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Identification of new gene products coded from X regions of human T-cell leukemia viruses

(retrovirus/synthetic peptide/antibody)

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ABSTRACT **Antibodies were raised against oligopeptides deduced from the nucleotide sequence in the conserved region located between *env* and the 3′ long terminal repeat in human T-cell leukemia virus type I (HTLV-I) and type II (HTLV-II) to detect a protein coded from this region in virus-infected cells. Two of these antibodies precipitated a protein of 41 kilodaltons in HTLV-I-infected cell lines and a protein of 38 kilodaltons in HTLV-II-infected cells. The protein in HTLV-I-infected cells was precipitated by plasma from patients with adult T-cell leukemia but not by plasma from a normal adult. These results indicate that these proteins were translated from new coding regions (X) present in HTLV-I and HTLV-II.**

Human T-cell leukemia viruses (HTLV), a family of exogenous retroviruses in humans, have been implicated as causative agents of several human diseases (1–8). HTLV type I (HTLV-I), the most prevalent type found to date, is associated with an aggressive form of adult T-cell leukemia or lymphoma (ATL) (1–3, 6). HTLV type II (HTLV-II), which has been isolated only occasionally, was obtained from a patient with a T-cell variant of clinically benign hairy cell leukemia (5, 9, 10). Recently, other viruses—LAV and HTLV-III—were isolated from patients with lymphadenopathy and the acquired immune deficiency syndrome, respectively (11, 12).

Nucleotide sequence analyses of these viral genomes indicated that HTLV-I and HTLV-II do not carry a typical *onc* gene derived from normal cells (13, 19). Instead, they have a sequence of 1.6 kilobases between *env* and the 3′ long terminal repeat (LTR), named the X region, with an unknown function.

Analysis of the integration site of HTLV-I in fresh leukemic cells from patients with ATL gave no evidence for a common integration site(s), suggesting the unlikelihood of a *cis*-acting function of LTR as an enhancer or a promotor insertion to activate cellular *onc* genes adjacent to provirus, as shown in avian lymphoma induced by infection with avian retrovirus (15–17).

To obtain information on the molecular mechanism of leukemogenesis induced by HTLV, we examined the nucleotide sequence of HTLV-II provirus. Comparison of the nucleotide sequence of HTLV-II with that of HTLV-I reported by others (13) showed a highly conserved sequence in the 3′ two-thirds of the X regions in the two viral genomes (18). We predicted the presence of a translational product(s) from this region from the following findings (18): (*i*) The presence of an open reading frame in the same regions of X of HTLV-II and HTLV-I that can code for a protein of >35 kDa. (*ii*) Conservation of nucleotide sequences within this frame but divergent sequences in the immediately flanking regions. (*iii*) Frequent changes of the third nucleotide relative to the first and second nucleotides of codons in this frame, suggesting that the predicted protein was conserved during evolution of HTLV. (*iv*) The presence of a splice acceptor site in the 5′ region of these frames, suggesting that the predicted protein is a fused protein translated from an mRNA spliced between this acceptor site and a donor site present upstream in viral RNA. The similarity of the X regions of the two viruses has been confirmed by others (19). To identify the predicted protein in virus-infected cells, we raised antibodies against four synthetic peptides corresponding to various regions of the predicted protein of HTLV-I. With these antibodies, we detected proteins of 41 and 38 kDa in cells infected with HTLV-I and HTLV-II, respectively. Some of these findings have been published elsewhere (20).

MATERIALS AND METHODS

Cell Line. The establishment of the T-cell line, ATL-5S, from fresh lymphatic cells of a patient with ATL has been described (21). HUT-102 is a lymphocytic cell line derived from a patient with cutaneous T-cell lymphoma (22). Cat-12 and CaL-2 cells were obtained by co-culture of cat fibroblasts and cat lymphocytes, respectively, with the HTLV-producing cell line ATL-2M (23). Ton-1 is a T-cell line established by co-culture of human tonsil cells with HTLV-II-producing cells, which was kindly supplied by Weiss and coworkers (24). ATL-5S, HUT-102, CaL-2, and Cat-12 carry HTLV-I proviruses in their genomes, Ton-1 is a HTLV-II-producing cell line and HL60 is a cell line not infected with these viruses.

