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Molecular cloning of a unique human T-cell leukemia virus (HTLV-II_{Mo})

(human type C retrovirus/integrated provirus)

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ABSTRACT **Human T-cell leukemia virus II_{Mo} (HTLV-II_{Mo}) is a human retrovirus isolated from a patient with a T-cell hairy cell leukemia. This virus has been shown to have core protein (gag) antigens similar to, but distinct from, those of all known isolates of the prototype human T-cell leukemia virus (HTLV-I). We have used a subgenomic clone of the HTLV-I *env–pX* region to detect and characterize HTLV-II_{Mo} proviral sequences by performing Southern blot hybridization under conditions of low stringency. Using the HTLV-I probe, we cloned a partial integrated HTLV-II_{Mo} provirus from a genomic library of the producer Mo cell line. These sequences could be characterized by low stringency hybridization with different subgenomic clones of HTLV-I. An HTLV-II_{Mo}-specific subclone was made by isolating a 3.6-kilobase *Bam*HI fragment of the partial provirus. This was used to clone two full-length integrated viral genomes. Using the HTLV-II_{Mo} viral probe, we also showed by hybridization under stringent conditions to DNA and RNA of various infected and uninfected cell lines that these HTLV-II_{Mo} sequences are unique.**

Human T-cell leukemia virus (HTLV), a human retrovirus, was originally isolated in the United States from some patients with leukemia and lymphoma involving relatively mature T cells (1–3). Several additional isolates of HTLV were subsequently identified in other patients from the United States, England, and elsewhere (4–8), and recent evidence has shown that HTLV is endemic in certain geographical areas. HTLV is different from all the animal retroviruses isolated as determined by comparison of viral nucleic acids by nucleic acid hybridization (9), by immunological analyses of the structural proteins (10, 11) and reverse transcriptase (12), and by amino acid sequence analysis of structural proteins (13). That HTLV is acquired by infection is suggested by the findings of high-titer antibodies to HTLV structural proteins in sera of patients and in some normal people (14–18) and is conclusively shown by the finding of proviral sequences in the DNA of neoplastic T cells but not in various normal human cells (3, 9). HTLV sequences are also absent from normal B cells from patients whose neoplastic T cells contain HTLV proviral sequences (3).

A significant feature of the various HTLV isolates from throughout the world is the lack of identifiable differences among them by tests of nucleic acid homology, by immunological crossreactivity of their structural proteins, and by the presence in all viral isolates of identical cleavage sites for at least four restriction enzymes (ref. 18 and unpublished observations). In addition to the epidemiologic link between HTLV and T-cell leukemia, studies of *in vitro* transmission and T-cell transformation (19) further underscore the role of this virus as a leukemogenic agent. The disease associated with this agent in most cases can be characterized clinically as an acute T-cell leukemia that has a predisposition for skin involvement, can cause lytic bone lesions and hypercalcemia, and is rapidly fatal (20).

We have recently identified a second human retrovirus, which was isolated from the cultured cells of a patient (Mo) with a chronic T-cell leukemia that had the histopathologic appearance of hairy cell leukemia (21, 22). This virus was shown to be antigenically related to HTLV by demonstrating crossreactions with antibodies to HTLV core proteins p24 (10) and p19 (11). However, competition radioimmunoassay comparing the prototype HTLV (HTLV-I_{CR}) and this new virus (HTLV-II_{Mo}) showed the latter to compete at only 60% the level of the homologous reaction. Furthermore, the slopes of the two competition curves were different, indicating that the antigenic crossreaction between HTLV-I_{CR} and HTLV-II_{Mo} was not type specific (22). These antigenic differences were not found in the characterization of many other HTLV-I isolates from patients with acute T-cell leukemia worldwide. Since all the HTLV-I isolates thus far examined are very closely related antigenically and are indistinguishable by restriction enzyme analysis of proviral sequences, we wished to study in greater detail the HTLV-II_{Mo} virus.

This report describes molecular biological studies carried out to characterize the HTLV-II_{Mo} provirus. We identified HTLV-II_{Mo} proviral sequences in Mo DNA by using a cloned HTLV-I probe that contains *env* and *pX* sequences (23, 24) hybridized to a Southern blot under conditions of low stringency. This same probe was used to clone an integrated defective provirus from an Mo genomic library. HTLV-II_{Mo} was characterized by using different subgenomic probes from HTLV-I and was shown to be related to but distinct from HTLV-I. An HTLV-II_{Mo} subgenomic clone was constructed and used to screen the Mo genomic library. Two complete HTLV-II_{Mo} proviral copies were isolated and mapped. Also, the HTLV-II_{Mo} subclone was used in hybridization experiments to confirm that this is a second unique human retrovirus.

