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Autoantigen Ku protein is involved in DNA binding proteins which recognize the U5 repressive element of human T-cell leukemia virus type I long terminal repeat

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Abstract We have identified and analyzed a 27-nucleotide sequence (U5 repressive element, designated as U5RE) at the U5 region of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) which is required for HTLV-I basal transcriptional repression. The basal promoter strength of constructs that contained deletions in the U5 region of the LTR was analyzed by chloramphenicol acetyltransferase (CAT) assays following transfection of HeLa cells or Jurkat T-cells in the presence or absence of viral transactivator *tax* protein. We consistently observed a 2- to 5-fold increase in basal promoter activity when sequences between +277 to +306 were deleted. In vivo competition experiments suggested that the U5 DNA fragment from +269 to +295 contains a functional repressive element (U5RE). Using gel mobility shift assays, we have purified a highly enriched fraction that could specifically bind U5RE. This DNA affinity column fraction contained three major detectable proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining: 110-, 80- and 70-kDa proteins. The 110-kDa protein appeared to be a novel DNA-binding protein whose characteristics are still obscure, while the 70- and 80-kDa proteins were shown to be related to the human autoantigen Ku, the Ku (p70/p80) complex, as demonstrated by amino acid sequencing and immunological analyses. As Ku is known to be involved in transcriptional regulation, the specific interaction of Ku with U5RE raises intriguing possibilities for its function in HTLV-I basal transcriptional repression.

Key words: Human T-cell leukemia virus type I (HTLV-I); DNA binding protein; Ku protein; Transcriptional repression

1. Introduction

Human T cell leukemia virus type I (HTLV-I) is an exogenous human retrovirus that has been shown to be the etiologic agent of a type of acute T-cell leukemia, known as adult T-cell leukemia (ATL), as well as a neurological disorder, known as HTLV-I-associated myelopathy or tropical spastic paraparesis (HAM/TSP) [1–6], and an eye disease, HTLV-I uveitis [7]. The latter two diseases are considered to be outcomes of immune disorders caused by HTLV-I infection. After infection into humans, the virus requires a long latent period until the onset of such diseases [8]. The full mechanism of the viral latency has not been uncovered yet. Analysis of HTLV-I gene expression in vitro, however, provides some important information on the mechanisms of the viral latent infection and activation from the latent state.

After integrating into host chromosomal DNA, the expression of the viral genes is known to be regulated by various viral and host nuclear factors through the viral 5′ long terminal repeat (LTR). The 21-bp repeat elements, TRE-1 (HTLV-I-*tax* protein responsive element 1), are required for the transactivation of the HTLV-I *tax* protein that is reported to bind indirectly to the enhancer elements through host cell nuclear factors [9–11]. The cellular nuclear factors such as SP1, TIF-1, Ets and Myb interact with the LTR at the region located between two proximal 21-bp repeats, TRE-2 (Tax responsive element 2) [12–15].

Moreover, sequences downstream of the RNA initiation site, including the R and the U5 regions, have been shown to be important for HTLV-I expression. A 137-bp fragment (+104 to +240) [16] or a 235-bp fragment (+32 to +266 [17] was reported to increase the level of HTLV-I gene expression. An 82-bp fragment (+266 to +347) was shown to function as an RNA element for post-transcriptional repression [17]. A 45-bp fragment at the boundary of the R/U5 region functions for HTLV-I basal transcriptional activation [18]. The R region of the LTR also contains the enhancer activity, and cellular binding factors (YB-1) to this enhancer region were characterized [19,20]. HTLV-I LTR suppression was recently reported to be associated with a cyclic-AMP responsive element binding protein (CREB) binding in the R region (+205 to +227) [21].

The HTLV-I *rex* protein is known to be the viral post-transcriptional regulator that recognizes the *rex*-responsive element (RXE) of the viral transcripts and enhances unspliced and singly spliced forms of viral transcripts resulting in the increase of the production of *gag/pol* and *env* proteins, respectively [22]. Since *tax* and *rex* proteins are produced from a doubly spliced viral transcript (designated as *tax/rex* mRNA), the accumulation of *rex* proteins induces a decrease in the level of *tax/rex* mRNA, resulting in the down-regulation of viral gene expression. Thus, in the viral latency, some contribution that gene expression is regulated at the post-transcriptional levels by *rex*-proteins has been suggested [23].

