Identification of $p26^{Xb}$ and $p24^{Xb}$ of human T-cell leukemia virus type II

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Human T-cell leukemia virus type II (HTLV-II) isolated from a T-cell variant of hairy cell leukemia contains *gag*, *pol* and *env* genes as well as a fourth gene termed *X*, which can code three major open reading frames Xa, Xb and Xc. Proteins with molecular masses of 26 kDa ($p26^{Xb}$) and 24 kDa ($p24^{Xb}$) encoded by the Xb open reading frame were identified with antisera directed against synthetic peptides corresponding to the N-terminal and C-terminal amino acid sequences deduced from the structure of the Xb open reading frame. More than half the Xb products were found to be located in the nuclear fraction of HTLV-II-infected cells.

Human T-cell leukemia *HTLV-II* *X gene* *X gene product* *Nuclear protein*

1. INTRODUCTION

Human T-cell leukemia virus type II (HTLV-II) was isolated from a patient (Mo) with a T-cell variant of hairy cell leukemia [1]. Like HTLV-I, HTLV-II is capable of transforming normal human peripheral blood lymphocytes in vitro [2–5]. However, the mechanism of HTLV-associated leukemogenesis is unknown. Nucleotide sequence analysis [6,7] and hybridization experiments (unpublished) indicated that HTLV-II does not carry a typical *onc* gene derived from normal cells but a sequence of 1.6 kb designated as the *X*, between *env* and 3′LTR, that has three major open reading frames, Xa, Xb and Xc. We have already detected p38 ($p38^{Xc}$) as a protein encoded by Xc [8], but no protein corresponding to the Xb open reading frame has yet been reported.

Here, we identified, in HTLV-II-infected cells, the proteins encoded by the Xb open reading frame (fig.1) and determined the subcellular localization of these proteins. This finding should be helpful in studying the function of the X proteins in the mechanism of transformation by HTLV.

2. MATERIALS AND METHODS

2.1. *Cells*

An HTLV-II producing T-cell line, Ton1, was kindly supplied by Dr R. Weiss [9]. Raji and Wil-2 are B-cell lines and Wil-2/43 is a Wil-2 cell line transfected with pH 6-neo which contains the entire HTLV-II provirus [6]. Expression of HTLV-II antigens by Wil-2/43 was confirmed by indirect immunofluorescence (unpublished). All lymphoid cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS).

2.2. *Sera*

The synthetic octadecapeptide, MPKTRRQRTRRARRNRPP (N-peptide), corresponding to the amino-terminal region of the open reading frame for Xb of HTLV-II, conjugated with keyhole limpet hemocyanin, and the synthesized C-terminal tridecapeptide, NQPSGISSPPSPSPNLASVPKTSTPPGEKP (C-peptide), conjugated with porcine thyroglobulin, were injected into guinea pigs (fig.1). The titer of the antibody was checked by Western blotting and serum with a high

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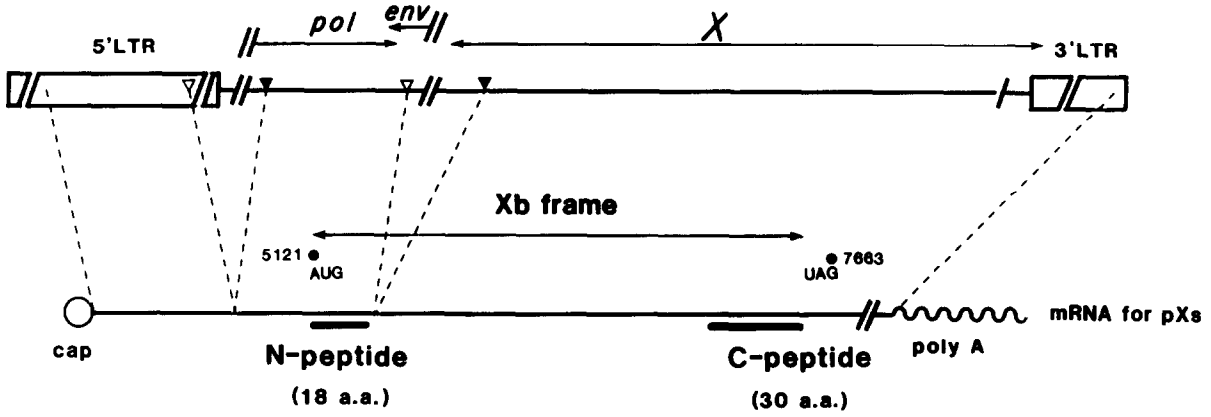

Fig.1. Localization of the N-peptide and C-peptide in the predicted protein coded from the Xb open reading frame of HTLV-II. Structure of HTLV-II provirus (upper) and mRNA for X proteins of HTLV-II (lower). Splicing sites (▽, donor site; ▼, acceptor site) for mRNA of pXs.

antibody titer was obtained after several immunizations.