MATERIALS AND METHODS

Cell Lines. DNA and RNA from the Mo cell line were gifts of Stephen Clark (Genetics Institute, Boston, MA). UK and MJ, HTLV-I-infected cell lines used for comparison, have been described (5).

Filter Hybridization. Restriction enzyme analysis was done with digests of 1 μg of cloned phage DNA or 5 μg of cellular genomic DNA; the digests were applied to an agarose gel for electrophoresis and Southern blotting to a nitro-

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Abbreviations: HTLV, human T-cell leukemia-lymphoma virus; kb, kilobase(s); LTR, long terminal repeat.

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cellulose membrane (25). Filter hybridization was carried out in 0.45 M NaCl/0.045 M sodium citrate/50% (vol/vol) formamide, at 37°C. Filters were washed at 60° with either 0.15 M NaCl/0.015 M sodium citrate (stringent conditions) or 0.45 M NaCl/0.045 M sodium citrate conditions (nonstringent conditions). Cloned DNA probes were radiolabeled with ^{32}P by nick-translation (26). RNA was selected on an oligo(dT)-cellulose column, electrophoresed in 2.2 M formaldehyde/1.2% agarose gels, and electroblotted onto GeneScreen filters (New England Nuclear). Hybridization of filters was performed as described (27).

Library Construction and Cloning. Mo cellular DNA was subjected to partial digestion with *Mbo* I. Twenty-kilobase (kb) fragments were isolated by sucrose gradient centrifugation (10–40% sucrose/10 mM Tris·HCl, pH 7.5/1 M NaCl in a Beckman SW 27 rotor at 25,000 rpm at 20°C for 24 hr). This fraction was ligated to *Bam*HI-cut λ-Jl phage DNA arms and packaged. Phage λ-Jl is a λ47.1 (28) derivative designed by J. Mullins to contain DNA with a 21.8-kb left arm and a 7.7-kb right arm bordered internally by polylinkers with *Eco*RI, *Hin*dIII, *Bgl* II, *Xba* I, and *Bam*HI restriction sites. Then 300,000 phage plaques were screened with pCR_{CH}, an HTLV *env–pX* probe (23) and, after washing at low stringency, one clone was isolated. Screening of the library with the $HTLV\text{-}II_{Mo}$ subclone, pMo1A-A, was done under stringent washing conditions.

RESULTS

Identification of the HTLV-II Provirus. The first step in our study was to identify the HTLV-II provirus in the infected Mo DNA. Since HTLV-II had been shown to have antigenic determinants related to HTLV-I core proteins, we extended this observation to other parts of the viral genome by hybridizing a cloned *env–pX* subgenomic fragment of HTLV-I (23) to Mo DNA. A diagram of the probe, pCR_{CH}, is shown in Fig. 1. The location of the retroviral structural genes, *gag–pol–env–pX* is based on the complete nucleotide sequence of an HTLV-I published recently (28). pCR_{CH} is a 2.4-kb *Hin*dIII/*Cla* I fragment isolated from a partial HTLV-I provirus clone, λ-CR-1 (23). This probe is specific for HTLV and identifies in a *Bam*HI digest of HTLV-I-infected cells a 1.05-kb band and in a *Pst* I digest a 2.5-kb band, both of which have been seen in every case of HTLV-I-infected cells (18). When this probe was hybridized to Mo DNA, bands were seen only when nonstringent washing conditions were used (Fig. 2). Moreover, the *Bam*HI restriction pattern, for example, differed from the pattern common to the HTLV-I provirus. *Bam*HI generated a 3.6-kb viral fragment in Mo that hybridized to pCR_{CH} as compared to the 1.05-kb fragment found in the HTLV-I producer line, HUT102. Another enzyme, *Sst* I, cleaved in Mo a 4.8-kb band detected by the probe. *Bam*HI and *Sst* I thus cleaved internal viral fragments detected by pCR_{CH} that were judged by band intensity to represent multiple copies cut from several proviral sequences. Under the conditions used in the hybridization of Fig. 2, no bands were detected in uninfected human DNA.

