cycling conditions comprised a initial step at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The absolute kinetic method was applied by using standard curves constructed from 5-fold serial dilutions of a plasmid containing the GAPDH, 18S rRNA amplicon, or HTLV-1 transcript amplicon.

The absolute copy number of each transcript was determined and normalized (normalized copy number, NCN) for the copy number of the 18S rRNA in the case of patients' or GAPDH in the case of transfected cells and C91PL. To better analyze the relative variation of each transcript over time, in the case of patients' we scaled the NCN of each transcript in each time point against the maximum value measured for the same mRNA during the time course of the experiment (scaled copy number, SCN) while in the case of transfected cells and C91PL cell line we calculated the Export Ratios by dividing the NCN in the cytoplasmic fraction by the NCN in the total lysate.

GAG	forward primer	SK110	5'-CCCTACAATCCAACCAGCTCAG-3'	900 nM
	reverse primer	SK111	5'-GTGGTGAAGCTGCCATCGGGTTTT-3'	300 nM
	probe	GAG	5'(FAM)-CTTTACTGACAAACCCGACCTAC-3'(TAMRA)	100 nM
ENV	forward primer	Env s	5'-GTCCGCCGTCTAG^CTTCC-3'	1000 nM
	reverse primer	Env as	5'-GAGGGGGCAGAACTGGAAG-3'	300 nM
	probe	ENV-G	5'(FAM)-CCCAGTGGATCCCGTGGAG-3'(TAMRA)	100 nM
p12	forward primer	1-B	5'-GTCCGCCGTCTAG^CACTATG-3'	900 nM
	reverse primer	3'6552	5'-GGAGGAAGCAGGAAGAGC-3'	300 nM
	probe	TMP-1	5'(FAM)-TTCGCCTTCTCAGCCCCTTGTCT-3'(TAMRA)	100 nM
p13	forward primer	p13 s	5'-GTCCGCCGTCTAG^CAGGTC-3'	300nM
	reverse primer	p13 as	5'-GGTTAACTTTGTATCTGTAGGGCTGTT-3'	600nM
	probe	p13	5'(FAM)-TCCGGGCATGGCACAGGCA-3'(TAMRA)	100 nM
p21rex	forward primer	p21rex s	5'-CCGCCGTCTAG^CCCACTT-3'	900nM
	reverse primer	p21rex as	5'-GAGTCGAGGGATAAGGAAC-3'	900nM
	probe	p21Rex	(FAM)-AAGGCGACTGGTGCCCCATCTCTGGG-(TAMRA)	100 nM
HBZ SP1	forward primer	HBZ s	5'-CTCAG^GGCTGTTTCGATGCT-3'	900 nM
	reverse primer	HBZ as	5'-GCCCGTCCACCAATTCCT-3'	900 nM
	probe	HBZ	5'(FAM)-CCTGTGTCATGCCCGGAGGACC-3'(TAMRA)	100 nM
HBZ US	forward primer	HBZ US s	5'(FAM)-GTTAACTTTGTATCTGCAGGG-3'(TAMRA)	900 nM
	reverse primer	HBZ as	5'(FAM)-CCTGTGTCATGCCCGGAGGACC-3'(TAMRA)	900 nM
	probe	HBZ	5'(FAM)-CCTGTGTCATGCCCGGAGGACC-3'(TAMRA)	100 nM
TaxRex	forward primer	Env s	5'-GTCCGCCGTCTAG^CTTCC-3'	900 nM
	reverse primer	TaxRex as	5'-CTGGGAAGTGGG^CCATGG-3'	900 nM
	probe	ENV-G	5'(FAM)-CCCAGTGGATCCCGTGGAG-3'(TAMRA)	100 nM
p30Tof	forward primer	2-Bs	5'-TCCAACACCATGG^CACTATG-3'	900 nM
	reverse primer	3'6552	5'-GGAGGAAGCAGGAAGAGC-3'	900 nM
	probe	TMP-1	(FAM)-TTCGCCTTCTCAGCCCCTTGTCT-(TAMRA)	100 nM

Table 1. Splice-junction-specific Real Time RT-PCR.

4. RESULTS

4.1 Quantitative analysis of HTLV-1 gene expression in peripheral blood mononuclear cells (PBMCs) obtained from HTLV-1-infected individuals

To investigate the expression profile of HTLV-1 we first employed an *ex vivo* virus reactivation model based on peripheral blood mononuclear cells (PBMCs) isolated from HTLV-1-infected patients. As previously described, the depletion of CD8 positive (CD8+) T-cells from unstimulated PBMCs, results in a sharp upregulation of viral expression in the remaining cell population (Hanon et al., 2000; see also Materials and Methods, Par. 3.6). We analyzed a total of 9 patients, including 3 ATLL and 6 TSP/HAM patients. Table 2 shows the clinical features, the percentage of Tax positive (Tax+) CD4 positive (CD4+) T-cells in the peripheral blood and the proviral loads (PVL) of each patient. The samples were obtained from patients participating in Institutional Review Board-approved studies (SMH LREC 02.31) following written informed consent.

Patient Code	Clinical Diagnosis	% Tax + CD4+	PVL (n°copies/ 100 PBMC)
ATLL-1	ATLL	15.5	170
ATLL-2	ATLL	5.7	47.5
ATLL-3	ATLL	ND	25.4
TSP-1	TSP/HAM	13	50.7
TSP-2	TSP/HAM	0.4	22
TSP-3	TSP/HAM	8	8.9
TSP-4	TSP/HAM	6.7	21.6
TSP-5	TSP/HAM	1.6	15
TSP-6	TSP/HAM	1.5	41.6

Table 2. Clinic-pathologic features of the HTLV-1 patients. ND: not determined

Splice-junction-specific Real Time RT-PCR was used to measure the expression levels and the timing of expression of HTLV-1 transcripts (see Materials and Methods, Par. 3.7 and Tab. 1). The absolute copy number determined for each

transcript was normalized for the copy number of 18S rRNA (normalized copy number, NCN).

4.1.1 Expression of the different HTLV-1 transcripts in PBMCs obtained from HTLV-1 infected individuals

Figure 6A shows the NCN of the different viral mRNAs after 2 and 48 hours (black bars and white bars, respectively) of culture *in vitro* following CD8+ T-cell depletion (patient ATLL-1, left-hand panel; patient TSP-1, right-hand panel). Expression of all transcripts was greatly upregulated upon culture *in vitro*. The most abundant plus-strand transcripts were tax/rex (coding for Tax and Rex), gag and env, followed by 1-3 (coding for p21rex), 1-2-B (coding for p30tof), 1-C (coding for p13) and 1-B (coding for p12); the minus-strand transcripts (coding for HBZ) were also expressed at high levels.

