ening of overall open times to the increase in the probability of large conductance events, which usually have short open times. However, there is another important factor contributing to the observed decrease in the mean open times. As shown in Fig. 2, E and F, the open times of the small conductance events alone are much longer at stage 24 (mean, 15.5 msec) than at stage 45 (mean, 5.3 msec).

The pooled results of several experiments (Fig. 3) indicate an approximately threefold reduction in the mean open time of the small conductance channels between stages 21 to 25 and stage 45. In contrast, the mean open time of the large conductance class does not appear to change during development. Other physiological characteristics, namely, single-channel conductance (determined from the differences of mean currents at different voltages, usually over the voltage range between resting potential and a 50-mV hyperpolarization), reversal potential, and voltage dependence of channel open time for both classes, do not appear to be influenced by development.

The observed shortening of open time is probably not the result of changes in resting membrane potential during development because (i) the reversal potential measured from the resting level did not change during development, (ii) the change in open time was in the opposite direction from what would be predicted by an increase in membrane potential, and (iii) the open time of only the small conductance events was affected.

In previous studies of AChR development in cultured *Xenopus* muscle a redistribution of receptor populations was reported (*3, 5*), but not a direct influence of development on the channel open times of either conductance class. One possible explanation for this is that most of the change in open time may have occurred before the time of the earliest recordings made by other investigators. Our evidence suggests that, after 1 day in culture, the mean open time of the small conducting channels (11.4 ± 1.9 msec at −50 mV; $n = 3$ cells) is already 50 percent shorter than that at stages 21 to 25.

Our results demonstrate a remarkable decrease in the open time of AChR channels during the earliest stage of development in the absence of innervation. This is caused partly by the redistribution of the receptor population from one class to the other (a mechanism observed previously), and partly as the result of a shortening of the open time of one class of receptor (a new mechanism). We do not know whether these changes are due to modification of existing receptor molecules or to replacement by new receptor molecules during development. Nor do we know whether there is more than one class of small conductance events. The observation that only one of the two classes is affected reduces the possibility of a nonspecific effect of membrane maturation.

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Antigens Encoded by the 3′-Terminal Region of Human T-Cell Leukemia Virus: Evidence for a Functional Gene

Abstract. *Antibodies in sera from patients with adult T-cell leukemia-lymphoma or from healthy carriers of type I human T-cell leukemia virus (HTLV) recognize an antigen of approximately 42 kilodaltons (p42) in cell lines infected with HTLV-I. Radiolabel sequence analysis of cyanogen bromide fragments of p42 led to the conclusion that this antigen is encoded in part by LOR, a conserved portion of the "X" region that is flanked by the envelope gene and the 3′ long terminal repeat of HTLV-I. It is possible that this novel product mediates the unique transformation properties of the HTLV family.*

The human T-cell leukemia viruses (HTLV) are a family of exogenous human retroviruses with three known types (*1, 2*). HTLV type I (HTLV-I) is etiologically associated with adult T-cell leukemia-lymphoma (ATLL) (*2, 3*). HTLV type II (HTLV-II) was isolated from a patient with a T-cell variant of hairy cell leukemia (*4*). HTLV type III (HTLV-III) refers to prototype virus isolated from patients with acquired immune deficiency syndrome (*5*).

HTLV-I and HTLV-II have several unusual features that distinguish them from the replication-competent retroviruses of mice and chickens. These include lack of chronic viremia in infected individuals, absence of common proviral integration sites in tumors (*6*), *trans*-activation of HTLV long terminal repeat (LTR)–directed transcription in infected cells (*7*), and ability to immortalize T cells in vitro (*8*). In addition to the *gag*, *pol*, and *env* genes of animal retroviruses, the HTLV genome contains a 1.5-kilobase region, initially described as the "X" region, and located between the *env* gene and the 3′ LTR (*9*). Sequence comparisons of this region between HTLV-I and HTLV-II demonstrate that it can be divided into a 5′ nonconserved region and a 3′ highly conserved region designated LOR (*10*).

