Volume 295, number 1,2,3, 141–145 FEBS 10530 December 1991

Alternatively spliced mRNA of the *pX* region of human T lymphotropic virus type I proviral genome

Keiko Furukawa, Koichi Furukawa and Hiroshi Shiku

Department of Oncology, Nagasaki University School of Medicine, 12-4, Sakamoto-machi, Nagasaki 852, Japan

Received 25 October 1991

The *pX* region of human T lymphotropic virus type I (HTLV-I) is believed to be expressed as a consequence of a 2 step splicing. It is conceivable, however, that a donor site of the 1st splicing and an acceptor site of the 2nd splicing results in the production of an alternatively spliced mRNA which is capable of coding $p21^{X\text{-}III}$. This possibility was examined by amplifying cDNA derived from HTLV-I$^+$ cells between the 5′ LTR and *pX* region. Bands of 2 different sizes were consistently observed. Sequencing of the longer band corresponded to a cDNA derived from a double-spliced *pX* mRNA as previously reported. The shorter band was derived from a single-spliced mRNA. HTLV-I$^+$ cell lines had both mRNAs to a varying degree. Expression of $p40^{tax}$ and $p21^{X\text{-}III}$ seem to be well correlated with a double-spliced and a single-spliced mRNA, respectively.

HTLV-I; *tax* Gene; *rex* Gene; *pX* Protein; Alternative splicing

1. INTRODUCTION

Human T lymphotropic virus type I (HTLV-I) is a human retrovirus which preferentially infects mature human T cells. Many lines of research evidence suggest its close association with adult T cell leukemia (ATL), a unique mature T cell malignancy [1–3], and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4] that is most likely caused by immune dysfunction involving T cells. HTLV-I has a unique sequence termed *pX*, in addition to *gag*, *pol* and *env* regions [5]. The *pX* region encodes 3 different molecules $p40^{tax}$, $p27^{rex}$ and $p21^{X\text{-}III}$. The $p40^{tax}$ molecule is a *trans*-activator of the HTLV-I LTR [6] as well as a variety of cellular genes such as those of IL-2, IL-2R [6], IL-3, IL-4, granulocyte-macrophage colony stimulating factor [7], and *c-fos* [8,9]. The $p27^{rex}$ molecule is considered to be a post-transcriptional *trans*-regulator for HTLV-I gene expression [10,11], whereas $p21^{X\text{-}III}$ shares an amino acid sequence identical to that of $p27^{rex}$ except that it lacks the N-terminal 78 amino acids. The biological function of $p21^{X\text{-}III}$ remains unknown.

Seiki et al. [12] previously showed that the *pX* region is expressed as a consequence of a 2-step splicing which generates the 2.1 kilobase (kb) mRNA. This *pX* mRNA has 2 overlapping open reading frames termed *tax*1, encoding $p40^{tax}$ [13–16], and *rex*1, encoding $p27^{rex}$ and $p21^{X\text{-}III}$ [17]. Two AUGs for $p40^{tax}$ and $p27^{rex}$ are located in the second exon originating from the 3′ region of the *pol* gene, whereas the AUG for $p21^{X\text{-}III}$ is located in the third exon originating from the *pX* region. The 2.1 kb single mRNA of *pX* produces 3 *pX* molecules. However, it is possible that a donor site of the 1st splicing and an acceptor site of the 2nd splicing proceeds to the production of an alternatively spliced mRNA. Although such mRNA cannot code for $p40^{tax}$ and $p27^{rex}$ due to the lack of the middle exon, it should be capable of coding for $p21^{X\text{-}III}$. In that case, production of $p21^{X\text{-}III}$ might be independent of the regulation of the other 2 *pX* molecules.

We demonstrate here that an alternatively spliced mRNA does exist in HTLV-I-infected cells, among which expression levels of a double-spliced mRNA and a single-spliced mRNA of *pX* vary considerably.

