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Repetitive structure in the long-terminal-repeat element of a type II human T-cell leukemia virus

(retrovirus/RNA tumor virus/hairy cell leukemia/enhancer)

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ABSTRACT **The majority of human T-cell leukemia virus isolates (HTLV-I) are associated with clinically aggressive adult T-cell leukemia/lymphomas. By contrast, HTLV-II has been isolated from a patient with a relatively benign hairy T-cell leukemia. To characterize differences in the viral genomes that might contribute to these different pathologies, we determined the nucleotide sequence of the long terminal repeat (LTR) of a HTLV-II provirus. Comparison with the type I HTLV LTR reveals that, whereas the overall structural features are similar, the two sequences differ markedly throughout most of the length of the LTR. Despite the overall differences, the sequences of several functional regions of the two LTRs are conserved. These include the 5′ boundary of U3, the RNA cap site, and the $tRNA^{Pro}$-binding site immediately 3′ to the LTR. Another point of similarity is a 21-base sequence that is repeated four times in the U3 region of HTLV-II and three times in the U3 region of HTLV-I. This sequence has a formal analogy to, but no common sequence with, viral transcriptional enhancers. The U3 region of HTLV-II possesses a series of imperfect tandem direct repeats, 42 bases long, 21 bases long, 19 bases long, and 7 bases long. These structures differ from those of HTLV-I except for the 21-base repeat sequence. Thus, the structure of HTLV-II differs substantially from that of HTLV-I in the region that governs transcriptional initiation and tissue specificity. Such differences may account for some of the differences in clinical presentation of HTLV-associated adult T-cell leukemia/lymphomas and hairy T-cell leukemia.**

The human T-cell leukemia viruses (HTLV) comprise a family of exogenous retroviruses of man (1–8). Two HTLV types have been defined, based on immunocompetition analysis using the *gag*-encoded p24 protein and on nucleic acid hybridization studies (8–10). HTLV-I represents the most prevalent type found to date and is associated with adult T-cell leukemia/lymphomas and, more recently, with acquired immune deficiency syndrome (1–7). HTLV-I-associated diseases tend to exhibit aggressive clinical courses and, in the great majority of cases, involve mature $OKT4^+$ T cells (1–7).

The HTLV-II strain reported here is derived from a patient with a clinically benign hairy T-cell leukemia, also involving the $OKT4^+$ population (8, 10). Recently, a second independent isolation of HTLV-II has been made (unpublished data).

Evidence from avian and murine systems indicates that the structure of the long terminal repeat (LTR) can profoundly influence the leukemogenicity, tissue tropism, and specific disease induced by slow-acting leukemia viruses (11–14). Structural variations in the repetitive elements of the U3 region of the LTR have been shown to account for the pathogenicity of particular virus isolates (11–14). To understand whether such changes might characterize the LTRs of HTLV variants associated with different diseases, we determined the complete nucleotide sequence of the LTR of a HTLV-II isolate.

MATERIALS AND METHODS

Clones. MO-1A and MO-15A are bacteriophage clones derived from a cell line established from the bone marrow and peripheral blood lymphocytes of a hairy T-cell leukemia patient (M.O.) (15). Both 5′ and 3′ LTRs, along with flanking sequences, were subcloned into pBR322 derivatives or into the replicative form of M13 phage for ease of sequencing.

DNA Sequencing. The nucleotide sequence of the HTLV-II LTR was determined using both the Maxam and Gilbert technique (16) and the dideoxy method of Sanger *et al.* (17). The strategy used for sequencing is outlined in Fig. 1. The sequences derived were analyzed by computer for areas of repetition, homology, or secondary structure.