In a previous study, serum samples from adult T-cell leukemia-lymphoma patients and from healthy carriers living in the HTLV-I endemic area of Japan were examined for the presence of antibodies to HTLV-associated membrane antigen (HTLV-MA). We reported that HTLV-specific antigens detected in an HTLV-I–infected tumor cell line, Hut 102, could be grouped into three categories (*11*). These included unglycosylated antigens encoded by the *gag* gene, glycosylated antigens encoded by the *env* gene, and an unglycosylated 42-kilodalton species (p42) whose coding origin

was unknown. Antigens sharing biochemical and immunological properties with the *gag* antigens and *env* antigens of Hut 102 cells were also expressed in four other HTLV-I–infected cell lines, although antigens encoded by the *env* gene varied somewhat in size and number in different cells, and the unglycosylated antigens of the p42 class exhibited slight variations in size (*11, 12*). Since p42 did not appear to be recognized products of the *gag* or *env* genes, we investigated the possibility that it might be encoded by the "X" region of HTLV. Using radiolabel sequence analysis (*13*), we found that a portion of the p42 protein appears to be encoded by LOR.

To analyze the generality of p42 expression, we used a competition assay in which 200 times more nonlabeled cell lysate from different cell lines was used to compete with [^{35}S]cysteine-labeled p42 of Hut 102 for specific antibody. As shown in Fig. 1A, antigens that were serologically related to p42 of Hut 102 appeared to be expressed in MT2, MJ, C5/MJ, and C91/PL cell lines (lanes 7 to 10) and in a nonproducer cell line, C81-66-45 (lane 11). The C81-66-45 cell line, also designated C63/CR$_{II}$-4, was defined as nonproducer by its lack of expression of *gag* antigens and the absence of reverse transcriptase activity and viral particles in the spent culture media (*14*). Lymphoid cell lines of different lineage and different stages of differentiation, but not infected with HTLV (lanes 3 to 6), did not complete with p42, thus reaffirming the HTLV-specific nature of this antigen.

Further studies of the p42 protein were done with the C81-66-45 cell line. Immunoprecipitation of [^{35}S]cysteine-labeled proteins with sera from people infected with HTLV-I reveals that a 42-kD antigen is the only major HTLV-specific antigen readily detected in this cell line. This is consistent with the observation that extracts of C81-66-45 cells compete for p42 but not for HTLV *gag*- or *env*-encoded proteins (Fig. 1A). When total cell lysate was applied to a lentil lectin–Sepharose 4B column, the p42 species was detected in the effluent (Fig. 1B) but not in the eluent (data not shown). Sera from individuals who do not have antibodies to HTLV-MA could not precipitate the p42 antigen from C81-66-45 (Fig. 1B) or from other HTLV-producer cell lines (*11, 12*).

To determine the coding sequence of p42, we labeled C81-66-45 cells with selected amino acids. The radiolabeled p42 was isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to automated Edman degradation to determine the NH_2-terminal protein sequence. For this purpose, 30×10^6 to 40×10^6 cells were metabolically labeled for 10 to 12 hours with either 5 mCi of [^{35}S]cysteine plus 10 mCi of [^{3}H]serine or 5 mCi of [^{35}S]cysteine plus 10 mCi of [^{3}H]alanine, or 5 mCi of [^{35}S]cysteine plus 10 mCi of [^{3}H]proline, in 25 ml of RPMI 1640 medium with 15 percent fetal bovine serum and depleted with the appropriate amino acids. Radiolabeled cell lysates prepared as described earlier (*11*) were precipitated with a reference human serum known to have antibody to p42. The detailed procedures for immunoprecipitation, electroelution, dialysis, and automated protein sequence analysis were described earlier (*11, 13*). In three trials, no radiolabeled amino acids were detected in the first 35 degradation cycles, indicating that either the NH_2-terminus of p42 was inaccessible to Edman degradation or there were no cysteine, serine, proline, and alanine residues present in the first 35 NH_2-terminal residues of p42.

