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A novel alternatively spliced viral mRNA transcribed in cells infected with human T cell leukemia virus type 1 is mainly responsible for expressing p21X protein

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The pX sequence of human T cell leukemia virus type 1 (HTLV-1) has been thought to be expressed as a doubly spliced mRNA that codes for p40tax, p27rex and p21X. However, we identified a novel alternatively spliced mRNA in the HTLV-1 infected cells by using reverse transcription followed by the polymerase chain reaction. This mRNA contains only the first and third exons of the doubly spliced mRNA and encodes only p21X. Our data that this mRNA is responsible for expressing p21X exists in most of HTLV-1 infected cells strongly suggests that p21X may play a crucial role for HTLV-1 replication.

Human T cell leukemia virus 1 (HTLV-1); RNA splicing; PX gene; Gene product, rex; Polymerase chain reaction

1. INTRODUCTION

The human T cell leukemia virus type 1 (HTLV-1) is a human retrovirus that has been shown to be a causative agent of a human malignant disorder, adult T cell leukemia (ATL) [1–3], and associated with a subgroup of chronic progressive myelopathies, tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) [4,5]. It has a unique regulatory sequence termed pX in addition to the *gag*, *pol* and *env* sequences present in all replication-competent retroviruses [6]. This region is expressed as a 2.1 kb doubly spliced mRNA [7], and the mRNA (*tax/rex* mRNA) has two overlapping open reading frames, designated *tax* and *rex* [8]. *tax* encodes p40tax, which was shown to be a trans-activator of the long terminal repeat (LTR) of HTLV-1 [9–13] and several cellular genes [14–17]. *rex* encodes p27rex and p21X [18], and p27rex is a post-transcriptional regulator, which induces the accumulation of unspliced viral *gag-pol* mRNA [19,20]. The complete amino acid sequence of p21X is contained within the C-terminal portion of p27rex [6], but the function of p21X is still not known [8,21].

Although a possibility had been implied to be that p27rex and p21X might be independent translation products from differently spliced mRNA [8], no evidence for alternatively spliced mRNA other than *tax/rex* mRNA was found by blotting or by a nuclease S1 protection assay [21]. Furthermore, from other data, *tax/rex* mRNA had been concluded to also code for p21X as well as p27rex as described elsewhere [18].

Recently, to detect mRNA that is expressed in small amounts, a highly sensitive method involving polymerase chain reaction coupled to reverse transcription (RT-PCR) was developed [21,22]. Until quite recently, no expression of HTLV-1 was detected by immunofluorescence analysis or RNA blot analysis in most fresh peripheral blood mononuclear cells (PBMC) of patients with ATL [23–26]. However, applying RT-PCR, recent reports indicated that the expression of *tax/rex* mRNA could be detected in their fresh PBMC [27,28]. Also, in another report, RT-PCR has been applied to analysis for a complex series of splicing events of viral mRNA transcripts in human immunodeficiency virus type 1 (HIV-1) infected cells [29]. HIV contains seven additional genes (*tat*, *rev*, *tev*, *vpu*, *vpr*, *vif* and *nef*) in addition to the signatory retroviral genes *gag*, *pol* and *env* [30]. The report has showed that the two-exon *nef* specific transcript represented the final product of the complex series of splicing pathways of HIV-1 infected cells [29], although after this discovery, it is still documented that the conventional double spliced mRNA is only responsible to code for *nef* [30,31].

In this report, the possibility that p27rex and p21X are independent translation products from differently spliced mRNA other than *tax/rex* mRNA was examined by RT-PCR or by a highly sensitive detection procedure of modified RT-PCR coupled to a double PCR with nested primers. We focused on analyzing the expression-mechanisms of p21X to understand its func-

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tion. Thereby we first found a novel mRNA that alternatively spliced the second exon from *tax/rex* mRNA and was composed of the first and third exon, like the *nef* specific mRNA in HIV as described [29]. Second, this new two-exon mRNA was demonstrated to specifically code for p21X, so it is named p21X mRNA, signifying the p21X specific mRNA. In addition to these, other similar alternatively spliced mRNA in addition to p21X mRNA detected by highly sensitive RT/TS-PCR in HTLV-I infected cells were described.

2. MATERIALS AND METHODS

2.1. *Cells*

Human T-cell lines carrying HTLV-I (MT-1, MT-2, MT-4, TL-Su, and H582) and not-carrying HTLV-I (Molt-4) were cultured in a medium, RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). The MT-1 [33] and H582 (a gift from H. Sato, Kyushu University) (Hayai in [8]) cell lines were derived from leukemia cells of the PBMC from patients with ATL. The MT-2 and MT-4 cell lines were established by cocultivation of human cord blood lymphocytes of normal subjects with the PBMC of ATL patients [33,34]. The TL-Su cell line was established from the PBMC of an asymptomatic HTLV-I carrier [26]. The Molt-4 cell line was established from PBMC of a patient with acute lymphoblastic leukemia (ATCC CRL-1582).