2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

Six HTLV-I-infected cell lines were used. HUT102 [2], MT-2 [18] and TL-Su [19] have been described previously. Three cell lines, Oka, Ish and Kaw, were recently established in our laboratory. In addition, MOLT-4 and HL60, an HTLV-I-negative T cell line, were also utilized. Peripheral blood mononuclear cells (PBMC) from 5 HAM patients, 2 healthy HTLV-I carriers and a normal HTLV-I-negative individual were also utilized. HUT102, MT-2, TL-Su and MOLT-4 were maintained in RPMI 1640 medium with 10% fetal calf serum, and Oka, Ish and Kaw were maintained in RPMI 1640 medium with 20% fetal calf serum plus 1 U/ml recombinant IL-2 (Takeda Pharm. Co.). PBMC were isolated from heparinized venous blood by density gradient centrifugation and cultured in RPMI 1640 medium with 20% fetal calf serum and 1 U/ml recombinant IL-2 for 10 days.

Abbreviations: HTLV-I, human T lymphotropic virus type I; ATL, adult T cell leukemia; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; PBMC, peripheral blood mononuclear cells; RT, reverse transcription; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence.

Correspondence address: H. Shiku, Department of Oncology, Nagasaki University School of Medicine, Nagasaki 852, Japan.

2.2. *RNA extraction and RT-PCR*

Total RNA was prepared according to Chomczynski and Sacchi [20]. Cells were washed twice in phosphate-buffered saline and lysed in solution D consisting of 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Lysate was extracted with 1.3 vol. of 0.2 M sodium acetate (pH 4.0)/phenol/chloroform (0.1:1:0.2). After extraction, RNA was precipitated by adding an equal volume of isopropanol, resuspended in solution D, and re-precipitated with isopropanol. After washing with 75% ethanol, the RNA was dissolved in DEPC-treated distilled water. Oligonucleotide primers and probes were synthesized using an automated DNA synthesizer, Model 380B DNA Synthesizer (Applied Biosystems).

Amplification of *pX* mRNA was achieved with oligonucleotide primers derived from the long terminal repeat and *pX* domain of HTLV- I, i.e. pX-1 and pX-2, respectively, as shown in Fig. 1 [21].

Single-strand cDNA was synthesized with oligo-dT_{14} primer. Total RNA (3 μg) was dissolved in 50 μl of 50 mM Tris-HCl (pH 8.3) containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dATP, dGTP, dCTP, dTTP, 0.5 μg oligo dT_{14}, and 200 U of Moloney murine leukemia virus reverse transcriptase (BRL), then incubated for 90 min at 37°C. 5 μl of reverse transcription products were diluted in a 50 μl reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, all 4 dNTPs (0.2 mM each), 0.002% gelatin, 0.325 μg pX-1 primer and 0.325 μg pX-2 primer. The mixture was heated at 94°C for 5 min, quickly cooled on ice, and supplemented with 1 U of Taq DNA polymerase (Perkin-Elmer Cetus). Samples from HTLV-I-infected cell lines and control lines were subjected to 25 cycles of amplification and those from HAM patients, HTLV-I healthy carriers and the normal individual to 40 cycles. For semi-quantitation of RT-PCR products, samples obtained after 20 cycles of amplification were analyzed. Each cycle consisted of 3 steps; denaturation for 1 min at 94°C, primer annealing for 1 min at 53°C and polymerization for 2 min at 72°C.