Sera. Four oligopeptides, deduced from the nucleotide sequence of the open reading frame for pX-IV of HTLV-I, were synthesized (Fig. 1). Oligopeptide-1 is Cys-Pro-Glu-His-Gln-Ile-Thr-Trp-Asp-Pro-Ile-Asp-Gly-Arg, corresponding to a region of the NH_2 terminus, oligopeptide-2 and -3 are Lys-Arg-Ile-Glu-Glu-Leu-Leu-Tyr-Lys and Ser-Gly-Pro-Cys-Pro-Lys-Asp-Gly-Gln-Pro-Ser, respectively, corresponding to internal regions in the open reading frame, and oligopeptide-4 is Ser-Leu-Leu-Phe-Asn-Glu-Lys-Glu-Ala-Asp-Asp-Asn, corresponding to a region near the COOH terminus. These sequences of the oligopeptides are either identical or similar to the corresponding regions of HTLV-II. In oligopeptide-1, isoleucine at position 6 is substituted with leucine in HTLV-II. In oligopeptide-2, isoleucine at position 3 is substituted with leucine in HTLV-II. In oligopeptide-3, cysteine and aspartic acid at positions 4 and 7 are substituted with tyrosine and alanine, respectively, in HTLV-II. In oligopeptide-4, leucine, glutamic acid, and lysine at positions 2, 6, and 7 are substituted with isoleucine, lysine, and glutamic

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Abbreviations: HTLV, human T-cell leukemia virus; LTR, long terminal repeat; ATL, adult T-cell leukemia.