4.1.2 Temporal analysis of HTLV-1 gene expression in PBMCs obtained from HTLV-1 infected individuals

Figure 6 (B, C) shows the timing of expression of the different viral transcripts in the two representative patients (ATLL-1, left-hand panels and TSP-1, right-hand panels) over a 48-hour time period. To better analyze the relative variation of each transcript over time, we scaled the NCN of each transcript in each time point for the maximum value measured for the same mRNA during the time course experiment (scaled copy number, SCN). Results of this analysis showed that tax/rex was the earliest transcript followed by a rise in gag expression whose curve intersected that of tax/rex between 8 and 24 hours (Fig. 6B), suggesting an "early-late" switch in HTLV-1 gene expression (indicated by a grey box in the figures). Analysis of the SCN of all mRNAs (Fig. 6C) confirmed the "early-late" switch (grey box) and suggested a distinct temporal sequence of expression among the "late" mRNAs. We measured the expression levels and the timing of expression of HTLV-1 transcripts in all the patients' listed in Table 2, obtaining similar results.

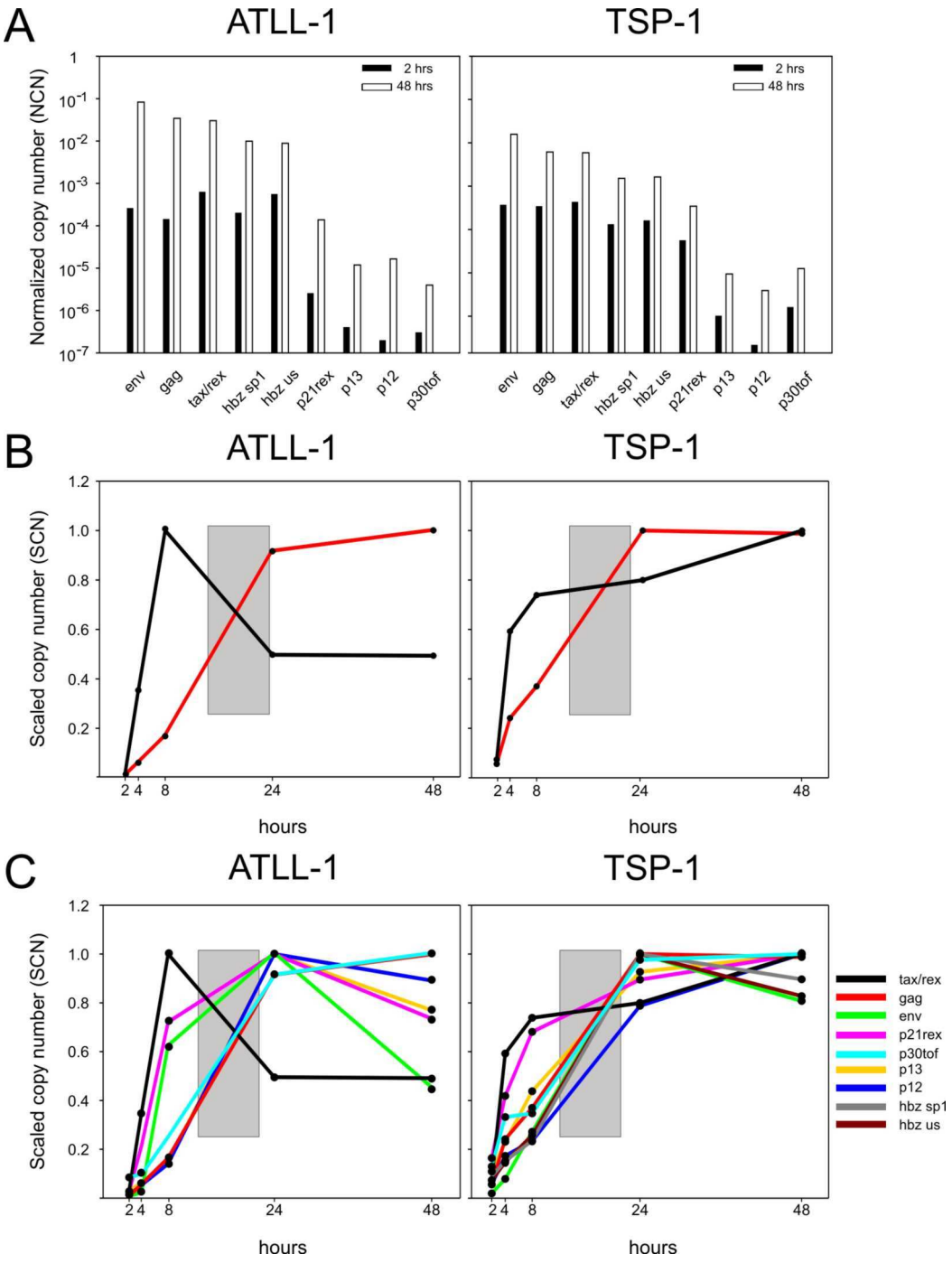

Figure 6. Quantitative analysis of HTLV-1 gene expression in PBMCs obtained from HTLV-1-infected individuals.

A. Bar graphs show the normalized copy numbers (NCN) of the indicated mRNAs after 2 hours (black bars) and 48 hours (white bars) of culture *in vitro* following CD8+ T-cell depletion measured in representative ATLL (left-hand panel) and TSP/HAM (right-hand panel) patients. NCN values were calculated by dividing the absolute copy number of each transcript by the copy number of the 18S rRNA. **B, C.** Line graphs show the variation in the tax/rex and gag mRNAs (B) and all measured transcripts (C). Scaled copy numbers (SCN) are plotted over a 48-hour time period (harvesting at 2, 4, 8, 24 and 48 hours following depletion of CD8+T-cells and culture *in vitro*). SCN values were calculated by dividing the NCN of each transcript at each time point by the maximum NCN value measured for that mRNA during the time course. mRNAs are indicated by colours as shown in C.

4.2 Quantitative analysis of HTLV-1 gene expression in transfection models

The abundance and timing of expression of the HTLV-1 mRNAs were further investigated in cells transfected with HTLV-1 molecular clones. This system permitted the quantitation of transcripts in the cytoplasmic and nuclear fractions, which was not possible with patient samples due to limited amounts of material. Considering that Rex acts at a post-transcriptional level controlling the nuclear export of unspliced and partially spliced transcripts, the quantitation of the nucleo-cytoplasmic partitioning of HTLV-1 mRNAs appeared to be an appropriate system for studying the kinetics of expression of HTLV-1 mRNAs *in vitro*. The absolute copy number determined for each transcript was normalized for the copy number of the GAPDH mRNA (normalized copy number, NCN).

4.2.1 Expression of the different HTLV-1 transcripts in transfection models

Figure 7A shows NCN in the cytoplasmic (left-hand panel) and nuclear fractions (right-hand panel) 24 hours after transfection of the wild-type HTLV-1 molecular clone, ACH (see Materials and Methods, Par. 3.2). The most abundant plus-strand transcripts were tax/rex and gag, followed by env and p21rex; p12, p13, and p30tof were expressed at lower levels. The plus-strand transcripts showed comparable abundance in the nucleus and cytoplasm; in contrast, the NCN of the hbz transcripts was over 10-fold higher in the nucleus.

