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Review Article

Human T-lymphotropic virus type 1 non-structural proteins: Requirements for latent infection

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It has been more than 30 years since the discovery of human T-lymphotropic virus type 1 (HTLV-1), the first human retrovirus identified. Human T-lymphotropic virus type 1 infects 15–20 million people worldwide causing two major diseases: adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis. Human T-lymphotropic virus type 1 establishes several decades of latent infection, during which viral–host interaction determines disease segregation. This review highlights non-structural proteins that are encoded on the viral genome and manage latent infection. Latent infection is a key in HTLV pathology, so that effective inhibition of these proteins might lead to successful disease management. (*Cancer Sci* 2013; 104: 983–988)

Human T-lymphotropic virus type 1 (HTLV-1) is the first *retroviridae* that has been identified to infect humans[1,2] and causes adult T-cell leukemia/lymphoma (ATL)[3] and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[4–6] Adult T-cell leukemia/lymphoma in its acute/lymphoma phases is highly aggressive and resistant to current intensive chemotherapy, with median survival time of approximately 1 year after onset.[7,8] HTLV-1-associated myelopathy/tropical spastic paraparesis is a neuro-degenerative disease in which patients suffer from chronic pain and loss in motility that progress irreversibly over years resulting in a low quality of life on wheelchairs. HTLV-1 has also been found in other conditions such as infectious dermatitis, HTLV-associated uveitis, arthritis, polymyositis and pneumonitis,[9,10] suggesting diverse and different degrees of roles in inflammation. While the diseases that are associated with HTLV-1 infection become evident, current management is not sufficient to foresee, prevent or cure any one of those conditions.

Discovery of HTLV-1[1,11] and its association with ATL[11] opened the field of human retrovirus research. It proved that the *retroviridae* infection to humans was not fictional; further, it could be quite common and pathogenic. It became known that HTLV-1-associated diseases required long latent infection, which had been making the viral etiology less apparent. While HTLV-1 was found in conditions other than ATL, the idea of human retroviruses has led to the discovery of other *retroviridae* family members to date, including: HTLV-2,[12] HTLV-3[13] and HTLV-4[14] within the same genus *deltaretroviruses*, as well as human immunodeficiency viruses (HIV) that belong to the genus *lentiviruses*. All of these viruses can be transmitted by blood,[15] breast milk[16] and seminal fluid,[17] and establish latent infection. Natural co-infection of HTLV-1/2 and HIV-1 can occur and has been reported in the areas where both viruses are endemic, causing complications.[18] Since there is no successful vaccine to any retroviruses, better guidelines in controlling HTLV-1 are needed, especially in the developing world where uncontrolled infections remain.

As a pathogen, HTLV-1 produces "non-structural proteins" (NSP) that do not become parts of viral virion but are essential in causing diseases. The NSP hijack cellular machinery, which on one hand immortalizes and transforms infected cells, but on the other hand optimizes viral replication and repels immune surveillance that finds and kills infected cells in asymptomatic carriers (AC). Both of these activities are essential, enabling HTLV-1 to establish decades of latent infection while inducing diseases.

Retroviruses other than HTLV-1 also encode NSP that are unique to each one of them and are required for latent infection. A high homology seen between HTLV-1 and HTLV-2 might implicate their conserved strategies in persistence whose termination might halt disease progression.

Virus and Non-Structural Proteins

Human T-lymphotropic virus type 1 equips multiple genes in its limited genome size (Fig. 1).[19] There are numbers of cellular and viral mechanisms that enable the proviral genome, a form of virus integrated in the host cell chromosome, to selectively express all genes and mature their end products.[20] Reported mechanisms include bidirectional transcription initiated by 5′- and 3′-long terminal repeats (LTR) located at both ends of the proviral genome,[21,22] alternative splicings that serve for overlapping open reading frames (ORF) with or without frame shifts,[23] post-transcriptional regulation,[24] ribosomal frameshift during translation and post-translational processing.[25]

Among several transcripts and their encoding proteins, some compose viral virion enclosing its contents, which are structural components, enzymes (integrase, reverse transcriptase and protease) and linear single-stranded RNA (+) diploid genome; while others remain in the host that produced them as non-structural proteins (NSP). They are also referred to as "regulatory proteins" or "accessory proteins" in other reports. Importantly, cytotoxic T-lymphocytes (CTL) directed to NSP have been detected in viral carriers.[26] A list of ORF for the NSP under investigation is summarized in Table 1. In general, HTLV are conserved viruses relative to HIV.[27] Human T-lymphotropic virus type 1 and HTLV-2 share approximately 70% homology in the nucleotide sequence,[28] so that the corresponding ORF products also score a distant homology in amino acid (AA) levels.