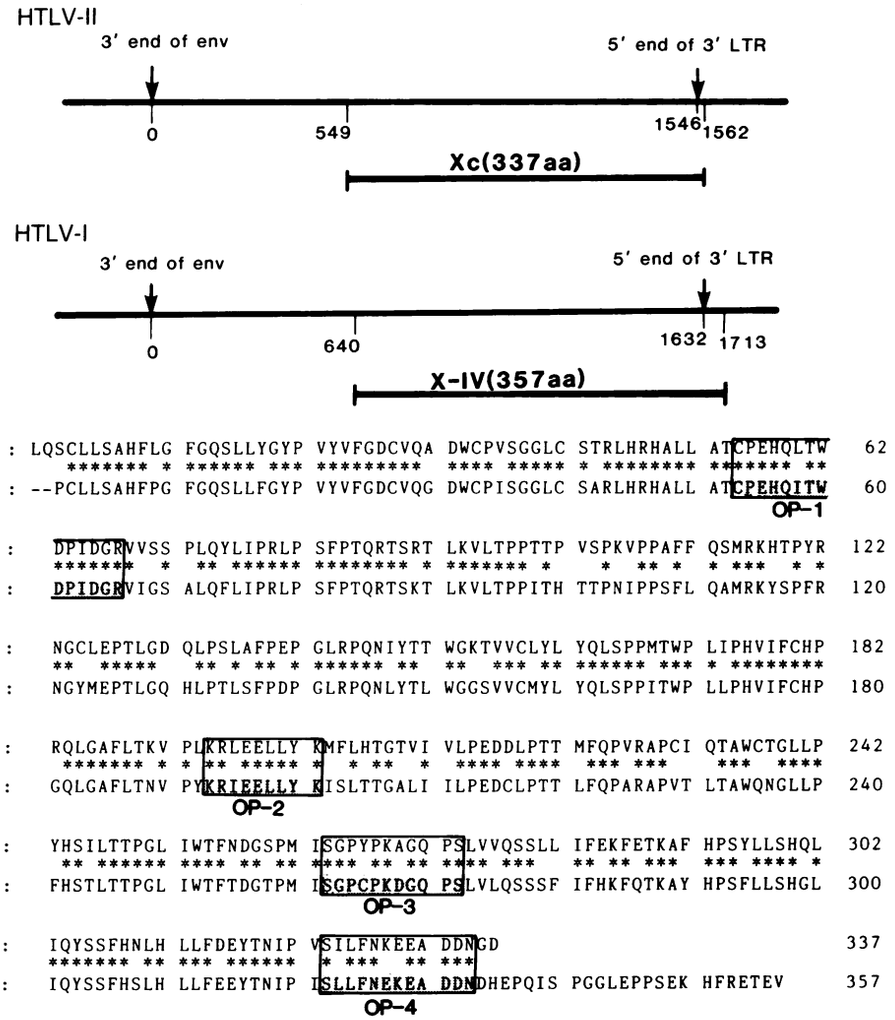

FIG. 1. (*Upper*) Location of the open reading frames, Xc and X-IV in HTLV-II and HTLV-I genomes, respectively. Numbers indicate nucleotide positions from the 3' ends of *env*. Numbers in parentheses indicate numbers of amino acids coded from these open reading frames. (*Lower*) Location of the synthesized oligopeptide-1 to -4 (OP-1 to OP-4) in the predicted protein coded from the open reading frame in the X region of HTLV. Oligopeptides in shaded areas in frames were synthesized. Data on the comparison of amino acid sequence coded from the open reading frame Xc of HTLV-II and X-IV of HTLV-I are from Shimotohno *et al.* (18). Data of HTLV-I are from Seiki *et al.* (13). Amino acids are represented by standard one-letter abbreviations.

acid. These peptides were conjugated with keyhole limpet hemocyanin and injected into rabbits to raise antibodies. Antibodies with high titers against these peptides were obtained after several immunizations. Plasma from patients with ATL and a normal adult were obtained by centrifugation on a Ficoll-Paque gradient.

Immunoprecipitation and Acrylamide Gel Electrophoresis. Approximately 2×10^6 cells were cultured for 16 hr in 3 ml of RPMI-1640 medium containing 200 μCi of [^{35}S]cysteine per ml (1500 Ci/mmol; 1 Ci = 37 GBq) supplemented with 20% of dialyzed fetal calf serum. The cells were washed twice with phosphate-buffered saline and lysed in 0.5 ml of 50 mM Tris·HCl, pH 7.2/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$/1 mM phenylmethylsulfonyl fluoride. The lysate was frozen at −20°C for 2 or 3 hr and then centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was used for immune complex formation. The supernatant ($\approx 2 \times 10^6$ cpm) of the acid-insoluble fraction was incubated with 3 μl of serum at 4°C for 12 hr. Then, 10 mg of protein A Sepharose was added to the reaction mixture to adsorb the immune complex. The immunoprecipitate bound to protein A Sepharose was washed twice with 1 ml of 20 mM Tris·HCl, pH 7.4/0.5 M NaCl/1 mM EDTA/0.5% Nonidet P-40/1% sodium desoxycholate, and twice with 1 ml of 10 mM Tris·HCl, pH 7.4/10 mM NaCl. Then, it was extracted with 50 μl of 0.25 M Tris·HCl, pH 6.8/1.2 M 2-mercaptoethanol/1% $NaDodSO_4$/20% (vol/vol) glycerol/0.01% bromphenol blue, at 90°C for 2 min and subjected to gel electrophoresis on 12% polyacrylamide containing 0.1% $NaDodSO_4$ (25). The gel was dried and autoradiographed. For competition experiments on immune complex formation, 100 μg of the synthetic peptide was added in the reaction mixture and the incubation was carried out as described above.

Materials. [^{35}S]Cysteine (1500 Ci/mmol) was purchased from Amersham. Protein A Sepharose was from Pharmacia. Dialyzed fetal bovine serum was from GIBCO.

RESULTS

Detection of a Protein Coded from the X-Region of HTLV-I in HUT-102 Cells. A ^{35}S-labeled HUT-102 cell lysate was incubated with 3 μl of preimmune serum or antiserum of rabbits immunized with synthetic oligopeptide-1 to -4. The immunoprecipitates were subjected to electrophoresis on 12% polyacrylamide gel containing 0.1% $NaDodSO_4$. The results are shown in Fig. 2*A*. Only antisera against oligopeptide-1 and -4 precipitated a protein of 41 kDa while antisera