RESULTS AND DISCUSSION

Sequencing of the HTLV-II LTR. Cell lines established from the hairy cell T-cell leukemia patient (M.O.) shown to be expressing HTLV were used as a source of cloned integrated provirus (15). For DNA sequence analysis of the LTR, clones MO-15A (apparently a complete provirus) and MO-1A (a 3′ LTR) were used. Using the *Bam*HI and *Sac* I sites within the LTR for end labeling and for subcloning into M13 phage, we obtained the complete nucleotide sequence of the HTLV-II 5′ and 3′ LTRs using both the Maxam and Gilbert and the dideoxy methods of DNA sequencing (Fig. 1) (16, 17).

The boundaries of the LTR were defined by sequencing the entire 5′ and 3′ LTRs of MO-15A and noting the points of sequence divergence. The HTLV-II LTR is 764 nucleotides long, which is longer than that of most retroviruses (Table 1). The U3 region of retroviral LTRs extends from the 5′ limit of the LTR to the RNA cap site. By locating HTLV-II LTR sequences that conform to consensus sequences established for the promoter $\begin{pmatrix}\text{G-T-A-T-T-T-A-A-G}\\ \text{C-A}\qquad\quad\text{A-A}\end{pmatrix}$, we were able to place the probable RNA cap site of HTLV-II at the guanosine residue at position 314. The R–U5 border was identified by matching our sequence to that obtained from a cDNA clone derived from HTLV-II-infected cells (10). Thus, the HTLV-II LTR consists of a 314-bp U3, a 247-bp R region, and a 203-bp U5.

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Abbreviations: HTLV, human T-cell leukemia virus; LTR, long terminal repeat; bp, base pair; BLV, bovine leukemia virus.

