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Protein isoforms encoded by the *pX* region of human T-cell leukemia/lymphotropic virus type I

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ABSTRACT **The *pX* region of the human T-cell leukemia/lymphotropic virus type I (HTLV-I) contains at least four open reading frames (*orf I–orf IV*). *orf III* and *orf IV* encode the regulatory HTLV-I proteins Rex and Tax, which together modulate viral expression, and the p21^{rex} protein of unknown function. By using the reverse transcriptase and polymerase chain reaction techniques on the RNA of an HTLV-I-infected cell culture, we uncovered the existence of alternatively spliced mRNAs generated through the use of three splice acceptor sites. These mRNAs encoded protein isoforms derived from the HTLV-I *orf I* (p12^{I}) and *orf II* (p13^{II} and p30^{II}). An additional acceptor splice site, used in the processing of the env and tax/rex mRNAs and a singly spliced mRNA for the p21^{rex} protein, was also identified. All of these HTLV-I mRNAs were also detected in freshly isolated cells from HTLV-I-infected individuals. Thus HTLV-I, like the human immunodeficiency virus type 1, has developed fine posttranscriptional mechanisms to increase the complexity of its genome.**

Human T-cell leukemia/lymphotropic virus type I (HTLV-I) (1, 2) is the causative agent of adult T-cell leukemia (ATL) (3–5) and a progressive myelopathy designated tropical spastic paraparesis/HTLV-I-associated myelopathy (TSP/HAM) (6–8). The 3′ end of the HTLV-I genome, originally called *pX* (9), carries at least four open reading frames (*orf I–orf IV*). Tax, the viral transcriptional transactivator (10, 11), and Rex, a posttranscriptional regulator of viral expression (12, 13), are both synthesized from the same polycistronic doubly spliced mRNA and are encoded by *orf IV* and *orf III*, respectively (14–17).

We investigated the existence of other viral proteins that could play a role in virus–host interaction and latency and in the various diseases associated with HTLV-I infection by studying viral transcripts in infected cells from TSP/HAM and ATL patients and healthy carriers. Our study indicated the presence of at least three proteins synthesized from the 3′ end of the viral genome.

MATERIALS AND METHODS

Cell Sources and Oligonucleotide DNA Sequences. Fresh cells from TSP/HAM and ATL patients and from HTLV-I-seropositive individuals were obtained from the French West Indies and Japan. The human T-cell line infected with the HTLV-I$_{LAF}$ isolate has been described (18). Total and cytoplasmic RNA was extracted as described (19).

The primers and the probes used for reverse transcriptase/polymerase chain reactions (RT/PCRs) have the following sequences. The numbers correspond to the nucleotide (nt) positions on HTLV-I RNA.

RPX3, 5′-ATCCCGTGGAGACTCCTCAA-3′ (nt 4743–4762)
RPX4, 5′-AACACGTAGACTGGGTATCC-3′ (nt 7004–6985)
IK1, 5′-GCTGGGCAGGATTGCAGG-3′ (nt 4963–4946)
IK4, 5′-GTGAGCGCAAGTGGAGAC-3′ (nt 6525–6508)
ToII, 5′-ATGGCACTATGCTGTTTCGCCTTC-3′ (nt 6478–6497)
orf1, 5′-CACCTCGCCTTCCAACTG-3′ (nt 6413–6430)
pX1, 5′-GCTGTGCTTGACGGTTTGC-3′ (nt 6805–6787)
rex, 5′-GAGACTCCTCAAGC-3′ (nt 4751–4764)
orf2, 5′-CGAAACAGCCCTGCAGATAC-3′ (nt 6906–6925)
pX2, 5′-GTAAGGACCTTGAGGGTC-3′ (nt 7217–7200)
S1, 5′-ATCCGCGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGCCGCGGAT-3′
S2, 5′-ATCCGCGGCCAAGCTAGCGTAATCTGGAACATCGTATGGGTAAGCCGCGGAT-3′
C1, 5′-ATACATCGATACCCATACGATGTTCCAGATTACGCTAGCTTGCATCGATAT-3′
C2, 5′-ATATCGATGCAAGCTAGCGTAATCTGGAACATCGTATGGGTATCGATGTAT-3′
IK0, 5′-CTGCCCCCTCATCTTCGG-3′ (nt 4869–4886)
IK3, 5′-GCAGGAGTTGGGGATTG-3′ (nt 6461–6445)
IK5, 5′-CCCGCCTGTGGTGCCTCC-3′ (nt 81–98)
IK17, 5′-CCTCCAACACCATGG/CAACTTCCTCCGTTCAGC-3′ (nt 4831/6383)
IK7, 5′-GCGTCCGCCGTCTAG/CAACTTCCTCCGTTCAGC-3′ (nt 119/6383)
IK19, 5′-CGAAACAGCATAGTG/CCATGGTGTTGGAGG-3′ (nt 6478/4831).