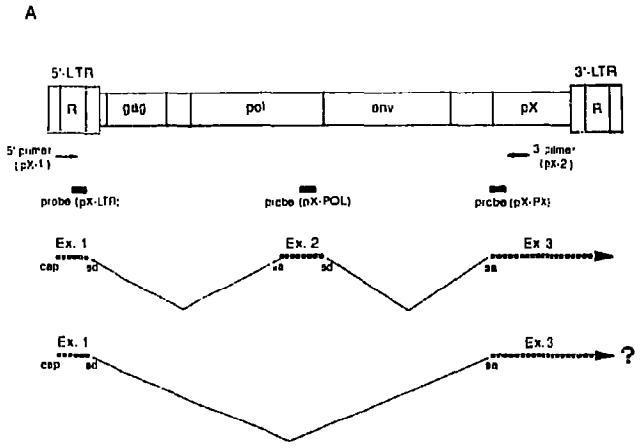

Fig. 1. (A) Genomic structure of HTLV-I provirus and double-spliced mRNA for *pX* gene products and a putative single-spliced mRNA. Sd, splice donor site; Sa, splice acceptor site. The location of the primers used for PCR, pX-1 and pX-2, are shown under the genomic structure. The pX-1 sense primer was derived from the R region of the long terminal repeat domain, and the pX-2 antisense primer was complementary to a 5′ sequence within the *pX* domain. (B) Sequences of primers for PCR and oligonucleotides used as probes for Southern blot analysis. Three internal sense oligonucleotide probes, pX-LTR, pX-POL and pX-PX derived from the long terminal repeat, *pol*, and *pX* domains, respectively, were used to detect the amplified *pX* cDNA.

2.3. *Southern blot hybridization*

10 μl of RT-PCR products were electrophoresed on a 15% polyacrylamide gel, and treated with an alkaline solution (0.4 N NaOH, 0.6 M NaCl) prior to transfer onto nylon filters. Filters were prehybridized in a solution containing 3.0 M tetramethyl ammoniumchloride, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1% SDS, 5× Denhardt's solution and denatured salmon sperm DNA. Samples were hybridized overnight at 58°C in the same buffer containing an oligonucleotide probe labeled with [γ-^{32}P]ATP (NEN) using polynucleotide kinase (Takara Shuzo Co.) as shown in Fig. 1. After hybridization, filters were washed twice in 2× SSC with 0.1% SDS for 5 min at room temperature, twice in 5× SSC with 0.1% SDS for 15 min at 58°C, and exposed to XAR-5 film (Kodak) for 10 h at −80°C.

2.4. *DNA sequencing*

PCR products were cloned into pBluescript II SK$^+$ vectors (Stratagene) and used as sequencing templates. 40 PCR cycles were performed as previously described using a pX-1 primer containing a *Bam*HI site and a pX-2 primer containing an *Eco*RI site, that is, primers pX-1′ and pX-2′. Amplified products were electrophoresed on a 15% polyacrylamide gel and specific bands extracted by electroelution were digested with *Bam*HI and *Eco*RI restriction enzymes (BRL), then inserted into pBluescript II SK$^+$ vectors. Cloned inserts were sequenced using the Taq Dye Primer Cycle Sequencing kit with the DNA Sequencer Model 370A (Applied Biosystems).

2.5. *Western blot analysis*

Western blot analysis for $p40^{tax}$, $p27^{rex}$ and $p21^{X\text{-}III}$ proteins was performed according to the method of Towbin et al. [22]. Cells were lysed with lysis buffer consisting of 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% DOC, and 1 mM PMSF and separated by SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred to a PVDF membrane (Immobilon, Millipore), pretreated with 5% skim-milk (Morinaga, Tokyo) in PBS at 4°C overnight. After pretreatment, the PVDF membrane was incubated with anti-$p40^{tax}$ mAb (Lt4) [23] or anti-$p27^{rex}$/$p21^{X\text{-}III}$ peptide rabbit antibodies [24] for 1 h at room temperature.

Bands were detected in $p40^{tax}$ by using VECTASTAIN ABC kit (Vector Laboratories) according to the manufacturer's instructions, and by protein A-peroxidase conjugate (Zymed) at 1:500 dilution for $p27^{rex}$ and $p21^{X\text{-}III}$. Each band was detected by the ECL (enhanced chemiluminescence) Detection System (Amersham). The membrane was exposed to XAR-5 film (Kodak) for 30–60 s at room temperature.