To obtain protein sequence information for p42, we subsequently prepared cyanogen bromide (CNBr) fragments from radiolabeled p42 for protein sequence analysis. For this purpose, p42 was isolated from C81-66-45 cells which were metabolically labeled with [^{35}S]cysteine, [^{3}H]leucine, [^{3}H]isoleucine, and [^{3}H]lysine (using the amounts and procedures listed above for the other amino acids). Radiolabeled p42 in 0.5 ml of 70 percent formic acid was cleaved with 0.01 mg of CNBr for 24 hours at room temperature. Cyanogen bromide–cleaved fragments of p42 were separated by Sephacryl S-200 chromatography (Fig. 2A). Five pooled fractions, A to E, of estimated size 15, 12, 10, 3, and 1 kD, respectively, were obtained from this separation. The results of automated Edman degradation for pools C to E are presented in Table 1. As shown in Table 2, at least five CNBr fragments, designated CNBr-1 to CNBr-5, can be expected for the CNBr cleavage products encoded by the LOR region of HTLV-I (*10*). Comparison of radioactive protein sequence data with the deduced amino acid sequence for the LOR region reveals that pool E contains the CNBr-2 fragment, pool D contains CNBr-3 fragment, and pool C contains both CNBr-4 and CNBr-5 fragments. Repetitive yield analysis indicated that the amounts of the two CNBr fragments in pool C occurred at a ratio of 4 to 1. Sequence analysis of the NH_2-terminal positions of pool A yielded no radioactive peaks in the first 18 degradation cycles. Sequence analysis of pool B suggested that it con-

Table 1. Amino terminal radiosequence of CNBr fragments of p42. The probabilities of having the observed sequence to occur by chance alone are: 5.3×10^{-14} (pool C), 8.0×10^{-11} (pool D), 6.3×10^{-3} (pool E), and 2.5×10^{-12} (pool 1). All assignments were made by HPLC analysis of PTH amino acid derivatives (*13*). The one-letter designations for the amino acids are I, isoleucine; C, cysteine; K, lysine; L, leucine; and S, serine.

Chromatography	Pooled fractions	Position of radiolabeled amino acid																														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
S-200	Pool C (major)*	I				C	K							L		L																							
S-200	Pool C (minor)		L			L				I				(L)†	(L)†				I		C																	
S-200	Pool D				L				L			L							L					L			L											
S-200	Pool E		K																																			
RPLC	Pool 1		S			C							S					S	S	S					S								S			S	

*The major sequence was present at four times the level of the minor sequence. †Although leucine residues were anticipated in these positions (see Table 2), definitive assignments could not be made because of the presence of the leucine residue at positions 13 and 15 in the major sequence.

tained a mixture of CNBr fragments. Although not enough radioactivity was present for high-performance liquid chromatographic (HPLC) analyses of the phenylthiohydantoin (PTH) amino acid derivatives, radioactive peaks occurred at positions where amino acids were identified in pools C to E, suggesting that pool B contained partial cleavage products.

The CNBr cleavage products of radiolabeled p42 were also separated by reverse phase liquid chromatography (RPLC). The profile of RPLC separation of [^{35}S]cysteine- and [^{3}H]serine-labeled

Table 2. Amino terminal protein sequence for the predicted CNBr-cleavage products encoded by the LOR region of HTLV. The one-letter symbols for the amino acids are: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; and V, valine.

CNBr-1 NH_2	——— P† C L L S A H F P G‡ F G Q S L L F G Y P V Y V F G N C V
CNBr-2	R K* Y S P F R N G Y M
CNBr-3	E P T L* G Q H L* P T L* S F P D P G L* R P Q N L* Y T L* W G G S V V C M
CNBr-4	Y L* Y Q L* S P P I* T W P L L P H V I* F* C H P G Q L G A F L T N V P Y
CNBr-5	I* S G P C* P K* D G Q P S* L* V L* Q S* S* S* F I F H K F Q T K A
	Y H P S* F L L S* H G

*Residues determined by radiolabel sequence analysis. †Corresponding to the nucleotide sequence 7286 to 7288 in (9). ‡A consensus splice acceptor site lies in the nucleotide sequence bearing this residue.