2.2. *Synthetic oligonucleotides and other reagents*

Oligonucleotide primers for PCR and probes for Southern hybridization analysis were synthesized on a Cyclone DNA synthesizer (Biosearch Inc., CA, USA) by the phosphoramidite method and purified with oligonucleotide purification cartridges (Applied Biosystems Inc., CA, USA). A random hexadeoxynucleotide primer, avian myeloblastosis virus reverse transcriptase, restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, and *Thermus aquanticus* DNA polymerase (Taq polymerase) were purchased from Takara Shuzoh (Kyoto, Japan). [α-^{32}P]dCTP and [γ-^{32}P]ATP were from Amersham (England).

2.3. *RNA preparation and Northern analysis*

RNA from cell lines was isolated by the guanidinium–acid–phenol method as described previously [35]. The amount and quality of RNA were estimated by measuring the A_{260} and A_{280} and by analyzing a sample on a formaldehyde-agarose gel and comparing the relative intensities of the bands of 18 S and 28 S rRNAs visualized by staining with ethidium bromide.

15 μg total RNA was fractionated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane filter (Hybond-N+; Amersham). The filter was prehybridized in a prehybridization buffer (6× SSC, 0.5% SDS, 5× Denhardt's, 100 μg/ml calf thymus DNA) at 37°C for 2 h and hybridized for 16 h at 37°C in the prehybridization buffer including a oligonucleotide probe (oligo-probe) (1–1.5×10^7 cpm) labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase or at 65°C in the prehybridization buffer including a double strand DNA fragment probe (dsDNA fragment-probe) (1–2×10^7 cpm) labeled with [α-^{32}P]dCTP by polymerase chain reaction with Taq DNA polymerase. Then the filter hybridized with an oligo-probe was washed in 1× SSC and 0.5% SDS for 15 min at 37°C, followed by 30 min washes at 55°C in 3 M tetramethylammonium chloride and 0.5% SDS solution, and exposed to Kodak XAR film with a single intensifying screen at −80°C for 7 days. These conditions did not allow hybridization of sequences carrying a single mismatch [36]. The other filter hybridized with a dsDNA fragment-probe was washed in 1× SSC and 0.5% SDS for 10 min at 65°C, followed by 15 min washes at 65°C in 0.1× SSC and 0.5% SDS, and exposed to Kodak XAR film with a single intensifying screen at −80°C for 2 days.

2.4. *RT-PCR, Southern hybridization and RT/two step-PCR (RT/TS-PCR)*

RT-PCR was carried out as described [23] with minor modifications. Briefly, 0.25 μg of total RNA was annealed with 500 ng of a random hexadeoxynucleotide primer and was reverse transcribed with 15 units of avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. Then 8.3 μl of 6× PCR buffer (1× PCR buffer consists of 16.6 mM ammonium sulfate, 67 mM Tris-HCl (pH 8.8 at 25°C), 6.7 mM magnesium chloride, 10 mM 2-mercaptoethanol, 7.7 μM EDTA, and bovine serum albumin at 170 μg/ml), 5 μl each of 15 mM dATP, 15 mM dCTP, 15 mM dGTP, and 15 mM TTP, and 50 pmol of each PCR primer were added to the reaction vessel in a final volume of 50 μl. The sequences of the primers for PCR (PX1, PX2, PX3, PX4 and PX10) and of probes for Southern hybridization analysis (PX5 and PX6) are shown in Fig. 1. PX1 and PX2 are located upstream and downstream, respectively, of the putative splice junction site of p21X mRNA. The amplified DNA fragments obtained with PX1 and PX2 after PCR were expected to be 508-base pairs (bp) for *tax/rex* mRNA and 317-bp for p21X mRNA. The probe PX6 is a 20-mer of the sequence surrounding the putative splice junction site of p21X mRNA. PX3 and PX4 are identical to RPX3 and RPX4, respectively, as described [27]. PX3 and PX4 are located upstream and downstream, respectively, of the second splice junction site of *tax/rex* mRNA. The amplified DNA fragments obtained with PX3 and PX4 after PCR were 144-bp for *tax/rex* mRNA. The probe PX5 identical to RPXPR1 as described previously [27] is a 20-mer of the sequence surrounding the second splice junction site of *tax/rex* mRNA. Then PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer/Cetus, CT, USA) for 35 cycles with 2.5 units of Taq polymerase. The reaction conditions were 1 min at 94°C for denaturation, 1 min at 55°C for primer annealing, and 2 min at 72°C for primer extension. After the reaction, one-fifth of the amplified DNA product was electrophoresed in a composite gel of 2% Nusieve agarose and 1% standard agarose (FMC BioProducts, ME, USA) and stained with ethidium bromide (0.5 μg/ml). The presence of a band with the expected size for each primer pair