2.3. *Cell extracts and immunoblot analysis*

Cell extracts were obtained by resuspending the pellet (10^7 cells) with 100 μl of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.05% SDS, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) followed by centrifugation at 10000 × *g* for 10 min at 4°C. The supernatant was mixed with the same volume of doubly concentrated sample buffer described by Laemmli [10], analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and electrophoretically transferred to a Durapore filter [11].

The filter was treated in buffer A [10 mM Tris-HCl (pH 7.4)/150 mM NaCl/1% gelatin/10% normal goat serum] for 30 min at room temperature to eliminate non-specific binding of proteins. The filter was then incubated with appropriately diluted antiserum in buffer A for 60 min at room temperature. After washing twice for 5 min in buffer B [10 mM Tris-HCl (pH 7.4)/150 mM NaCl/0.1% Tween 20] the filter was incubated with biotinylated goat anti-guinea-pig IgG in buffer B for 30 min at room temperature, and washed with buffer B as described above. The filter was then treated with avidin-biotin-peroxidase complex [12] for 30 min at room temperature. After washing with buffer B three times, each for 5 min, the filter was incubated with 0.05% diaminobenzidine tetrahydrochloride and 0.05% H_2O_2 in 0.05 M Tris-HCl (pH 7.4) for 2–3 min.

2.4. *Subcellular fractionation*

Ton1 cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 20 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 2 mM PMSF and 0.5% NP-40. These cells were then incubated on ice until the nuclei became microscopically free from cytoplasmic contamination, and the suspension was centrifuged for 5 min at 600 × *g*. The pellet was washed once with the same buffer. The supernatant was termed the cytoplasmic/membrane fraction and the pellet denoted as the nuclear fraction.

2.5. *Assay of β-N-acetylglucosaminidase*

The nuclear and cytoplasmic/membrane fractions were sonicated and centrifuged for 5 min at 5000 × *g* to remove debris. Samples were incubated in 0.1 M sodium citrate (pH 4.5), 0.16% Triton X-100 and 2 mM *p*-nitrophenyl-*β*-*N*-acetylglucosaminide at 37°C. *β*-*N*-Acetylglucosaminidase activity was determined by the change in absorbance at 420 nm [13].

2.6. *Labelling of DNA with [^{3}H]thymidine*

Ton1 cells were cultured in RPMI medium supplemented with 10% FBS and 1 μCi [^{3}H]thymidine/ml for 18 h and washed twice with PBS and fractionated to the nuclear fraction and the cytoplasmic/membrane fraction as described in section 2.5. [^{3}H]Thymidine-labelled DNA was precipitated with 5% trichloroacetic acid and radioactivity of the precipitate measured using a scintillation counter.

3. RESULTS

The extracts from various cell lines were tested by immunoblot analysis with antiserum to C-peptide. We found proteins of 26 kDa and 24 kDa in the HTLV-II-infected cell lines, Ton1 and Wil-2/43 (fig.2A, lanes a,d) but not in the uninfected cell lines, Raji and Wil-2 (fig.2A, lanes b,c). the 26 kDa (p26) and 24 kDa (p24) proteins were also detected by antiserum to N-peptide only in these HTLV-II-infected cells (fig.2B). These results indicated that p26 and p24 are not cellular proteins but virally coded ones.

The specificities of the reactions to p26 and p24 of the antiserum to C-peptide were examined by investigating their competition with the C-peptide. As shown in fig.3A, bands of p26 and p24 were detected in Ton1 cell extract with the antiserum to C-peptide (fig.3A, lane a) but not with normal guinea-pig serum (fig.3A, lane c). The intensities of these bands were greatly diminished when treatment with the antiserum to C-peptide was carried out in the presence of 1 μg C-peptide (fig.3A, lane b). Therefore, the reactions of p26 and p24 with the antiserum to C-peptide seemed to be specific.