2.6. *Quantitative estimate of RT-PCR products and* pX *proteins*

RT-PCR products were semi-quantified by exposing the filter to an imaging plate (BAS-III, Fuji Photo Film Co.) for 1 h after Southern blot hybridization, then the radioactivity of each band was measured using the Bio-Imaging Analyzer, BAS 2000 (Fuji Photo Film Co.).

In order to quantify the amount of *pX* proteins, the densitometric analysis of autoradiogram was performed by using UNIGRAPHY UHG-101 (Unique Medical Co.).

3. RESULTS AND DISCUSSION

RNA from HTLV-I-infected cell lines, PBMCs of HAM patients, healthy carriers and a normal control

was analyzed by RT-PCR as described in Materials and Methods (Fig. 1). Using 3 sets of probes as shown in Fig. 1, RT-PCR products were Southern-blotted. When probes pX-LTR for exon 1 and pX-PX for exon 3 were used, 2 major bands were detected essentially in all samples, although the intensity of the 2 bands varied depending on the samples (Fig. 2A,C). The upper band was about 343 bp long corresponding with the double-spliced *pX* mRNA previously reported [21]. The lower band was about 150 bp long by comparison with molecular size markers. When probe pX-POL for exon 2 was used, only the upper 343-bp band was detected and none of the samples showed the lower band (Fig. 2B).

In order to confirm the identity of the upper band and to elucidate the derivation of the lower band, the 2 components were cloned and sequenced as described in Materials and Methods. The results are summarized in Fig. 3. The sequence of the upper component comprised those from the *Bam*HI site to the end (position 5183 in the HTLV-I genomic sequence [12]) of exon 2 and from the beginning (position 7302) of exon 3 to the primer pX-2′ as shown in Fig. 3A. This sequence was consistent with that of a double-spliced mRNA previously described [12]. Three clones derived from the lower band were also sequenced. All of them comprised the sequences from the primer pX-1′ to the end (position 470 in the HTLV-I genomic sequence) of exon 1 and from the beginning (position 7302) of exon 3 to the primer pX-2′. Consequently, it was demonstrated that the whole of exon 2 was deleted and that the splice donor site of exon 1 bound directly with the splice acceptor site of exon 3, thus producing a single-spliced *pX* mRNA. Since exon 2 comprises only 191 nucleotides (position 4993–5183), it is not surprising that the single-spliced mRNA has been missed by standard Northern blot analysis.

HTLV-I-infected cells seem to have both mRNAs to varying degrees as already shown in Fig. 2. In some cell lines, like Oka, the double-spliced mRNA is apparently dominant, whereas in others, such as MT-2 and Ish, the single-spliced mRNA is more dominant. Alternatively, both mRNAs are equally recognizable in HUT102, TL-Su and Kaw. Variations in *pX* expression also occurs in short-term-cultured PBMCs derived from HAM patients and HTLV-I carriers, which indicates that the presence of the single-spliced mRNA is not restricted to long-term-cultured HTLV-I$^+$ cell lines.

Since the double-spliced mRNA is capable of coding for $p40^{tax}$, $p27^{rex}$ and $p21^{X\text{-}III}$ and the single-spliced