Primers and probes MZ2, -3, -4, -5, -9, -10, and -14 have been described (20).

RT/PCR on Cellular RNA and cDNA Characterization. The RT reaction conditions have been described (20). Thirty and 40 cycles of PCR amplification were performed for the cell line and the peripheral blood mononuclear cells (PBMCs), respectively, in a DNA thermal cycler (Perkin–Elmer/Cetus) as follows: denaturation at 94°C for 1 min, annealing at 60°C for 2 min, extension at 72°C for 2 min with a 2-sec increase in this incubation time per cycle, and extension in the final cycle at 72°C for 7 min. The amplified fragments were hybridized with DNA oligonucleotide splice junction probes, as described (20).

cDNA libraries were constructed by ligating the PCR products cleaved with the *Eco*RI and *Not* I restriction endonucleases at the equivalent sites of the Bluescript vector (Stratagene) (19) and selected molecular clones were sequenced using the Sanger method (21).

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Abbreviations: HTLV-I, human T-cell leukemia/lymphotropic virus type I; PBMC, peripheral blood mononuclear cell; ATL, adult T-cell leukemia; TSP/HAM, tropical spastic paraparesis/HTLV-I-associated myelopathy; RT, reverse transcriptase; nt, nucleotide(s).

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Generation of "Tagged" orf I and orf II cDNAs. cDNA corresponding to the mRNA pX–orf I, pX–rex–orf I, pX–orf II, and pX–tax–orf II were derived from the HTLV-I_{LAF} cell line (using the oligodeoxynucleotide primers orf1, rex, pX1, orf2, ToII, and pX2) and cloned in the expression plasmid vector HCMV-HSPA (22). The S1 and S2 and the C1 and C2 oligonucleotide pairs, containing the genetic information for the HA1 epitope (YPYDVPDYASL) (23), were synthesized and ligated at the *Sac* II and *Cla* I restriction sites of the *pX–orf I* and *rex–orf I*, and the *pX–orf II* and *tax–orf II* genes, respectively. DNA transfection and protein analysis was performed on COS-1 (24) and HeLa–*tat* cell (25) lines as described (26).

RESULTS

Identification of Alternatively Spliced mRNAs for *env* and *orf I*, *orf II*, *orf III*, and *orf IV*. The characterization of molecular clones generated by the use of MZ4/IK1 primers and selected with the IK0 and IK5 probes revealed that the HTLV-I env mRNA could be processed using a splice donor site at position 119 and two splice acceptor sites located 17 nt apart at positions 4641 and 4658 in the viral RNA (Fig. 1).

The screening of the cDNA library obtained using the MZ4/IK4 primers with the IK3 and IK5 probes revealed the existence of two acceptor splice sites. The first is located at position 6383 and is used in the generation of a doubly spliced pX–rex–orf I and a singly spliced pX–orf I mRNAs (Fig. 1). In the first mRNA the first coding exon of the Rex protein is joined in-frame to orf I and this mRNA could encode a protein of 152 amino acids (see Fig. 2). In contrast, the singly spliced pX–orf I mRNA could encode a 99-amino acid protein initiated at the internal AUG of the orf I RNA (Figs. 1 and 2).