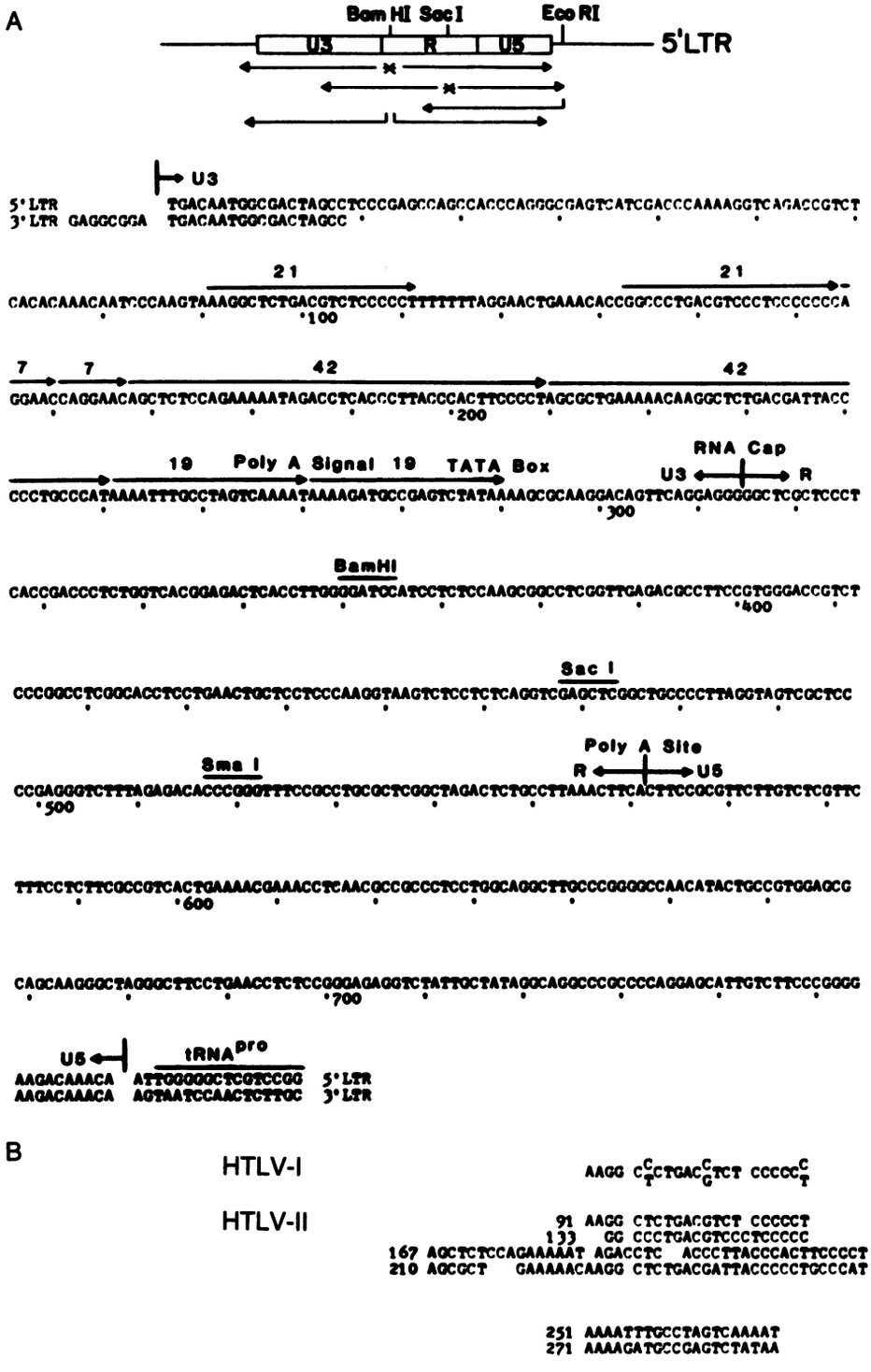

FIG. 1. (*A*) Nucleotide sequence of the HTLV-II LTR. The sequencing strategy is shown at the top. Asterisks designate those sites used for cloning into M13 for dideoxy sequencing. The remaining sites were end labeled and sequenced by the method of Maxam and Gilbert. The nucleotide sequence of the entire 5′ HTLV-II LTR is given. The sequence of the 3′ LTR is identical except at the 5′ and 3′ points of divergence that mark the limits of the LTR. The U3, R, and U5 boundaries are noted, as are the potential Proudfoot–Brownlee sequence (18) [poly-(A) signal], promoter (TATA box), RNA cap site, polyadenylylation [poly(A)] site, and binding site for $tRNA^{Pro}$. A purine-rich sequence is located in the pX region immediately 5′ to the 3′ LTR. The imperfect tandem repeat elements are marked with arrows with numbers signifying the length of the repetitive element. (*B*) Repetitive elements of HTLV LTRs. The top line presents the consensus sequences of three 21-base-pair (bp) imperfect repeat elements of HTLV-I. These span nucleotides 102–122, 150–170, and 251–270 of the sequence of Seiki *et al.* (19). This repeat element of HTLV-I is aligned with four similar elements in the HTLV-II U3 region. In HTLV-II, two of these sequences are imbedded in larger 42-bp repeat elements. The sequences of another 19-bp imperfect repeat element in the U3 region of HTLV-II are given at the bottom of the figure. Numbers refer to the position of the 5′ most nucleotide in the element (*A*).

Consensus Sequences Within the LTR. We examined the HTLV-II LTR sequence for known characteristics of retroviral LTRs. LTRs typically have short inverted repeats at their limits, most often beginning with the sequence T-G at the 5′ end and ending with C-A at the 3′ end. The LTR inverted repeats have been shown to be important for proviral integration in animal retroviruses (24). The HTLV-II LTR begins with T-G and ends with C-A. The LTR of HTLV-I also has unusually short (2 bp) inverted repeats at its termini (19, 20). This suggests that, in the HTLV family, sequence requirements for recognition by endonucleases mediating proviral integration may be less stringent than those of other retroviruses.