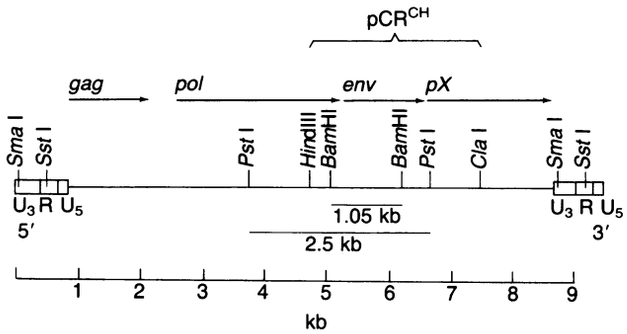

FIG. 1. Location of probe pCR_{CH} in the HTLV-I genome. The orientation is 5'→3'. pCR_{CH} is a plasmid clone of the *Hin*dIII/*Cla* I 2.4-kb fragment extending from 4.2 to 6.6 kb in the map of HTLV-I. This region overlaps the *env* and *pX* regions as shown. A 1.05-kb *Bam*HI fragment is included within the CR_{CH} fragment. A 2.5-kb internal *Pst* I fragment overlaps sequences with CR_{CH}. The boxes indicate long terminal repeats (LTRs).

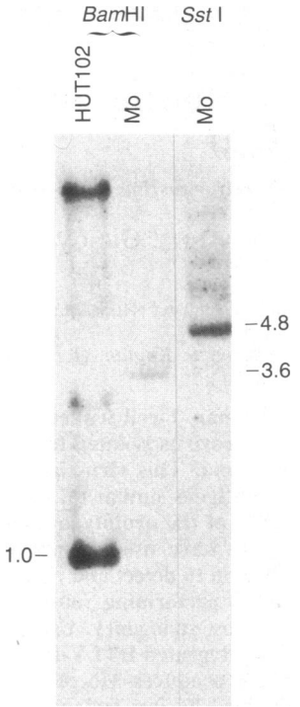

FIG. 2. Detection of $HTLV\text{-}II_{Mo}$ provirus with pCR_{CH}. pCR_{CH} was hybridized under nonstringent conditions to Southern blots containing the DNAs indicated. HUT102 is an HTLV-I producer cell line that, when digested with *Bam*HI, contains the internal 1.05-kb fragment. *Bam*HI and *Sst* I digestion of Mo DNA cleave internal bands as shown. Fragment lengths are in kb.

Cloning of the HTLV-II Provirus. Since we were able to use pCR_{CH} as a hybridization probe to detect $HTLV\text{-}II_{Mo}$, we used this probe to clone integrated $HTLV\text{-}II_{Mo}$ proviral sequences. A genomic library of Mo DNA in λ-Jl was screened and washed under nonstringent conditions. One clone was isolated. Using HTLV-I probes pCR_{CH} and $pCR_{5\text{-}1}$, a 600-nucleotide clone of the LTR R and U_5 regions with adjacent 5' sequences (23), we mapped by nonstringent hybridization homologous viral sequences on this $HTLV\text{-}II_{Mo}$ cloned genomic fragment. The restriction map of this clone, λ-Mo1A, with the highlighted viral sequence, is shown in Fig. 3*A*. The 18-kb insert contained approximately 4 kb of viral DNA flanked on both sides by cellular sequences. Different restriction fragments of the clone could be detected with the HTLV-I *env–pX* and LTR probes, allowing us to orient the partial provirus in a 3'→5' direction within the clone.

The 3.6-kb *Bam*HI fragment encompassing most of the sequences homologous to pCR_{CH} was inserted into the *Bam*HI site of pBR322. This probe, named pMo1A, was used to screen the Mo genomic library and detected two additional phage plaques that represented independent clones of complete integrated $HTLV\text{-}II_{Mo}$ provirus. Fig. 3*B* shows restriction enzyme maps of these two clones. λ-Mo15A contained a 13.5-kb insert and λMo15B contained a 20.2-kb insert. The 3.6-kb *Bam*HI fragment of pMo1A-A was readily identified in both clones. Both clones had a common 8.6-kb region bordered on the left by a *Bam*HI site and on the right by an *Sst* I site. These matching sequences represented the $HTLV\text{-}II_{Mo}$