The second splice acceptor site, located at position 6478, was used in the processing of a doubly spliced mRNA called pX–tax–orf II (Fig. 1). In this mRNA, the splicing event (positions 4831/6478) provides the initiation codon (AUG) of the env RNA to the orf II RNA. Thus, the AUG of the env RNA is used not only to provide an initiation codon to the tax RNA as shown (15–17) but also to the orf II RNA. Translation of this mRNA would generate a protein of 241 amino acids (see Fig. 2).

A previously described splice acceptor site (20), located downstream at nt 6875 in the orf II RNA, was also identified using the MZ4/MZ5 set of primers. This singly spliced mRNA designated pX–orf II used the donor splice site at position 119 and could encode for a 87-amino acid protein (Figs. 1 and 2).

Finally, the screening of the cDNA library generated by the use of the MZ4 and MZ5 primers yielded the expected splice junctions for the pX–tax/rex/$p21^{rex}$ mRNA (positions 119/4641 and 4831/6950) and an alternative splice acceptor site for the second exon at position 4658 (Fig. 1). This acceptor splice site was also found in the alternatively spliced env mRNA. Furthermore, a singly spliced mRNA species (positions 119/6950) that could encode the $p21^{rex}$ protein was identified. This mRNA, designated pX–$p21^{rex}$ (Fig. 1), has also been described as a prevalent mRNA species in ATL (20).

Detection of Alternatively Spliced mRNA from *ex vivo* RNA Samples. To ascertain whether the splice junctions were part of true mRNA rather than merely cell culture artifacts, we extended our study to 20 *ex vivo* RNA samples obtained from the PBMCs of healthy HTLV-I carriers or patients with TSP/HAM and/or ATL. As shown in Fig. 3 and summarized in Table 1, amplified DNA fragments of the expected size for the pX–tax/rex mRNA were generated not only from the total and cytoplasmic RNA of cultured PBMCs but also from 50% of the *ex vivo* samples. It has been shown (27–29) that the pX–tax/rex mRNA is detected consistently in TSP/HAM. By using DNA primers (RPX3/RPX4) that amplify only the splice junction between the second and third exons (28, 29), we detected the mRNA for these regulatory proteins in almost 100% of the samples studied (Table 1).

Several samples scored positive for the singly spliced pX–$p21^{rex}$ mRNA, suggesting a functional role for the p21 protein *in vivo*. The detection of the mRNA species either doubly or singly spliced for *orf I* and *orf II* was far less frequent (Fig. 3 and Table 1). Nevertheless, the presence of these mRNAs from *ex vivo* samples supports the hypothesis

