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X-I and X-II Open Reading Frames of HTLV-I Are Not Required for Virus

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In contrast to other retroviruses of the oncovirinae subgroup, the primate and bovine leukemia viruses (HTLV, STLV, and BLV) encode genes in the X-region of the genome, between the env gene and the 3′ long terminal repeat. In HTLV-I, two overlapping open reading frames (ORFs) in the distal half of the X-region encode tax and rex genes, while two ORFs (X-I and X-II) in the proximal half of this region potentially encode proteins designated $p12^{XI}$ (or rof) and $p30^{XII}$ (or tof). The biological functions and mechanisms of tax and rex have been studied extensively whereas the roles of the other ORFs have not yet been established. To identify possible functions for ORFs X-I and X-II, an infectious molecular clone of HTLV-I and a mutant provirus lacking these ORFs were compared with respect to virus replication, gene expression, and ability to immortalize primary T-cells. When transiently transfected into 293 cells, both intact and deleted proviruses directed the synthesis of virus mRNAs and proteins that were quantitatively and qualitatively identical. These viruses were also indistinguishable in their abilities to infect and replicate in DBS-FRhL cells, which are permissive for HTLV-I propagation. Immortalized T-cell lines were established after cell-free or coculture methods for infection of activated, human peripheral blood or cord blood lymphocytes with each of the cloned viruses. The growth kinetics, cytokine dependence, and cell surface markers of the infected T-cell cultures were similar for each provirus clone. Thus, ORFs X-I and X-II are not essential for virus infectivity, replication, gene expression, or T-cell immortalization *in vitro*.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia and progressive neurological disorders collectively referred to as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al.,* 1985; Poiesz *et al.,* 1980; Osame *et al.,* 1986; Yoshida *et al.,* 1982; Franchini, 1995). HTLV-I transforms predominantly $CD4^+$ T-cells *in vivo* although other cell types are infected both *in vivo* and *in vitro* (Poiesz *et al.,* 1980; Hattori *et al.,* 1981; De Rossi *et al.,* 1985; Richardson *et al.,* 1990; Clapham *et al.,* 1983; Ho *et al.,* 1984; Hoxie *et al.,* 1984; Longo *et al.,* 1984; Mann *et al.,* 1984; Hoffman *et al.,* 1992). HTLV-I, with HTLV-II, simian T-cell leukemia viruses, and bovine leukemia virus form a unique subgroup of oncoretroviruses characterized by a unique genome organization and coding potential. Unlike the less complex oncoretroviruses, the HTLV group of retroviruses contains several open reading frames in a region located between the envelope gene and the 3′ long terminal repeat (Fig. 1). The overlapping tax and rex genes, located in the distal half of this region, have been extensively characterized and shown to regulate transcription of cellular and virus promoters and to mediate nuclear export of viral mRNAs, respectively (Felber *et al.,* 1985; Inoue *et al.,* 1987; Fujisawa *et al.,* 1985; Sodroski *et al.,* 1984; McGuire *et al.,* 1993). HTLV-I contains two additional open reading frames (ORFs) in the proximal half of the X-region that have been referred to as ORFs X-I and X-II (Seiki *et al.,* 1983). Alternative splicing produces unique mRNAs containing ORFs X-I and X-II whose predicted translation products have been designated $p12^{X-I}$ (rof), $p30^{X-II}$ (tof), and $p13^{X-II}$ (Ciminale *et al.,* 1992; Koralnik *et al.,* 1992; Berneman *et al.,* 1992). The mRNAs capable of encoding ORFs X-I and X-II have been detected in infected cell lines, in lymphocytes from infected individuals, and in cells transfected with HTLV-I provirus clones. Relative to other viral mRNAs, the levels of these alternatively spliced mRNAs are extremely low and their putative proteins have not been detected in infected cells (Caputo and Haseltine, 1992). The p12 and p30 proteins of HTLV-I have been ectopically expressed from plasmids in transfected cells in order to study their subcellular localization and interactions with cellular proteins (Koralnik *et al.,* 1993, 1995). The conservation of predicted amino acid sequences of ORFs X-I and X-II among HTLV-I isolates as well as the presence of distantly related ORFs in HTLV-II and BLV suggest that they are important for virus propagation in nature (Ciminale *et al.,* 1992, 1995, 1997; Alexandersen *et al.,* 1993; Willems *et al.,* 1994). However, their functions have yet to be established.