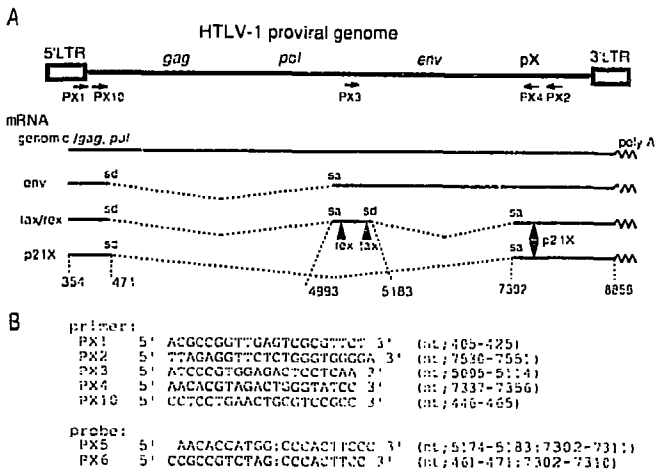

Fig. 1. Transcription map of HTLV-I with location and sequences of oligonucleotide primers and probes for RT-PCR analysis. (A) Genomic structure of HTLV-I provirus and three typical RNA transcripts and a novel p21X-specific transcript. Sd, splice donor site; sa, splice acceptor site. The p40tax, p27rex and p21X initiation codons (AUG) are indicated by the vertical arrowheads. The locations and orientation of the primers for PCR, PX1, PX2, PX3, PX4 and PX10, are shown by the horizontal arrows. (B) Sequences of primers and probes used in PCR and Southern blot analysis. DNA sequence information from Seiki et al. [6] is used. PX5 and PX6 are identical to the sequences surrounding the splice junction sites of the *tax/rex* and the p21X-specific transcripts, respectively. The colons in these probe-sequences indicate the splice junction sites.

was determined to be positive. To confirm the specificity by hybridization, the gel was denatured in alkaline solution and transferred to a nylon membrane filter (Hybond-N+). After fixation of UV irradiation, the filter was hybridized at 42°C with a ^{32}P-end-labeled oligoprobe (1–1.5×10^7 cpm) for 16 h. Prehybridization and hybridization were carried out as described [37]. Then the filter was washed in 5× SSPE at 48°C and exposed to Kodak XAR film with a single intensifying screen for 2 h at −80°C.

To determine easily and specifically the presence of the HTLV-1 proviral sequences, we have developed the two-step-PCR (TS-PCR) method as described previously [38], which is a modified nested double PCR method [39] used with an optimal concentration of primer pairs in the first amplification step. So, to detect mRNA, a very highly sensitive method involving the TS-PCR coupled to reverse transcription (RT/TS-PCR) was developed. Briefly, after the RT reaction as mentioned above, the first step PCR was performed for 25 cycles under the same reaction conditions as mentioned above except for using one picomole of each primer (PX1 and PX2). One-tenth of the first step PCR DNA product was used as template DNA for the second step PCR with 100 pmol of each primer (PX10 and PX4 for targeting p21X mRNA, and PX3 and PX4 for *tax/rex* mRNA) for 35 cycles. The other reaction conditions were the same as those mentioned above. After the reaction, the amplified DNA product was determined by staining with ethidium bromide and Southern hybridization. The final amplified DNA fragments after RT/TS-PCR were expected to be 272-bp for *tax/rex* mRNA and 81-bp for p21X mRNA when amplified with primers PX10 and PX4 and 144-bp for *tax/rex* mRNA when amplified with primers PX3 and PX4.

2.5. *Cloning and DNA sequencing*

In some experiments, gel-purified PCR products were sequenced directly by a modification of the dideoxy chain termination procedure [40] with a Sequenase kit (United States Biochemical, OH, USA). When subcloning before sequencing was required, the 317-bp fragment amplified from p21X mRNA in H582 by RT-PCR with primers PX1 and PX2 was separated from unincorporated primers and the 508-bp fragment derived from *tax/rex* mRNA in H582 by electrophoresis in 2% agarose gels. The purified fragment was ligated with T4 DNA ligase in buffer supplied by the manufacturer into a pUC18 vector (Takara Shuzoh) cut with the restriction endonuclease *Sma*I. A small portion was transfected into competent host cells (JM109) and the resultant clones were analyzed by hybridization with the 317-bp amplified fragment and by DNA sequence analyses using the dideoxy chain termination method.

2.6. *Detection of p27rex and p21X proteins by Western blot analysis*

Western blot analysis of p27rex and p21X was performed as described previously [42]. Proteins were extracted from cells, separated by 18% SDS-polyacrylamide gel electrophoresis, and transferred to a membrane (Clear Blot membrane-P; ATTO Co., Tokyo, Japan). Blotted proteins were incubated with rabbit antibody against C-terminal peptide, which reacts with both p27rex and p21X (a gift from H. Shida, Kyoto University) [43]. The immunocomplexes were detected by binding with horseradish peroxidase-conjugated protein A and colored with a Konika immunostain kit (Konika Co., Tokyo, Japan).

3. RESULTS

3.1. *Finding of a novel alternatively spliced mRNA in HTLV-1 infected cell lines*

RNA from HTLV-1-infected cell lines was analyzed to evaluate the sensitivity and specificity of RT-PCR with primers PX3 and PX4 to detect *tax/rex* mRNA as described previously [27]. After RT-PCR, we confirmed the specific signal and sufficient sensitivity was observed to detect the amplified band, 144-bp, derived from *tax/rex* mRNA in HTLV-1 infected cell lines, MT-2 and H582 and not to detect any bands from an HTLV-1 uninfected cell line, Molt-4 (Fig. 2a). On the other hand, when using primers PX1 and PX2, a 508-bp fragment was expected to be amplified from *tax/rex* mRNA by RT-PCR, but only a 317-bp fragment was observed to be amplified in MT-2 and H582 (Fig. 2b). The 317-bp band was not hybridized with the probe PX5 (data not shown) but was done with the oligo-probe PX6 (Fig. 4). From these findings, we postulated that the 317-bp fragment might be amplified from a putative alternatively spliced mRNA transcript which contains only the first and third exons of the *tax/rex* mRNA and codes for p21X.