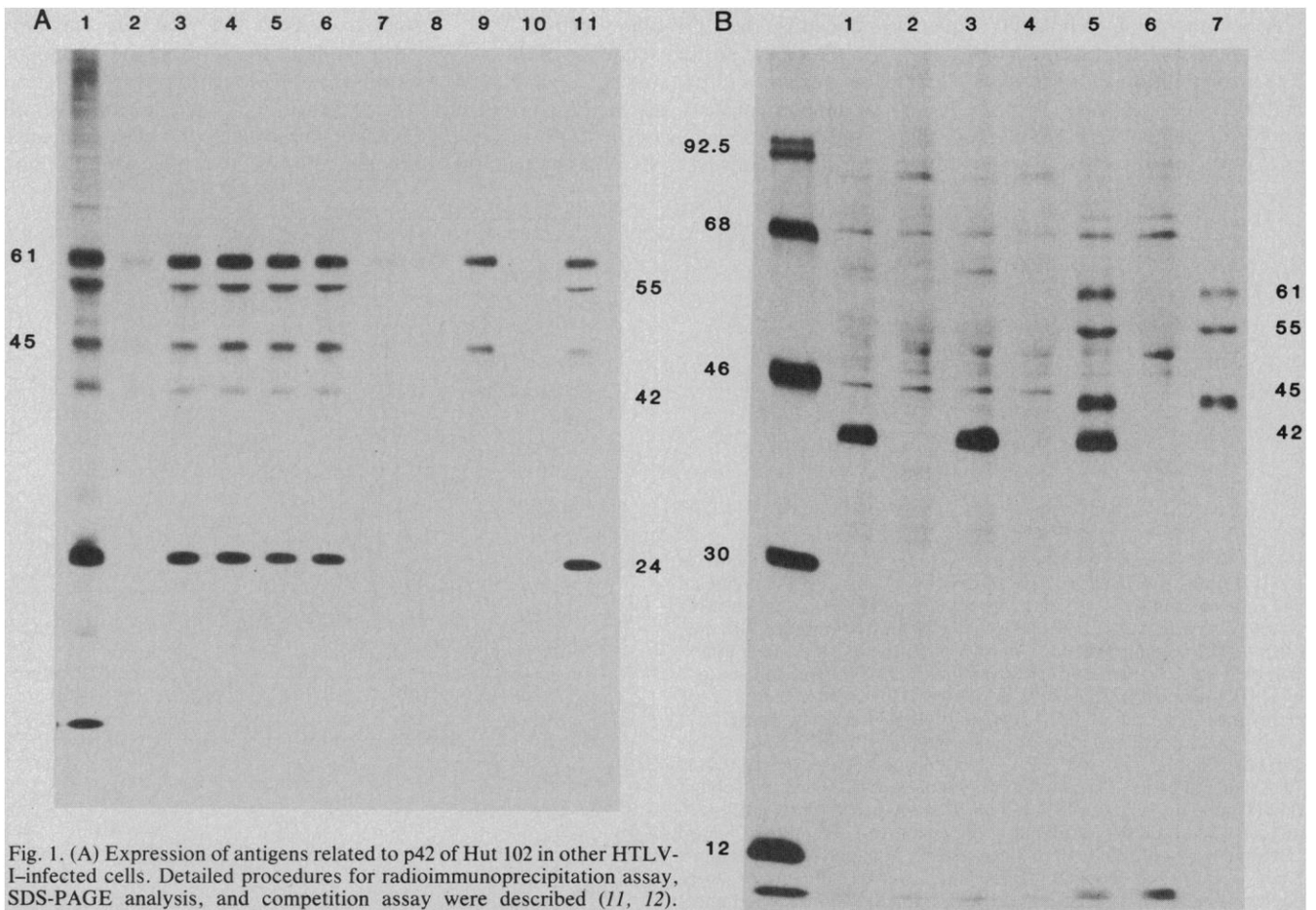

Fig. 1. (A) Expression of antigens related to p42 of Hut 102 in other HTLV-I–infected cells. Detailed procedures for radioimmunoprecipitation assay, SDS-PAGE analysis, and competition assay were described (*11, 12*). Briefly, 5 μl of reference serum known to have antibody to p42 from a Japanese healthy carrier was preabsorbed to protein A–Sepharose 4B. Approximately 100 × 10^6 cells equivalent of nonradiolabeled cell lysates from the following cell lines, Hut 102 (lane 2), T8402 (lane 3), NC37 (lane 4), Jurkat (lane 5), Hut 78 (lane 6), MT2 (lane 7), MJ (lane 8), C5/MJ (lane 9), C91/pL (lane 10), C81-66-45 (lane 11), and phosphate-buffered saline (lane 1) were incubated with the positive reference serum absorbed to protein A–Sepharose 4B for 1 hour at 4°C before 0.5 × 10^6 cells equivalent of [^{35}S]cysteine-labeled cell lysate from Hut 102 was added. After 1 to 4 hours of incubation with intermittent gentle swirling, antigen-antibody complexes were washed and then eluted from immunoprecipitates by boiling for 2 minutes; they were analyzed in a 11.5 percent SDS–polyacrylamide gel. (B) Detection of p42 in C81-66-45 cells. Radioimmunoprecipitation, competition assay, and SDS-PAGE were done exactly as described above. [^{35}S]Cysteine-labeled soluble cell lysate used in lanes 1 and 2 was from C81-66-45 cells. [^{35}S]Cysteine-labeled soluble cell lysate from C81-66-45 cells was also incubated with lentil lectin–Sepharose 4B (Pharmacia) at the ratio of 10 × 10^6 cells to 0.2 ml of lectin for 1 to 2 hours. The unbound fraction was used as the source of antigen for lanes 3 and 4. [^{35}S]Cysteine-labeled Hut 102 cells prepared as described (*11, 12*) was the source of antigen for lanes 5, 6, and 7, except that in lane 7 nonradiolabeled cell lysate from 30 × 10^6 C81-66-45 cells was incubated with the serum for 1 hour before a portion of radiolabeled Hut 102 cell lysate (0.5 × 10^6 cells equivalent) was added. A positive reference serum from a healthy Japanese carrier (*11, 12*) was used in lanes 1, 3, 5, and 7. A negative reference serum (*11, 12*) from a healthy Japanese subject was used in lanes 2, 4, and 6.

Table 3. Summary of radiolabel sequence analysis of CNBr fragments of p42.

Item	Gel filtration (S-200)				Total	RPLC