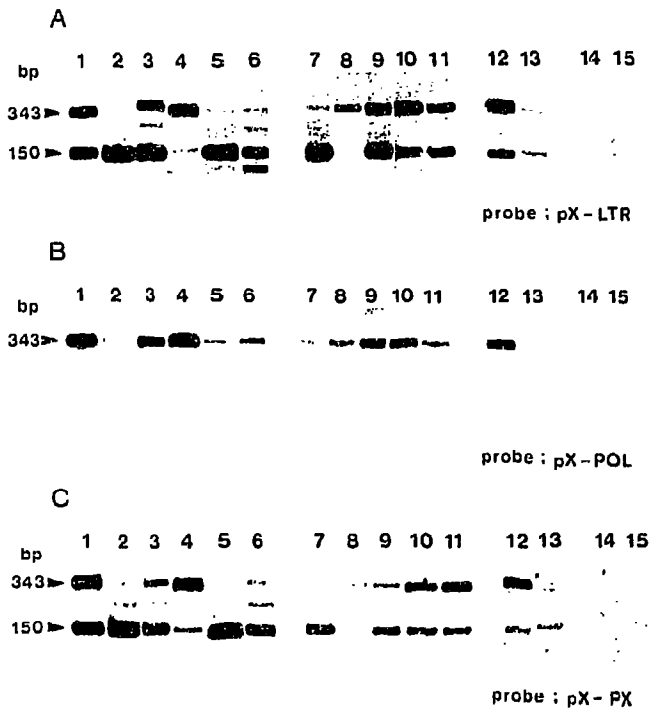

Fig. 2. Southern blot analysis of amplified DNA fragments derived from HTLV-I *pX* mRNA: (lanes 1–6) RT-PCR products from the following HTLV-I infected cell lines; (1) HUT102; (2) MT-2; (3) TL-Su; (4) Oka; (5) Ish; (6) Kaw; (lanes 7–11) RT-PCR products from PBMC of HAM patients; (lanes 12–13) from healthy carriers; (lane 14) from an HTLV-I-negative control; (lane 15) from MOLT-4 cell line. Each set was hybridized with 3 probes, i.e. (A) pX-LTR, (B) pX-POL, and (C) pX-PX. The exposure time was 3 h for lanes 1–6, and 10 h for lanes 7–15. bp, base pairs; M, molecular size markers.

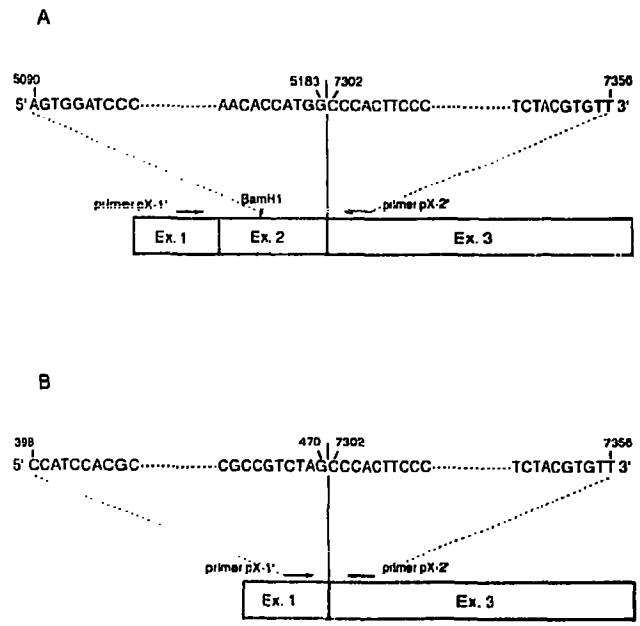

Fig. 3. DNA sequencing of RT-PCR products. Forty PCR cycles were performed on an HUT102 sample using a pX-1′ sense primer containing a *Bam*HI site, 5′-GGGGATCCCCATCCACGCCGGTTGA-3′, and a pX-2′ antisense primer containing an *Eco*RI site, 5′-GGGA-ATTCAACACGTAGACTGGGTAT-3′. (A) The amplified upper band (343 bp) was electroeluted and digested with *Bam*HI and *Eco*RI. A *Bam*HI site was also present in exon 2 of the double-spliced mRNA, therefore the longer fragment from the *Bam*HI site in exon 2 to the position of the 3′-antisense primer, pX-2′, was cloned into pBluescript II SK+ vectors and sequenced. (B) The amplified lower band (151 bp) was electroeluted and digested with *Bam*HI and *Eco*RI restriction enzymes, cloned into pBluescript II SK+ vectors, then sequenced as described in Materials and Methods.