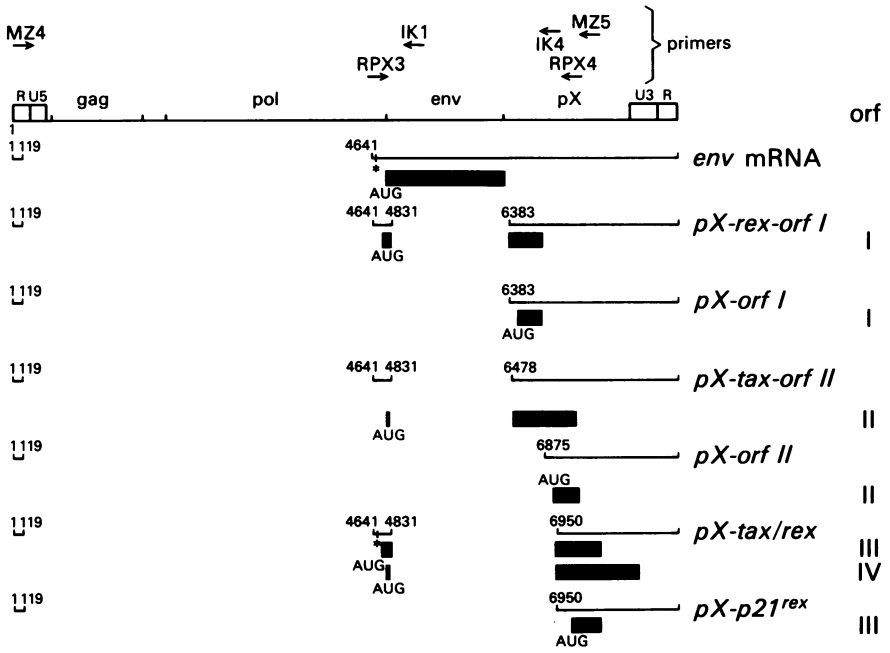
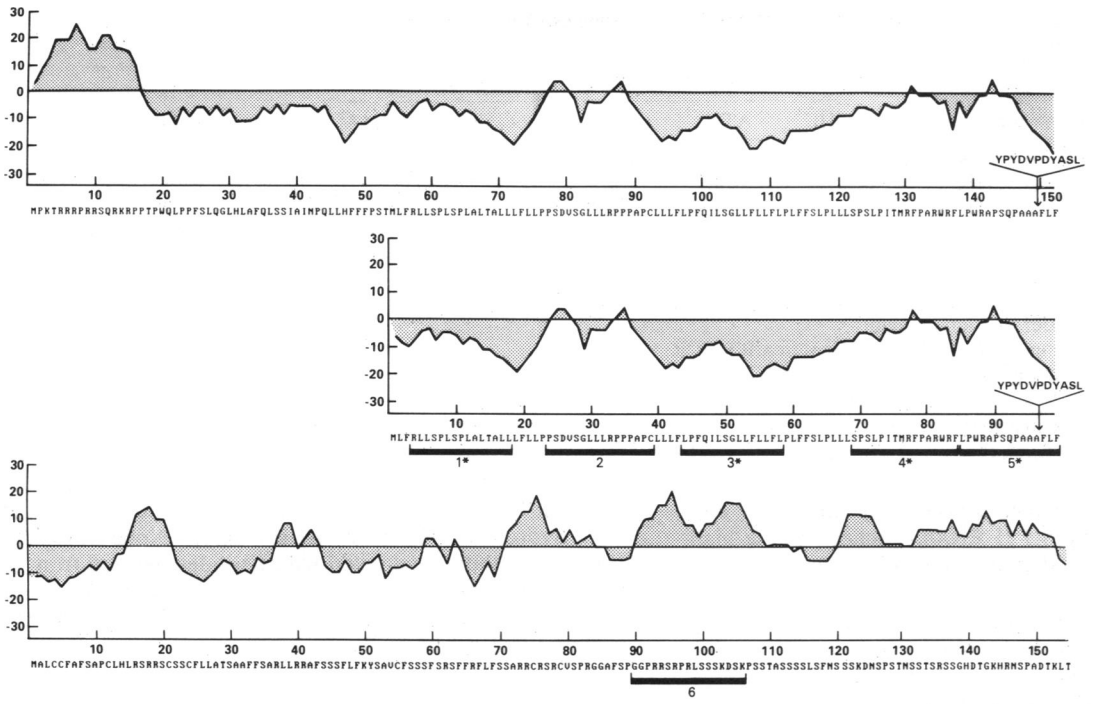


FIG. 1. Schematic representation of the spliced mRNA identified in HTLV-I-infected cells (HTLV-I_{LAF}). The numbers correspond to the nucleotide position of the exons with respect to the HTLV-I RNA. The asterisk corresponds to a splice acceptor site located at position 4658, which is used in the alternatively spliced env mRNA and the pX–tax/rex mRNA.