As mentioned above, the sequences within the U5 region have been identified as a *cis*-acting repressive element of the LTR-directed gene expression at post-transcriptional level [17]. In this report, we show that a U5 sequence in the LTR has an ability to repress the HTLV-I basal transcription in the absence of *tax*-protein as well as in the presence of *tax*-protein. Several nuclear proteins from Jurkat T cells interacted with this U5 region, which contains a functional repressive element (U5RE),

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were determined. Moreover, we report that the autoantigen Ku protein is involved in the U5RE-binding proteins. Ku is a heterodimer of about 70- and 80-kDa proteins that characteristically binds the ends of DNA fragments in vitro and translocates to form multimeric complexes [24,25], and is suggested to play a role in transcription [26,27]. Possibilities for involvement of Ku in the U5-mediated repression are discussed.

2. Materials and methods

2.1. Cell lines

The following human cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (R10F medium): T-cell lines; Jurkat, Molt-4, CEM, MT-2, MT-4, TL-Om1, TL-Su, and H582, and non T-cell lines; Daudi, K562, U937 and NB9. A human T-cell line, TOM-1, was cultured in R10F medium containing 100 units/ml of human recombinant interleukin-2 (Shionogi & Co., Ltd., Osaka, Japan). Other human non-T-cell lines, HeLa and U251, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. MT-2, MT-4, TL-Om1, TL-Su, H582, and TOM-1 cells are HTLV-I infected T-cell lines. NB9, U251, CEM, K562, U937, and Daudi cells were kindly provided by the Japanese Cancer Research Resources Bank. The other cell lines were described previously [28].

2.2. Oligonucleotides for probes and competitors

The following oligonucleotides were synthesized using a DNA synthesizer (Cyclone Plus DNA Synthesizer, Model 391 PCR-MATE DNA Synthesizer, Applied Biosystems Inc., CA, USA): for U5RE DNA (nucleotide numbers from +269 to +295), a sense strand; 5'-AGCTTAAGTTCCACCCCTTTCCCTTTCATTCA-3' and an antisense strand; 5'-AGCTTGAATGAAAGGGAAAGGGGTGGAACTTA-3', for non-specific control DNA, a sense strand; 5'-AGCTTCAGGTAGACTGCTTCGATCACTAGAGA-3' and an antisense strand; 5'-AGCTTCTCTAGTGATCGAAGCAGTCTACCTGA-3' (an additional linker sequence corresponding to a restriction endonuclease *Hin*dIII site is indicated by underlining), and for U5(+255 to +294) DNA, a sense strand; 5'-GCCGTTACAGATCGAAAGTTCCACCCCTTTCCCTTTCATT-3' and an antisense strand; 5'-AATGAAAGGGAAAGGGGTGGAACTTTCGATCTGTAACGGC-3'. DNA sequence information from Seiki et al. [4] was used and nucleotide numbers referred to nucleotide positions relative to the cap site (+1). The synthesized DNAs were purified by electrophoresis in a 20% polyacrylamide/7 M urea gel and DE52 ion-exchange columns, precipitated in cold ethanol, and suspended in TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

The recessed 3' end of double-stranded DNA was labeled by [α-^{32}P]dCTP (Amersham, Buckingshamshire, UK) with the Klenow fragment of *E. coli* DNA polymerase (Takara Shuzoh, Kyoto, Japan). The 5' end of synthetic oligonucleotides was labeled by T4 polynucleotide kinase (Takara Shuzoh) end labeling with [γ-^{32}P]ATP (Amersham).

2.3. CAT assay and in vivo competition assay

Three chloramphenicol acetyltransferase (CAT) expression plasmids derived from the HTLV-I LTR promoter with the deletion mutants within the U5 region were constructed as follows (Fig. 1A); pBLTR-1, -2 and -9, in which the CAT gene was under the control of the U3-R-U5 (−325 to +316), (−325 to +276) and (−325 to +246) region of the LTR, respectively. pCHL4 and pRSV-CAT plasmids, in which the CAT gene is under the control of the U3-R-U5 (−325 to +306) region of HTLV-I LTR [16] and under the control of the Rous sarcoma virus LTR, respectively, were also used.

HeLa or Jurkat cells were transfected with the LTR-CAT expression plasmids with or without the *tax* protein expression plasmid pMAXneo [9] by using a Lipofectin reagent (GIBCO BRL; Gaithersburg, USA) in serum-free culture medium for 16 h, and further cultured for 48 h in medium containing 10% FBS. In in vivo competition experiments, DNA competitors were cotransfected into cells with the CAT expression plasmids. Thereafter cells were recovered, and whole cell lysates in 0.25 M Tris-HCl (pH 7.8) were prepared by five cycles of freezing and thawing. The lysates were cleared by centrifugation at 15,000 × *g* for 10 min. The supernatants were recovered, and assayed for CAT activities as previously described [29] using L-threo-[dichloracetyl-1-^{14}C]chloramphenicol (Amersham). The degree of acetylation of ^{14}C-chloramphenicol was quantified by autoradiography of by using a Bio-Image analyzer (Model BA100; Fuji Film, Tokyo, Japan).