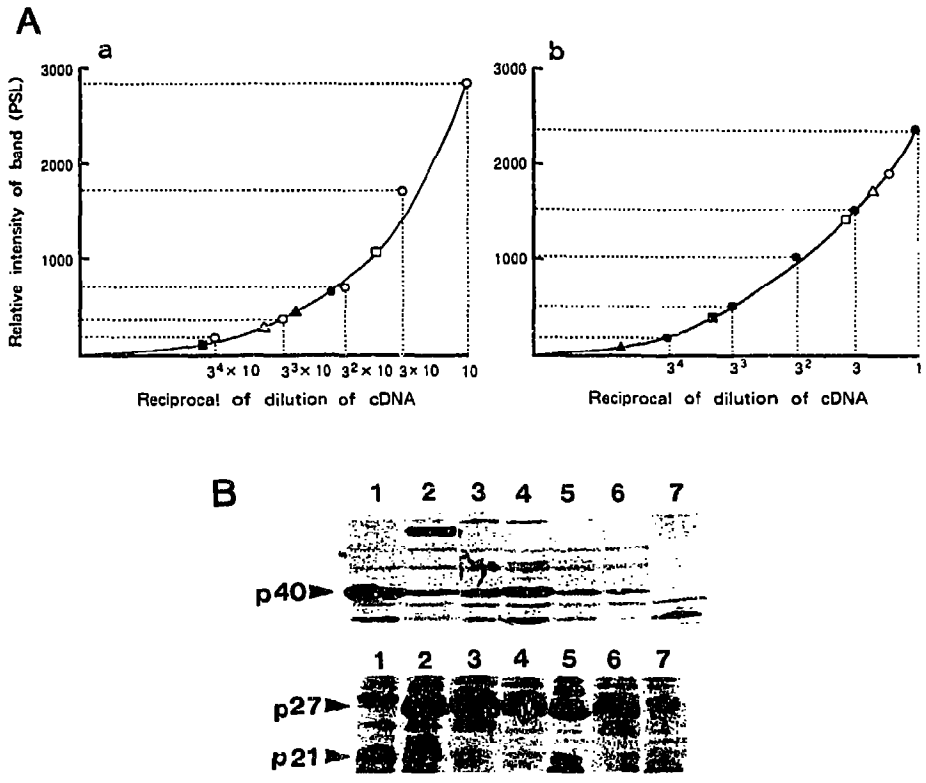

Fig. 4. Semi-quantitation of RT-PCR products and Western blot analysis of $p40^{tax}$, $p27^{rex}$ and $p21^{X\text{-}III}$. (A) The following 6 HTLV-I$^+$ cell lines were analyzed; HUT102, ○; MT-2, ●; TL-Su, □; Oka, ▲; Ish, △; Kaw, ■. Two RT-PCR products after 20 cycles of amplification were analyzed by Southern blotting using the pX-PX probe. The radioactivity of two bands, i.e. 343 bp (a) and 151 bp (b), was measured by a Bio-Imaging Analyzer and plotted. (PSL corresponds to cpm in Bio-Imaging Analyzer.) Standard curves were drawn by applying a 3-fold-diluted cDNA of HUT102 (for 343 bp bands in Fig. Aa) and MT-2 (for 151 bp bands in Fig. Ab). Since HUT102 expressed the extremely high amount of RT-PCR products (343 bp band), a dilution was started at 1:10. (B) Cell extracts from each cell line containing 20 μg protein for $p40^{tax}$ and 30 μg for $p27^{rex}$ and $p21^{X\text{-}III}$ were analyzed by Western blotting; (lane 1) HUT102; (lane 2) MT-2; (lane 3) TL-Su; (lane 4) Oka; (lane 5) Ish; (lane 6) Kaw; (lane 7) HL60.

