single action potential, but also makes it possible to separate and measure the contributions of various Ca^{2+} transport systems to generation of muscle tension.

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16. In Fig. 1, A to C and G, the weighted average is $\Sigma = 0.20\ (\Delta I_{600}/I_{600}) - (\Delta I_{718}/I_{718}) + 0.85\ (\Delta I_{788}/I_{788})$, where $\Delta I/I$ is the relative change in light intensity and subscripts are wavelengths in nanometers. The weight chosen for the Ca^{2+} signal (in this case, 718 nm) is −1. The two other weights are calculated by making the simplifying assumption that the contraction artifact of the perfusate or the tissue at any wavelength is proportional to their respective absorptions. Thus, at 600 nm the weight is 1/5 = 0.2 since the dye absorption is five times larger than at 718 nm. At 788 nm the dye is transparent and a weight of 0.85 is calculated to compensate for tissue motion at the two other wavelengths (tissue absorption is roughly proportional to the inverse square of the wavelengths). Different weights were used when the wavelengths or the composition of the perfusate were altered.
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Expression of the 3′ Terminal Region of Human T-Cell Leukemia Viruses

Abstract. *Human T-cell leukemia viruses (HTLV) are closely associated with some human T-cell leukemias and lymphomas. A unique 3′ region of the HTLV genome is believed to be involved in HTLV-induced cellular transformation, although the function of this region has yet to be determined. A subgenomic messenger RNA transcribed from this region of HTLV has now been characterized. These results provide direct evidence for the expression of a novel gene in HTLV.*

The human T-cell leukemia viruses (HTLV) types I and II are lymphotropic retroviruses that have been isolated from patients with specific T-cell malignancies (*1–4*). Infection of normal leukocytes by HTLV-I or HTLV-II in vitro results in the transformation of mature T cells, as shown by their continued proliferation (*5–8*). The mechanism of HTLV-induced leukemogenesis remains obscure. No known viral oncogene has been identified in HTLV. However, analyses of HTLV-I and HTLV-II nucleic acid sequences have identified a region, termed X, whose function is, as yet, unknown (*9–11*). The X region is bounded by *env* and the 3′ terminal repeat (LTR), is approximately 1.6 kilobase pairs long, and contains several open reading frames. The conservation of the X sequence in both HTLV-I and HTLV-II suggests that it encodes a protein that may play a critical role in HTLV replication or HTLV-induced cellular transformation, or both.

The HTLV-II–infected Mo-T cell line was established by primary culture of splenic tissue from a patient with a T-cell variant hairy cell leukemia (*12, 13*). Mo-T cell RNA was assayed for the presence of X-specific sequences by the hybridization–S_1 nuclease technique. Nucleic acid sequence analyses of the HTLV X region revealed three open reading frames in HTLV-II (X-a to X-c) and four open reading frames in HTLV-I (X-I to X-IV) (*9–11*). Three of the open reading frames in HTLV-I are homologous with those in HTLV-II, namely, X-II with X-a, X-III with X-b, and X-IV with X-c. Comparison of nucleic acid sequences in the X region of HTLV-I and HTLV-II reveals only 33 percent homology in the first third of the sequences and an abrupt shift to 75 percent homology in the 3′ two-thirds, where the homologous open reading frames are located (*10, 11*). The Hha I–Cla I probe prepared from cloned HTLV-II DNA (see Fig. 1B) is a 431–base-pair fragment that spans the junc-

