300 m from the wastewater discharge pipe. The average annual concentration of total dissolved phosphorus in the wastewater effluent at the outflow pipe in the wetland is 3.3 ± 0.6 mg liter^{-1} (85 percent of the total dissolved phosphorus is available to organisms as DIP). Approximately 6300 kg of phosphorus had been placed in the fen during the treatment period (1978 to 1982) (Kadlec, 1983). This represents phosphorus additions of nearly 9 kg ha^{-1}, but the wastewater was not distributed evenly over the entire 716-ha wetland. The large size of the fen in comparison to the input volumes of water and phosphate has resulted in the confinement of all phosphate additions within the ecosystem. Outputs, however, calculated for the entire peatland, do not give a realistic picture of the removal efficiency per hectare. An estimate of the phosphorus removal capacity of a portion of the fen was determined by a mass-balance approach for an area of 19.5 ha adjacent to the discharge pipe during 1978 to 1982. This size area was chosen since the average area of wetlands used for wastewater disposal in the midwest is 16 ha (*4*). The hydrologic budget and phosphorus concentrations for inputs and outputs were estimated for this area from data reported by Kadlec (*14*, *17*) and D. E. Hammer and R. H. Kadlec [in *Sixth International Peat Congress* (International Peat Society, Helsinki, Finland, 1981), pp. 563–569.

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Studies of the Putative Transforming Protein of the Type I Human T-Cell Leukemia Virus

Abstract. *The putative transforming protein of the type I human T-cell leukemia virus (HTLV-I) is a 40-kilodalton protein encoded by the X region and is termed p40^{x1}. On the basis of both subcellular fractionation techniques and immunocytochemical analysis, it is now shown that p40^{x1} is a nuclear protein with a relatively short half-life (120 minutes). It is synthesized de novo in considerable quantities in a human T-cell line infected with and transformed by the virus in vitro, and it is not packaged in detectable amounts in the extracellular virus.*

The human T-cell leukemia viruses, HTLV-I and HTLV-II, are closely associated with specific malignancies of T cells in humans (*1*) and are capable of transforming normal peripheral blood T lymphocytes in vitro (*2*). The putative transforming gene of these viruses, termed *x*, is located between the *env* gene and the 3′ long terminal repeat (LTR) (*3*). The proteins encoded by this gene in both HTLV-I and HTLV-II have been identified (*4*, *5*). A 40-kilodalton (kD) protein called p40^{xI} and a 37-kD protein called p37^{xII} were found in cells infected with HTLV-I and HTLV-II, respectively (*4*). The same proteins have been called p42^{lor} and p38^{lor} (*5*). Attention has been focused on the biology of these proteins because of their possible role in induction of T-cell malignancies. In this report we describe studies on the amount of p40^{xI} in infected cells, the kinetics of intracellular turnover of the protein, and its subcellular localization.

Previously, we used synthetic peptides representing determinants of the predicted translation products of the *x* genes of HTLV-I and HTLV-II to generate antisera to these proteins (*4*). Earlier studies had shown that antibodies to proteins produced in bacteria via expression vectors are useful in detecting the products of viral transforming genes (*6*, *7*). Here, we generated antibodies to the COOH-terminus of the p40^{xI} protein. The antiserum was tested in an immunoprecipitation assay with cells that had been infected with HTLV-I (SLB-I) and labeled with [^{35}S]methionine as described (*4*). As with the antisera to the peptides, the antiserum to the COOH-terminus recognized a 40-kD protein in HTLV-I–infected cells (Fig. 1). The antibody titers achieved in rabbits that had been injected with either the synthetic peptides or the bacterially promoted protein (fusion protein) were similar (*8*). However, in experiments with a fixed amount of isotopically labeled cell lysate and an equivalent amount of antiserum, antiserum to the fusion protein was three to five times better at immunoprecipitating the p40^{xI} protein than antiserum to the peptide (Fig. 1, lanes b, c, e, and f). This may have been due to the greater number of potential epitopes in the bacterially promoted 54-amino-acid x^{I} polypeptide compared to the 14- or 17-amino-acid synthetic peptides. The antiserum to the promoted protein, however, did not recognize the p37^{xII} protein in HTLV-II–infected cells (JLB-I). It is known that the proteins encoded by the *x* genes have less sequence homology at the COOH-termini than at the NH_2-termini (*9*).