Viral and eukaryotic sequences known to function as transcriptional promoters often contain the sequence C-C-A-A-T 70–80 bp 5′ to the cap site and the sequence $\begin{matrix}\text{G-T-A-T-T-T-A-A-G}\\ \text{C-A}\qquad\quad\text{A-A}\end{matrix}$ 20–30 bp 5′ to the cap site (25, 26). Although no C-C-A-A-T sequence is evident in the appropriate position in the HTLV-II LTR, a "TATA" box exists 25 bp 5′ to a series of guanosine residues that presumably represents the RNA cap site (Fig. 1).

Polyadenylylation of retroviral RNAs occurs within the 3′ LTR, often at $\begin{matrix}\text{C-T-T-T-G-C-N-C-T-T-G-C-A}\\ \text{T}\qquad\qquad\qquad\qquad\qquad\qquad\text{G}\end{matrix}$ sequences 20–30 bp 3′ to a Proudfoot–Brownlee consensus sequence

A-A-T-A-A-A
G (18). In the HTLV-II R region, a sequence distantly related to the former sequence can be identified, with polyadenylylation occurring at the C-A residues as in other retroviral LTRs (Fig. 1). However, the only Proudfoot–Brownlee sequence is distant from the polyadenylylation site, in the U3 region of the LTR (position 269). Seiki *et al.* (20) proposed a secondary structural model for the HTLV-I LTR in which multiple stem-loop structures allowed the juxtaposition of a similar Proudfoot–Brownlee sequence in the U3 region with the site of polyadenylylation at the R–U5 boundary. We examined the HTLV-II LTR for ability to form potential secondary structures of the type described for HTLV-I. No complex structure such as that proposed for HTLV-I appears to be energetically favored.

Furthermore, in the region immediately 5′ to the polyadenylylation site, no significant homology between HTLV-I and HTLV-II exists. However, a distantly related pyrimidine-rich tract exists in the 3′ vicinity of the polyadenylylation site of HTLV-I, HTLV-II, and bovine leukemia virus (BLV):

poly(A) site

HTLV-II ↓------C-G-T-T-C-T-T-−-G-T-C-T-C-G-T-T

HTLV-I ↓------↓C-G-−-T-C-T-T-T-G-T-T-T-C-G-T-T

BLV ↓--C-G-C-G-−-T-T-T-G-T-T-T-C-C-T

Two sites of HTLV-I polyadenylylation are indicated because differences have been observed between HTLV-I isolates (our unpublished data and ref. 20). Although the exact mechanism of polyadenylylation of HTLV remains unclear, the U3 Proudfoot–Brownlee sequence and the pyrimidine-rich tract in U5 may play a role in this process.

The sequence flanking the 5′ LTR of the retroviral genome contains the binding site of a specific tRNA, which serves as a primer for reverse transcription (27). The HTLV-II genome contains a 19-bp sequence in this region that is identical to the $tRNA^{Pro}$ binding site used by murine leukemia viruses (Fig. 1).

The left-hand boundary of 3′ retroviral LTRs is usually located adjacent to a purine tract, thought to act as a plus-strand binding site (28). A purine-rich sequence is similarly positioned in HTLV-II (Fig. 1), within the region of the virus designated pX (20).

Open Reading Frames. We examined the HTLV-II LTR protein-coding potential by searching for open reading frames. Although proteins up to 99 amino acids long could be encoded by the HTLV-II LTR, none of the open reading frames encoded a methionine at the initial position (data not shown).

Repetitive Structure of the HTLV-II U3 Region. The U3 region of retroviral LTRs usually contains repetitive elements distant from the C-C-A-A-T and TATA promoter sequences that act to enhance RNA transcription (23, 29). We examined the sequence of the HTLV-II U3 region for such repetitive structures. The HTLV-II U3 region can be organized into a series of imperfect tandem direct repeat elements that span the region from nucleotide position 91 to position 290 (including the TATA box) (Figs. 1 and 3). The region surrounding the TATA box is included in a 19- or 20-nucleotide imperfect direct repeat. Immediately 5′ are two 42-bp imperfect direct repeats. These are flanked 5′ by two tandem perfect 7-bp direct repeats. Within the 42-bp repeats is a 21-nucleotide sequence that is again repeated upstream beginning at positions 91 and 133. The proximity of these repetitive elements to the RNA cap site is unusual among retroviral LTRs. Furthermore, this highly repetitive structure distinguishes the HTLV-II LTR from that of HTLV-I.