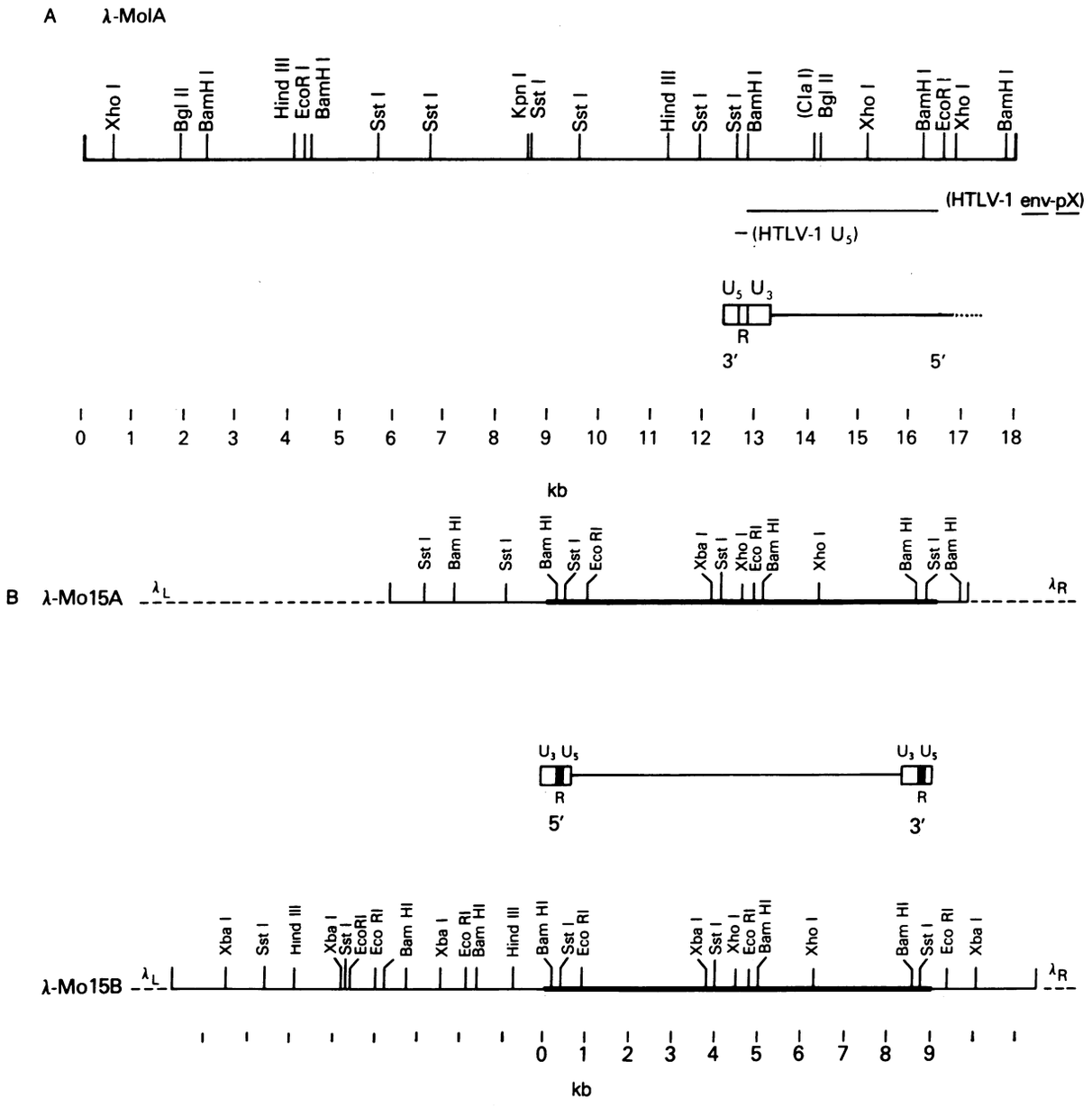

FIG. 3. (*A*) Restriction map and location of proviral sequences in λ-Mo1A. The 18-kb cellular insert was mapped with the enzymes shown. pCR_{CH} and $pCR_{5\text{-}1}$ (see text) were used as probes to detect viral sequences and determine orientation. Fragments that hybridized to the specific probes are indicated by the short lines on the right. A map of a partial provirus is drawn below and is inferred from the hybridization data. The *Cla* I site within the proviral DNA is in parentheses because other *Cla* I sites in the clone outside the viral DNA were not mapped. (*B*) Restriction maps of two complete proviral genomes selected by screening the Mo genomic library with pMo1A-A. λ-Mo15A contains a 13.5-kb insert within which the viral genome is oriented 5′→3′ within the λ-Jl phage arms. λ-Mo15B contains a 20.2-kb insert also with a 5′→3′ orientation of the viral genome. The two maps are drawn so as to align the identical viral genomes. The schematic drawing between the two maps is derived as described in the text. The total lengths of the LTRs and the viral genome are approximate.

provirus integrated at two different locations since there were no common restriction sites in the cellular flanking sequences of the two clones. The position of the far right and left *Bam*HI and *Sst* I sites in the viral genome suggested that they were in the LTRs. This was proved by the mapping with the HTLV-I U_5 probe in Fig. 3*A* and by hybridizing λ-Mo15A and -B with pMo1A-A. In Southern blots of the cloned DNA the plasmid pMo1A-A always hybridized to viral restriction fragments with which it overlapped and also to the fragments that contained the left-hand virus–cell junction. Thus pMo1A-A contained sequences present at both ends of the viral genome. The combined mapping data allowed us to predict that *Bam*HI cleaves in the U_3 region of the LTR and *Sst* I cleaves in the R or U_5 region. We have drawn a schematic viral genome in Fig. 3*B* that outlines this conclusion. The size of the LTR was chosen by analogy to HTLV-I and may not be accurate. The HTLV-II viral genome is 8.6–9.0 kb in length, depending on the exact size of the LTR.