The level of p40^{xI} protein synthesis compared to that of other cellular and viral proteins in HTLV-I–infected cells was estimated by immunoprecipitation. A known amount of isotopically labeled cell lysate was assayed and the radioactivity in the 40-kD protein band was eluted, counted, and compared to the total trichloroacetic acid–precipitable material in the cell lysate (*10*). Approximately 0.15 percent of the total [^{35}S]methionine incorporation was asso-

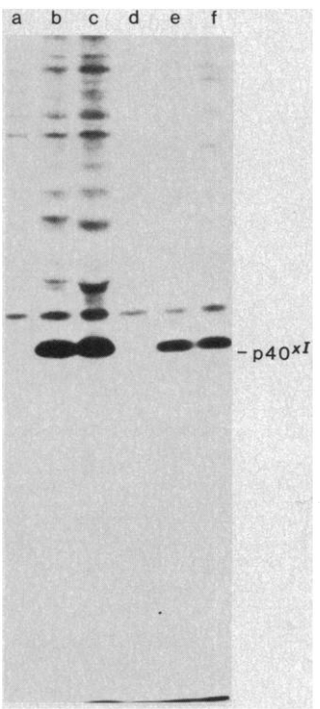

Fig. 1. Immunoprecipitation with antibodies against a p40^{xI} fusion protein. The derivative "runaway" plasmid pCFM516 (*25*), containing a bacterial tryptophan synthetase promoter and a synthetic bovine growth-hormone (bGH) gene (*26*), was used to generate a bGH-p40^{xI} fusion protein in *Escherichia coli*. A Sca I–Hinc II DNA fragment coding for the COOH-terminus of the p40^{xI} protein was ligated into M13 mp11 (*27*). The COOH-terminal p40^{xI} DNA fragment was excised from M13 mp11 with Sst I and Bam HI and placed in the bGH expression vector to form a fusion protein containing the NH_2-terminal 76 amino acids of bGH and the COOH-terminal 54 amino acids of the p40^{xI} protein. The fusion protein was purified from bacterial whole cell lysate by SDS-PAGE (*28*). The purified bGH-p40^{xI} fusion product was then used to immunize rabbits by the method previously described (*4*). Antisera were tested in an immunoprecipitation assay with HTLV-I–infected cells (SLB-I) metabolically labeled with [^{35}S]methionine for 4 hours (*4*). (Lane a) SLB-I cell lysate and sera from unimmunized rabbits; (lane b) SLB-I cell lysate and 5 μl of antiserum to p40^{xI} fusion protein; (lane c) SLB-I cell lysate and 15 μl of antiserum to the p40^{xI} fusion protein; (lane d) SLB-I cell lysate and 5 μl of sera from unimmunized rabbits; (lane e) SLB-I cell lysate and 5 μl of antiserum to the pX IV-6 peptide (*4*); and (lane f) SLB-I cell lysate and 15 μl of antiserum to the pX IV-6 peptide.