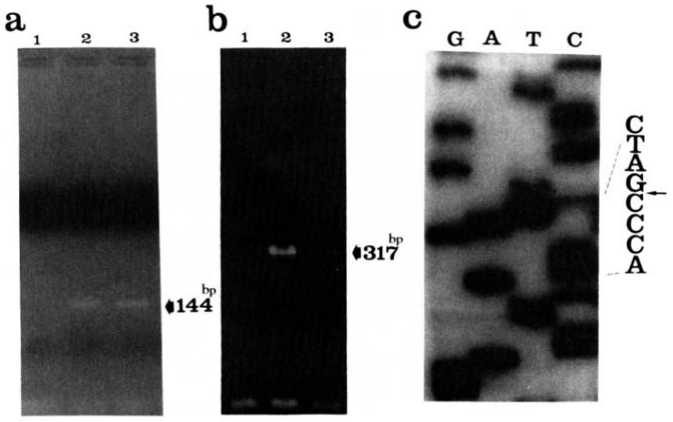

Fig. 2. Ethidium bromide staining and sequencing of an amplified DNA fragment derived from the HTLV-1 p21X mRNA. A 144-bp (a) and a 317-bp (b) amplified DNA by RT-PCR with primers PX3 and PX4, and PX1 and PX2, respectively, are shown. RNA derived from Molt-4 (lane 1), H582 (lane 2) and MT-2 (lane 3) were used as template RNA. (c) Sequencing autoradiogram of the 317-bp fragment prepared from H582 cells. The arrow indicates the position of the splice junction of the alternatively spliced pX mRNA (p21X mRNA shown in Fig. 1A). The sequence shown here is consistent with the sequence of probe PX6 (Fig. 1B).

To characterize this 317-bp-fragment, the fragment was subcloned into the pUC18 vector and sequenced by the chain-termination method with dideoxy nucleotide (Fig. 2c) [40]. The sequence data confirmed that the fragment did not contain the second exon of *tax/rex* mRNA but just connected the first and third exons of *tax/rex* mRNA, and revealed that the amplified 317-bp fragment was derived from a novel alternatively spliced mRNA transcript (p21X mRNA shown in Fig. 1).

To determine p21X mRNA by an additional technique, Northern analysis was carried out using the oligo-probe PX6, which hybridized specifically to p21X mRNA but not to *tax/rex* mRNA as mentioned above. In total RNA from H582 and MT-2, typical viral RNA (8.8, 4.2, 2.1 kb) was detected with the pX dsDNA fragment-probe, which can hybridize all viral RNA (Fig. 3a). However, p21X mRNA (1.9 kb) was detected with the PX6 probe only in RNA from H582 (Fig. 3B).

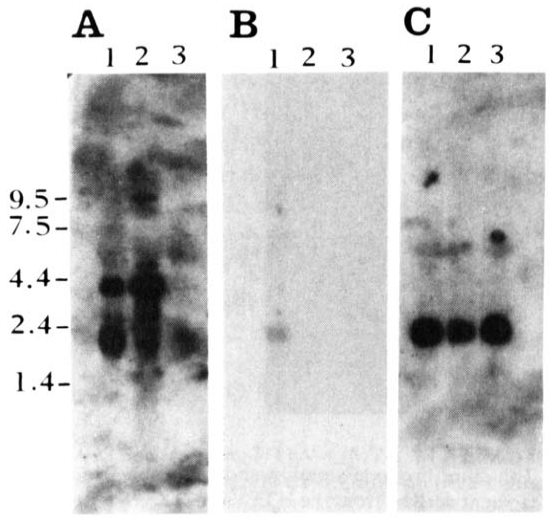

Fig. 3. Determination of the length of p21X mRNA by Northern analysis with the specific oligo-probe. Total RNA (15 μg) from H582 (lane 1), MT-2 (lane 2) and Molt-4 (lane 3) was fractionated on a formaldehyde-gel, transferred to a nylon membrane filter and hybridized with the HTLV-1 pX dsDNA fragment-probe (429-bp; 7386–7814 nt [6]) (A), the oligo-probe PX6 (B) and the β-actin dsDNA fragment-probe (419-bp; 281–1141 nt [46]) (C). (A) RNA from H582 and MT-2 were analyzed for HTLV-1 genomic/gag-pol (8.5 kb), env (4.2 kb), and tax/rex mRNA (2.1 kb) RNA from H582 was analyzed for p21X mRNA (1.9 kb). (C) RNA from H582, MT-2 and Molt-4 was analyzed for β-actin mRNA (1.9 kb). This experiment was performed to verify that an equal amount of total RNA was used for these analyses. The molecular standard bands (0.24–9.5 kb RNA Ladder; Bethesda Research Laboratories, MD, USA) are indicated at the left.

It might be considered that the sensitivity of mRNA detection by Northern analysis is lower than that by the RT-PCR method and p21X mRNA expression in MT-2 is low-level as shown in Fig. 2. The negative data in RNA from MT-2 is consistent with the RT-PCR data, showing that the expression levels of p21X mRNA in MT-2 were lower than that in H582 (Fig. 2). From the data of this Northern analysis, the presence of p21X mRNA was also proven and the size (1.9 kb) of p21X mRNA was consistent with the predicted one (1675 bases + polyA tail (~300 bases) = ~1.9 kb; Fig. 1).