mRNA only for $p21^{X\text{-}III}$, the expression of 2 different *pX* mRNAs with that of *pX*-encoded proteins should correlate. Two different mRNA were detected by Southern blot analysis with a pX-PX probe after 20 cycles of RT-PCR. Intensity of bands was measured by the Bio-Imaging Analyzer which images and quantitates with a degree of sensitivity equal to the combined use of autoradiography and scintillation counting. Standard curves were drawn by applying a 3-fold-diluted cDNA of HUT102 (for 343 bp bands in Fig. 4A(a)) and MT-2 (for 151 bp bands in Fig. 4A(b)), both of which showed the maximal expression of corresponding RT-PCR products among examined cell lines. Relative amounts of 2 different RT-PCR products of 5 cell lines to HUT102 or MT-2 were obtained as shown in Fig. 4A and summarized in Table I. The presence of $p40^{tax}$, $p27^{rex}$ and $p21^{X\text{-}III}$ was confirmed by Western blotting with appropriate antibodies (Fig. 4B). Expression of 3 different proteins in 6 different HTLV-I$^+$ cell lines was also measured by UNIGRAPHY and summarized in Table I.

Expression of $p40^{tax}$ seems to be correlated with that of the 343-bp band. On the other hand, expression of $p21^{X\text{-}III}$ seems to be more associated with that of the 151-bp, but not the 343-bp bands, whereas $p27^{rex}$ does

Table I

Comparison of two different mRNAs from the *pX* region with expression of $p40^{tax}$, $p27^{rex}$ and $p21^{X\text{-}III}$

PCR products and proteins	HUT102	MT-2	TL-Su	Oka	Ish	Kaw
PCR products						
343 bp	1000*	11	18	3.8	2.5	1
151 bp	56*	100	32	0.3	44	3.2
Proteins						
p40	230**	57	78	90	34	21
p27	0**	204	214	105	145	138
p21	65**	56	6	0	25	3

*Amounts of RT-PCR products relative to HUT102 (for 343-bp bands) and to MT-2 (for 151-bp bands) were estimated in Fig. 4A.
**Unit (corresponding to the O.D. in UNIGRAPHY UHG-101) based on the intensity of bands detected in Western blot analysis (See Fig. 4B).

not seem to associate with either. Thus it is possible that $p21^{X\text{-}III}$ is more dependent on the single- rather than the double-spliced *pX* mRNA.

Inoue et al. [10] have reported that $p27^{rex}$ acts as a post-transcriptional regulator for the expression and replication of HTLV-I. Induction of $p27^{rex}$ expression results in increased expression of *gag/pol* mRNA, as well as that of *env* mRNA, whereas expression of *tax/rex* mRNA (the double-spliced mRNA of *pX*) is greatly suppressed. Despite the fact that $p21^{X\text{-}III}$ is a part of the $p27^{rex}$ peptide, in their experiments it failed to show a similar molecular effect, which leaves the biological roles of $p21^{X\text{-}III}$ to be elucidated. Since the entire $p21^{X\text{-}III}$ shares an identical amino acid sequence with the C-terminal portion of $p27^{rex}$, analogous or antagonistic molecular functions between the 2 molecules may be speculated upon. In fact, recent experimental data suggest a possible antagonistic role of $p21^{X\text{-}III}$ against $p27^{rex}$ [25].

It has yet to be determined whether or not $p27^{rex}$ regulates both the single- and double-spliced mRNA of *pX*. Shifted expression of $p21^{X\text{-}III}$, independently of $p40^{tax}$ and $p27^{rex}$, may result in an alteration of the molecular regulation of HTLV-I genomes and/or cellular genomes. Elucidation of the biological function of $p21^{X\text{-}III}$ is indispensable for further understanding of their relationship.

Acknowledgements: We thank Dr. Y. Tanaka for providing anti-$p40^{tax}$ mAb (Lt4) and anti-$p27^{rex}$/$p21^{X\text{-}III}$ peptide rabbit antibodies, and Dr. M. Mori for supplying the HAM patients' samples. This work was supported by Special Coordination Funds from the Science and Technology Agency of the Japanese Government and by a grant from the Ministry of Health and Welfare of Japan.