4.2.2 Temporal analysis of HTLV-1 gene expression in transfection models: Rex-dependence of the "two-phase" kinetics

The timing of HTLV-1 expression following the transfection of molecular clones was investigated by calculating "Export Ratios": the NCN in the cytoplasmic fraction was divided by the NCN in the total lysate for all HTLV-1 transcripts.

Figure 7B (left hand panel) shows the Export Ratios for the different transcripts expressed from the wild-type HTLV-1 molecular clone ACH over a time course of 48 hours. Results indicated that tax/rex was expressed earliest, with a peak at 24 hours post-transfection, while gag, env, p12, p13, p21rex and p30tof reached their maximal expression levels at later time points. Consistent with results obtained in the *ex vivo* model, ACH showed a “two-phase” expression kinetics with "early" tax/rex

expression (measured as a sharp increase in the Export Ratio) followed by a rise in the Export Ratios of all the other transcripts at approximately 36 hours (shaded area). Using a Rex knock-out derivative of ACH (ACH-Rex-KO, see Materials and Methods, Par. 3.2) we also tested the Rex-dependence of the “two-phase” mRNA expression kinetics. Figure 7B (right-hand panel) shows the Export Ratios for the different transcripts expressed from ACH-Rex-KO over a time course of 48 hours. Results indicated that the "two-phase" kinetics were completely abolished in the absence of Rex, demonstrating the critical role of this protein in regulating these kinetics.

4.2.3 Nuclear retention of hbz transcripts

Interstingly we observed that the Export Ratios of the hbz transcripts remained remarkably low throughout the time course after ACH transfection (Fig. 7B, left-hand panel) and their expression profile was not affected by Rex (Fig. 7B, right-hand panel). The nuclear retention of hbz transcripts that we first observed after the transfection of the wild-type molecular clone (see Par. 4.2.1 and Fig. 7A of this Section) was also confirmed in the chronically infected cell line C91PL (Fig. 7C).

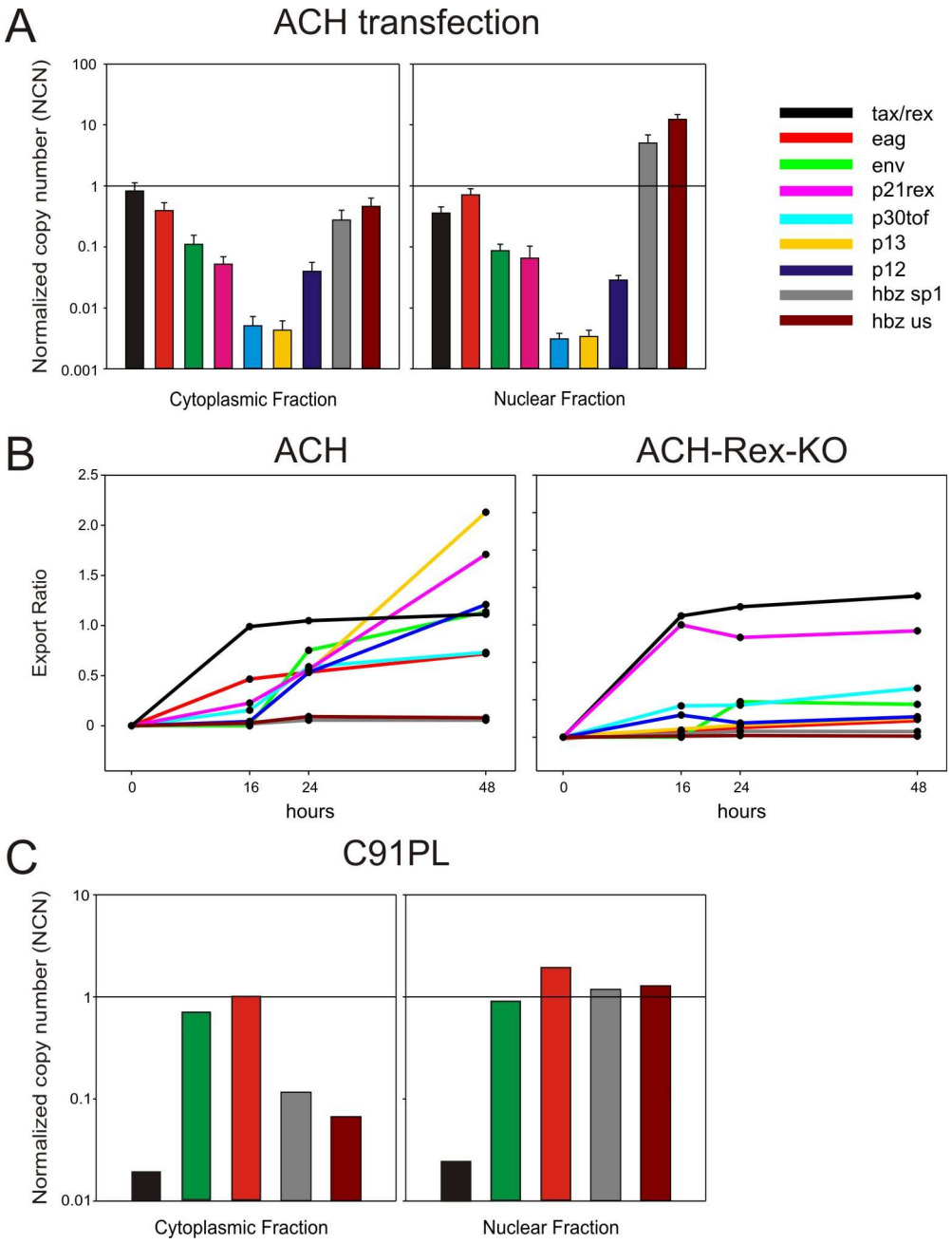

Figure 7. Quantitative analysis of HTLV-1 gene expression in transfection models.
A. Bar graphs show the normalized copy numbers (NCN) of all HTLV-1 mRNAs in the cytoplasmic (left-hand panel) and nuclear fractions (right-hand panel) 24 hours after transfection of HLtat cells with wild-type HTLV-1 molecular clone ACH (means of 3 independent experiments and standard error bars are shown). NCN values were determined by dividing the absolute copy number of each transcript by the copy number of the GAPDH mRNA. mRNAs are indicated by colours as shown to the right of the graphs. **B.** Line graphs show the temporal variations in the nucleo-cytoplasmic export of all HTLV-1 mRNAs expressed from ACH (left-hand panel) and from the Rex knock-out molecular clone (ACH-Rex-KO, right-hand panel) in transfected HLtat cells. Export Ratios are plotted over a 48-hour time period (harvesting at 0, 16, 24 and 48 hours) and were calculated as the ratio between cytoplasmic and total NCN. mRNAs are indicated by colours as shown in A. **C.** NCN of all HTLV-1 mRNAs in the cytoplasmic and nuclear fractions of the chronically infected cell line C91PL. NCN values were determined by dividing the absolute copy number of each transcript by the copy number of the GAPDH mRNA. mRNAs are indicated by colours as shown in A.