Comparison with the HTLV-I LTR. The nucleotide sequences of several HTLV-I isolates from leukemia cells of United States, Caribbean, and Japanese patients with adult T-cell malignancies and from United States cases with acquired immune deficiency syndrome are available (refs. 19 and 20 and our unpublished data).

Although similar in general organization, the HTLV-I and HTLV-II LTRs exhibit only limited areas of homology. At the 5′ border of the LTR is a 6-bp region of homology (T-G-A-C-A-A) whose conserved nature suggests a function, perhaps in proviral integration.

The region surrounding the RNA cap site, including the TATA box, is also highly conserved:

		RNA cap site ↓
HTLV-I	G-A-G-T-C-T-A-T-A-A-A-A-G-C-T	T-G-G-A-G-A-C-A-G-T-T-C-A-G-G-A-G-G-G-G-G-C-T-C-G-C
HTLV-II	G-A-G-T-C-T-A-T-A-A-A-A-G-C-G-C-A-A-G-G-A	C-A-G-T-T-C-A-G-G-A-G-G-G-G-G-C-T-C-G-C

In HTLV-I, HTLV-II, and BLV, the most energetically favored secondary structure in the LTR is a stem loop that can be formed near the RNA cap site (Fig. 2). In all three cases, a sequence immediately 3′ to the TATA box and extending slightly beyond the cap site exhibits dyad symmetry with a sequence in the R region. The position of this latter region differs in all three cases so that the size of the loop formed also varies. However, despite differences in nucleotide sequence in the involved regions, the positions of both the TATA box at the base of the stem and the RNA cap site at the terminus of the stem appear to be conserved. Since this structure could not form at the 5′ end of viral RNA, its potential function likely occurs at the DNA level—e.g., transcriptional control. Similar stem-loop structures adjacent to the TATA box have been noted for the simian virus 40 early promoter (30), but we have not been able to identify analogous structures in other retroviral LTRs.

A 19-bp sequence 3′ to the 5′ LTRs of both HTLV types, which represents the binding site of $tRNA^{Pro}$ during reverse transcription of the viral RNA genome (27), is exactly conserved.

The U3 region of the HTLV-II LTR is smaller than that of HTLV-I (Table 1). The organization of the repetitive elements in the U3 region differs considerably between HTLV-I and HTLV-II LTRs (Fig. 3). In HTLV-I, a tandem pair of approximately 50-nucleotide imperfect direct repeats is located 262 bp 5′ to the RNA cap site. A 21-bp internal se-

Table 1. Length (in bp) of HTLV and BLV LTR regions

Virus	U3	R	U5	Total	Ref.
HTLV-I	353	221	180	754	19, 20
HTLV-II	314	247	203	764	
BLV	(215)	233 or 234	86 or 87	(535)	21, 22
Feline leukemia	338	69	75	482	23
Murine leukemia	372–445	60–68	72–87	515–588	23
Mouse mammary tumor	1194	16	120	1330	23
Avian leukosis	227–245	21	78	326–344	23