The bands corresponding to the p26 and p24 detected by the antiserum to the C-peptide were found, upon examination, to be identical to the proteins detected by the antiserum to the N-peptide. An extract from Ton1 cells was immunoprecipitated with normal guinea-pig serum, with the antiserum to N-peptide or antiserum to C-peptide. The precipitates were analyzed by SDS-

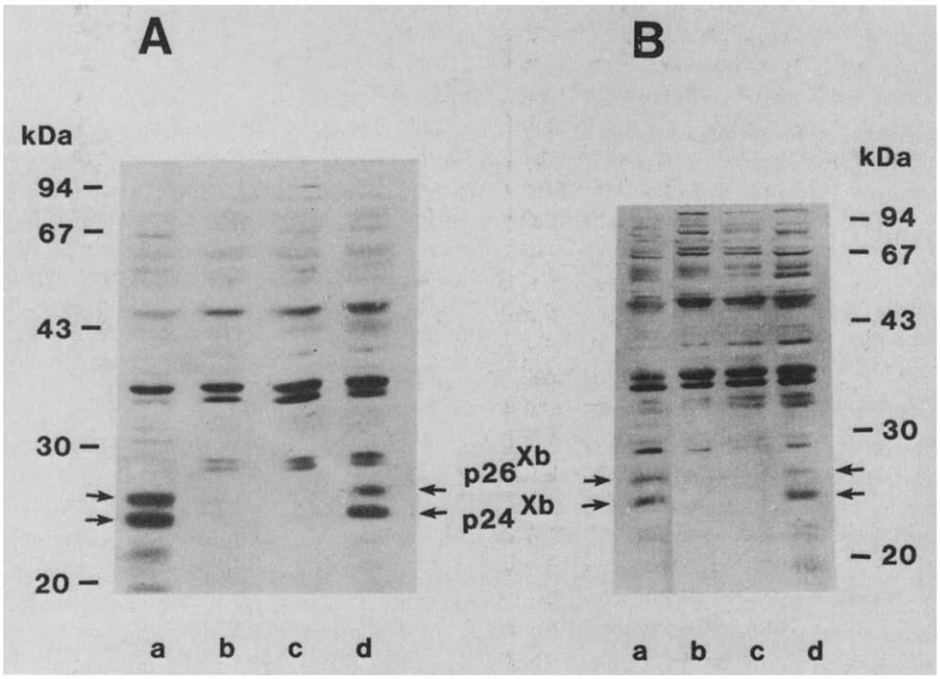

Fig.2. Detection of p26 and p24 with antisera to the synthetic peptides. Extracts of 2×10^5 cells were analyzed by SDS-PAGE of 12% polyacrylamide gel followed by immunoblotting with antisera to C-peptide (A) and N-peptide (B). Details are given in section 2. Lanes: a, Ton1; b, Raji; c, Wil-2; d, Wil-2/43.