4.3. Analysis of the Rex-dependence of HTLV-1 transcripts

Extrapolating from results obtained for the tax/rex, env and gag transcripts (Inoue et al., 1986; Inoue et al., 1987; Hidaka et al., 1988; Seiki et al., 1988; Inoue et al., 1991), current models of HTLV-1 regulation predict that multiply-spliced mRNAs such as tax/rex and p30tof should be Rex-independent, while unspliced (gag-pro-pol) and singly-spliced mRNAs (env, p12, p13 and p21rex) should be Rex-dependent. Hbz transcripts, which are expressed from the minus strand, do not contain the RxRE sequence and are thus predicted to be Rex-independent.

To test the validity of this prediction, we measured the steady-state levels of the HTLV-1 mRNAs following transfection of wild-type vs. Rex knock-out HTLV-1 molecular clones in the total, cytoplasmic and nuclear fractions to calculate the Export Ratios for all HTLV-1 transcripts.

Figure 8 shows a comparison of the Export Ratio of the different viral mRNAs (mean of 8 independent experiments, standard error bars are shown) expressed from ACH (white bars) or ACH-Rex-KO (black bars) 24 hours after transfection. Consistent with previous studies, there was no statistically significant difference in the Export Ratio of the tax/rex mRNA between ACH and ACH-Rex-KO (Mann-Whitney Rank Sum Test; $p=0.13$), while the export of the env and gag mRNAs was significantly impaired in the Rex knock-out virus (statistically significant difference between ACH and ACH-Rex-KO Export Ratios, Mann-Whitney Rank Sum Test; $p<0.001$). Regarding the other transcripts, our study revealed that the singly-spliced p21rex mRNA, as well as the hbz sp1 and hbz us mRNAs, were Rex-independent (no statistically significant difference in the Export Ratios between ACH and ACH-Rex-KO, Mann-Whitney Rank Sum Test; $p=0.92$, $p=0.694$ and $p=0.343$, respectively). Interestingly, while the p21rex mRNA was efficiently exported both in the absence and in the presence of Rex, the nucleo-cytoplasmic export of the hbz mRNAs was highly impaired both in the presence and in the absence of Rex. The Rex-independence of hbz transcripts is consistent with the fact that these mRNAs are transcribed from the minus strand and thus do not contain the RxRE sequence. The export of the p12, p13 and p30tof mRNAs was significantly impaired in the absence of Rex (statistically significant difference in the Export Ratios between ACH and ACH-Rex-KO, Mann-Whitney Rank Sum Test; $p<0.001$, $p<0.001$ and $p<0.007$, respectively).

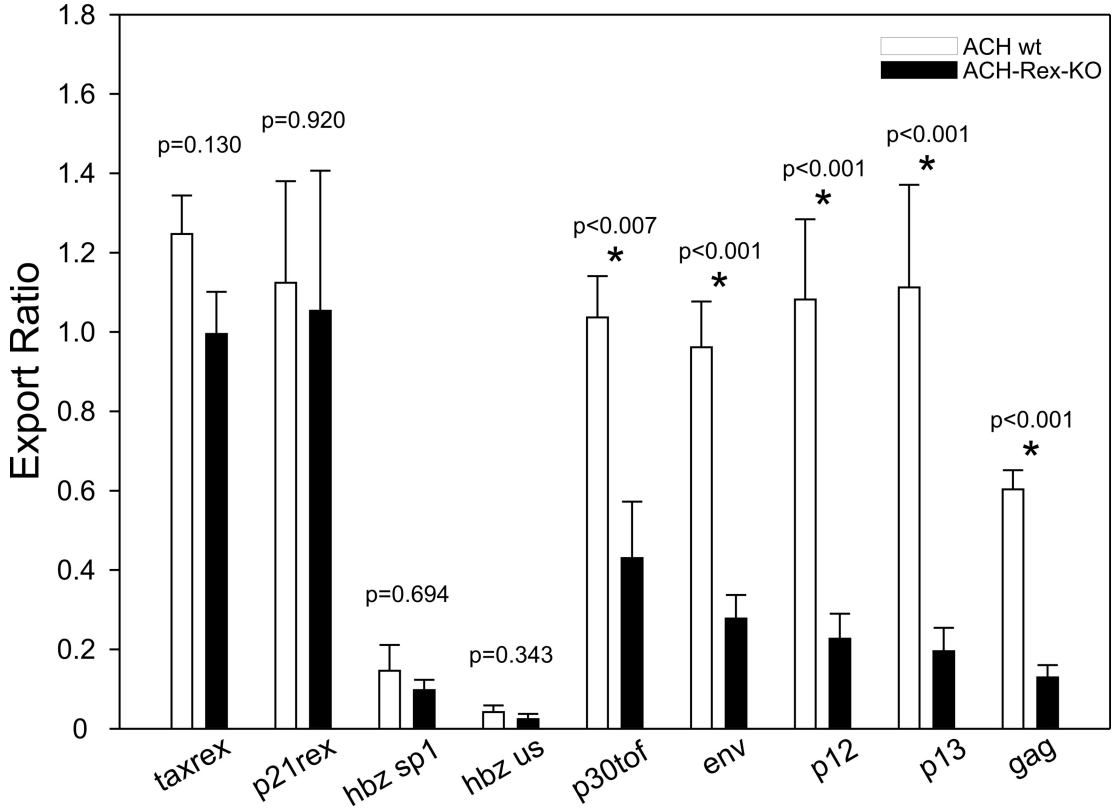

Figure 8. Rex-dependence of HTLV-1 transcripts.
Export Ratios of HTLV-1 mRNAs 24 hours after transfection of HLtat cells with ACH (white bars) or ACH-Rex-KO (black bars). Mean values of 8 independent experiments, standard error bars and p-values are shown. The asterisks in the figure indicate a statistically significant difference calculated with the Mann-Whitney Rank Sum Test.

4.4. Insights into the mechanisms controlling Rex-responsiveness of HTLV-1 mRNAs

Rex function is mediated through the binding to a cis-acting RNA element, named the Rex-responsive element (RxRE). Since the RxRE is present in the 3′ LTR of all the positive-strand HTLV-1 transcripts (Baydoun et al., 2008), it is unclear how Rex can exert a selective action enhancing the expression of some mRNAs (gag, env, p13, p12 and p30tof) and not others (tax/rex and p21rex). In light of the knowledge gained from studies of other complex retroviruses, we hypothesized that the Rex-dependence of a transcript might be determined by a distinct cis-acting regulatory sequence present on some transcripts and absent on others, while the RxRE could mainly function as a docking site for Rex binding.

4.4.1 Mapping a novel regulatory element determining Rex-responsiveness

To test this hypothesis, we first aligned the sequence of the Rex dependent and Rex-independent mRNAs. As shown in Figure 9, all transcripts containing a region (shaded area) comprised between splice acceptors C and 3 (nucleotide 6875 and 6950, respectively) are Rex-dependent (p12, p13, p30tof, gag and env), while transcripts lacking this region are Rex-independent (p21rex and tax/rex), regardless of being produced through a single or double splicing event. The hbz transcripts, which are transcribed from the minus strand, are not predicted to contain this region; in fact they were observed to be Rex-independent. Our data thus indicate that the Rex-dependence of HTLV-1 transcripts would not be determined by the number of splicing events that produced them, as suggested by current models, but by the intronic regions retained in the mature transcript.