Furthermore, we examined the expression of p21X mRNA in other HTLV-1 infected cell lines, MT-1, MT-4 and TL-Su, as well as MT-2, H582, and Molt-4 by RT-PCR and Southern hybridization. It is shown in Fig. 4a that the 144-bp fragment hybridized with the PX5 probe was amplified with primers PX3 and PX4 in RNA from MT-1, MT-2, MT-4, TL-Su and H582 but not from Molt-4, suggesting that *tax/rex* mRNA was specifically expressed in all HTLV-1 infected cell lines used here. After RT-PCR with primers PX1 and PX2 and hybridization with the PX6 probe, the 317-bp specific signal amplified was observed in RNA from MT-2, MT-4, TL-Su, and H582 but not from MT-1, Molt-4 (Fig. 4b). Interestingly, a very high-level expression of p21X mRNA was observed in H582, compared with that in MT-2, MT-4, and TL-Su. p21X mRNA was not detected in MT-1, regardless of the presence of *tax/rex* mRNA.

3.2. *The novel alternatively spliced mRNA translates p21X protein*

From the viral genomic structure, this alternatively spliced mRNA could code for p21X (Fig. 1). To investigate this possibility, Western blot analysis using antibody against the C-terminus oligopeptide of p27rex/p21X was carried out. In H582, a larger amount of p21X was detected than in MT-2, MT-4 and TL-Su (Fig. 5). However, no p21X was detected in MT-1 (Fig. 5), confirming the published data as described previously [8]. We already mentioned above that the expression of p21X mRNA was strongly positive in H582 and negative in MT-1 (Fig. 4), indicating that the amount of p21X in the HTLV-1 infected cell lines tended to be

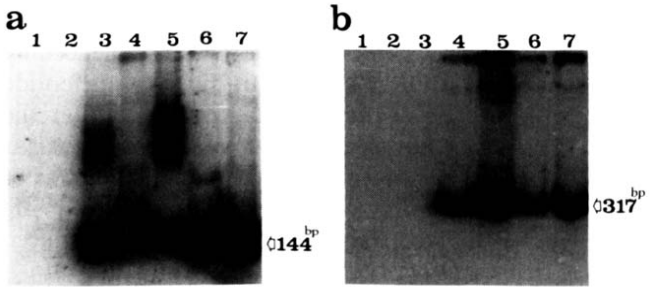

Fig. 4. RT-PCR and Southern analysis of an amplified DNA fragment derived from p21X mRNA in HTLV-1 infected cells. (a) The 144-bp amplified DNA as described in the legend of Fig. 2 is determined by hybridization with the oligo-probe PX5 in RNA from MT-1 (lane 3), MT-2 (lane 4), H582 (lane 5), MT-4 (lane 6), and TL-Su (lane 7), but not in distilled water (lane 1) and Molt-4 (lane 2). (b) The 317-bp amplified DNA as described in the legend of Fig. 2 is determined by hybridization with the oligo-probe PX6 in RNA from MT-2 (lane 4), H582 (lane 5), MT-4 (lane 6), and TL-Su (lane 7), but not in distilled water (lane 1), Molt-4 (lane 2), and MT-1 (lane 3).

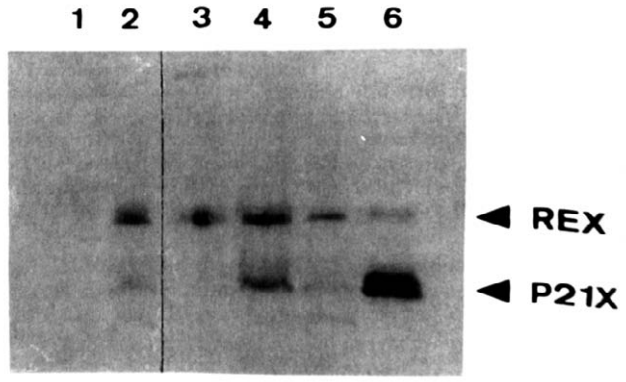

Fig. 5. Western blot analysis for p27rex and p21X in HTLV-1 infected cell lines. Total cell extracts from HTLV-1 infected cell lines, TL-Su (lane 2), MT-1 (lane 3), MT-2 (lane 4), MT-4 (lane 5), and H582 (lane 6), and an HTLV-1 uninfected cell line, Molt-4 (lane 1), were separated by SDS-polyacrylamide gel electrophoresis and transferred to a filter. p27rex and p21X were revealed with rabbit antiserum against both proteins.