Both HTLV-1 and HTLV-2 are capable of infecting and immortalizing normal human peripheral blood cells by co-cultivation with pre-infected donor cells.[2,29,30] Although there is a continuous debate,[31] HTLV-1 *in vivo* is more infectious

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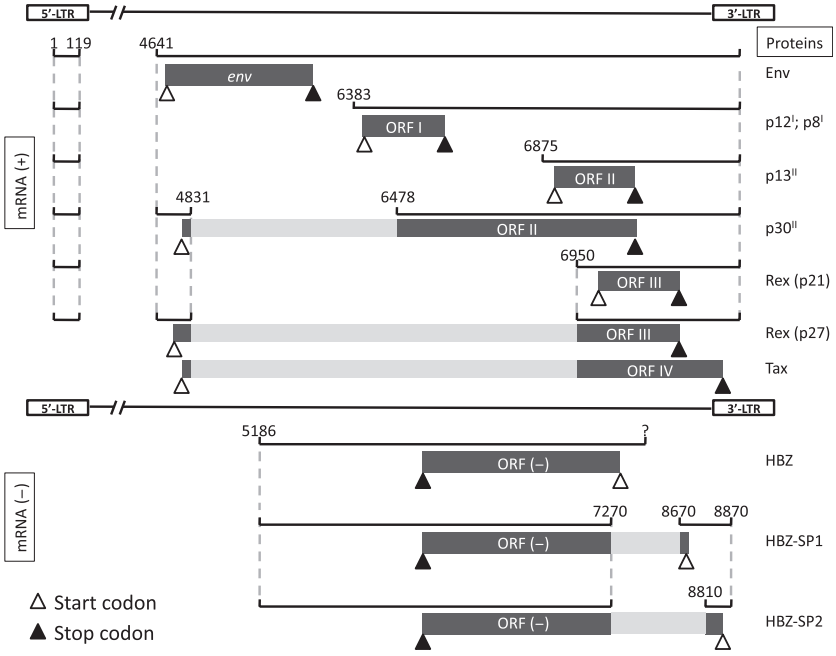

Fig. 1. Human T-lymphotropic virus type 1 (HTLV-1) genome organization. The HTLV-1 provirus uses complicated mechanisms to express mRNA and proteins. 5′-long terminal repeat (LTR) and 3′-LTR located on both ends operate singly or doubly spliced mRNA species with their open reading frames (ORF). The ORF relevant to non-structural proteins are depicted. The unspliced mRNA from 5′-LTR (not shown) encodes structural proteins other than Env and also becomes a genome in a newly formed viral virion. The origin of reported unspliced HTLV-1 bZIP factor (HBZ) mRNA is currently unknown.

Table 1. Human T-lymphotropic virus type 1 (HTLV-1) ORF and their products (NSP) in comparison with HTLV-2

Transcription	ORF	NSP	Mechanism†	HTLV-2
5′-LTR	I	$p12^{I}$; $p8^{I}$	Proteolytic cleavage	$p10^{I}$
5′-LTR	II	$p30^{II}$; $p13^{II}$	Doubly; singly spliced mRNA	$p28^{II}$
5′-LTR	III	Rex (p27; p21)	Doubly; singly spliced mRNA	Rex-2
5′-LTR	IV	Tax		Tax-2
3′-LTR	(–)	HBZ; HBZ-SP1 or -SP2	Un-; singly spliced mRNA	APH-2

†Each ORF might give rise to multiple proteins by the given mechanism. HBZ, HTLV-1 bZIP factor; LTR, long terminal repeat; NSP, non-structural protein; ORF, open reading frame.