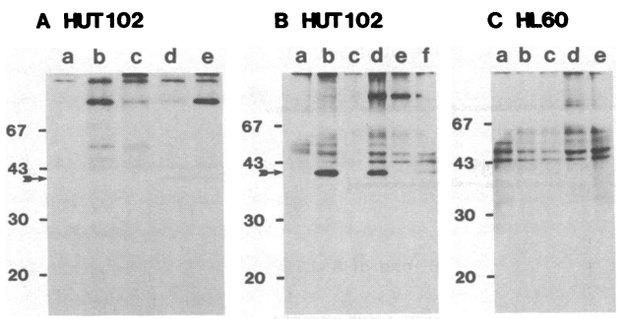

FIG. 2. Immunoprecipitation of cell lysates with antibodies against the synthetic peptides. (*A*) ^{35}S-labeled lysate of HUT-102 cells precipitated with serum from a normal rabbit (lane a), and antisera against oligopeptide-2 (lane b), -3 (lane c), -1 (lane d), and -4 (lane e). Numbers represent sizes (in kDa) of albumin (67), ovalbumin (43), carbonic anhydrase (30), and trypsin inhibitor (20) used as markers. (*B*) ^{35}S-labeled lysate of HUT-102 cells precipitated with serum from a normal rabbit (lane a) and antisera against oligopeptide-1 (lanes b and c) and -4 (lanes d and e). Reaction was carried out in the presence of 100 μg of synthetic oligopeptide-1 (lane c) or -4 (lane e). Immunoprecipitation of supernatant from the reaction in lane b with antiserum against oligopeptide-4 (lane f). (*C*) Immunoprecipitation of a lysate of HL-60 cells with normal serum of a rabbit (lane a) and antisera against oligopeptide-1 (lanes b and c) and -4 (lanes d and e). Reaction was carried out in the presence of 100 μg of oligopeptide-1 (lane c) or -4 (lane e). Arrows indicate position of 41-kDa protein.

against oligopeptide-2 and -3 and normal serum did not. Thus, antisera against oligopeptide-1 and -4 were used for further investigations. The specificities of the reactions of antisera against oligopeptide-1 and -4 with 41-kDa protein were examined by tests on competition with the synthetic peptides, oligopeptide-1 and -4, respectively. Addition of 100 μg of oligopeptide-1 to the reaction mixture completely inhibited the precipitation of 41-kDa protein by antiserum to oligopeptide-1 (Fig. 2*B*, lane c). Similarly, addition of oligopeptide-4 blocked immunoprecipitation of 41-kDa protein by antiserum against oligopeptide-4 (Fig. 2*B*, lane e). Therefore, the reaction of 41-kDa protein with antisera against these synthetic peptides seemed to be specific. To demonstrate that these antisera precipitated the same protein of 41 kDa, a HUT-102 cell lysate was first treated with antiserum against oligopeptide-1, and the immune complex was removed by adsorption on protein A Sepharose. The supernatant was then mixed with antiserum against oligopeptide-4, and the immune complex that bound to protein A Sepharose was subjected to gel electrophoresis on 12% polyacrylamide containing 0.1% NaDodSO$_4$. These procedures resulted in reduction in intensity of the band of 41 kDa, indicating that the two antisera recognized the same protein (Fig. 2*B*, lane f). Since the 41-kDa protein was not precipitated with normal rabbit serum, it seemed to react specifically with antisera against oligopeptide-1 and -4.

Immunoprecipitation of Extracts from Various Cell Lines with Antisera Against Oligopeptide-1 and -4. Extracts from various cell lines containing integrated HTLV-I or HTLV-II provirus were tested for immunoprecipitation with these antisera. In extracts from Cat-12 and ATL-5S, which carry HTLV-I provirus, 41-kDa protein was precipitated with antisera against oligopeptide-1 and -4 (Fig. 3 *A* and *C*, lanes b and d), and oligopeptide-1 and -4 competitively inhibited these reactions (Fig. 3 *A* and *C*, lanes c and e). The 38-kDa peptide, but not the 41-kDa peptide, in an extract from the HTLV-II infected cell line, Ton-1, was precipitated with antisera against oligopeptide-1 and -4 (Fig. 3*D*, lanes b and d), and the reaction was inhibited by the presence of either oligopeptide-1 or -4 (Fig. 3*D*, lanes c and e), suggesting that this 38-kDa protein, like the 41-kDa protein in HTLV-I infected cells, is precipitated specifically by these antisera. The fact that neither 41- nor 38-kDa protein was detected in an extract of HL-60 cells suggests that these proteins are encoded by the viruses (Fig. 2*C*).