The development of an infectious molecular clone of

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HTLV-I, combined with improved infection and transient transfection methods, provides new opportunities to examine HTLV-I gene function (Derse *et al.,* 1996). In an attempt to define biologic functions for the X-region ORFs, we constructed HTLV-I proviruses in which ORFs X-I and X-II have been mutated or deleted. These viruses were compared with respect to their gene expression, infectivity, and replication *in vitro.* We have established immortalized T-cell lines after infections of primary human lymphocytes by both cell-free and coculture infection methods with cloned HTLV-I proviruses and have tested the ability of the mutated proviruses to immortalize primary T-cells *in vitro.*

MATERIALS AND METHODS

Plasmids. The cloning and construction of the plasmid pCS-HTLV has been described previously, and it was demonstrated to encode an infectious HTLV-I provirus (Derse *et al.,* 1995, 1996). The *Pst*I to *Sst*II fragment, spanning nucleotides 6750 to 7136 of pCS-HTLV and containing the ORFs X-I and X-II, was replaced with a synthetic *Xba*I linker to produce pHTLV-ΔPSX. ORF X-I of the provirus clone pCS-HTLV was found to contain a premature stop codon. Therefore, the *Pst*I to *Sst*II fragment containing ORFs X-I and X-II of pCS-HTLV was replaced with the homologous fragment generated by RT-PCR of mRNA derived from MT2 cells. MT2 cell mRNA was converted to cDNA with random hexamer primers and then amplified with primers flanking the *Pst*I and *Sst*II sites at positions 6750 and 7136, respectively. The PCR product was digested with *Pst*I and *Sst*II and ligated into pCS-HTLV. Sequence analysis confirmed that ORFs X-I and X-II of pCS-X1MT matched published sequences and are thus theoretically capable of translation.

Cell lines and transfections. Human 293 cells and DBS-FRhL cells (clone B5) were maintained in Dulbecco's modified medium supplemented with 10% fetal calf serum. HTLV-I-transformed T-cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and 10% partially purified human IL-2 (Cellular Products, Buffalo, NY). For transfections, 293 cells were plated at 2×10^6 cells in a 10-cm dish the day prior to transfection. Plasmid DNA (10 μg) was applied to the cells for 6 hr as calcium phosphate coprecipitates. Two days after transfection cells and supernatants were collected for analysis of viral mRNAs and proteins.

Infections. DBS-FRhL (clone B5) cells were infected with filtered supernatants from transiently transfected 293 cells. Supernatants were collected from 293 cells 2 days after transfection, cleared of cell debris by low-speed centrifugation, filtered through a 0.45-μm filter, and applied to 3.5-cm dishes containing DBS-FRhL cells. DBS-FRhL cells were plated the previous day at 3×10^5 cells per dish. Five days after infection, cells from each 3.5-cm dish were trypsinized and transferred to 10-cm dishes and then passaged every 5 days at a 1:5 dilution. Aliquots were examined by p19 antigen capture ELISA (Cellular Products) at each passage.

Normal human peripheral blood and cord blood mononuclear cells were obtained by centrifugation of heperanized blood on a Ficoll density gradient (Organon Technika, Durham, NC). Cells were activated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg each of penicillin and streptomycin, 1 μg per ml phytohemagglutinin (Burroughs Welcome, Research Triangle Park, NC), and 10% delectinated, partially purified human IL-2 (Cellular Products, Buffalo, NY) for 72 hr at 37° in a 5% CO_2 incubator. Cell-free infections of lymphocytes were performed by two different methods, both resulting in establishment of immortalized T-cell lines. Supernatants from transiently transfected 293 cells were filtered through 0.45-μm filters (Millipore). Lymphocytes (5×10^6) were suspended in 1 ml of filtered supernatant and incubated for 4 hr at 37°. Cells were collected by centrifugation, suspended in 10 ml of complete medium, and transferred to T25 culture flasks. Cells were maintained at a density of 5×10^5 cells per milliliter and medium was replenished weekly. The second approach for cell-free infections utilized transwell plates (Costar). DBS-FRhL cells persistently infected with HTLV-I were plated at approximately 30% confluence in the bottom chamber of a 6-well plate. Transwells (0.45 μm) containing activated lymphocytes (2×10^5 per milliliter) were placed in contact with the fluid in the lower chambers and cells were allowed to proliferate in the presence of continuously produced virus for 3 days. Lymphocytes were then transferred to T25 flasks and cultured as above.