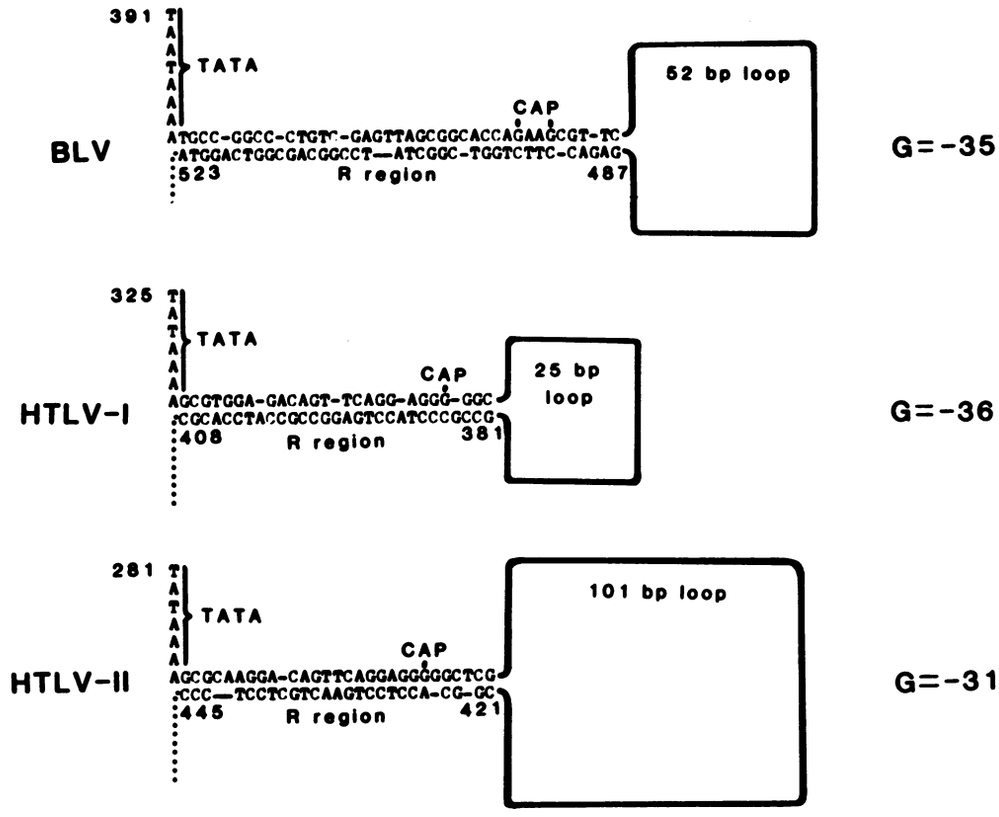

FIG. 2. Potential secondary structure in the BLV and HTLV LTRs. The most energetically favored secondary structure in the BLV and HTLV LTRs is shown. Nucleotide positions for BLV are from Tsimanis *et al.* (21) and those for HTLV-I are from Seiki *et al.* (19). Note the conserved position, despite sequence divergence, of the TATA boxes (TATA) and RNA cap site (CAP) with respect to the stem structure. The size of the loop and the free energy (*G*) of the structure in kilocalories (1 cal = 4.18 J) is shown. The complementary sequence of the dyad arises within different portions of the R region of all three viruses.

quence in this repeat element can be identified beginning 104 bp 5′ to the cap site. However, no other repetitive structures like those found in the HTLV-II U3 have been identified in the HTLV-I LTR. Of possible functional significance is the fact that the 21-bp internal sequence of the repetitive elements of both types of LTR is highly conserved (Fig. 1*B*). The position, conserved status, and repetitive nature of this element are reminiscent of viral transcriptional enhancer elements. However, no homology between this sequence and known viral or immunoglobulin enhancers has been identified.

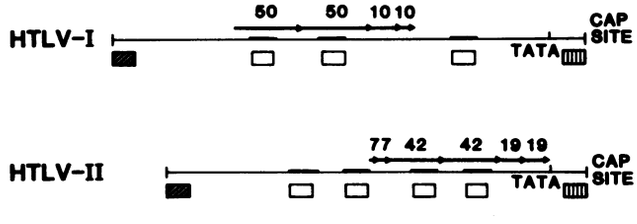

FIG. 3. Comparison of U3 regions of the HTLV-I and HTLV-II LTRs. The organization of the repetitive elements of the HTLV-I and HTLV-II U3 regions is shown. Arrows indicate the repetitive elements and numbers indicate approximate length of the element in nucleotides. Areas of homology between the U3 regions of these two isolates are indicated by the boxes underneath each figure. The open boxes depict the positions of conserved 21-bp elements in the repetitive structures of HTLV-I and HTLV-II. The promoter (TATA) and RNA cap site are noted. Note that conservation of sequences also occurs near the 5′ boundary of the LTR (boxes with diagonal hatching) and near the cap site (boxes with vertical hatching).