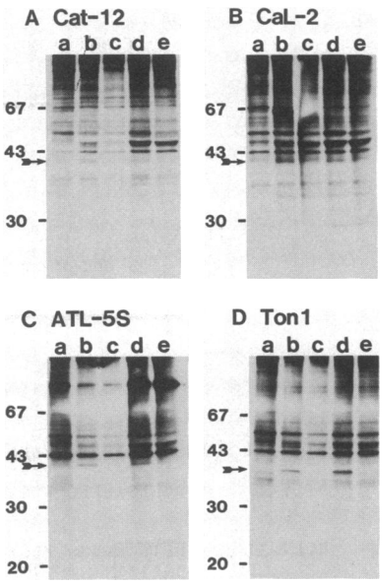

FIG. 3. Immunoprecipitation of lysates from various cell lines. ^{35}S-labeled cell lysates were treated with antiserum of a normal rabbit (lane a) or antisera against oligopeptide-1 (lanes b and c) or -4 (lanes d and e). Reaction was carried out in the presence of 100 μg of oligopeptide-1 (lane c) or -4 (lane e). Arrows indicate the position of 41- or 38-kDa protein. Numbers represent size markers as described in Fig. 2.

Precipitation of 41-kDa Protein in a HUT-102 Cell Extract by Plasma of Patients with ATL. A ^{35}S-labeled extract of HUT-102 cells was incubated with plasma from patients with ATL, and the immunoprecipitates were analyzed by electrophoresis on 12% polyarylamide gel containing 0.1% NaDodSO$_4$ (Fig. 4). A faint band was seen at the position of 41 kDa in the lanes as well as other specific bands of virally encoded protein (Fig. 4, lanes a and c). This band was not detected in a precipitate with normal human plasma (data not shown). The intensity of this band became weak when the lysate was adsorbed with antiserum against oligopeptide-4 before reaction with plasma of the ATL patient (Fig. 4, lanes b and d). These results suggest that an antibody specific to the 41-kDa protein coded from the X region of HTLV-I is present in peripheral blood of patients with ATL.

DISCUSSION

In this work, we identified proteins encoded by the X regions of HTLV-I and HTLV-II. The 41-kDa protein in

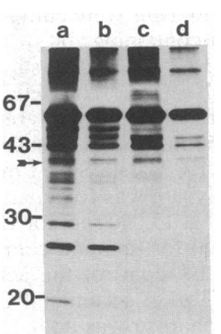

FIG. 4. Immunoprecipitation of a lysate of HUT-102 cells with plasma from patient S (lanes a and b) or patient M (lanes c and d) with ATL. Lysate was absorbed by antiserum against oligopeptide-4 before the reactions (lanes b and d).

HTLV-infected cells was precipitated not only by antiserum against oligopeptide-1, which corresponds to the NH_2 terminus region, but also by antiserum against oligopeptide-4, which corresponds to the COOH terminus in the predicted protein encoded by the X-IV open reading frame in HTLV-I (13, 14). Therefore, we believe that this protein was translated from the open reading frame of X-IV in the X region. In addition, these antisera precipitated a protein of 38 kDa in HTLV-II-infected cells, indicating the expression of a similar protein but of different size from the open reading frame Xc in the X region of HTLV-II. This result is consistent with our previous prediction of a new gene product of HTLV (18). The difference in size of the two proteins could be explained by the difference in size of the coding frames for these proteins and/or by the difference in size of the NH_2 terminus region derived from another part of the viral gene as a fused protein. The fact that these proteins were not precipitated with antiserum against oligopeptide-2 or -3 may be due to internal localization of these oligopeptide portions in the three-dimensional structure of the proteins so that they cannot be detected by these antisera. Alternatively, the 41- or 38-kDa proteins may be translated from mRNA truncated by splicing within the open reading frame Xc or X-IV, in such a way that the regions encoding oligopeptide-2 and -3 are not present in the protein products. In this regard, it is noteworthy that there are putative splice donor and acceptor sites in these frames. However, we think that this possibility is unlikely because of the high homology of amino acid sequences throughout the frames of the two viruses (18).