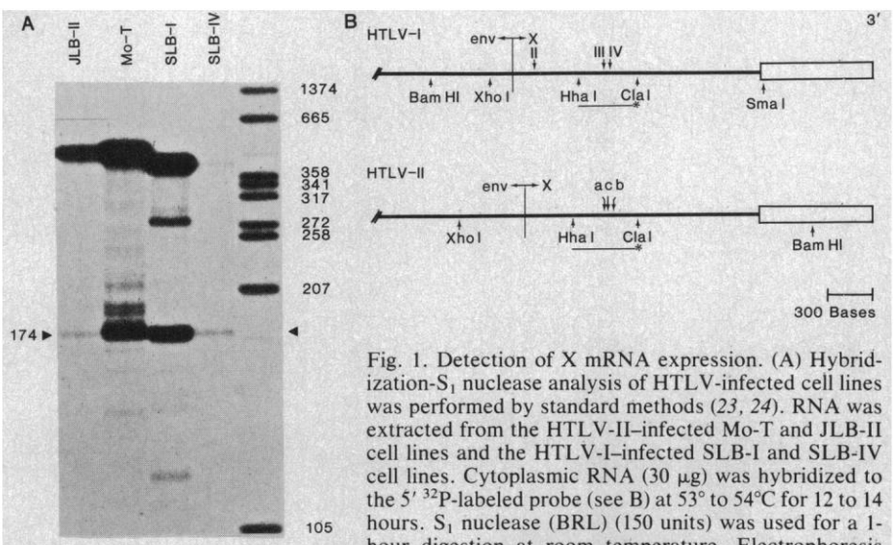


Fig. 1. Detection of X mRNA expression. (A) Hybridization-S_1 nuclease analysis of HTLV-infected cell lines was performed by standard methods (*23, 24*). RNA was extracted from the HTLV-II–infected Mo-T and JLB-II cell lines and the HTLV-I–infected SLB-I and SLB-IV cell lines. Cytoplasmic RNA (30 μg) was hybridized to the 5′ ^{32}P-labeled probe (see B) at 53° to 54°C for 12 to 14 hours. S_1 nuclease (BRL) (150 units) was used for a 1-hour digestion at room temperature. Electrophoresis was performed on an 8 percent acrylamide–8*M* urea sequencing gel. The size of the marker DNA fragments is shown in nucleotides. The 174-base fragment is indicated by the arrow. Control S_1 nuclease studies with RNA from uninfected cells and either HTLV probe reveal no protected DNA fragment (data not shown). (B) Partial restriction enzyme map of the pHT-1 HTLV-I and pH-6 HTLV-II DNA clones. The 3′ long terminal repeat is shown by an open box. The division between X and *env* sequences is indicated. The 5′ ends of the open reading frames are shown for HTLV-I X-II, X-III, and X-IV and for HTLV-II X-a, X-b, and X-c. The Hha I–Cla I hybridization probe utilized for S_1 nuclease analysis is shown as a thin line below the map with an asterisk denoting the site of 5′ ^{32}P-labeling. The sizes of the HTLV-I pHT-1 and HTLV-II pH-6 probes are 396 and 431 nucleotides, respectively.

tion between the high- and low-homology X sequences. Figure 1A shows the DNA fragments protected by hybridization to Mo-T cell RNA. A band indicative of a 174-nucleotide protected fragment is observed. This fragment terminates close to the 5′ end of the X-c open reading frame near the junction between sequences of low and high homology. The large protected fragment of 431 nucleotides corresponds to hybridization of full-length probe with either genomic