$HTLV\text{-}II_{Mo}$ Sequences Are Unique to That Virus. To confirm that these cloned sequences represented a unique viral genome, we hybridized pMo1A-A to genomic DNA and cellular RNA of infected and uninfected cells. When pMo1A-A was used as a probe against a Southern blot of cellular DNAs and the hybridized product was melted under stringent conditions, the probe did not hybridize to uninfected cellular DNA or to HTLV-I-infected cellular DNA (Fig. 4). In Mo DNA the plasmid detected the 4.8-kb internal *Sst* I fragment, but this restriction fragment was not seen in the λ-Mo1A clone since the 5′ *Sst* I site bordering this fragment was ab-

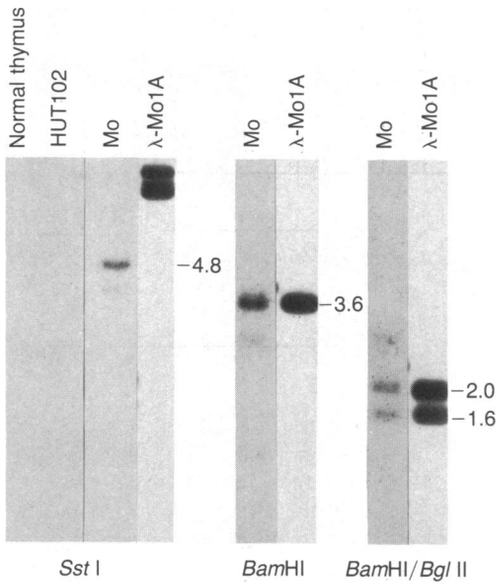

FIG. 4. Hybridization of pMo1A-A to infected and uninfected cellular DNA. pMo1A-A, the *Bam*HI viral subclone of λMo1A, was hybridized under stringent conditions to the DNAs shown.

sent. The pMo1A-A plasmid hybridized to its homologue 3.6-kb *Bam*HI fragment in both Mo DNA and the λ-Mo1A clone. This internal viral restriction fragment is cleaved by *Bgl* II in both cases as seen in the *Bgl* II/*Bam*HI double digests of Fig. 4. The pMo1A-A probe was also hybridized to a blot of RNA from HTLV-I-infected cells and Mo (Fig. 5). The HTLV-I producer lines used represented viral isolates from two different regions, the United States and Israel. The hybridization showed that the pMo1A-A probe was specific for HTLV-II_{Mo} viral mRNA. The 3′ genomic fragment was transcribed in the genomic-length 9-kb mRNA and in a subgenomic 4.2-kb message. The two lanes of Mo RNA represent 5 μg and 25 μg, respectively; the larger amount was used to detect any minor message-size classes.