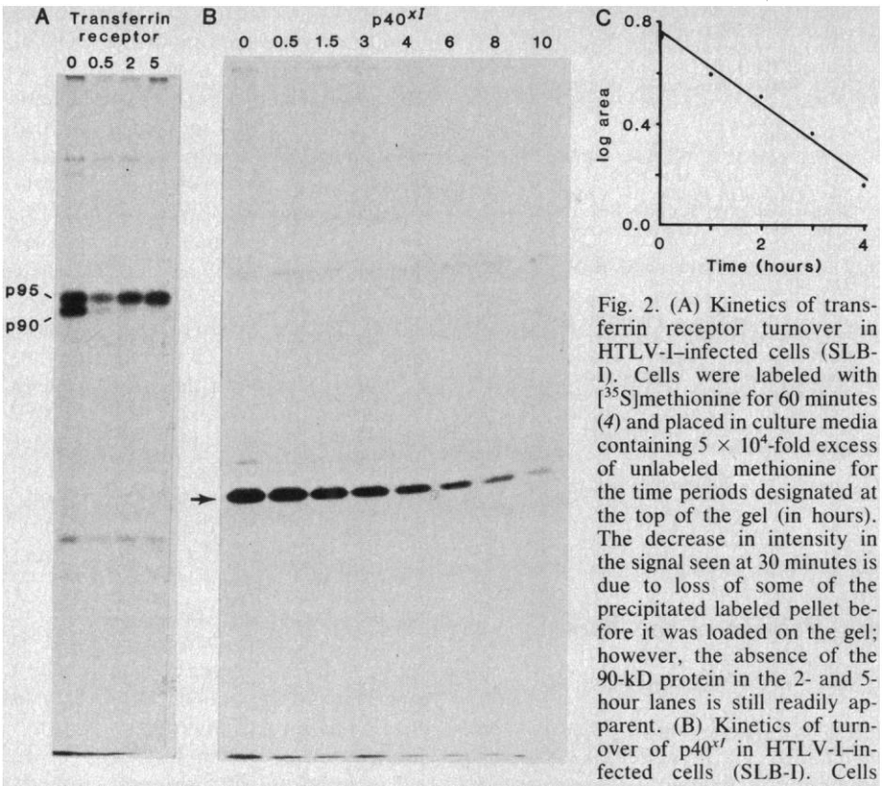

Fig. 2. (A) Kinetics of transferrin receptor turnover in HTLV-I–infected cells (SLB-I). Cells were labeled with [^{35}S]methionine for 60 minutes (*4*) and placed in culture media containing 5×10^4-fold excess of unlabeled methionine for the time periods designated at the top of the gel (in hours). The decrease in intensity in the signal seen at 30 minutes is due to loss of some of the precipitated labeled pellet before it was loaded on the gel; however, the absence of the 90-kD protein in the 2- and 5-hour lanes is still readily apparent. (B) Kinetics of turnover of p40^{xI} in HTLV-I–infected cells (SLB-I). Cells were labeled as above and chased in medium containing unlabeled methionine for the designated periods of time (in hours). Arrow indicates p40^{xI}. The resulting radioautograph was subjected to soft laser densitometry scanning and the rate of disappearance of the p40^{xI} signal corresponded to a half-life of 120 minutes (C).

ciated with p40^{xI}. The amount of p40^{xI} precipitated was not limited by the amount of antiserum in the experiment, as increasing the amount of antiserum (lane c) did not result in an increased amount of precipitated p40^{xI}.

The kinetics of intracellular turnover of the p40^{xI} protein was determined by pulse-chase labeling experiments. HTLV-I–infected cells were labeled with [^{35}S]methionine for 60 minutes, then chased with excess unlabeled methionine (Fig. 2). As a control, the half-life of a protein with known kinetics was studied in the same cells. The transferrin receptor is a 95-kD protein with a half-life of 60 hours (*11*). It is initially seen as a doublet of 95 kD and 90 kD. The smaller protein has a much shorter half-life, being converted into the larger protein within 4 hours (*11*). This pattern was demonstrable in the HTLV-I–infected cells. With the same whole cell lysate, the half-life of the p40^{xI} protein was 120 minutes (Fig. 2).