Table 2. Non-structural protein (NSP)-mediated viral spread and cell growth

NSP	Major target	Impact
$p8^{I}$	Cell–cell contact	Increases viral transmission[43]
$p12^{I}$	Calreticulin	Induces Ca^{++} secretion and cell activation[40]
$p12^{I}$	IL-2R β and γ_c chains	Increases sensitivity to IL-2 of host cells[37]
Tax; Rex	5′-LTR; viral mRNA	Increases viral replication[21,24]
Tax	NFκB; SRF	Increases IL-2Rα, IL-6, GM-CSF, *c*-fos, *c*-egr[58]

GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; NFκB, nuclear factor kappa B; LTR, long terminal repeat; SRF, serum response factor.

to $CD4^{+}$ than to $CD8^{+}$ T-cells and vice versa for HTLV-2.[32] Only HTLV-1 causes T-cell outgrowth and malignant transformation *in vivo*, indicating that viral factors unique to HTLV-1 are essential in causing ATL. In contrast, HTLV-2 can cause neurological disorders that might resemble HAM/TSP.[33] No association of HTLV-3/4 with diseases has been established presently, which is due to the limited number of known infections since their discoveries. Human T-lymphotropic virus type 3 shares significant homology with Simian T-lymphotropic virus type 3 (STLV-3) found in non-human primates,[13] while HTLV-4 reveals much less homology with any other known HTLV or STLV, except that antiserum developed in a carrier exhibited reactivity to HTLV-1 antigens.[14]

Non-Structural Proteins in Viral Persistence

Much like other retroviruses, HTLV-1 integrates into host cell chromosomes after infection, that is, provirus, and persists permanently. It is true that there is no need for the virus to keep a productive replication cycle by producing infectious virions, as long as the host cell undergoes mitosis, which results in daughter cells with the same number and integration sites of the proviral copies. Given a well-known fact that HTLV-1 poorly self-replicates *in vivo*,[27] immortalization of the host appears to be a prerequisite for life-long infection. Human T-lymphotropic virus type 1 expresses viral proteins in the absence of adaptive immunity during primary infection. While Tax above all is believed to have the most powerful and indispensable roles in the host immortalization process through the modulation of numerous cellular transcriptions (see Open reading frame IV section), $p12^{I}$ was also found to potentiate activation and proliferation of T-cells in response to different extracellular stimuli (see Open reading frame I section). Tax, in the meantime, also activates viral gene transcription through 5′-LTR and induces viral replication (Table 2).[21]

On one hand the replication benefits HTLV-1 to spread infection *de novo*; however, on the other hand it increases the risk of viral antigens exposed to the immunity in AC. In fact, Tax is one of the major viral antigens that CTL target.[34] Some NSP resulting from Tax-mediated viral induction in turn self-limit viral expression much like negative feedback mechanisms. One exception is the HTLV-1 bZIP factor (HBZ), whose expression relies on 3′-LTR and does not depend on Tax.[35] The NSP also sabotage host immune surveillance to the virus, which ultimately promotes propagation of infected cells within AC. These adjustments would be important particularly during the primary and early infection stages, slowing infected individuals to acquire adaptive immunity against

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Table 3. Non-structural protein (NSP)-mediated viral defense against host immunity

NSP	Major target	Impact
$p8^{I}$	T-cell antigen receptor	Causes anergy; inhibits viral 5′-LTR induction[25]
$p12^{I}$	MHC-I	Inhibits viral antigens presented to CTL[38]
$p13^{II}$	Tax	Inhibits viral replication by direct binding[50]
$p30^{II}$	Tax/Rex mRNA	Inhibits Tax/Rex production and viral replication[46]
HBZ	CREB-2	Inhibits viral transcription by Tax[51]

CREB, CRE-binding protein; CTL, cytotoxic T-lymphocytes; HBZ, HTLV-1 bZIP factor; LTR, long terminal repeat; MHC-I, major histocompatibility complex class I.