Northern blotting. Transfected or infected cells were collected and suspended in lysis buffer containing: 10 m*M* Tris–HCl, pH 7.5; 150 m*M* NaCl; 0.5% Nonidet P40; and 1.5 m*M* $MgCl_2$. The cytoplasmic supernatant was extracted with RNA STAT 50LS (Tel-Test Inc.) and RNA was precipitated with ethanol. Poly(A)$^+$ mRNA was prepared and run on 1% agarose/0.67 *M* formaldehyde gels and blotted with labeled tax probe as previously described (Martarano *et al.,* 1994).

Western immunoblotting. Culture media from HTLV-I-infected cells were collected and cleared of cell debris by low-speed centrifugation and filtration through a 0.45-μm filter. Supernatant was then underlaid with 2.5 ml of 20% glycerol in phosphate-buffered saline in 10 ml Oakridge tubes and centrifuged for 90 min at 30,000 RPM in a Ti70.1 rotor. Virus pellets were dissolved in 100 μl of SDS sample buffer; 50 μl was loaded onto 12.5% acrylamide–SDS gels and then electrophoretically transferred to Immobilon (Millipore) filters as previously described (Martarano *et al.,* 1994). Filters were incubated with rabbit antipeptide antisera to HTLV-I gag proteins p24 (CA), p19 (MA), and p15 (NC) followed by goat anti-rabbit antibody conjugated to horseradish peroxidase