In cellular fractionation studies, HTLV-I–infected cells were disrupted mechanically in a hypotonic detergent buffer and separated into nuclear, cytoplasmic, and membrane fractions (Fig. 3). The nuclear fraction was monitored microscopically (*12, 13*) to ensure that all intact cells had been eliminated and that only nuclei remained (Fig. 3A). However, microscopic evaluation cannot be used to reliably determine the integrity of cytoplasmic and membrane fractions. Immunoprecipitation studies with lysates from all three fractions and an antiserum to a known cytoplasmic protein, tubulin, resulted in detection of a 50-kD protein corresponding to the size of human tubulin (*14*) in the cytoplasmic fraction, but not in the nuclear or membrane fractions (Fig. 3a). Likewise, immunoprecipitation with antiserum di-

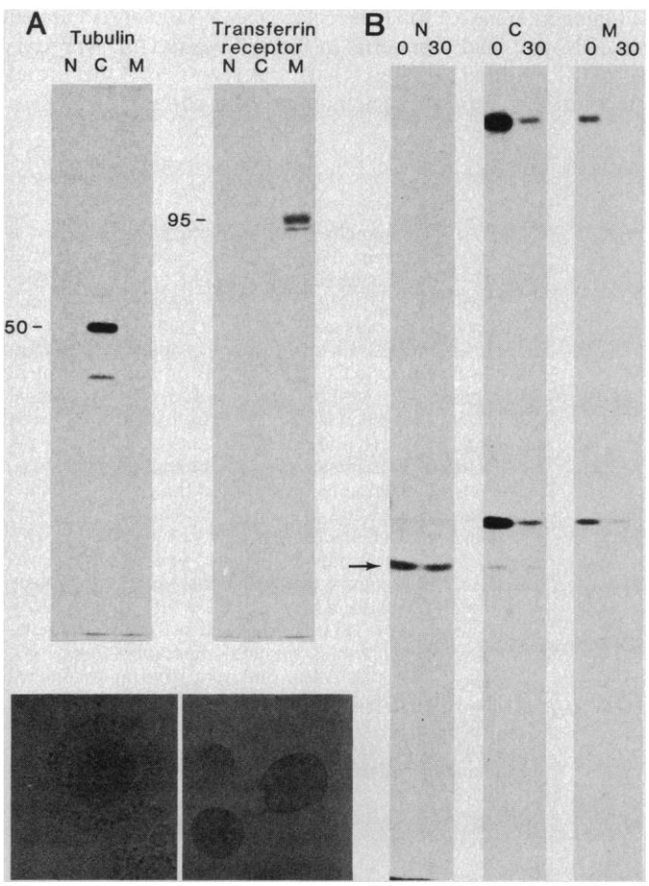

Fig. 3. (A) Subcellular localization of the p40^{xI} protein; N, nucleus; C, cytoplasm; M, membrane. HTLV-I–infected cells were labeled for 2 hours and fractionated with a modification of a previous method (*29*). In brief, 2×10^7 cells in hypotonic homogenization buffer (5 m*M* KCl, 1 m*M* $MgCl_2$, 20 m*M* Hepes, *p*H 7.4) were disrupted with 30 strokes in a glass dounce homogenizer (0.002 inches clearance). The lysate was centrifuged at 1000 rev/min for 5 minutes at 4°C and the nuclear pellet was further clarified by centrifugation (1100 rev/min, 6 minutes, 4°C) through a 200-μl sucrose cushion (30 percent sucrose, 1 m*M* EDTA, 25 m*M* tris, *p*H 7.5). The pellet was washed once in a Kyro EOB detergent buffer (*30*) and a sample was examined microscopically to assess purity. (Bottom left) Cells before lysis; (bottom right) nuclei after lysis. The nuclear pellet was then lysed in RIPA buffer (*4*), passed through a 25-gauge needle, and subjected to immunoprecipitation with either 1 μl of monoclonal antiserum to tubulin or 2 μl of a monoclonal antiserum to the transferrin receptor. The supernatant from the initial dounce homogenization, containing the cytoplasmic and membrane fractions, was centrifuged (40,000 rev/min in a Beckman Ty65 rotor, 1 hour, 4°C) to produce a pellet of the membrane fragments. The resulting supernatant was designated the cytoplasmic fraction. Both the cytoplasmic and membrane fractions were placed in RIPA buffer (*4*) and analyzed by immunoprecipitation assay. (B) Subcellular localization of the p40^{xI} protein. Lysates described in (A) were also immunoprecipitated with the antiserum to p40^{xI} fusion protein. Most of the p40^{xI} protein (arrow) was found in the nuclear fraction of cell lysates after either 2 hours of metabolic labeling or 2 hours of labeling and 30 minutes of chase with cold methionine.