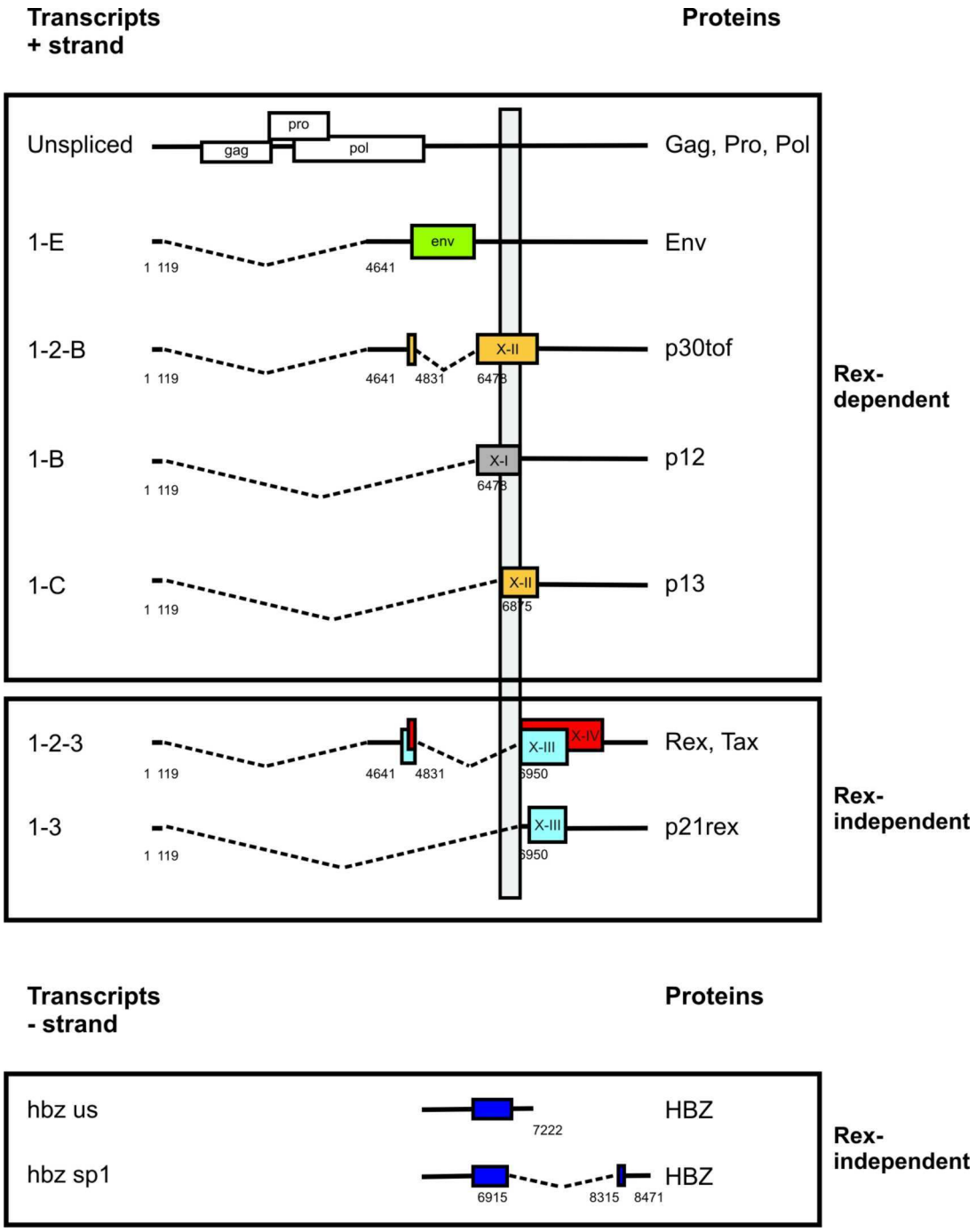

Figure 9. Mapping a novel regulatory cis-acting element determining Rex-responsiveness.
Mapping of the putative regulatory cis-acting Rex-control region, located between splice acceptor C (nt 6875) and 3 (nt 6950).

4.5. Recognizing a pattern: mathematical modelling of HTLV-1 gene expression and regulatory networks

Since Tax and Rex are coded by a bicistronic mRNA, one might expect the two proteins to be produced and to act simultaneously. We explored this hypothesis using the data obtained from HTLV-1-infected patient ATLL-1 to test a mathematical model of HTLV-1 expression composed of non-linear differential equations (Corrradin et al., 2010). This model, performed in collaboration with Dott. Alberto Corradin (Department of Information Engineering, University of Padova), was based on the following assumptions: (1) the tax/rex transcript is translated into the Tax and Rex proteins; (2) Tax is the master activator of viral mRNA expression; (3) Rex prevents splicing, so expression of tax/rex mRNA decreases while transcription of Rex-dependent genes increases. This generates positive and negative feedback regulatory loops determined, respectively, by Tax and Rex: the viral transactivator Tax drives transcription of the viral genome and the Rex-mediated post-transcriptional regulatory loop enhances the nuclear export and expression of a subset of mRNAs coding for the virion-associated proteins (Fig. 5). As shown in Figure 10 (left-hand panel), assuming the simultaneous action of Tax and Rex, the model predicts a pattern of expression (black line: tax/rex, red line: 1-B) that did not match the observed values (black diamonds: tax/rex, red circles: 1-B) but instead predicts an initial rise of the tax/rex mRNA, followed by a silencing of viral expression due to the opposite action of the positive (Tax) and negative (Rex) regulatory loops. Modification of the model by assuming that Rex function would ensue with a temporal delay compared to that of Tax results in a very close match between the prediction and the observed data (Fig. 10, right-hand panel). However, this temporal delay is in apparent conflict with the fact that both proteins are synthesized from the same mRNA.