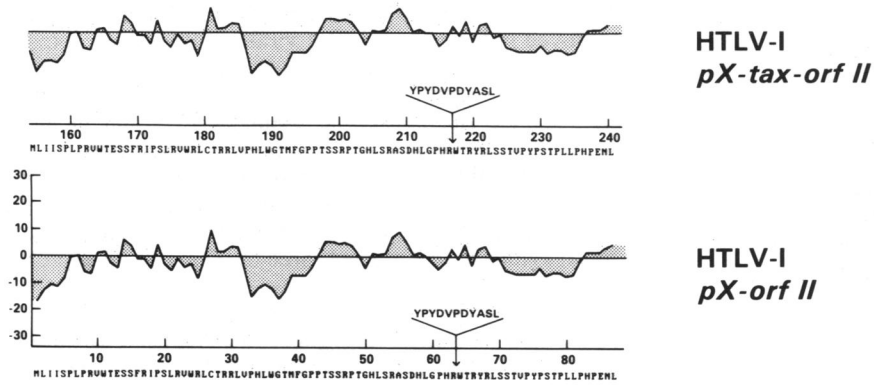


FIG. 2. Hydropathy profiles of the following HTLV-I proteins: the putative 152-amino acid protein from pX–rex–orf I mRNA, the 99-amino acid protein from the pX–orf I mRNA, and the two proteins of 241 and 87 amino acids generated by the alternative splicing in orf II mRNAs. On the *y* axes, the areas below and above the horizontal line correspond to hydrophobic and hydrophilic amino acids, respectively. The capital letters indicate the amino acid residues (using the single letter amino acid code). The bars encompass the sequence of the peptides used to immunize rabbits and the asterisk indicates that a tail of three amino acids (KKC) has been added to increase the solubility of the peptides. The positions where the HA1 epitope (YPYDVPDYASL) has been inserted in each protein are indicated by an arrow.

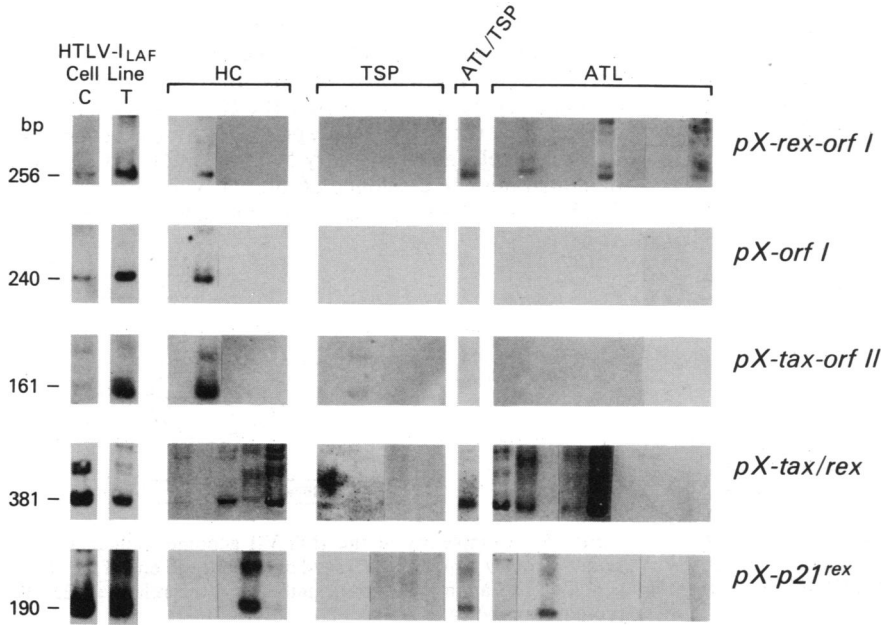


FIG. 3. Southern blot hybridization of the RT/PCR products with spliced junction probes. C, cytoplasmic RNA; T, total cellular RNA; bp, base pairs; HC, healthy carrier. Lanes TSP and ATL are from patients with only one disease; lane ATL/TSP is from a patient who had both diseases concurrently.

Table 1. Detection of splice-junction DNA fragments in RNA species from freshly isolated cells from HTLV-I-infected individuals

HTLV-I mRNA	Splice junction probe	No. positive/no. tested				Protein(s)
		HC	ATL	TSP/HAM	ATL–TSP/HAM	
pX–rex–orf I*	IK17	1/5	3/9	0/5	1/1	152^{aa}
pX–orf I	IK7	1/5	0/9	0/5	0/1	$p12^{I}$
pX–tax–orf II*	IK19	1/5	0/9	1/5	0/1	$p30^{II}$
pX–tax–rex	MZ3	4/5	4/9	1/5	1/1	$p40^{Tax}$, $p27^{rex}$, $p21^{rex}$
pX–tax–rex†	MZ3	5/5	8/9	5/5	1/1	$p40^{Tax}$, $p27^{rex}$, $p21^{rex}$
pX–$p21^{rex}$	MZ10	2/5	2/9	1/5	1/1	$p21^{rex}$