rected against a known membrane protein, the transferrin receptor, resulted in detection of a 90- and 95-kD protein in the membrane, but not in the nuclear or cytoplasmic fractions (Fig. 3a). Immunoprecipitation of lysates with antiserum to $p40^{x/}$ showed that most of this protein in HTLV-I–infected cells is in the nucleus, with smaller amounts of $p40^{x/}$ found in the cytoplasmic and membrane fractions (Fig. 3b). Since we have demonstrated that the nuclear fraction was not appreciably cross-contaminated with cytoplasmic or membrane proteins, this result can be interpreted as true nuclear localization of the $p40^{x/}$ protein.

We confirmed the nuclear localization of $p40^{x/}$ by incubating antiserum to $p40^{x/}$ with fixed, frozen sections of HTLV-I–infected cells and then subjecting the sections to an indirect immunoperoxidase staining procedure. Examination of the sections by light microscopy revealed specific staining of the $p40^{x/}$ protein in the nuclei of approximately 80 percent of the cells (Fig. 4, a and b). The intensity of staining in individual cells was variable, but all staining was nuclear. Staining did not occur when HTLV-I–infected cells were incubated with preimmune sera (Fig. 4c), nor did it occur when uninfected, transformed human T cells (MOLT-4) were incubated with antiserum to $p40^{x/}$ (Fig. 4d).

The cytoplasmic $p40^{x/}$ could be explained by de novo synthesis of the protein in the cytoplasm. In immunoprecipitation experiments in which we used a lactoperoxidase bead technique (Enzymobead, Bio-Rad) to iodinate outer surface membrane proteins of HTLV-I–infected cells, no $p40^{x/}$ was found. Thus, the small amount of $p40^{x/}$ found in the membrane fraction is not displayed on the extracellular membrane.

Finally, to determine if any of the $p40^{x/}$ protein was a component of the virion itself, we purified extracellular virus from the culture medium of HTLV-I–infected cells, disrupted the viral particles, and labeled the component virion proteins with ^{125}I. These proteins were then subjected to immunoprecipitation either with patient sera that were reactive with *gag* proteins or with antiserum to $p40^{x/}$ and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The p24 *gag* proteins of the virions were easily detected (Fig. 5, lane b). However, $p40^{x/}$ protein was not observed (Fig. 5, lane a), indicating that the $p40^{x/}$ is not packaged in detectable amounts in the HTLV-I virus.

Our data show that an antiserum to the bacterially promoted COOH-terminus of the x^{I} gene product of HTLV-I recognized the same 40-kD protein that was identified by antisera to potential epitopes from the NH_2-terminus (*4*). This confirms that the 40-kD protein is the gene product of the *x* gene. Because the synthetic peptides used to generate antisera against the NH_2-terminus of the protein were from epitopes upstream of the first methionine in the *x* open reading frame of HTLV-I, we predicted that the 40-kD protein represented the product of a spliced messenger RNA consisting predominantly of *x* sequences, as well as 5′ viral sequences (*4*). This has been confirmed with data indicating that a methionine initiation codon from *env* and one nucleotide of the next codon is spliced to the *x* open reading frame (*15, 16*). From this information, the exact size of the protein encoded by the *x* message is predicted to be 39.89 kD, which is consistent with the 40-kD protein identified previously (*4*) and studied in greater detail here.

By means of cellular fractionation techniques as well as immunocytochemistry, most of the intracellular $p40^{x/}$ protein was found to be in the nucleus of transformed cells (Figs. 3 and 4). The subcellular localization of $p40^{x/}$ to the nucleus places the protein in a cellular compartment consistent with its proposed function as a *trans*-acting enhancer of gene transcription (*17–19*).