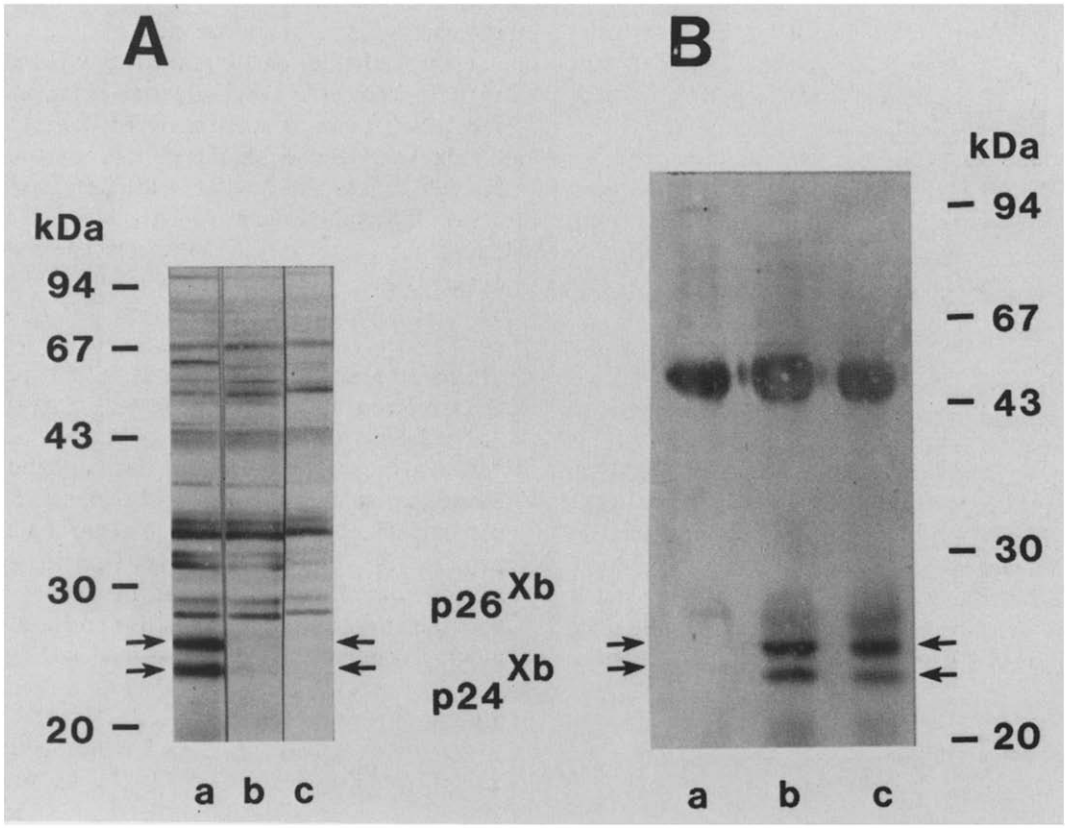

Fig.3. (A) Specificity of antiserum against C-peptide. Ton1 cell lysate was analyzed by SDS-PAGE on a 12% gel. After proteins were transferred to a Durapore filter, the filter was cut into three strips. Each strip was treated with antiserum to C-peptide (lane a) or antiserum to C-peptide in the presence of 1 μg of C-peptide (lane b) or with normal guinea-pig serum (lane c). $p26^{Xb}$ and $p24^{Xb}$ were detected by immunoblotting as described in section 2. (B) Comparison between immunoprecipitates with antisera to N-peptide and C-peptide by immunoblotting analysis. An extract of 2×10^6 Ton1 cells was reacted with 2 μl normal guinea-pig serum (lane a) or 2 μl antiserum to C-peptide (lane b) or 2 μl antiserum to N-peptide (lane c) for 10 h at 4°C, and immunoprecipitated after incubating with 5 mg protein A-Sepharose CL-4B for 10 h at 4°C. Immunoprecipitates were washed 6 times with phosphate-buffered saline and analyzed by SDS-PAGE on a 14% gel. For immunoblotting, antiserum to C-peptide was used as the first antibody.

PAGE on a 14% gel and subjected to immunoblotting with the antiserum to C-peptide (fig.3B). p26 and p24 bands are not detected in the fraction precipitated by normal guinea-pig serum (lane a) but are in the fractions precipitated by the antisera to C-peptide (lane b) and N-peptide (lane c). These results show that the proteins which reacted with the antisera to N-peptide and C-peptide are the same proteins.

Ton1 cells were fractionated into nuclear and cytoplasmic/membrane fractions. The nuclear fraction was free of cytoplasmic contamination as judged by microscopic examination. Cross-contamination of the two fractions was assumed to be negligible, because only 7% of the β-*N*-acetylglucosaminidase, a biochemical marker of the cytoplasmic fraction, was found in the nuclear fraction, while 93% was found in the cytoplasmic/membrane fraction and, also, because more than 99% of [^{3}H]thymidine-labelled DNA was found in the nuclear fraction and less than 1% in the cytoplasmic/membrane fraction. When, in the immunoblotting experiments with the antiserum to C-peptide, the amounts of the cell extract applied