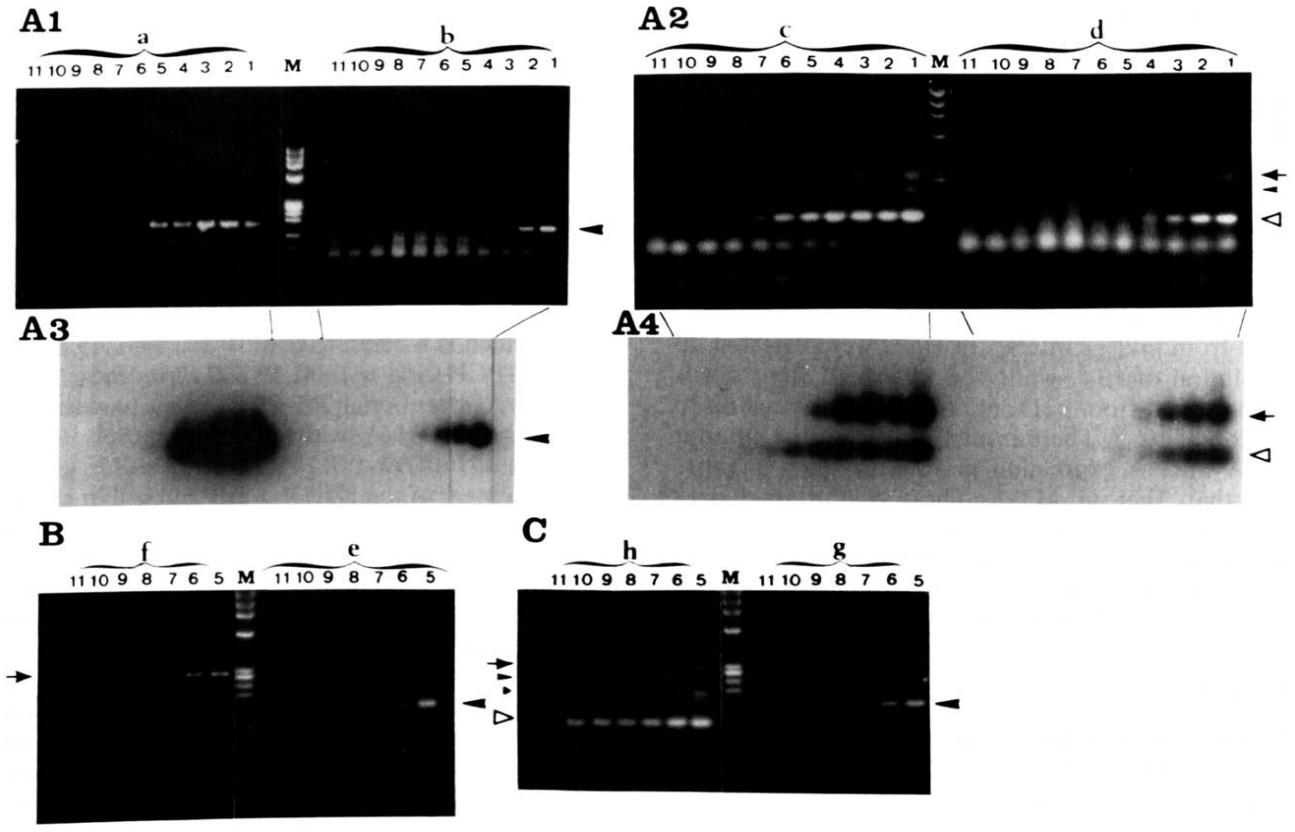

Fig. 6. Quantitative detection of *tax/rex* mRNA and p21X mRNA by RT/TS-PCR method. (A) Serial-diluted MT-2 total RNA with Molt-4 total RNA were amplified by RT-PCR (b and d) or RT/TS-PCR (a and c), followed by ethidium bromide-staining (A1 and A2) or Southern hybridization with PX5 and PX6 mix-probe (A3 and A4). In each amplification, 0.5 μg of Molt-4 cell total RNA plus 5×10^{4} pg, 5×10^{3} pg, 5×10^{2} pg, 5×10^{1} pg, 5×10^{0} pg, 5×10^{-1} pg, 5×10^{-2} pg, 5×10^{-3} pg, 5×10^{-4} pg, 5×10^{-5} pg, or 0 pg to MT-2 cell total RNA (lanes 1–11, respectively) were used as template RNA. The series of RT-PCR products, 144-bp (◄), derived from *tax/rex* mRNA with primers, PX3 and PX4, are shown in A1b and A3b. The series of RT/TS-PCR products, 144-bp (◄), derived from *tax/rex* mRNA with primers, PX1 and PX2, for 1st step amplification and with primers, PX3 and PX4, for 2nd step amplification are shown in A1a and A3a. The series of RT-PCR products, 272-bp (◄–) and 81-bp (◁), derived from *tax/rex* mRNA and p21X mRNA, respectively, with primers, PX10 and PX4, are shown in A2b and A4b. The series of RT/TS-PCR products, 272-bp (◄–) and 81-bp (◁), derived from tax/rex mRNA and p21X mRNA, respectively, with primers, PX1 and PX2, for 1st step amplification and with primers, PX10 and PX4, for 2nd step amplification are shown in A2c and A4c. (B) Serial-diluted MT-1 total RNA with Molt-4 total RNA as described in (A) was amplified by RT/TS-PCR with primers, PX1 and PX2, for 1st step amplification and primers, PX3 and PX4 (e), or PX10 and PX4 (f), for 2nd step amplification. (C) Serial-diluted H582 total RNA with Molt-4 total RNA as described in (A) was amplified by RT/TS-PCR with primers, PX1 and PX2, for 1st step amplification and primers, PX3 and PX4 (g), or PX10 and PX4 (h), for 2nd step amplification. Two additional amplified products, about 200-bp (◄) and about 170-bp (◄), are shown in A2 and Ch. The molecular standard bands (*Hae*III digested φX174 phage DNA) are indicated by the letter, M.

correlated with the level of p21X mRNA expression but not with that of *tax/rex* mRNA expression. The evidence implies that p21X might be translated mainly from p21X mRNA instead of *tax/rex* mRNA in HTLV-1 infected cell lines.