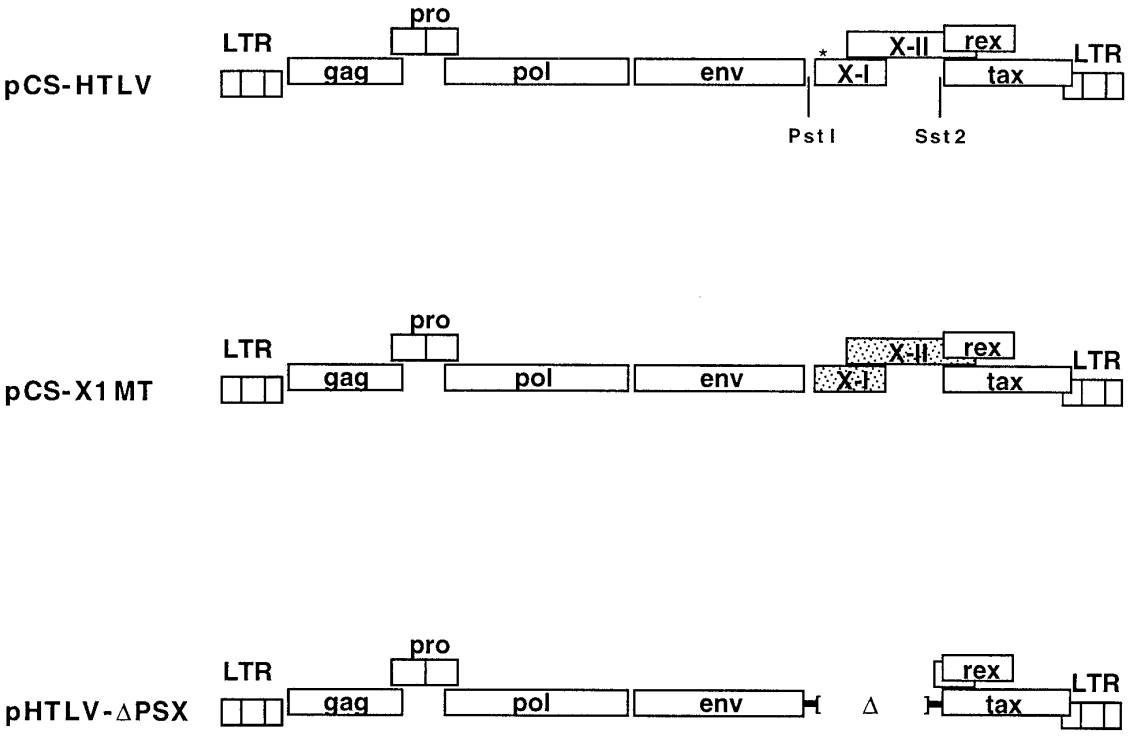

FIG. 1. Genomic organization and location of ORFs X-I and X-II in the molecular clones of HTLV-I. ORFs X-I and X-II are located in the proximal X-region of the virus, between the env gene and the 3′ LTR. Construction of pCS-HTLV was described previously. In the course of this work it was discovered that a premature stop codon is present in ORF X-I of this clone (marked by an asterisk). The persistently infected T-cell line, MT2, was used as the source of mRNA to prepare cDNA for amplification with primers flanking ORFs X-I and X-II. The PCR product was digested with *Pst*I and *Sst*II and replaced the analogous region in pCS-HTLV to yield pCS-X1MT. The deletion mutant, pHTLV-ΔPSX, was made by deleting the *Pst*I to *Sst*II fragment and replacing it with a synthetic *Xba*I linker.

and visualized by enhanced chemiluminescence (ECL, Amersham).

RESULTS

The cloning and construction of pCS-HTLV, which contains an infectious, full-length HTLV-I provirus, has been described (Derse *et al.,* 1995, 1996). Sequence analysis of the X region of pCS-HTLV revealed several differences compared with published sequences, including a premature stop codon in ORF X-I. ORFs X-I and X-II in pCS-HTLV were therefore replaced with HTLV-I sequences expressed in MT2 cells. Cytoplasmic mRNA from MT2 cells was converted to cDNA, and the proximal X region was amplified by PCR and ligated into the corresponding region of pCS-HTLV to generate pCS-X1MT (Fig. 1). Sequence analysis of pCS-X1MT confirmed that ORFs X-I and X-II were potentially viable. Another provirus clone, pHTLV-ΔPSX, was constructed by deleting the region containing ORF X-I and most of ORF X-II from pCS-HTLV (Fig. 1). These three proviruses were then compared with respect to gene expression, infectivity, and T-cell immortalization.

In order to determine whether deletion of the region containing ORFs X-I and X-II affected synthesis or processing of viral mRNA, human 293 cells were transfected with each of the three provirus clones. Two days after transfection cellular RNA was extracted and examined by Northern blotting with an HTLV-I tax/rex probe (Fig. 2). Nearly identical levels of virus mRNAs were expressed in transfected cells and typical patterns of the three major viral mRNAs were observed. The unspliced and singly spliced mRNAs produced in cells transfected with pHTLV-ΔPSX are predicted to be 386 nucleotides smaller than the mRNAs expressed from the other proviruses. At the same time, cells were collected for RNA preparation and supernatants from transfected cells were analyzed by HTLV-I p19 antigen capture ELISA assays, which revealed that virion protein synthesis was similar for the three proviruses (data not shown). Thus, the three proviruses were indistinguishable with respect to RNA and protein synthesis in transiently transfected cells, indicating that ORFs X-I and X-II did not affect viral gene expression.

A subclone of fetal rhesus lung cells (DBS-FRhL) designated B5 was previously demonstrated to be permissive for HTLV-I infection and replication (Derse *et al.,* 1996). B5 cells can be infected with cell-free virus or directly transfected with provirus DNA. Approximately 2 weeks after infection or transfection, HTLV-I gag antigens can be detected in culture supernatants; virus production plateaus after 4 weeks and remains at high levels there-

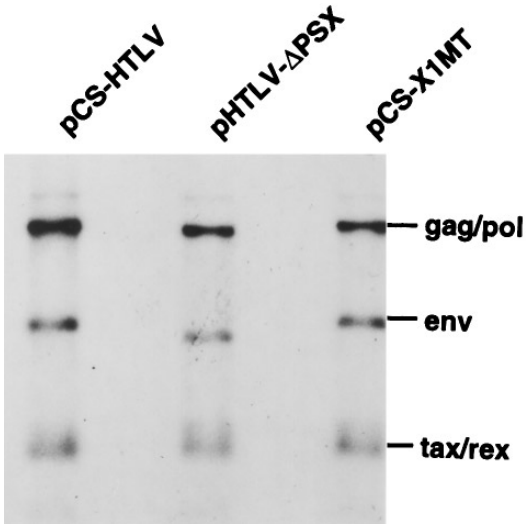

FIG. 2. Expression of proviral RNAs in transfected 293 cells. Human 293 cells were transfected with the indicated plasmid DNAs by calcium phosphate coprecipitation. Two days later cells were collected and poly(A)$^+$ mRNA was prepared and run on 1% agarose/0.67 *M* formaldehyde gels, transfered to nylon membrane, and hybridized to a tax fragment probe. The unspliced (gag/pol), singly spliced (env), and doubly spliced (tax/rex) mRNAs are indicated.