HTLV-1. Once the infection becomes latent, the level of antisera against HTLV-1 in general correlates with the proviral load, indicating active viral replication does induce the immune response and these two are balanced in AC.[36]

The NSP that have been found to limit viral replication are $p8^{I}$, $p30^{II}$, $p13^{II}$ and HBZ. $P12^{I}$ and $p8^{I}$ have also been found to interfere with cellular immune machineries (Table 3). These NSP target different phases in viral replication and immune processes and are discussed below with respect to each ORF.

Open reading frame I. It encodes hydrophobic protein of 99AA known as $p12^{I}$. This protein has been shown to bind directly to interleukin-2 receptor (IL-2R) β and γ_c chains,[37] major histocompatibility complex class I (MHC-I) heavy chain,[38] linker for activation of T-cells,[39] calreticulin,[40] vacuolar-ATPase[41] and is present in multiple cellular compartments, most abundantly in endothelial reticulum (ER)–Golgi organelles and to a lesser extent on the plasma membrane.[23,39] Each interaction above results in sensitized IL-2R signaling, reduction of MHC-I on the cell surface, impaired T-cell antigen receptor signaling and persistent leakage of Ca^{++} from the ER, which might confer the host aberrance in proliferation, immune evasion, anergy and activation in the absence of extracellular stimulation. $P12^{I}$ in the presence of phorbol myristate acetate activates nuclear factor of activated T-cells (NFAT).[42] These studies implicate significant roles of $p12^{I}$ during latency, but at the same time raise a question on how this protein can access different cellular compartments and exert those pleiotropic functions.

Ectopic expression of ORF I results in production of two major products by possible cleavage, whose sizes apparently differ as observed using immunoblots, that is, 12 kDa ($p12^{I}$) and 8 kDa ($p8^{I}$) forms.[25] Given multiple functions in the different cellular compartments reported, it became necessary to reevaluate the functions that belong to each form. More importantly, analysis of proviral genomes existing in 304 HTLV-1 carriers revealed polymorphisms in nucleotide sequences that would result in AA changes.[25] Expression of those variants *in vitro* demonstrated a spectrum of ratios in $p8^{I}/p12^{I}$ production unique to the variants. Further analyses using variants predominantly producing $p12^{I}$ or $p8^{I}$ indicated their distinct cellular distribution: $p12^{I}$ in the ER and Golgi organelles while $p8^{I}$ on the plasma membrane. It has been shown that immunological synapse (IS) recruits $p8^{I}$ when viral host T-lymphocytes engage antigen-presenting cells, causing anergic signaling. As IS induces 5′-LTR transcription and viral replication, $p8^{I}$ also inhibits viral reactivation by this route.[25] It was later found that $p8^{I}$ also increased cellular conduits and virus transmission.[43] The exact cleavage sites have not been determined using terminal peptide sequencing. However, it is clear that cleavage requiring no other viral proteins co-existed. The study of HTLV-2 $p10^{I}$ protein is much behind the study for $p12^{I}$; however, it has been found to bind the free chain of MHC-I, suggesting functional overlap.[44] There is no equivalent of $p8^{I}$ in HTLV-2 reported. The $p12^{I}$ protein also shares distant homology with Nef of HIV-1 that is important for viral infectivity. The HIV-1 clone, in which $p12^{I}$ replaced Nef, retained its infectivity *in vitro*, demonstrating functional substitution.[45]

Open reading frame II. It encodes two in-frame proteins known as $p30^{II}$ (241AA) and $p13^{II}$ (87AA). $P13^{II}$ is a carboxy-terminal of $p30^{II}$ protein, utilizing internal methionine for its initiation and termination of $p30^{II}$, operated by alternative splicing. $P30^{II}$ possesses nuclear localization signal (NLS) and upon expression resides in the nucleus/nucleolus. $P30^{II}$ does not access cytoplasm. $P30^{II}$ was found to specifically bind to Tax/Rex mRNA of proviral origin and retain it in the nucleus and prevent it being translated.[46] By doing so, $p30^{II}$ suppresses viral replication, as Tax/Rex are master viral regulators. Human T-lymphotropic virus type 2 $p28^{II}$ was reported to have a function similar to $p30^{II}$; it has been shown to bind Tax/Rex mRNA of both HTLV-1 and HTLV-2, and so has $p30^{II}$.[47] Under genotoxic stress induced by radiation, $p30^{II}$ was also found to modulate activity of ataxia telangiectasia mutated, which essentially sensors DNA damage and mediates cell cycle arrest and consequently apoptosis, and increased survival. It was also found to inhibit homologous recombination favoring unfaithful DNA repair.[48] $P13^{II}$, which lacks amino-terminal NLS of $P30^{II}$, accumulates mostly in the inner mitochondrial membrane.[49] $P13^{II}$ was shown to interact with Tax protein, to be rerouted in nuclear speckles where it inhibited viral replication.[50] Open reading frame II products by above control Tax production and activity at the post-transcriptional and post-translational levels. Whether HTLV-2 expresses HTLV-1 $p13^{II}$ equivalent is currently unknown.