3.3. *Highly sensitive and quantitative detection of* tax/rex *mRNA and p21X mRNA by RT/TS-PCR*

To evaluate the high sensitivity of the RT/TS-PCR method for detection of mRNA, we compared the sensitivity of RT/TS-PCR with that of RT-PCR using serial-diluted MT-2 cell total RNA with Molt-4 cell total RNA as described in Fig. 6A. For detection of *tax/rex* mRNA, the specific detection for the RT-PCR products (144-bp) was observed only up to a dilution of 10^{-4} (Fig. 6A1b), while the specific detection for the RT/TS-PCR products (144-bp) was observed up to a dilution of 10^{-7} (Fig. 6A1a). For detection of p21X mRNA, the specific detection for the RT-PCR products (81-bp) was observed only up to a dilution of 10^{-4} (Fig. 6A2d), while the specific detection for the RT/TS-PCR products (81-bp) was observed up to a dilution of 10^{-8} (Fig. 6A2c). These results demonstrated that RT/TS-PCR under these conditions is 1000 to 10 000 times more highly sensitive than RT-PCR. These RT/TS-PCR products were also qualitatively verified by the data of Southern hybridization experiments (Fig. 6A3 and 6A4).

Moreover, we applied RT/TS-PCR to determine

amounts of *tax/rex* mRNA and p21X mRNA in MT-1 and H582. As shown in Fig. 6B, the RT/TS-PCR products (144-bp) derived from *tax/rex* mRNA in MT-1 were detected up to a dilution of 10^{-6}, while no RT/TS-PCR product derived from p21X mRNA was detected up to a dilution of 10^{-5}. In other RT/TS-PCR experiments with undiluted total RNA of MT-1, the fact that no band derived from p21X mRNA was amplified (data not shown) strongly verified that MT-1 cells express p21X mRNA at an undetectable level or not at all. As shown in Fig. 6C, the RT/TS-PCR products (144-bp) derived from *tax/rex* mRNA in H582 were detected up to a dilution of 10^{-6}, while the RT/TS-PCR products (81-bp) derived from p21X mRNA were detected up to a dilution of 10^{-10}. These results show that the amount of p21X mRNA expression in H582 is over 10 times higher than that in MT-2. These quantitative data strongly suggest that p21X mRNA is mainly responsible for expressing p21X in HTLV-1 infected cells but *tax/rex* mRNA has very little or no responsibility for expressing p21X in infected cells.

4. DISCUSSION

To date, *tax/rex* mRNA of HTLV-1 is well known to encode for p40tax, p27rex and p21X, and has been interpreted to express all of them. On the contrary, Kiyokawa et al. [8] suggested that there might be a further possibility that p27rex and p21X were independent translation products from differently spliced mRNA. However, they found no evidence for alternatively spliced mRNA by blotting or nuclease S1 protection assay [44]. Nagashima et al. [18] later demonstrated that *tax/rex* mRNA was a common transcript for p40tax, p27rex and p21X. They cloned a cDNA clone derived from *tax/rex* mRNA, prepared pure mRNA of this cDNA clone using SP6 vector and the polymerase system, and translated it in vitro. They identified three pX proteins, p40tax, p27rex and p21X, as products translated from the pure mRNA. Mutagenesis on AUG codons demonstrated that the first AUG codon in mRNA is for p27rex translation, the second for p40tax and the fourth for p21X. These findings have clearly swept away the former possibility since then.

In order to investigate the expression of pX mRNA in more detail, we developed a new technique, RT/TS-PCR, which is a combination of the two-step PCR [38] and RT-PCR, and is 1000–10 000 times more sensitive than the conventional RT-PCR so as to detect small amounts of mRNA without using radioisotopes. In addition to *tax/rex* mRNA, a novel mRNA was detected in most of the HTLV-I infected cell lines studied here by using RT-PCR and RT/TS-PCR. The DNA sequencing analysis of cDNA derived from the novel mRNA showed that the novel mRNA contained only the first and third exons of the double spliced transcript (*tax/rex* mRNA) but not the second exon, suggesting that this RNA was an alternatively spliced mRNA transcript, and the third exon only contained the p21X coding frame, suggesting that this RNA could code for p21X but not p27rex nor p40tax. The alternatively spliced mRNA in our HTLV-1 infected cells is the two-exon mRNA, which is analogous to the two-exon *nef* specific mRNA in HIV-1 infected cells [29]. Moreover, by Northern analysis with an oligo-probe specific for this mRNA, we determined that the size of this mRNA is 1.9 kb, which is very close to that of *tax/rex* mRNA (2.1 kb) [7]. Because of the similarity in size, therefore, the analysis of the relative expression levels of pX transcripts by Northern blots was not feasible.