2.4. Cell nuclear extracts

Nuclear extracts were prepared from Jurkat cells according to the method of Dignam et al. [30]. Briefly, cells were washed with PBS, suspended in 5 packed volumes of ice-cold lysis buffer 1 (10 mM HEPES, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), kept on ice for 10 min, and centrifuged at 1,000 × *g* for 10 min. The cell pellet was resuspended in 2 volumes of ice-cold lysis buffer 1, homogenized by 10–20 strokes with a Dounce homogenizer, and centrifuged at 1,000 × *g* for 10 min at 4°C. After discarding the supernatant, the precipitate was recentrifuged at 25,000 × *g* for 20 min at 4°C. The nuclear pellet was resuspended in ice-cold extraction buffer 1 (20 mM HEPES, pH 8.0, 0.5 M NaCl, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) at a ratio of 2.5 ml/10^9 cells, homogenized by 10–20 strokes with a Dounce homogenizer, and kept on ice for 30 min. After centrifugation at 25,000 × *g* for 30 min at 4°C, the supernatant was dialyzed against dialysis buffer 2 (20 mM Tris-HCl, pH 8.0, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) for more than 8 h at 4°C. The dialyzed extract was cleared by centrifugation at 8,000 × *g* for 15 min, and the supernatant was kept in aliquots at −80°C until use.

A rapid preparation of nuclear extracts from various cell lines was performed according to Schreiber et al. [31]. In brief, 10^6 cells were washed with PBS, pelleted, and suspended in 0.4 ml of ice-cold lysis buffer 2 (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM DTT, 0.5 mM PMSF). After 15 min, 25 μl of a 10% solution of Nonidet P-40 (NP-40) was added, and the solution was vortexed vigorously for 10 s and centrifuged for 30 s in a microcentrifuge. The nuclear pellet was resuspended in 50 μl of ice-cold extraction buffer 2 (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), rocked vigorously for 15 min at 4°C on a shaking platform (model MT-360, Tomy, Tokyo, Japan), and centrifuged for 5 min in a microcentrifuge. The supernatant was collected, and kept at −80°C until use.

2.5. Gel mobility shift assay

The crude nuclear extracts or partially purified fractions were incubated with ^{32}P-end-labeled double strand DNA probes in binding buffer 1 (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) in the presence of poly(dI-dC)/poly(dI-dC) (Pharmacia P-L Biochemicals Inc., Wisconsin, USA) for 30 min at RT. In most cases, U5 fractions were incubated with the DNA probes without poly(dI-dC)/poly(CdI-dC). Aliquots of the reaction mixtures were loaded onto a 5% polyacrylamide gel, and the samples were electrophoresed for 90 min at 150 V in the electrophoresis buffer (TAE; 40 mM Tris acetate buffer, 1 mM EDTA). The mobility retarded DNA bands were visualized either by autoradiography or by using a BA100 Bio-Image Analyzer.

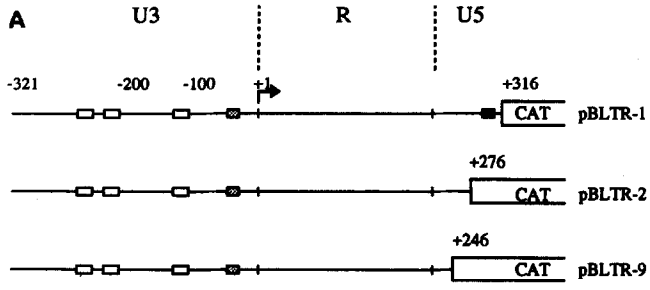
For supershift assays, 1 μl of the appropriate antiserum was added to the binding reaction 15 min prior to the loading of the gel.