	Pool E (CNBr-2)	Pool D (CNBr-3)	Pool C* (CNBr-4)	Pool C† (CNBr-5)		Pool 1 (CNBr-5)
Residues analyzed	11	34	20	20	85	38
Residues expected‡	1	6	7	5	19	8
Observed						
Matched	1	6	5§	5	17	8
Unmatched	0	0	0	0	0	0

*Minor sequence. †Major sequence. ‡Expected from nucleotide sequence in (*10*). §See second footnote to Table 1.

CNBr fragments of p42 is shown in Fig. 2B. Two major pooled fractions, designated pool 1 and pool 2, were obtained. Automated protein sequence analysis of pool 1 indicated that pool 1 contained the CNBr-5 fragment (Table 1). Repetitive yield analysis of radiosequence indicated that pool 2 contained a mixture of CNBr-2, CNBr-4, and CNBr-5 fragments.

A summary of radiolabel sequence analysis of p42 is presented in Table 3. The probabilities of observing such a match for pool E, pool D, pool C, and pool 1 by chance alone are no more than $(1/17)^1 \times (16/20)^{11-1}$, that is 6.3×10^{-3}; $(1/17)^6 \times (16/20)^{34-6}$, or 8.0×10^{-11}; $(1/17)^{10} \times (16/20)^{20-10}$, or 5.3×10^{-14}; and $(1/19)^8 \times (18/20)^{38-8}$, or 2.5×10^{-12}, respectively. Since it is highly improbable that the observed matches, which are located in a contiguous stretch of 245 amino acid residues in the LOR region, could occur by chance alone, we conclude that p42 is at least partially encoded by the LOR region of HTLV.

Since the protein sequence of the amino terminus of p42 was not available, we cannot rigorously discuss the origin of the amino terminus of the LOR-encoded protein. However, it is likely that the amino terminus of an LOR protein is encoded by another region of the HTLV genome, possibly the *gag* region. There is at least one potential splice donor site present in the 5′ end of the p19 *gag* coding region and one potential splice acceptor site present in the 5′ end of the LOR region that could conceivably yield an in-frame spliced transcript encoding a *gag*-LOR fusion product of 42 kD (*10*). Oroszlan and co-workers reported that the amino terminus of the HTLV *gag* gene products was inaccessible to Edman degradation, apparently due to myristylation of the *gag* product, p19, at its second residue (*15*). Thus, the inability to sequence the amino terminus of p42 may be due to the presence of amino terminal *gag* sequences containing similar posttranslational modifications.