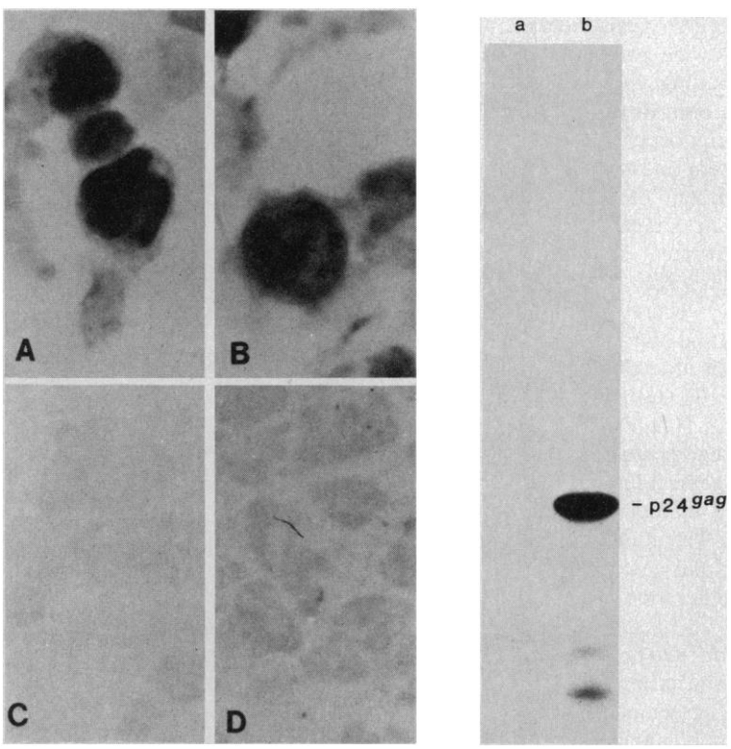

Fig. 4 (left). Immunocytochemical localization of the $p40^{x/}$ protein in HTLV-I–infected cells (SLB-I) with an indirect immunoperoxidase technique (*31*). Antiserum to the $p40^{x/}$ fusion protein was diluted 1:25, 1:50, or 1:100 and absorbed with both rat liver powder and human immunoglobulin G (IgG) and applied to frozen sections of cells that had been fixed with 95 percent alcohol, acetone, picric acid–paraformaldehyde (*32*), or periodate-lysine-paraformaldehyde (*33*). This was followed by treatment with goat antibody to rabbit IgG coupled to peroxidase (Sternberger-Meyer, 1:25 dilution). Each application of antiserum was followed by three 5-minute washes in phosphate-buffered saline (PBS), and treatment with a solution of diaminobenzidine (0.6 mg/ml) and 0.03 percent H_2O_2 in PBS. (A) Specific staining for $p40^{x/}$ in SLB-I cells that had been incubated with antiserum to the bGH-$p40^{x/}$ fusion protein. (B) Occasional cells had large, clear, unstained intranuclear areas which were interpreted as nucleoli. (C) SLB-I cells that were incubated with pre-immune sera and stained. (D) MOLT-4 (a transformed human T-cell line not infected with HTLV-I) were incubated with the antiserum to $p40^{x/}$ and stained. Fig. 5 (right). Extracellular HTLV-I virus was obtained from 1.5 liters of culture supernatant of SLB-I cells. The media was filtered through gauze and centrifuged (18,000 rev/min, Beckman type 19 rotor 2.5 hours, 4°C). The pellet was collected in cold TEN buffer (10 m*M* tris, 1 m*M* EDTA, 100 m*M* NaCl, *p*H 7.4) and gently homogenized. The virus was then purified by banding on a 20 to 50 percent sucrose gradient and the viral band was harvested and dialyzed against TEN buffer overnight at 4°C. Virus was pelleted by centrifugation (30,000 rev/min, SW40 rotor, 1 hour, 4°C) and disrupted into individual viral proteins (*34*). Protein concentration was determined with the Bio-Rad protein assay and 1 μg was iodinated by a modification of the chloramine-T method (*35*). The disrupted virion proteins (3×10^6 count/min) were immunoprecipitated with (lane a) 10 μl of the antiserum to $p40^{x/}$ fusion protein or (lane b) 10 μl of sera from patients with adult T-cell leukemia which recognize the p24 *gag* proteins.