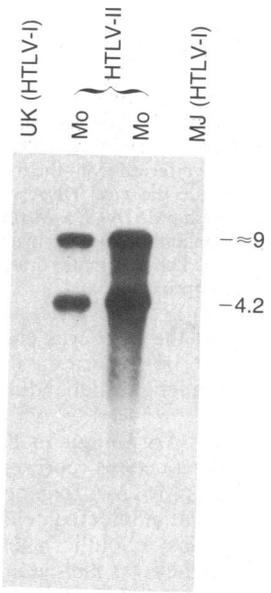

FIG. 5. Hybridization of pMo1A-A to HTLV-I and HTLV-II_{Mo} RNA. Poly(A)-selected cellular RNA (5 μg) was electrophoresed, blotted, and hybridized to the viral probe. The second lane of Mo RNA contained 25 μg.

DISCUSSION

We have described the cloning of the HTLV-II_{Mo} provirus, a unique human retrovirus that we were able to detect by using a heterologous nucleic acid hybridization probe from HTLV-I. The homology between the two viruses was sufficient to allow us to clone HTLV-II_{Mo} proviral sequences from a genomic library of the Mo producer cell line. HTLV-II_{Mo} differs in its restriction map from all known isolates of HTLV-I and the two viruses cross-hybridize only under nonstringent conditions. Previous antigenic comparison between HTLV-I and HTLV-II_{Mo} gag proteins showed that these viruses share group-specific but not type-specific determinants. Thus, HTLV-I and HTLV-II belong to a group of retroviruses that may, in time, include other agents. A possible candidate for a retrovirus related to this group is the bovine leukemia virus (BLV). HTLV-I has been shown to share homologous gag sequences with BLV (ref. 13 and unpublished observations).

The homology between these viruses has facilitated our derivation of the HTLV-II_{Mo} genomic map. By using probes specific for an internal coding region of HTLV-I and the R and U_5 regions of the HTLV-I LTR, we were able to identify fragments in the HTLV-II_{Mo} clones that contained homologous sequences and thus assign tentative map locations to these fragments. The fortuitous inclusion of HTLV-II_{Mo} LTR sequences in pMo1A-A made this probe useful for identifying the extreme ends of the proviral genome. Thus we were able to identify tentatively the limits of the viral genome, the LTRs, and the orientation of the genomic map. The regions of cellular DNA flanking the two complete and one partial provirus in the clones show no overlap in their restriction enzyme maps. Without the availability of fresh uncultured leukemia cells we were unable to determine whether any of these integration sites were present in the original malignant cells. As we have observed with HTLV-I producer cell lines, new sites of viral integration may appear after prolonged passage of infected leukemic cells in culture (unpublished observations). Since the genomic integration site may have pathogenic relevance via several mechanisms, including transcriptional activation of cellular sequences by viral promoter insertion, it is important to analyze the flanking cellular regions of the clones for evidence of transcription to mRNA.

Although we have shown substantial structural differences between HTLV-I and HTLV-II_{Mo}, there is remarkable similarity in their T-cell tropism and in the effect they have on cultured T lymphocytes. Both viruses have been isolated from mature T cells and are able to immortalize cord blood T cells after cocultivation with infected cell lines. Furthermore, infection with either virus causes similar morphologic changes in T cells, induction of the cellular receptor for T-cell growth factor (TCGF), and decreased TCGF requirement for cell proliferation (refs. 5 and 19 and unpublished observations). In contrast to the *in vitro* similarities, the two viruses are associated with diseases that have different clinical manifestations. The comparison is of course limited by the fact that only one individual, Mo, has been identified as having HTLV-II infection. However, HTLV-I disease is usually a fulminant acute leukemia often associated with lytic bone lesions, hypercalcemia, and a predisposition for leukemic involvement of the skin (20). The clinical spectrum includes a few patients with mature T-cell malignancies closely resembling the cutaneous T-cell lymphomas (2). Mo has a disease very much resembling hairy cell leukemia, usually a chronic leukemia characterized by a proliferation of malignant T cells with involvement of bone marrow, spleen, and liver (21). Only an extensive survey of patients with similar disorders will reveal if HTLV-II, a unique virus, can be associated with a distinct clinical entity.

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