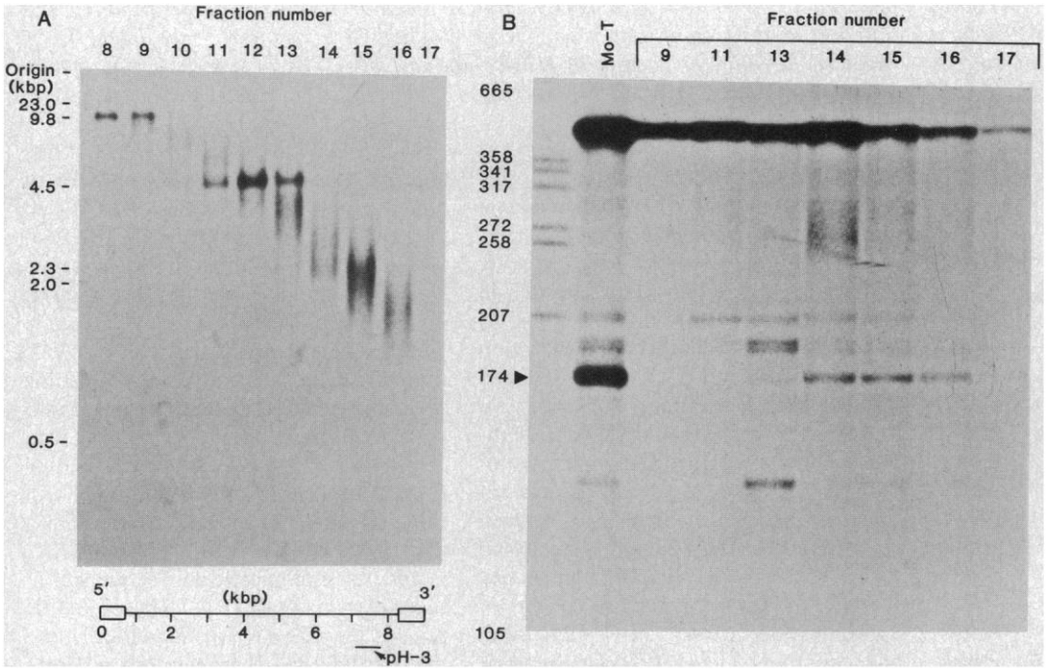
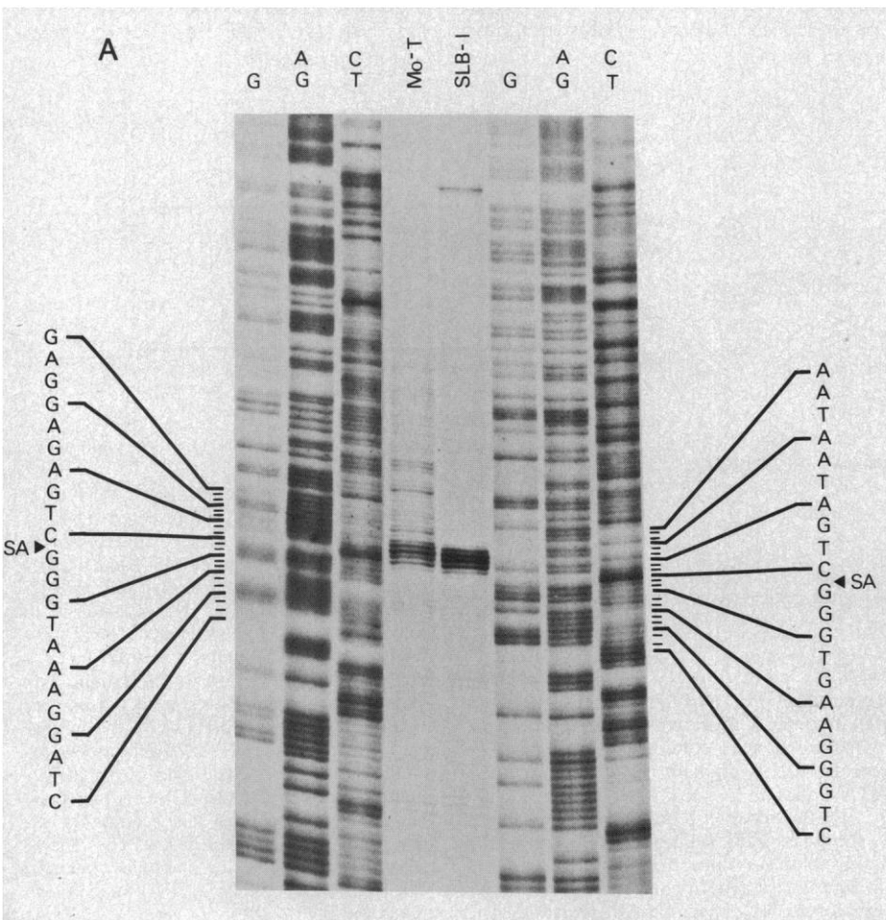