after. The B5 cell culture system thus provides a means to examine HTLV-I infectivity and replication since multiple rounds of infection are required for virus spread through the culture. B5 cells were infected with filtered supernatants from transiently transfected 293 cells, and HTLV-I p19 gag antigen in the culture supernatants was monitored at weekly intervals. Both the kinetics of appearance and levels of p19 were similar for the three viruses (data not shown). Virion proteins released from the persistently infected cell cultures were compared by immunoblotting with anti-gag antisera. Virion core proteins were qualitatively and quantitatively similar among the three virus-infected cultures (Fig. 3A). Virions released from B5 cells that are persistently infected with the three cloned viruses were shown to infect both naive B5 cells and primary human lymphocytes (see below). Thus, ORFs X-I and X-II did not influence virus infectivity or replication in DBS-FRhL cells.

Activated human PBMCs and cord blood lymphocytes were infected with HTLV-I produced from both B5 cells persistently infected with pCS-HTLV, pHTLV-ΔPSX, or pCS-X1MT and transiently transfected 293 cells. Either coculture of activated lymphocytes with HTLV-infected B5 cells or cell-free infection with transfected cell supernatants resulted in the continuous proliferation and outgrowth of IL-2-dependent T-cells; in contrast, uninfected lymphocytes ceased to proliferate after approximately 6 weeks. T-cells were considered immortalized after at least 12 weeks of continuous growth and in all cases, the HTLV-I immortalized lines have been in culture for at least 1 year. All HTLV-I-infected T-cell lines that were established remained IL2-dependent.

Virus expression in the immortalized T-cells was examined by immunoblotting supernatants with anti-gag antibodies (Fig. 3B). Again, expression and release of virus particles into the supernatants were similar for each provirus. Cell surface markers were examined by FACS analysis and revealed no differences among cultures infected with pCS-HTLV, pHTLV-ΔPSX, or pCS-X1MT (Table 1). The T-cell cultures were all CD4$^+$, CD8$^-$, and CD34$^+$, and CD3 was either absent or very low. All cultures showed similar growth kinetics and gave identical responses to cytokines. Thus, ORFs X-I and X-II did not appear to be involved in HTLV-I-mediated T-cell immortalization or proliferation *in vitro.*

DISCUSSION

HTLV-I ORFs X-I and X-II were dispensable for virus replication in cell culture and had no discernable effect on virus gene expression. Primary human lymphocytes were successfully infected and immortalized with cell-free virus produced from transiently transfected 293 cells or stably infected DBS-FRhL cells expressing the parental or mutant virus clones. The viruses produced from DBS-FRhL cells chronically infected with cloned proviruses were capable of infecting naive DBS-FRhL cells and primary T-cells by either cell-free or coculture routes.