The 42-kD product of the HTLV LOR region is a novel retroviral protein, distinct from both the viral *gag* and *env* proteins and from oncogene products exhibiting homology to host cell species. The presence of p42 in a large number of HTLV-I–immortalized cells indicates the generality of its production. The ab-

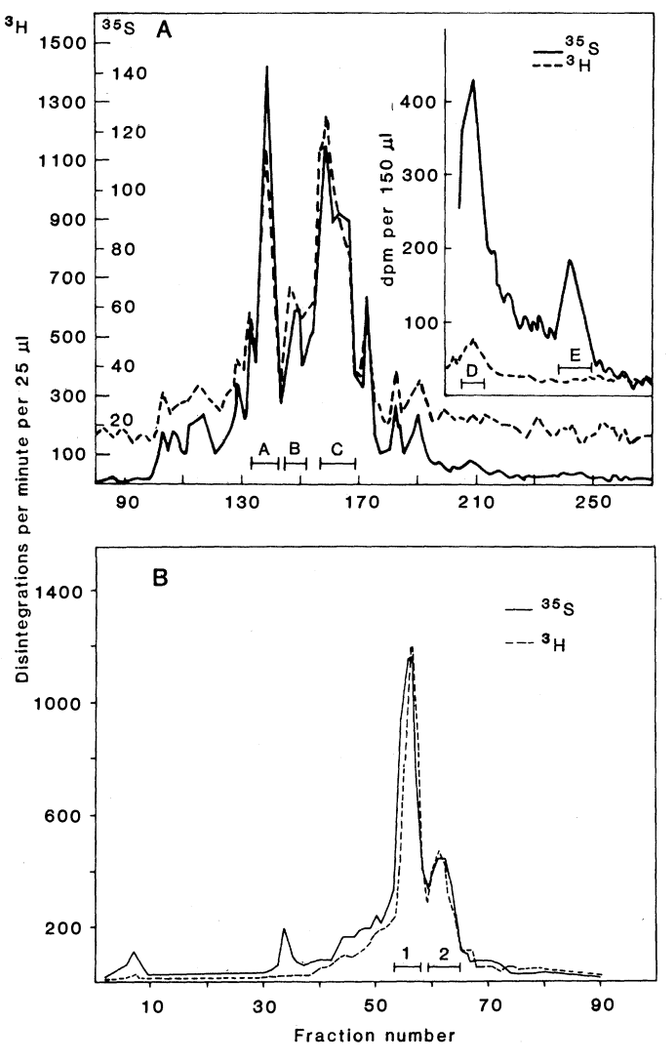

Fig. 2. (A) Sephacryl S-200 chromatography of CNBr fragments of [^{35}S]cysteine-, [^{3}H]leucine-, [^{3}H]isoleucine-, and [^{3}H]lysine-labeled p42. The column (2 by 195 cm) was equilibrated in 6*M* guanidine HCl plus 0.5 percent acetic acid. Fraction size was 2.0 ml, and the flow rate was 0.2 ml/min. Fractions were pooled as indicated. (B) Purification of CNBr fragments of [^{35}S]cysteine- and [^{3}H]serine-labeled p42 by RPLC. The CNBr fragments of p42 were dissolved in 0.3 ml of 6*M* guanidine HCl and chromatographed on a Beckman RPSC C-3 column with a Spectra-Physics model 8000B HPLC. Solvent A was 0.01*M* trifluoroacetic acid (TFA) in H_2O, and solvent B was 0.01*M* TFA in acetonitrile. The solvent program was 0 to 70 percent solvent B in 60 minutes, hold for 10 minutes, 70 to 100 percent solvent B in 10 minutes, and hold for 10 minutes. Flow rate was 0.5 ml/min; 0.5 ml fractions were collected; 10 to 30 μl of each fraction was counted in a liquid scintillation counter (Beckman LS-9000). Fractions were pooled as indicated [fractions A through E in (A) and fractions 1 and 2 in (B)].

sence of other previously characterized HTLV structural proteins in the nonproducer cell line, C81-66-45, provides circumstantial evidence for the importance of p42 in the maintenance of the immortalization in vitro and *trans*-acting transcriptional activation observed in this cell line (*7*). A 38-kD protein, which shares immunochemical properties with p42 and appears to be the LOR region product of HTLV-II, has also been identified in a HTLV-II–immortalized cell line, C3-44/MO (*16*). Further work aimed at characterizing the function of these proteins is likely to yield insight into the transformation-related properties of the HTLV family of retroviruses.