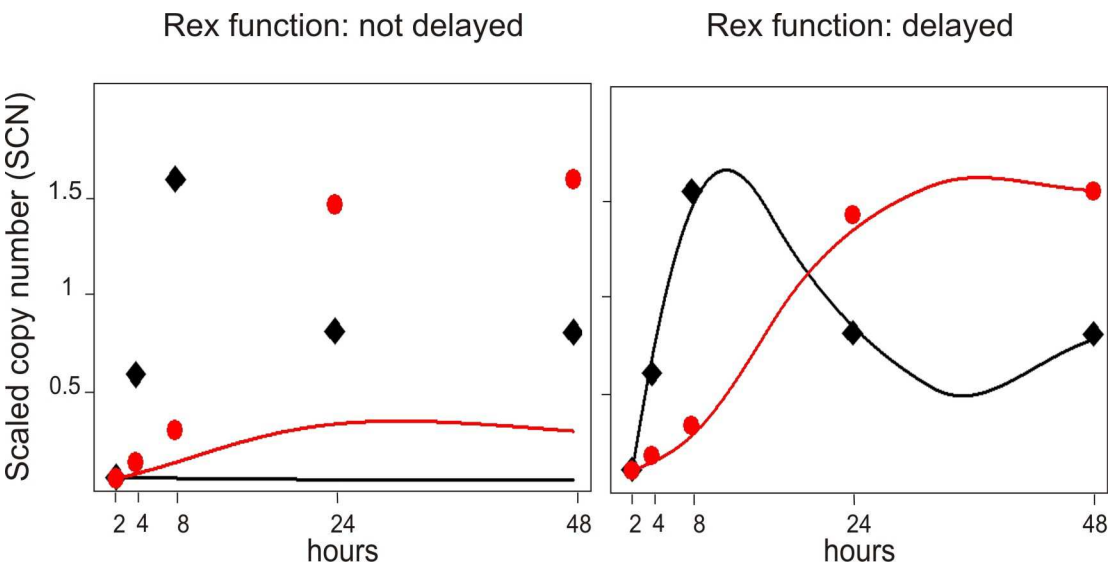

Figure 10. Mathematical modelling of HTLV-1 gene expression.
The left-hand panel shows the prediction of a model based on the simultaneous action of Tax and Rex proteins. The predicted kinetics of expression (black line: tax/rex, red line: 1-B) do not match experimentally values measured in patient ATLL-1 (black diamonds: tax/rex, red circles: 1-B). The right-hand panel shows the prediction of a model that assumes a temporal delay in Rex function compared to that of Tax. The predicted time course of expression (black line: tax/rex, red line: 1-B) gives a very close prediction of the experimentally values measured in patient ATLL-1 (black diamonds: tax/rex, red circles: 1-B).

4.6. Distinct kinetics of Tax and Rex protein turnover

Mathematical modelling (Fig. 10) underscored the importance of a delay in Rex function comapared to that of Tax. A possible explanation for the temporal separation of the Tax and Rex regulatory feedbacks could be a differential rate of synthesis/degradation of the two proteins. To test whether this could be the case we first investigated the time course of Tax and Rex protein expression from a plasmid that expresses the full-length mature tax/rex mRNA (pBS1-2-3; see Materials and Methods, Par. 3.2), or from the HTLV-1 molecular clone ACH. Flow cytometry analysis performed after the transfection of HLtat cells with these plasmids showed a relative accumulation of Rex compared to Tax at later time points (32, 48 hours) both from the full-length mature tax/rex mRNA (Fig. 11A) and from the HTLV-1 molecular clone (Fig. 11B), resulting in a progressive rise in the Rex/Tax ratio (Fig. 11C). Even if the kinetics of Tax and Rex accumulation appear to be similar between the two plasmids, the switch in Rex accumulation appears to be slower in the context of the complete viral genome.

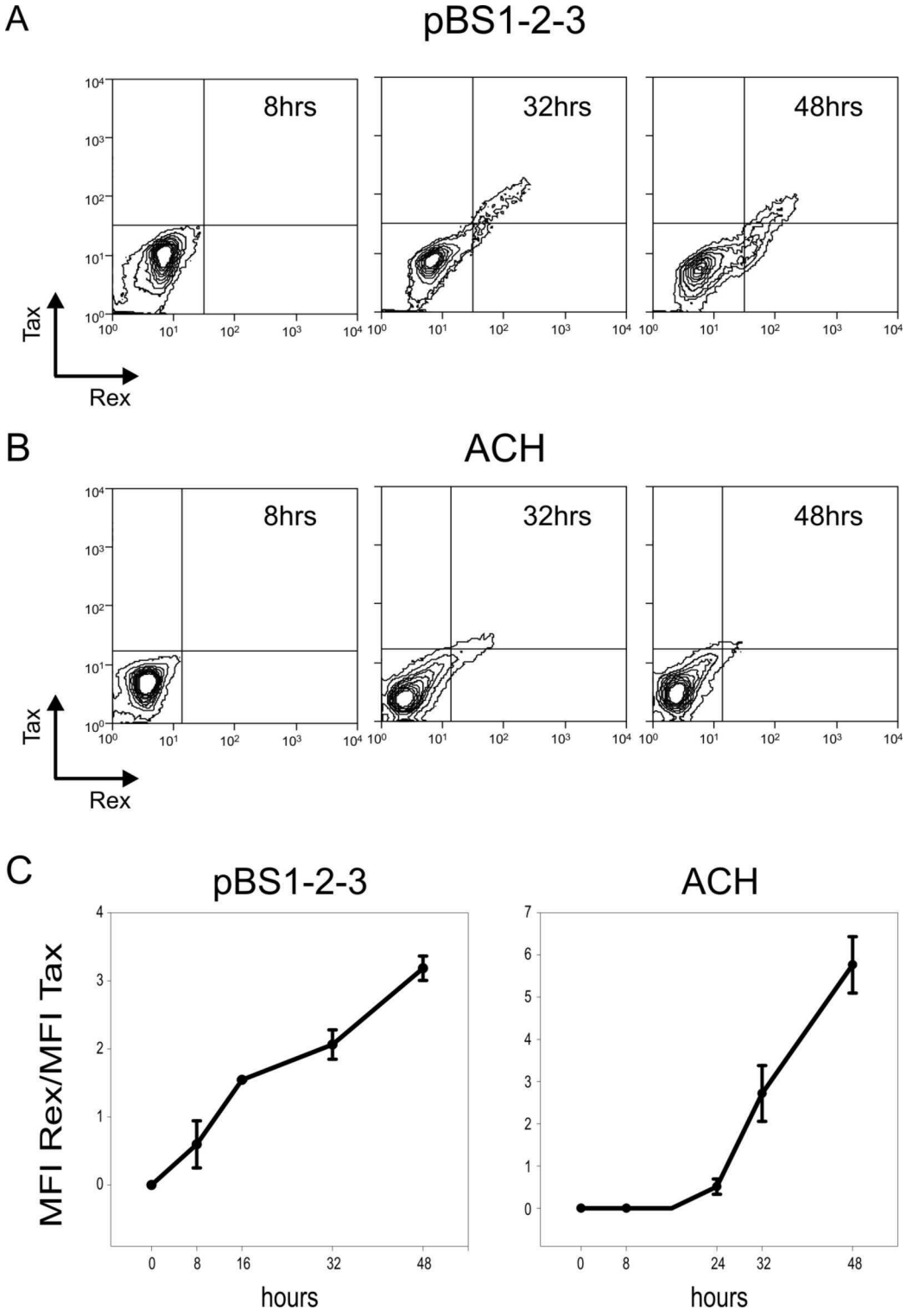

Figure 11. Kinetics of Tax and Rex protein accumulation.
A, B. Kinetics of Tax and Rex protein expression in HLtat cells from plasmid pBS1-2-3 or from the infectious molecular clone ACH. Data are represented as equal probability plots. **C.** Rex/Tax fluorescence intensity ratios after the transfection of the pBS1-2-3 plasmid (left-hand panel) or the ACH molecular clone (right-hand panel). Mean values of three independent experiments and standard error bars are shown.