HC, healthy carrier; aa, amino acid.
*Data obtained using the RPX3/IK4 primers in a RT/PCR.
†Data obtained using RPX3/RPX4 primers in a RT/PCR.

that they are not cell culture artifacts and that these viral mRNAs might encode proteins that are important in the viral life cycle.

Identification of the Proteins Encoded by the Singly and Doubly Spliced orf I and orf II RNAs. Because of the high hydrophobicity of the predicted amino acid sequence of Orf I and since the prediction of immunogenic epitopes in proteins is often uncertain, we studied the protein products of these putative HTLV-I genes by using two approaches. The first involved the addition of the hemagglutinin epitope YPYDVPDYASL (HA1) (23) to the C terminus of the proteins (Fig. 2).

Transfection of the tagged pX–orf I cDNA yielded, upon immunoprecipitation with the 12AC5 monoclonal antibody, which recognizes the HA1 epitope, a 12-kDa protein ($p12^{I}$) in both COS-1 and HeLa–*tat* cells (Fig. 4 *A* and *C*). Similarly, the tagged tax–orf II cDNA yielded a 30-kDa protein ($p30^{II}$) in both cell lines (Fig. 4 *B* and *C*). A 13-kDa ($p13^{II}$) protein was expressed by the pX–orf II cDNA in HeLa–*tat* cells (the COS-1 cells were not tested; Fig. 4*C*). Interestingly, the pX–rex–orf I cDNA yielded a 12-kDa protein that has the same relative migration as the $p12^{I}$ protein expressed by the singly spliced pX–orf I RNA (Fig. 4*C*).

The predicted protein encoded by the pX–rex–orf I mRNA would contain 152 amino acids (see Fig. 2) and the size of the protein detected experimentally is only 12 kDa. This mRNA could also encode the $p12^{I}$ protein since the internal orf I AUG is present in this mRNA. Therefore, it is likely that both the doubly and singly spliced mRNAs encode the $p12^{I}$ protein.

The second approach was to generate rabbit immune sera by using five peptides derived from Orf I and one from the most hydrophilic region of Orf II (Fig. 2). None of the sera from the rabbits immunized with peptides derived from the Orf I recognized the $p12^{I}$ protein, whereas rabbit 10, immunized with peptide 6, recognized the 30-kDa Tax–Orf II protein (data not shown).

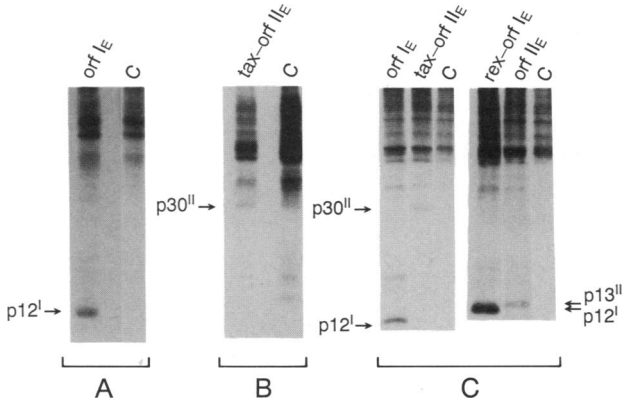


FIG. 4. Radioimmunoprecipitation of the $p12^{I}$, $p13^{II}$, and $p30^{II}$ from transfected human and monkey cells. The COS-1 cell line (*A* and *B*) and HeLa–*tat* cells (*C*) were used in the transfection assays. 12CA5 (23), a mouse monoclonal antibody directed against the HA1 epitope, was used in the immunoprecipitation assay (30). orf I_E, rex–orf I_E, tax–orf II_E, and orf II_E indicate the presence of the HA1 epitope (E) sequence in the transfected DNA. Lanes C contain control cell lysate from cells transfected with HCMV–HSPA vector DNA and immunoprecipitated with 12AC5.