Fig. 2. Determination of X mRNA size. Poly(A)$^+$ cytoplasmic RNA from Mo-T cells (150 μg) was sedimented on a 15 to 30 percent sucrose density gradient in a Beckman SW41 rotor. Twenty-three fractions were collected. (A) Northern hybridization of 10-μl portions of selected gradient RNA fractions was performed on a formaldehyde gel by standard methods (*25*). The nick-translated hybridization probe was prepared from the HTLV-II pH-3 subclone, which is shown diagramatically below the HTLV-II genome. The molecular size markers are indicated in kilobase pairs. (B) S_1 nuclease analysis of 30-μl portions of selected gradient RNA fractions. The methods are as described in Fig. 1A with the following modifications: 18 μg of L-cell RNA was added to each S_1 assay before the hybridization step and the amount of S_1 nuclease was reduced to 100 units. Control S_1 nuclease assay of uninfected Mo-T RNA is shown in parallel. The molecular size markers are shown in nucleotides. The protected fragment of 174 nucleotides is indicated.



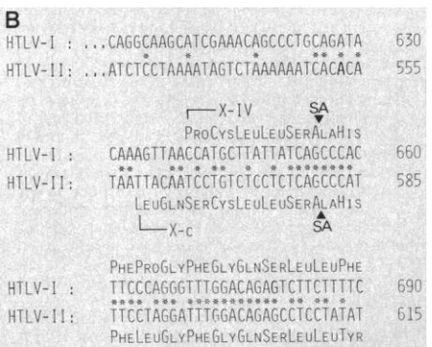


Fig. 3. Localization of the splice acceptor sequence. (A) The HTLV-II and HTLV-I Hha I–Cla I probe (see Fig. 1B) was sequenced by the method of Maxam-Gilbert (*26*). Electrophoresis was performed on an 8 percent acrylamide–8*M* urea sequencing gel in parallel with the respective S_1 analysis of HTLV-II (Mo-T) or HTLV-I (SLB-I) RNA (see Fig. 1A for details). The sequence of the DNA probe near the terminus of the 174-nucleotide protected fragment is shown. The sequence shown is complementary to the viral RNA. The position of the splice acceptor site (SA) is indicated. (B) Nucleic acid sequence of a portion of the X region showing the position of the X splice acceptor site (SA) at base 655 in HTLV-I and base 580 in HTLV-II, the nucleic acid sequence being numbered from the 5′ end of the respective X regions (*11*). Asterisks denote HTLV-I and HTLV-II nucleic acid sequence homology. The start of the predicted amino acid sequence for the HTLV-I X-IV and the HTLV-II X-c open reading frames is shown.

HTLV-II RNA or a subgenomic messenger RNA (mRNA) species, such as *env*, that would encompass the entire probe. Control S_1 nuclease studies with RNA from uninfected cell lines did not reveal any protected fragments (data not shown).

JLB-II is an HTLV-II–transformed T-cell line, derived by cocultivation of lethally irradiated Mo-T cells with normal human leukocytes (*8, 14, 15*). In contrast to Mo-T, which harbors multiple proviruses that differ in nucleic acid sequence (*11, 14*), JLB-II contains only the wild-type, replication-competent HTLV-II genome (data not shown). The protected fragments at 174 and 431 nucleotides are again observed in the S_1 nuclease assay with JLB-II RNA. These data suggest that a potential splice acceptor site is located at approximately 174 nucleotides upstream of the Cla I site. The other minor bands in Mo-T cells may be due to variant proviruses or to nonspecific cleavage of the hybrid.