Open reading frame (–). While the presence of transcription from 3′-LTR has been known since 1989,[22] the possibility of encoded proteins has not been tested or reported earlier than 2002 when HBZ of 209AA was found.[51] Since then, other groups have also described its splicing variants.[52,53] Human T-lymphotropic virus type 1 bZIP factor appears to have a bimodal effect through the mRNA and protein levels. As a protein, HBZ bound one of the activating transcription factor/CRE-binding protein (ATF/CREB) family members, CREB-2, and inhibited Tax-mediated viral transcription from 5′-LTR.[51] However, at the mRNA level, HBZ promoted proliferation of a T-cell line and, conversely, small interfering RNA against HBZ inhibited growth of ATL cells.[35] As many proviral genomes in ATL cells contain defects or otherwise epigenetic changes at their 5′-ends including LTR while 3′-LTR remains intact with mRNA expression, these transcripts and proteins might be of relevance during late ATL development.[54] Besides, the lack of 5′-LTR might help survival of infected cells over the course of latency, among which ATL develops (Fig. 2). An mRNA encoding antisense protein of HTLV-2 (APH-2) with 183AA was also found in lymphocytes of HTLV-2-infected individuals.[55] The APH-2 localizes in the nucleus and represses Tax-2 activity through interaction with CREB, despite the fact that it lacks the bZIP domain seen in HBZ.

NSP in Cell Transformation

Once it infects HTLV-1 remains lifelong regardless of its concomitance to disease, while general longevity of any blood cell types – including viral host T-cell subsets – would be considerably shorter.[56] Thus, HTLV-1 needs to either immortalize its host or repeat *de novo* infection. However, continuous replication and infection of HTLV-1 is unlikely *in vivo* because of strong immune pressure even though it occurred through cell–cell transmission where no virion is produced.[34] Early studies that outlined basic viral life cycle led to the findings in indispensable roles of ORF IV product Tax protein in cellular