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Identification of the Putative Transforming Protein of the Human T-Cell Leukemia Viruses HTLV-I and HTLV-II

Abstract. *The human T-cell leukemia viruses HTLV-I and HTLV-II are unique among the transforming retroviruses of vertebrates in their ability to transform human T cells in vitro and in their close association with human malignancies (T-cell lymphomas and leukemia). Their genomes are relatively simple, containing the genes* gag, pol, env, *and a 3′ region termed "X." This 3′ region may be responsible for the transforming potential of the viruses. The existence of proteins encoded by the 3′ region has been postulated on the basis of multiple open reading frames. In the present study this region is shown to contain a gene encoding a protein of 40 kilodaltons in HTLV-I and 37 kilodaltons in HTLV-II. It is proposed that these proteins be called, respectively, $p40^{xI}$ and $p37^{xII}$.*

Human T-cell leukemia virus type I (HTLV-I) and type II (HTLV-II) are closely associated with specific human leukemias and lymphomas involving T lymphocytes (*1–4*). Both of these retroviruses transform normal T lymphocytes in vitro, lending credence to their etiologic role in these human malignancies (*5–8*). Most of the known oncogenic retroviruses belong to one of two general groups, depending on the mechanisms by which they induce malignancy (*9*). The long latency of the chronic transforming retroviruses, such as the avian leukosis viruses and the murine mammary tumor virus, is thought to be due to transformation by insertional mutagenesis, a mechanism whereby the proviral genome is integrated at sites near a cellular gene with subsequent activation of the cellular gene (*10, 11*). The second group, known as the acutely transforming retroviruses, carry specific transforming sequences (viral oncogenes) derived from a cohort of normal cellular genes (cellular oncogenes or proto-oncogenes) (*9*). HTLV appears to be unique among the transforming retroviruses in that it does not have a viral oncogene with a normal cellular homolog, nor does it integrate at any preferential sites in tumors or in cells transformed in vitro (*12, 13*). These observations argue against a mechanism involving insertional mutagenesis, and the absence of sequences homologous to normal cellular DNA sequences separate HTLV from the acutely transforming retroviruses.

The nucleotide sequences of HTLV-I and HTLV-II contain a highly conserved region between *env* and the 3′ long terminal repeat (LTR) of the viruses. Such sequences have not been observed in animal retroviruses except for a similar sequence in bovine leukemia virus (*14–17*). This region, termed "X" by Seiki *et al.* (*14*) has no definitely assigned function but is believed to participate in HTLV-induced cellular transformation (*14, 16, 17*). Protein products encoded by this region have not been reported previously; however, the existence of such proteins has been predicted on the basis of four open reading frames (called X-I to X-IV) in HTLV-I (*14*) and three open reading frames (called pX-a to pX-c) in HTLV-II (*16, 17*). Comparison of the nucleotide sequences of the X regions from the two viruses reveals significant sequence homology (about 75 percent) between the X-IV region of HTLV-I and the X-c region of HTLV-II (*16, 17*). The molecular size of the predicted protein encoded by the X-IV region of HTLV-I is 24 kilodaltons if initiation of translation occurs at the first methionine codon of the X-IV region (*14*). Analysis of the sequence, however, reveals that the reading frame remains open for some 100 amino acid codons upstream from the methionine. A similar open reading frame is found in HTLV-II (*16, 17*). This extended open reading frame is sufficient to encode a protein of 39.6 kD in HTLV-I and 36.8 kD in HTLV-II. The high degree of conservation between the HTLV-I and HTLV-II genomes in this region suggested that this reading frame