DISCUSSION

Our data indicate that the alternative splicing mechanism(s) employed in the processing of viral mRNA greatly expands the complexity of the HTLV-I genome (Fig. 5). The splice acceptor sites identified in this study agree with the consensus 3′ splice site conserved sequence (31) that include a pyrimidine-rich region (U/C), a nonconserved position (N), another pyrimidine, and the absolutely conserved AG dinucleotide [U/C)$_n$N(C/U)AG/G]. Four additional putative proteins could be translated from these mRNAs, and we have shown evidence that at least three of them can be expressed in human and monkey cells.

HTLV-I expression *in vivo* in both ATL and TSP/HAM has been extensively investigated to establish a causal relationship between viral expression and disease manifestations. In ATL, it appears that the majority of the leukemic cells do not express viral antigens (32, 33) and viral mRNA (34). More recently, using the RT/PCR technique, we (20) and others (29) have shown that viral expression can be detected in the PBMCs of most ATL patients, although this particular technique does not allow discrimination between viral expression occurring in leukemic cells versus the infected circulating normal T cells. In TSP/HAM, viral expression can be demonstrated in the majority of cases and the number of expressing cells appears to range between 1 in every 100 cells and 1 in every 5000 cells (28, 35). Our study demonstrates the existence of alternatively spliced mRNAs for the *orf I* and *orf II* genes *in vivo* and indicates that HTLV-I

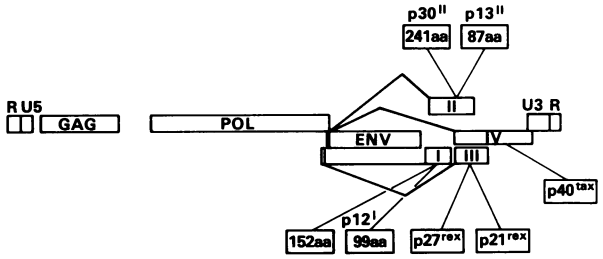


FIG. 5. Complexity of the HTLV-I genome. Schematic representation of *orf I*, *orf II*, *orf III*, and *orf IV* at the 3′ end of the HTLV-I genome. The amino acid (aa) number and the relative size of the proteins are indicated.

has a complex pattern of splicing that allows for the expression of at least three additional proteins, $p12^{I}$, $p13^{II}$, and $p30^{II}$. The $p12^{I}$ (derived from *orf I*) is highly hydrophobic; 32% of its amino acid residues are leucine and 17% are proline. However, in the doubly spliced mRNA (pX–rex–orf I), the first 20 amino acids of the $p27^{rex}$ protein are added to Orf I to generate a putative protein of 152 amino acid that could reside in the nucleolus since the first exon of the Rex protein has been shown to contain the nucleolar targeting sequence (36). In this study we were unable to detect the protein product of the doubly spliced mRNA using two approaches. However, others have shown in an *in vitro* translation system a protein of 27-kDa translated from this doubly spliced mRNA (37). It is possible that the removal of the internal AUG in the cDNA is necessary to obtain detectable expression of the 152-amino acid protein since in all experiments we could detect only the $p12^{I}$ protein. Alternatively, the 152-amino acid protein might undergo posttranslational modification (folding and/or cleavage) that renders the epitopes inaccessible to the immune sera. Further experiments aimed at studying the metabolism and the cellular localization of this putative protein are necessary. Both proteins synthesized from the *orf II*, $p30^{II}$ and $p13^{II}$, are arginine- and serine-rich, and preliminary data suggest that $p30^{II}$ is localized in the nucleolus, in agreement with others (37), whereas $p13^{II}$ is in the nucleus. Some of these proteins might have regulatory functions and be involved in viral–cell interactions. Future study on the proteins synthesized from these viral transcripts might help to elucidate the mechanisms of viral latency and the pathogenesis of HTLV-I in ATL and TSP/HAM.

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