In agreement with these observations, a comparison of the proteins' half-lives after blocking protein synthesis with cycloheximide revealed a slower rate of degradation of Rex compared to Tax (Fig. 12A, B), with half-lives of 19.6 hours and 6.6 hours respectively, after transfection of pBS1-2-3 plasmid, and 19 hours and 6 hours respectively, after transfection of the ACH molecular clone. These findings provide experimental grounds for the delay in Rex function postulated in the mathematical model and suggest a post-translational control of Tax and Rex activity.

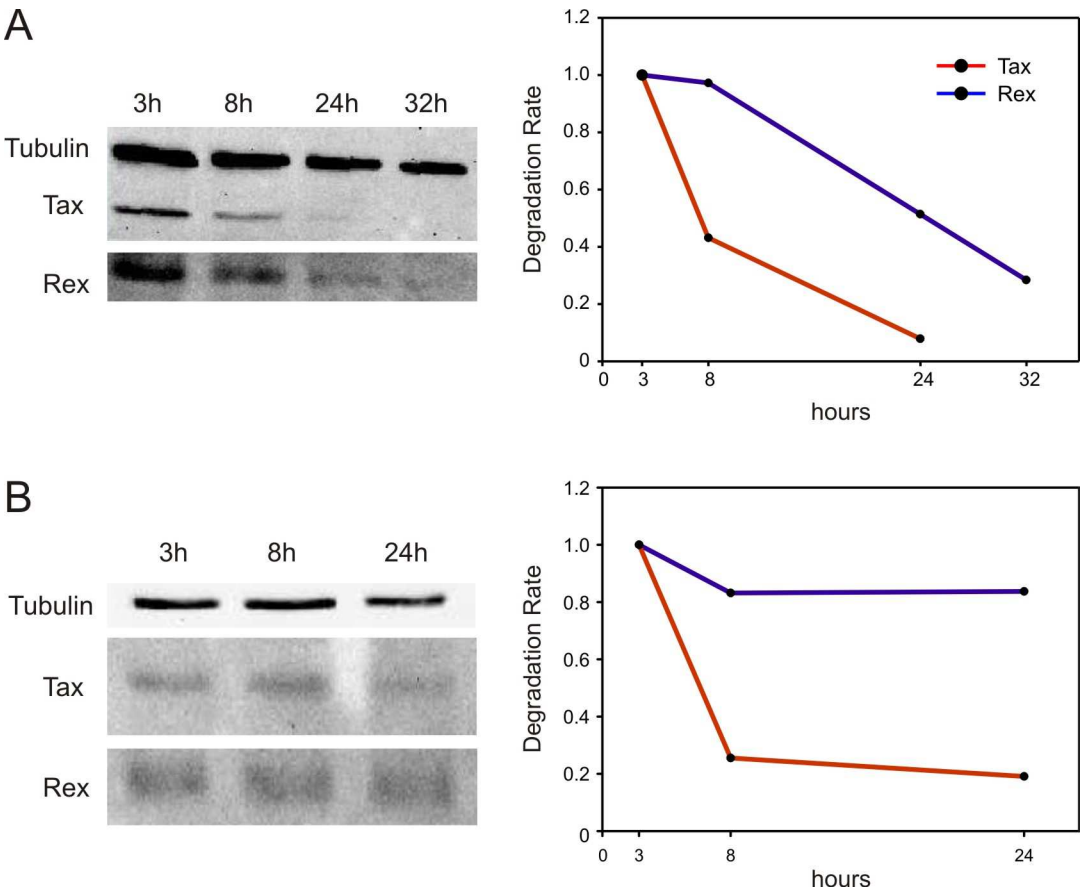

Figure 12. Kinetics of Tax and Rex protein degradation.
A, B. Degradation rates of Tax and Rex protein after transfecting the plasmid pBS1-2-3 or the ACH molecular clone and blocking protein synthesis by treating the cells with 10 µM cycloeximide 24 hours after transfection. Data were normalized by dividing Tax and Rex signals by the tubulin signal. Protein half-life (19.6 hours for Rex, 6.6 hours for Tax or 19 hours for Rex, 6 hours for Tax, from pBS1-2-3 and ACH, respectively) was estimated by fitting a linear decay model to the data, assuming a constant degradation rate.

5. DISCUSSION

Early studies established that HTLV-1 gene expression is controlled by two key regulatory circuits (Figure 5): a positive transcriptional regulatory feedback loop provided by the viral trans-activator Tax (Felber et al., 1985), and a negative post-transcriptional regulatory feedback loop provided by Rex. By binding to the Rex-responsive element (RxRE) present at the 3' end of HTLV-1 transcripts, Rex enhances the nuclear export and expression of a subset of mRNAs coding for the virion-associated proteins Gag, Pro, Pol and Env (Inoue et al., 1986; Inoue et al., 1987; Hidaka et al., 1988; Seiki et al., 1988; Inoue et al., 1991). More recently it has become apparent that the HTLV-1 expression strategy is characterized by production of positive- and negative-strand transcripts, alternative splicing and polycistronic translation (Lairmore and Franchini, 2007). This strategy greatly increases the coding potential of the virus resulting in expression of non-structural genes (Tax, Rex, p12, p13, p21rex, p30tof and HBZ) (Ciminale et al., 1992; Koralnik et al., 1992) in addition to the structural proteins and virion-associated enzymes common to all retroviruses (Gag, Pro, Pol and Env).

An implication of the feedback regulation of HTLV-1 gene expression mediated by Tax and Rex is that viral transcripts might be expressed with a distinct timing during the course of the viral life cycle, with a switch from early (Rex-independent) to late (Rex-dependent) genes. However, this switch in HTLV-1 expression has proven difficult to demonstrate experimentally, and the time-course of expression of alternatively spliced transcripts has not been examined in detail.

In the present work, we employed a Real Time RT-PCR method using splice-sites-specific primers to quantitate the levels of the different HTLV-1 transcripts and their kinetics of expression in unstimulated peripheral blood mononuclear cells (PBMCs) isolated from HTLV-1-infected individuals (ATLL and TSP/HAM patients) and cultured *in vitro* after CD8+ T-cell depletion. As previously described, depletion of CD8+ T-cells results in a sharp upregulation of viral expression in the remaining cell populations (Hanon et al., 2000). Results of our studies showed that, both in ATLL and in TSP/HAM patients, the most abundant plus-strand transcripts were tax/rex, gag and env, followed by p21rex, p30tof, p13 and p12; the minus-strand transcripts (hbz) were also expressed at high levels. Furthermore, the analysis of the timing of

expression of the different viral transcripts over a 48-hour time period showed that tax/rex was the earliest transcript followed by a rise in all other mRNAs expression whose curves intersected that of tax/rex, suggesting an "early-late" switch in HTLV-1 gene expression.