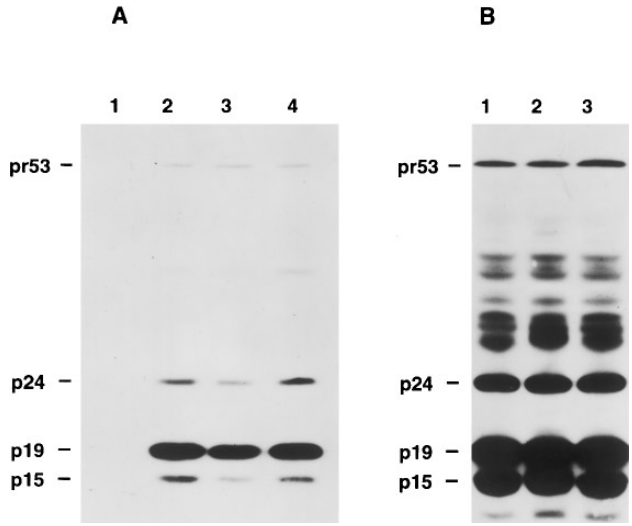

FIG. 3. Expression and processing of virion structural proteins released from HTLV-I-infected cell lines. (A) Immunoblot of virions from chronically infected DBS-FRhL (clone B5) cells. Uninfected cells (lane 1); cells persistently infected with pCS-HTLV (lane 2); pCS-X1MT (lane 3); or pHTLV-ΔPSX (lane 4) were grown to confluence in 10-cm dishes. The culture medium was replaced 24 hr before collection. Virus particles in culture supernatants were concentrated by ultracentrifugation through a 20% glycerol cushion and fractionated on 12.5% acrylamide–SDS gels. Membrane-bound proteins were probed with anti-HTLV-I gag antisera. The gag precursor polyprotein, pr53, and mature proteins, p24, p19, and p15, are indicated. (B) Immunoblot of virions from immortalized T-cell lines infected with: pCS-HTLV (lane 1); pCS-X1MT (lane 2); and pHTLV-ΔPSX (lane 3). Virus particles in cell culture supernatants were prepared and immunoblotted as in A.

TABLE 1

FACS Analysis of HTLV-I Immortalized T-Cells

	Cell surface expression[a]							
Cell line	CD2	CD3	CD4	CD8	CD14	CD16	CD25	CD19
CB/X1MT	95%	7%	95%	0	0	0	89%	0
PBL/X1MT	97%	12%	92%	0	0	0	88%	0
CB/pCS	98%	0	90%	0	0	0	89%	0
CB/ΔPSX#3	98%	0	90%	0	0	20%	90%	0
CB/ΔPSX#4	95%	14%	95%	0	0	16%	85%	0

[a] Immunophenotypic analyses of immortalized T-cell lines were performed 12 to 16 weeks after infection. Cell staining was done in buffer containing 2% human AB serum to block Fc receptors. All antisera were fluorescein-conjugated and obtained from commercial sources (Becton–Dickinson and Immunotech). Fluorescent-activated cell sorting was performed on a Coulter Profile instrument according to the manufacturer's standard protocols. Results are presented as percentage positive cells.

Immortalized T-cell lines that were obtained after infections with either the parental or deleted viruses were nearly identical and expressed cell surface markers typical of previously established HTLV-I-infected T-cell lines. All of the T-cell lines that were obtained with the cloned proviruses here were IL2-dependent and expressed CD4, CD2, and CD25 (IL2Ra) on their surface.

The predicted amino acid sequences of the X region ORFs are highly conserved among HTLV-I isolates and analogous ORFs are present in HTLV-II and BLV. There is little sequence similarity among the ORFs of HTLV-I, HTLV-II, and BLV, however, and the organization and splicing patterns are different for the three viruses. The X region gene products of HTLV-I (p12 and p30) and the analogous proteins from HTLV-II and BLV have not yet been detected in infected cells. In all three viruses, the mRNAs encoding these open reading frames are produced at levels much lower than the virus mRNAs encoding tax/rex and structural and enzymatic proteins. It is still unclear whether the alternatively spliced mRNAs are ever produced at higher levels in certain cell types or under specific conditions. It is also possible that these putative proteins are functional at very low levels. Studies have previously addressed the subcellular localization and interactions of p12 and p30 with cellular proteins in transfected cells (Koralnik *et al.,* 1993, 1995) but it is still unclear how these results relate to virus propagation and pathogenesis *in vivo.*

The potential functions of the proximal X-region ORFs in the different HTLV/BLV group of viruses may be similar and the *in vitro* results presented here for HTLV-I are consistent with those previously reported for HTLV-II and BLV (Cockerell *et al.,* 1996; Green *et al.,* 1995; Willems *et al.,* 1994). The latter viruses have been examined further in animal model systems where viruses lacking the X region genes appeared to have lower proviral DNA copy number. Although the functions of these putative genes remain unknown, they are likely to exert subtle effects on the infected cell *in vivo.* These could be manifested as effects on virus spread, lymphocyte trafficking, or immunological detection; alternatively they may directly affect cellular transformation or clonal expansion of infected cells. While studies of HTLV-I propagation and infectivity in animal model systems may show patterns similar to HTLV-II and BLV, the differences in pathogenesis effected by these viruses suggest that their respective X-region ORFs may have distinct characteristics.

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