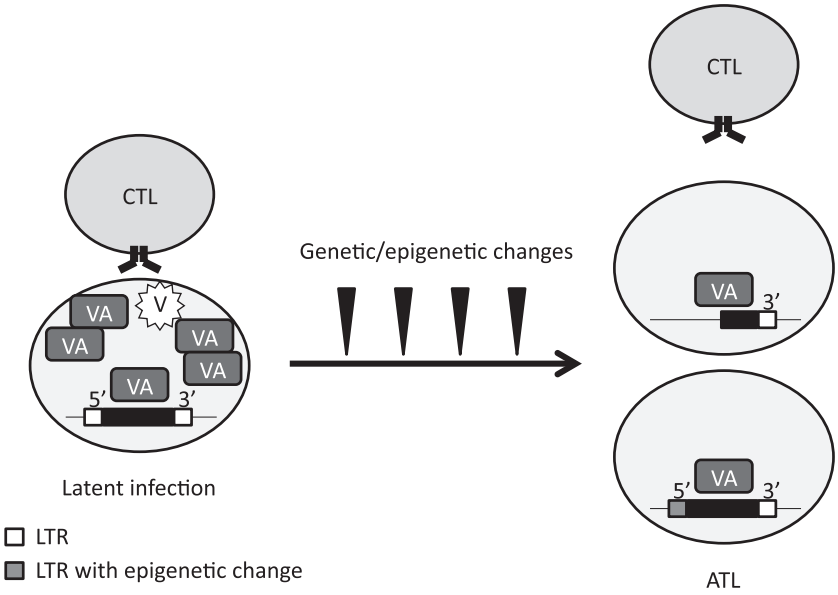

Fig. 2. Between latent infection and adult T-cell leukemia/lymphoma (ATL). Human T-lymphotropic virus type 1 (HTLV-1)-infected cells express viral antigens (VA), that is, all viral products including non-structural proteins, and virions (V) to survive and spread under host immunity (left). However, once immortalization of infected cells is complete the risk of having VA might exceed the benefit. In this model, the developing ATL acquires several genetic/epigenetic changes including a defect or methylation on proviral 5′-long terminal repeat (LTR), resulting in the absence of all 5′-LTR-operated VA. The chance of immune detection of such cells becomes smaller, from which ATL arises (right). The exact timing and mechanism for proviral defects remain elusive.

genetic changes that would substantially promote leukemia development, apart from in viral regulation in coordination with 27 kDa Rex of ORF III facilitating nuclear export of singly- and un-spliced viral mRNAs.[24]

Open reading frame IV. It encodes 353AA protein with molecular mass of 40 kDa, transcriptional activator of pX region (Tax). Tax immortalizes T-lymphocytes of healthy donors with high efficiency in the absence of any other viral proteins.[57] It is believed that Tax, not a mutagen *per se*, exerts antiapoptotic effect, dysregulates cell cycle and promotes proliferation in infected cells,[58] regardless of the presence of genotoxic stress and resulting mutations caused by other means. Tax-mediated activation of nuclear factor kappa B (NFκB) may explain IL-2Rα chain commonly overexpressed on ATL cells.[59] However, Tax-induced IL-2 autocrine loop is currently not supported.[60,61] Tax-2 of HTLV-2 was also found to immortalize T-lymphocytes associated with induction of IL-2 and IL-2R,[62] while it activates NFAT instead of NFκB, and autocrine loop is suggested.[63] Therefore, there is a significant diversity between these viral regulators even though they share 75% of homology in AA level.

Non-Structural Proteins Tested in Viral Molecular Clones

The full length of the HTLV-1 genome is available as molecular clones, so that ablation of specific proteins on the isogenic genome was also performed using genetic manipulation to test its effect on viral replication *in vitro*, *ex vivo* and in animal models. Loss of the ORF I message was associated with reduced infectivity in rabbit and human primary lymphocytes.[64,65] However, more recently it was reported that $p12^{I}$ and $p30^{II}$ are required for viral infectivity to human dendritic cells and in macaques, but not in rabbits.[66] Lack of $p13^{II}$,[67] Rex[68] and HBZ[69] each alone was associated with significant loss in viral infectivity and persistence in rabbit. The methods used for manipulation of proviral clones as well as infectious properties of resulting viruses are summarized in Table 4. While infection studies using such clones are valuable, these mutations caused simultaneous changes in AA of other overlapping NSP and gave us an indefinite conclusion. Thus, molecular studies of individual NSP are still needed to complement what is not available in animal infection models. Of note, some mutations were reverted back to wild-type forms in macaques, suggesting the requirement of ablated proteins during the early phase of infection.[66]

Table 4. Mutant HTLV-1 clones lacking specific non-structural protein (NSP)

NSP†	Manipulation	Property
$p12^{I}$	Splice acceptor	Decreased infectivity in human PBMC and rabbits[64,65]
$p12^{I}$	First codon	Decreased infectivity in human dendritic cells and in macaques[66]
$p13^{I}$	First codon	Loss of infectivity in rabbits[67]
$p30^{II}$	Third codon	Decreased infectivity in human dendritic cells and in macaques[66]
Rex	Third codon	Loss of infectivity in rabbits[68]
HBZ	Codons 11; 175	Decreased infectivity and persistence in rabbits[69]

†Knock out proteins are listed here. See references for the effects on overlapping NSP. HBZ, HTLV-1 bZIP factor.

Conclusion

There has been no acute disease known after HTLV-1 infection. The HTLV field in past decades saw what was thought "latent infection" was under the persistent control of NSP, many of which had previously been neglected. Relevance of NSP in HTLV pathology should therefore not be underestimated.

Disclosure Statement

The author has no conflict of interest.

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