The abundance and timing of expression of the HTLV-1 mRNAs were further investigated in cells transfected with HTLV-1 molecular clones. The *in vitro* system permitted the quantitation of transcripts in the cytoplasmic and nuclear fractions, which was not possible with patient samples due to limited amounts of material. Considering that Rex acts at a post-transcriptional level controlling the nuclear export of unspliced and partially spliced transcripts, the quantitation of the nucleo-cytoplasmic partitioning of HTLV-1 mRNAs appeared to be an appropriate system for studying the kinetics of expression of HTLV-1 mRNAs. Results obtained from transfections with the wild-type molecular clone (ACH), were consistent with the data obtained from the patients' samples. Interestingly, while the plus-strand transcripts showed a comparable abundance in the nucleus and cytoplasm, the hbz mRNAs were over 10-fold higher in the nucleus. The nuclear retention of hbz transcripts was also confirmed in the chronically infected cell line C91PL. Although the significance of this peculiar feature of hbz transcripts remains to be understood, we propose that it might favour viral persistence by reducing hbz transcript translation and thereby reducing exposure of the infected cell to the critical HBZ-specific host CD8+ T-cell response (Macnamara et al., 2010; Hilburn et al., in press) while allowing their function as non-coding transcripts driving T-cell proliferation (Satou et al., 2006).

To better understand the molecular mechanisms controlling the “two-phase” mRNA expression kinetics and, in particular, to test if the kinetics of HTLV-1 gene expression might be dependent on Rex function, we generated a Rex knock-out HTLV-1 molecular clone derived from ACH (ACH-Rex-KO) and analyzed the timing of its mRNA expression following transfection. Results revealed complete loss of the "two-phase" kinetics in the absence of Rex, demonstrating the critical role of this protein in regulating these kinetics.

Extrapolating from results obtained for the tax/rex, env and gag transcripts (Hidaka et al., 1988), current models of HTLV-1 regulation predict that multiply-spliced mRNAs such as tax/rex and p30tof should be Rex-independent, while unspliced and

singly-spliced mRNAs (env, p12, p13 and p21rex) should be Rex-dependent. Hbz transcripts, which are transcribed from the negative strand, do not contain the RxRE sequence and thus are predicted to be Rex-independent. To test the validity of this prediction, we measured the levels of the HTLV-1 mRNAs 24 hours following transfection of ACH vs. ACH-Rex-KO HTLV-1 molecular clones in the total, cytoplasmic and nuclear fractions and calculated the Export Ratios for all HTLV-1 transcripts. Consistent with previous studies, there was no statistically significant difference in the Export Ratio of the tax/rex mRNA between ACH and ACH-Rex-KO, meaning that this transcript is Rex-independent; in contrast the export of the env and gag mRNAs was significantly impaired in the Rex knock-out virus, meaning that these mRNAs are Rex-dependent. Regarding the other transcripts, results of our study revealed that the p21rex singly-spliced mRNA, hbz sp1 and hbz us mRNAs were Rex-independent. Interestingly, while the p21rex mRNA was efficiently exported both in the absence and in the presence of Rex, the hbz mRNAs were highly impaired in their nucleo-cytoplasmic export both in the presence and in the absence of Rex. The Rex-independence of hbz transcripts is consistent with the fact that these mRNAs are transcribed from the minus strand and thus do not contain the RxRE sequence. However it was somewhat intriguing that over 90% of the hbz transcripts were retained in the nucleus regardless of the presence of Rex, suggesting a major role of hbz as a non-coding nuclear transcript, as described above. Moreover, the export of the p12, p13 and p30tof mRNAs was significantly impaired in the absence of Rex, indicating their Rex-dependence. Our data thus indicate that the Rex-dependence of HTLV-1 transcripts is not determined by the number of splicing events that produced them, as suggested by current model; in fact, the p21rex mRNA, which is singly-spliced mRNA, was found to be Rex-independent instead of dependent, while the p30tof mRNA, which is doubly-spliced, was Rex-dependent instead of independent. In light of these new findings we propose that the Rex-dependence of HTLV-1 mRNAs might be determined by the intronic regions retained in the mature transcripts. To test this hypothesis, we aligned the sequence of the Rex-dependent and Rex-independent mRNAs and observed that all transcripts containing a region comprised between splice acceptors C and 3 (nucleotide 6875 and 6950, respectively) are Rex-dependent (p12, p13, p30tof, gag and env), while transcripts lacking this region are Rex-independent (p21rex and tax/rex), regardless

of being produced through a single or double splicing event. The hbz transcripts, which are transcribed from the minus strand, are not predicted to contain this region; in fact they are Rex-independent.

Although further experimental validation is needed, this finding suggests that the Rex-responsiveness of an mRNA would be determined by a cis-acting regulatory sequence present on some transcripts and absent on others, while the RxRE could mainly function as a docking site for Rex binding. These results provide clues to a long-standing paradox of HTLV-1 gene expression regulation represented by the different Rex-dependence of viral transcripts in spite of the presence of the RxRE in the 3' untranslated region of all mRNAs.

Another intriguing aspect of HTLV-1's genetic organization is represented by the fact that the two positive and negative regulatory feedback loops that regulates HTLV-1 gene expression are mediated by the Tax and Rex proteins, coded by the same dicistronic mRNA. Mathematical modelling showed that if Tax and Rex acted simultaneously, the positive and negative regulatory loops would work against each other, resulting in an inhibition of viral gene expression. On the other hand, modification of the model by assuming that Rex function would ensue with a temporal delay compared to that of Tax results in a very close prediction of the kinetic of expression observed in patient samples. This temporal delay, which is in apparent conflict with the fact that both proteins are synthesized from the same mRNA, could be determined by different mechanisms: (1) delayed or impaired translation from the Rex AUG; (2) different Ks for Tax and Rex function, resulting in full Tax activity at lower protein concentrations (i.e. earlier time) compared to Rex; (3) initial silencing of Rex function by an “early” viral inhibitor that is subsequently overcome; (4) Rex function might require the synthesis of an "activator" that would be produced as an "intermediate gene" (viral or cellular). To test whether a differential rate of synthesis/degradation of Tax and Rex could be a possible explanation for the temporal separation of the two proteins regulatory feedbacks, we first investigated the time course of Tax and Rex protein expression from a plasmid that encodes the full-length mature tax/rex mRNA, or from the HTLV-1 molecular clone ACH. Flow citometry analysis showed a delayed accumulation of Rex compared to Tax both from the full-length mature tax/rex mRNA and from the HTLV-1 molecular clone. Although the kinetics of Tax and Rex

accumulation appears to be similar between the two plasmids, the switch in Rex accumulation appears to be slower in the context of the complete viral genome, suggesting that transcription from the viral LTR could be delayed. In agreement with these observations, a comparison of the proteins' half-lives revealed a slower rate of degradation of Rex compared to Tax, both from the tax/rex cDNA and from the ACH molecular clone. These findings provide experimental grounds for the delay in Rex function postulated in the mathematical model, and suggest a post-translational control of Tax and Rex activity.

Current studies are aimed at verifying if the Rex-dependence could be determined by the synergic action of the cis-acting regulatory sequence (Fig. 9) and the RxRE by testing the combined effects of these two regions in the context of a heterologous reporter system. If this proves to be the case, our efforts will be aimed at the analysis of the molecular mechanism of function of this sequence by searching for its cellular or viral binding partner.

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