We then assumed that this new p21X mRNA might be a specific transcript for p21X. First, we isolated a specific cDNA clone derived from the p21X mRNA, ligated the cDNA fragment at a sense orientation into an expression vector, pCD-SRα [41], and examined the transient expression in COS cells (ATCC CRL-1650) transfected with the construct plasmid DNA. In the transfected COS cells, p21X but not p27rex was detected (data not shown), confirming that the cDNA from the p21X mRNA contains the p21 coding frame and has the ability to express p21X. Second, as summarized in Table I, we investigated the comparison between the amounts of the *tax/rex* and p21X mRNA and the amounts of p27rex and p21X in HTLV-I infected cell lines. Although the *tax/rex* mRNA and p27rex were expressed in all HTLV-I infected cell lines tested, H582, MT-1, MT-2, MT-4, TL-Su, but not in the HTLV-I uninfected cell line, Molt-4, the p21X mRNA and p21X were expressed in most of HTLV-I infected cell lines, such as H582, MT-2, MT-4, TL-Su, except for MT-1 and Molt-4. Interestingly, H582 cells express the largest amount of p21X and of p21X mRNA when compared

Table I

Positive correlation between the level of p21X mRNA expression and the amount of p21X protein expressed in HTLV-I infected cell lines

	mRNA[a]		Protein[b]	
Cell lines	*tax/rex*	p21X	p27rex	p21X
MT-1	10^6	$<10^0$	+	−
MT-2	10^7	10^8	++	++
MT-4	10^6	10^6	+	+
TL-Su	10^7	10^6	++	+
H582	10^6	10^{10}	+	+++
Molt-4	$<10^0$	$<10^0$	−	−

[a] Amounts of mRNA are quantitatively estimated as described in the text. RNA was titrated in ten-fold dilutions for RT/TS-PCR and the reciprocal numbers of the endpoint dilutions which gave a positive were indicated.

[b] Estimated the amount of each protein expressed in cells semiquantitatively by comparison of intensities of immunoreacted bands on filters of Western blot analysis (Fig. 5) with those of the most abundantly expressing cells and categorized them into four groups: (−) no band was visible; (+) <10% of the strongest; (++) >10% of the strongest; (+++) the strongest.

to other HTLV-1 infected cell lines, MT-2, MT-4 and TL-Su. On the contrary, in MT-1 cells, neither the p21X mRNA nor p21X was detected. Moreover, the level of this new p21X mRNA expression was also found to be correlated with the amount of p21X in the other HTLV-1 infected cell lines, MT-2, MT-4, TL-Su. These findings imply that p21X might be mainly translated from p21X mRNA instead of *tax/rex* mRNA.

However, according to another point of view, these results could indicate that a sequence alteration participated in p21X translating in *tax/rex* mRNA, which commonly existed in HTLV-1 infected cells, might be involved in the expression variation of p21X. Therefore, we determined the sequence of *tax/rex* mRNA surrounding the p21X initiation codon by sequencing directly DNA fragments produced by RT-PCR. The sequence of cDNA from *tax/rex* mRNA in the three HTLV-1 infected cell lines, MT-1, MT-2 and H582 (data not shown) was found to be completely identical to that of the HTLV-1 ATK clone described previously, indicating no presence of any sequence alterations of *tax/rex* mRNA surrounding the p21X initiation codon in MT-1, MT-2 and H582. In consequence, the data suggest that *tax/rex* mRNA is little, if at all, responsible for translating p21X in HTLV-1 infected cells. Thus, we can conclude that p21X is translated from p21X mRNA. Moreover, in *tax/rex* mRNA, the initiation codon of p21X is located downstream of the p27rex and p40tax initiation codons, and the translation initiation sequence surrounding the p21X initiation codon does not conform closely to the Kozak consensus sequence [45]. Thus, p21X is likely to be translated more efficiently from p21X mRNA than *tax/rex* mRNA, but such detailed mechanisms are not clear yet and remain to be determined. From these data, we conclude that p21X is translated from p21X mRNA in HTLV-1 infected-cultured cell lines. Although it is not yet clear whether the presence of p21X is an absolute requirement for the production of HTLV-1 viral antigens, the results indicate that p21X may play a crucial role in the regulation of HTLV-1 propagation.

After RT/TS-PCR, two other faint bands (an about 200-bp band between the 272-bp and 81-bp bands shown in Fig. 6A2c and an about 170-bp band between the 200-bp and 81-bp bands in Fig. 6Ch) was clearly found to be amplified additionally. These 200-bp and 170-bp fragments were not hybridized with the oligo(mix-probe) (PX5 + PX6) (Fig. 6A4 and data not shown), suggesting that these fragments are derived from different mRNA transcripts from *tax/rex* mRNA and p21X mRNA. Our preliminary data (unpublished data) suggest that one of these transcripts is also an alternatively spliced mRNA, which uses the same splice-donor site but not the different splice-acceptor site of p21X mRNA, and contains the p21X coding frame. However, this mRNA seems to be very little, if not at all, responsible for encoding p21X, because this mRNA expresses much smaller amounts than p21X mRNA in MT-2 and H582 cells. On the other hand, the other additional mRNA has not yet been characterized, but the mRNA also shows low-level (<1/1000 or <1/10 000) expression compared with p21X mRNA expression in MT-2 or H582. Further detailed analyses are required for understanding the role of these mRNA in HTLV-1 infection.

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