The half-life of the $p40^{x/}$ protein is relatively short (120 minutes) (Fig. 2), which is what might be expected for a protein with regulatory function. Proteins encoded by two other transforming viruses, the *myc* protein of the MC-29 virus and the E1A protein of adenovirus are involved in regulation of gene transcription (*20*). Both of these proteins also have short half-lives (*21, 22*), and both are found in the nuclei of infected cells (*12, 23, 24*).

If the $p40^{x/}$ protein is responsible for activation of the viral LTR and for viral gene transcription as postulated (*17–19*), a remaining question is how viral transcription is initially carried out in newly infected cells before the *x* gene is transcribed. At least two possibilities exist: (i) like reverse transcriptase, the $p40^{x/}$ protein may be packaged in the virion, thus facilitating viral gene transcription upon infection, or (ii) low levels of gene transcription occur in the absence of the $p40^{x/}$ protein and subsequently increase as the amount of $p40^{x/}$ increases. We found no detectable levels of $p40^{x/}$ protein in disrupted viral particles (Fig. 5), which argues against the first possibility. Moreover, we have found that low levels of viral transcription do occur in the absence of the x^{II} gene in HTLV-II–infected cells (*18*). A similar mechanism is likely for HTLV-I–infected cells.

It has been postulated that the product of the *x* gene interacts with viral LTR sequences to facilitate viral gene transcription. This function may also activate a cellular gene or genes involved in T-cell proliferation, thus inducing malignancy.

Note added in proof: After submission of this manuscript, Goh *et al.* published results in agreement with Fig. 3b of this study, that is, nuclear localization of the *x* protein (*36*).

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A Transcriptional Activator Protein Encoded by the x-*lor* Region of the Human T-Cell Leukemia Virus

Abstract. *Human T-cell leukemia viruses type I and II (HTLV-I and -II) exhibit several features characteristic of this retroviral family: the presence of an x-*lor *gene encoding a nuclear protein, transformation properties suggesting the involvement of a virus-associated* trans*-acting factor, and transcriptional* trans*-activation of the long terminal repeat (LTR) in infected cells. In the study described here the HTLV x-*lor *products, in the absence of other viral proteins, were able to activate gene expression in* trans *directed by the HTLV LTR. The regulation of the expression of particular genes in* trans *by HTLV x-*lor *products suggests that they play a role in viral replication and possibly in transformation of T lymphocytes.*

Human T-cell leukemia viruses (HTLV) comprise a retroviral family associated with lymphoid disorders. HTLV types have been identified on the basis of immunocompetition analysis of *gag* proteins (*1*). HTLV-I is associated with adult T-cell leukemia-lymphoma (ATLL) that is endemic in certain geographic regions (*2*). HTLV-II is a rare isolate associated with a benign form of hairy T-cell leukemia (*1*). HTLV-III has been identified as a probable cause of the acquired immune deficiency syndrome (AIDS) (*3*). Bovine leukemia virus (BLV), the etiological agent of enzootic leukosis in domestic cattle, demonstrates antigenic relatedness to the HTLV viruses in some of the virion proteins (*4*).

HTLV-I, -II, and BLV have been categorized as a separate family (HTLV-BLV) of transforming retroviruses (*5*) because of their characteristic structural and biological properties. The HTLV-BLV viruses can be distinguished from chronic murine leukemia viruses in that the former exhibit *trans*-acting transcriptional activation of the viral long terminal repeat (LTR), which governs viral gene expression, in infected cells (*6, 7*). Cells infected by these viruses contain factors that greatly augment steady-state levels of RNA produced by the viral LTR, without stimulating the replication of the template DNA (*7, 8*). The *trans*-acting factors act most efficiently on the LTR of the infecting virus (*7*), suggesting that a virus-specific rather than host cell-