Sera from patients with ATL precipitated a 41-kDa protein from a HUT-102 cell extract. The intensity of the band of the 41-kDa protein was reduced when the lysate was adsorbed with antiserum against oligopeptide-1 or -4 before the reaction, suggesting the presence of an antibody against the 41-kDa protein in ATL patient plasma.

From nucleotide sequence analysis of HTLV-I and HTLV-II, we previously predicted the existence of a protein encoded from the X region as a fused form from a spliced mRNA that uses a splice acceptor site located in the 5′ region of the open reading frame, Xc and X-IV in HTLV-II and HTLV-I, respectively. From the molecular size of the identified protein and the coding capacities of the open reading frames, Xc and X-IV, we conclude that a nucleotide sequence that is able to encode a peptide composed of 10–30 amino acids is spliced to the acceptor site present at the 5′ end of the open reading frame of Xc or X-IV to make a spliced mRNA for the protein. In this regard, it is interesting to note the presence of splice donor sites at position 59 from the first base of the initiation codon of *env*, or at position 820 from the 5′ end of the provirus of HTLV-I as proposed by others (13, 26) and at position 4 from the first base of the initiation codon of *env* in HTLV-II (14) (Fig. 5). Based on this assumption, the 41-kDa protein in HTLV-I could be explained as an *env*-X or *gag*-X protein of ≈40 or ≈39 kDa, respectively, and the 38-kDa protein in HTLV-II could be explained as an *env*-X protein of ≈37 kDa. The discrepancies between these predicted molecular sizes and the observed values may be explained by a different mobility of a protein with a tertiary structure. Since the possibility of splicing with another donor site was not excluded, further studies are needed on the mechanisms of expression of the 41- and 38-kDa proteins.

The function of these proteins coded from the X region in virus-infected cells is not known. However, introduction of a deletion in the coding region for the 38-kDa protein gene of an infectious HTLV-II provirus was found to reduce expression of viral antigen as well as viral production in provirus-transfected cells, suggesting that these proteins have some important function in viral replication, probably at the level of gene expression (unpublished results). Recently, the possible existence of a *trans*-acting regulatory factor(s) that stimulates gene expression directed by HTLV LTR in virus-infected cells was reported (27). The 41- or 38-kDa protein may act as one such factor and also control expression of host genes on infection of cells with HTLV, which may be an important step in leukemogenesis of T cells (28). In this regard, the similarity in nucleotide sequence upstream of the T-cell growth factor (interleukin 2) gene to the LTR of HTLV-I is noteworthy (14).

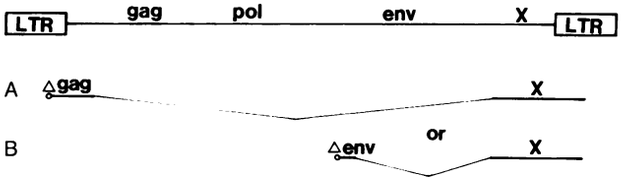

FIG. 5. A proposed spliced mRNA for 41- or 38-kDa protein encoded from the X region in HTLV. Putative splice donor sites were deduced from nucleotide sequences of HTLV-II and HTLV-I proviruses (13, 14). Model A or B in the case of HTLV-I and model B in the case of HTLV-II are discussed in the text.

The efficiency of translation of the 41-kDa protein seemed to differ in the different cell lines tested. The 41-kDa protein was expressed ≈10 times more in HUT-102 cells than in CaL-2 cells (Fig. 2*B*), in which the 41-kDa protein was hardly distinguished from a band of the same size present in the immunoprecipitate with normal rabbit serum (Fig. 3*B*). But in other experiments with different conditions of gel electrophoresis, we detected a faint band of the 41-kDa protein in a CaL-2 cell extract (data not shown).

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