Because of the high homology between the nucleic acid sequences of HTLV-I and HTLV-II in the distal two-thirds of the X region, an X splice acceptor site should also be present in an analogous position of the HTLV-I genome. The Cla I restriction enzyme site is conserved in homologous sequences of HTLV-I and HTLV-II (Fig. 1B). Thus, we prepared a 396-nucleotide probe utilizing the Cla I site in HTLV-I, allowing a direct comparison of the S_1 mapping data.

RNA was extracted from the SLB-I cell line, which was derived by infection of normal human leukocytes with HTLV-I in vitro (*15, 16*). As in the S_1 nuclease assay of HTLV-II RNA, a fragment that is virtually coincident with the 174-nucleotide HTLV-II fragment is protected in the HTLV-I system (Fig. 1A). Analysis of RNA from the HTLV-I–infected SLB-IV cell line resulted in a similar pattern of hybridization.

Taken together, the identity of the S_1 nuclease–resistant fragment seen with HTLV-I and HTLV-II RNA suggests that the X sequences are transcribed into a processed mRNA species. To confirm the presence and determine the size of the subgenomic mRNA predicted from the S_1 nuclease analysis, we performed further studies with size-fractionated polyadenylated [poly(A)$^+$] Mo-T cell RNA (Fig. 2). Northern hybridization data reveal three major species of RNA (Fig. 2A). The 9-kb band in fractions 8 and 9 is the size expected for genomic HTLV-II RNA. The 4.5-kb band in fractions 11 to 13 has been observed with probes specific for the *env* region of HTLV-II (data not shown). The third band, most prominent in fraction 15, is 2.0 to 2.3 kb in size. S_1 nuclease analysis of the fractions used for Northern hybridization demonstrate that the 2.0- to 2.3-kb species corresponds to the fractions containing the protected 174-nucleotide fragment (Fig. 2B).

Analysis of the HTLV-I and HTLV-II nucleic acid sequences in the vicinity of the splice acceptor site identified above reveals two potential consensus splice acceptor sites (*10, 11*). Each probe was sequenced by the method of Maxam-Gilbert and subjected to electrophoresis in parallel with the respective S_1 nuclease–resistant fragments. The data (Fig. 3) reveal the splice acceptor site in HTLV-I to be ACTCAG/CCCA at base pair 655 and in HTLV-II to be CTCAG/CCCA at base pair 580, consistent with consensus sequences for mRNA splice acceptor sites (*17*).

These results are direct evidence for expression of the X region in HTLV. The high homology of HTLV-I and HTLV-II distal to the splice acceptor site (Fig. 3) makes it likely that these sequences encode an X-derived protein. On the basis of the position of the splice site, we predict that these X-encoded products will be from the longest open reading frame, X-IV and X-c in HTLV-I and HTLV-II, respectively. Although other reading frames cannot be excluded, the identification of 40- and 37-kilodalton proteins encoded by X-IV and X-c, respectively, are consistent with this interpretation (*18*).

The location of the first methionine start codons in the X-IV and X-c open reading frames are more than 300 nucleotides downstream from the splice acceptor site (*9–11*), which would be expected to occur nearly midway in the 2- to 2.3-kb X mRNA. Consequently, we believe that the true methionine start codon for both the HTLV-I and HTLV-II X mRNA will be derived from the upstream donor sequencs. Primer extension analyses can be used to determine these leader sequences and identify the methionine start codon for the X message.

HTLV-I and HTLV-II are atypical among replication-competent retroviruses in both their functional and structural features. These viruses have a limited host range (*8, 19, 20*) and exclusively transform T cells. They do not contain known viral oncogenes, nor are they found to be specifically integrated near cellular oncogenes in tumors or transformed cells (*21, 22*). The HTLV-I and HTLV-II genomes are distinguished by the presence of the X sequences. It is likely that the protein encoded by these sequences is related to the unusual biology of HTLV. The expression of these sequences as a unique subgenomic mRNA species supports the classification of these sequences as a new gene distinct from other genes of naturally occurring retroviruses.

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