REFERENCES

[1] Hinuma, Y., Nagata, K., Hanoka, M., Nakai, M., Matsumoto, T., Kinoshita, K., Shirakawa, S. and Miyoshi, I. (1981) Proc. Natl. Acad. Sci. USA 78, 6476–6480.
[2] Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.B., Minna, J. and Gallo, R.C. (1980) Proc. Natl. Acad. Sci. USA 77, 7415–7419.
[3] Yoshida, M., Miyoshi, I. and Hinuma, Y. (1982) Proc. Natl. Acad. Sci. USA 79, 2031–2035.
[4] Osame, M., Usuku, K., Izumo, S., Ijiti, N., Amitani, H., Igata, A., Matsumoto, M. and Tara, M. (1986) Lancet I, 1032–1033.
[5] Seiki, M., Hattori, S., Hirayama, Y. and Yoshida, M. (1983) Proc. Natl. Acad. Sci. USA 80, 3618–3622.
[6] Yoshida, M. and Seiki, M. (1987) Annu. Rev. Immunol. 5, 541–559.
[7] Miyatake, S., Seiki, M., Malefiji, R.D., Heike, T., Fujisawa, J., Takebe, Y., Nishida, J., Shromai, J., Yokota, T., Yoshida, M., Arai, K. and Arai, N. (1988) Nucleic Acids Res. 16, 6547–6566.
[8] Fujii, M., Sassone-Corsi, P. and Verma, I.M. (1988) Proc. Natl. Acad. Sci. USA 85, 8526–8530.
[9] Nagata, K., Ohtani, K., Nakamura, M. and Sugamura, K. (1989) J. Virol. 68, 3220–3226.
[10] Inoue, J., Yoshida, M. and Seiki, M. (1987) Proc. Natl. Acad. Sci. USA 84, 3635–3657.
[11] Hidaka, M., Inoue, J., Yoshida, M. and Seiki, M. (1988) EMBO J. 7, 519–523.
[12] Seiki, M., Hikikoshi, A., Taniguchi, T. and Yoshida, M. (1985) Science 288, 1532–1534.
[13] Kiyokawa, T., Seiki, M., Imagawa, K., Shimizu, F. and Yoshida, M. (1984) Gann 75, 747–751.
[14] Slamon, D.J., Shimotohno, K., Cline, M.J., Golde, D.W. and Chen, I.S.Y. (1984) Science 226, 61–65.
[15] Miwa, M., Shimotohno, K., Hoshino, H., Fujino, M. and Sugimura, T. (1984) Gann 75, 752–755.
[16] Lee, T.H., Coligan, J.E., Sodroski, J.G., Haseltine, W.A., Salahuddin, S.Z., Wong-Staal, F., Gallo, R.C. and Essex, M. (1984) Science 226, 57–61.
[17] Kiyokawa, T., Seiki, M., Iwashita, S., Imagawa, K., Shimizu, F. and Yoshida, M. (1985) Proc. Natl. Acad. Sci. USA 82, 8359–8363.
[18] Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K. and Hinuma, Y. (1981) Nature 294, 770–771.
[19] Gotoh, Y., Sugamura, K. and Hinuma, Y. (1982) Proc. Natl. Acad. Sci. USA 79, 4780–4782.
[20] Chomczynski, P. and Sacchi, N. (1987) Analyt. Biochem. 162, 156–159.
[21] Tendler, C.L., Greenberg, S.J., Blattner, W.A., Manns, A., Murphy, E., Fleisher, T., Hanchard, B., Morgan, O., Burton, J.D., Nelson, D.L. and Waldmann, T.A. (1990) Proc. Natl. Acad. Sci. USA 87, 5218–5222.
[22] Towbin, H., Stachelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[23] Lee, B., Tanaka, Y. and Tozawa, H. (1989) Tohoku J. Exp. Med. 157, 1–11.
[24] Siomi, H., Shida, H., Nam, S.H., Nosaka, T., Maki, M. and Hatanaka, M. (1988) Cell 55, 197–209.
[25] Akayama, H., Nosaka, T., Adachi, Y., Sato, T., Maki, M. and Hatanaka, M. (1991) Proc. 64th Meeting Jpn. Biochem. Soc.