No extended homology exists between the HTLV-I and HTLV-II R and U5 regions, including the region surrounding the sites of RNA polyadenylylation. In general, except for a few conserved areas, the nucleotide sequences of these LTRs differ considerably. This is surprising in view of the well-conserved status of the LTR among members of animal retrovirus families. The differences in the HTLV LTRs suggest that they may be adapted to different cellular environments or contexts.

Comparison with the BLV LTR. HLTV and BLV share features that distinguish them from other retroviruses. Both retroviruses have in common a *gag*-encoded p24 protein sharing some antigenic determinants, a reverse transcriptase that exhibits greater catalytic activity in the presence of magnesium than of manganese, a major envelope glycoprotein apparently smaller than the typical 70-kilodalton long "strong stop" DNAs, and inefficient infectivity of extracellular virus (3, 19, 31, 32). We compared the HTLV-II LTR with the LTR sequence of BLV (21, 22). Although repetitive structures are present in the U3 region of BLV, they exhibit no homology to those of HTLV-II. Both BLV and HTLV-II lack properly positioned C-C-A-A-T boxes, although BLV does have a similar sequence about 90 bp 5′ to the RNA cap site. The most energetically favored secondary structure in the BLV LTR occurs near the RNA cap site as does that of HTLV-I and HTLV-II (see above). BLV, like HTLV-I and -II, lacks a Proudfoot–Brownlee sequence near its polyadenylylation site but has such a sequence in the U3 region. Distant homology exists near the polyadenylylation sites of HTLV-II and BLV, including the pyrimidine-rich sequence in the U5 region mentioned previously. Thus, the LTRs of BLV and HTLV have similar organizational features, al-

though at the nucleotide level, homology between the two is minimal.

It is likely that the HTLV LTRs will serve functions similar to those described for other retroviral LTRs. These include proviral integration and transcriptional enhancement/promotion of viral and, in some cases, of adjacent cellular sequences. In both murine and avian systems, it is clear that changes in the LTRs, often consisting of rearrangements of the repetitive elements of the U3 region, can profoundly affect the tissue tropism and pathogenicity of the retrovirus (11–14). This report demonstrates that similar structural changes occur within the U3 region of LTRs of HTLV isolates associated with diseases that differ in pathology and degree of malignancy. The similarities and differences between the HTLV-I and HTLV-II LTRs are consistent with a role for HTLV LTR in both early and chronic events in leukemogenesis. Both HTLV-I and HTLV-II can rapidly immortalize primary T cells *in vitro* (33–36), suggesting that this function is encoded by a viral protein, possibly the product of the pX region (19). The regions of similarity between the LTRs of HTLV-I and HTLV-II, notably the 21-bp U3 repeats, might restrict expression of the immortalizing protein to particular target lymphocytes. The period between HTLV infection and the development of a particular type of leukemia/lymphoma is long, suggesting that a rare, possibly disease-specific, event is involved. The dissimilarities between the HTLV-I and HTLV-II LTRs could account for differences in this latter stage of HTLV tumorigenesis, resulting in adult T-cell leukemia/lymphoma or the more benign hairy cell leukemia of a T-cell subtype, respectively. Since the known rare events in animal viral leukemogenesis involve transcriptional enhancement/promotion of cellular sequences by the LTR, a functional comparison of these HTLV LTRs with respect to transcription may provide clues to the different clinical consequences of infection by these viruses.

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