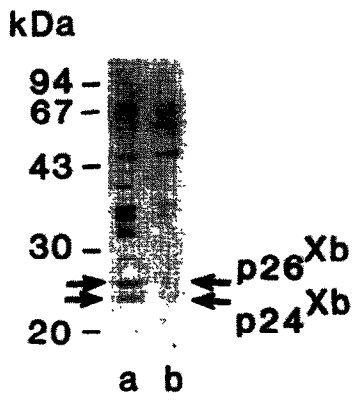

Fig.4. Subcellular localization of the $p26^{Xb}$ and $p24^{Xb}$. Tonl cells were lysed in the hypotonic buffer containing 0.5% NP-40 and fractionated as described in section 2. The volume of each fraction applied was adjusted so that the extracts from equal cell numbers were loaded on a 12% polyacrylamide gel. After electrophoresis, $p26^{Xb}$ and $p24^{Xb}$ were detected by immunoblotting with antiserum to C-peptide. Lanes: a, nuclear fraction; b, cytoplasmic/membrane fraction.

to each lane were adjusted to correspond to equivalent cell numbers, more than half the p26 and p24 was found in the nuclear fraction (fig.4).

4. DISCUSSION

A protein encoded by the Xb frame of HTLV-II was predicted to be translated from the same single spliced mRNA as $p38^{Xc}$ of HTLV-II encoded by the Xc frame [14–17]. Using the antisera to the octadecapeptide corresponding to the NH_2 terminus and to the tridecapeptide corresponding to the COOH terminus of the Xb frame (nucleotide 5121–5174 and 7571–7660) [7], the same proteins, p26 and p24 were detected. These proteins were found only in cells infected with HTLV-II (fig.2A,B) and the reactions between them and the antisera were specific. Therefore, we concluded that p26 and p24 were virally coded proteins ($p26^{Xb}$ and $p24^{Xb}$) encoded by the Xb open reading frame of HTLV-II. The putative protein encoded by the Xb open reading frame consists of 170 amino acids and has a molecular mass of 19 kDa. The discrepancies in the molecular sizes of the observed immunoreacted p26 or p24 and the predicted p19 may be due to the high content of proline residues in the predicted amino acid sequence, since proteins with high proline contents were shown to move more slowly than expected on SDS-PAGE [18]. Although we do not know how these proteins are coded from the Xb open reading frame, there are at least three possible reasons for the two proteins, $p26^{Xb}$ and $p24^{Xb}$, being identified by the antisera to the synthetic peptides: (i) there is another X mRNA which has a structure other than that shown in fig.1 which may have a truncated Xb frame; (ii) $p26^{Xb}$ has the same peptide backbone as $p24^{Xb}$ but has a larger molecular mass in SDS-PAGE due to post-translational modification such as glycosylation or phosphorylation; (iii) $p24^{Xb}$ is a degraded product from $p26^{Xb}$ in which a small portion from the C-terminus of $p26^{Xb}$, which corresponds to about 17 amino acid residues judging from the difference in the molecular masses of p26 and p24, may be cleaved off but in which the remaining protein, $p24^{Xb}$, still has an epitope which is reactive to the antibody to C-peptide.

More than half the $p26^{Xb}$ and the $p24^{Xb}$ was found to be present in the nuclear fraction in Tonl cells (fig.4). The nuclear localization of these proteins seems consistent with them having a cluster of arginine residues (8 out of the 18 amino acid residues at the N-terminal region are arginine residues). A cluster of such basic amino acids is thought to act as a nuclear location signal as it has been shown that a cluster of basic amino acid residues regulates the localization of SV40 large T antigen [19]. The function of Xb proteins is unknown, but their nuclear localization suggests that they may be involved in regulating viral and/or cellular gene expression.

There is much homology between the amino acid sequence of the pX-III frame in HTLV-I and that of the Xb frame in HTLV-II. We reported a sequence, CCCATTTCCTAG, from nucleotides 7214 to 7225 in HTLV-II but, on resequencing the X gene, T at nucleotide 7223 was found to be C (unpublished). Thus, the first termination codon at the 5′-end of the Xb frame of the spliced mRNA must be glutamine and the Xb open reading frame continuous to the termination codon at nucleotides 7661–7663. Recently, proteins encoded by the pX-III frame in HTLV-I, named $p27^{X-III}$ and $p21^{X-III}$, have been reported in HTLV-I-infected cells and were detected mainly in the nuclear fraction [20]. These findings suggest that both the

$p26^{Xb}$ or $p24^{Xb}$ of HTLV-II and the $p27^{X-III}$ or $p21^{X-III}$ of HTLV-I have important functions in viral replication and/or cellular transformation.

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