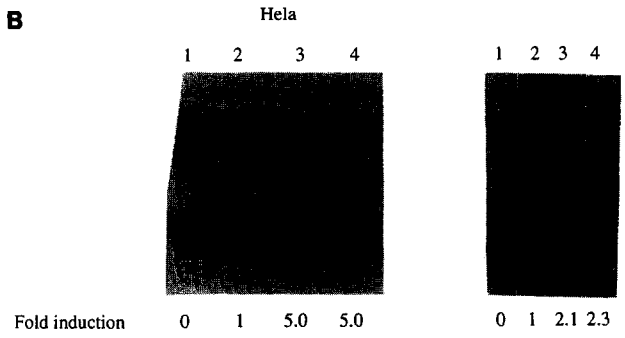
2.6. U5RE DNA-coupled Sepharose beads

One gram of activated CNBr-Sepharose 4B (Pharmacia, Uppsala, Sweden) was treated with 15 ml of 1 mM HCl for 15 min, washed with 150 ml of 1 mM HCl and then with 10 mM phosphate buffer (PB, pH 8.0), and suspended in 3.5 ml of PB. Immediately, 200 μg of the U5RE double strand DNA fragment were added to the Sepharose solution. After incubation for 2 h at 25°C, 0.8 ml of 1 M Tris (pH 8.0) was added to the solution and further incubated for 4 h at 25°C. After washing extensively with Tris buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 8.0), the U5RE DNA-coupled Sepharose beads were suspended in Tris buffer containing 0.02% sodium azide, and kept at 4°C until use.

2.7. Purification of U5RE DNA binding proteins

Frozen stocks of the Jurkat crude nuclear extracts were thawed on ice and applied to the heparin-agarose (GIBCO BRL; Gaithersburg, USA) column (2 ml extract/1 ml heparin-agarose) which was prewashed with TM buffer (50 mM Tris-HCl, pH 8.0; 12.5 mM $MgCl_2$, 1 mM





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	Hela cells	Jurkat cells
pBLTR-2/pBLTR-1	4.2 ± 0.7	2.3 ± 0.4
pBLTR-9/pBLTR-1	4.3 ± 0.5	2.4 ± 0.7

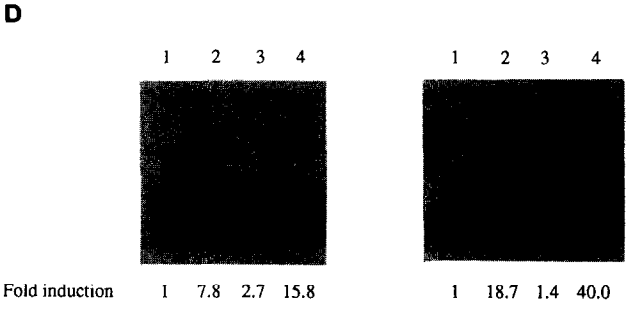

Fig. 1. (A.) Schematic diagram of three CAT expression plasmids driven by the downstream deletion mutants of the HTLV-I LTR promoter, pBLTR-1, -2, and -9. All constructs contained intact U3 and R sequences and various sequences of the U5 region are as described in the text. Numbers refer to nucleotide positions relative to the cap site (+1). The TATA box and three 21-bp repeat elements, TRE-1, in the U3 region are indicated by a stippled box and open boxes, respectively. Another small stippled box in the U5 region of pBLTR-1 indicates the U5RE region. CAT, the open reading frame of the chloramphenicol acetyltransferase gene. (B) Results of a representative experiment in the absence of *tax* protein are shown. HeLa or Jurkat cells were transfected with 2 μg of control pUC13 (lane 1), pBLTR-1 (lane 2), -2 (lane 3), and -9 (lane 4), and 48 h later CAT activity in the cell lysates (500 μg protein per assay) was determined. For each sample, the percent conversion of chloramphenicol to its acetylated forms is measured. For pBLTR-2 and -9, the percentage of chloramphenicol acetylation compared with that of pBLTR-1 was determined and the fold induction was calculated. (C) Significant differences in the promoter activity of pBLTR-1 compared with those of pBLTR-2 and -9. HeLa or Jurkat cells were transfected with the plasmids, pBLTR-1, -2, and -9. Promoter activity was determined by CAT analysis. The CAT activity of each deletion mutant construct was compared with the activity of the pBLTR-1 construct in each cell line. Results are the means ± S.E. of the mean for at least three independent experiments. (D) Results of a representative experiment in the presence of *tax* protein are shown. pBLTR-1 (lanes 1 and 2) or pBLTR-2 (lanes 3 and 4) were co-transfected into cells with (lanes 2 and 4) or without the *tax* protein expression plasmid pMAXneo (lanes 1 and 3). 48 h later, 50 μg protein of the cell lysates were assayed. For pBLTR-1 in the presence of *tax* protein and pBLTR-2 in the presence or absence of *tax* protein, the percentage of chloramphenicol acetylation compared with that of pBLTR-1 in the absence of *tax* protein was determined and the fold induction was calculated.

EDTA, 20% glycerol, 1 mM DTT) containing 1.0 M KCl and equilibrated with ice-cold 0.1 M KCl-TM buffer. The column was washed with 2 bed volumes of 0.1 M KCl-TM buffer, and stepwise eluted with 0.2 M KCl-TM, 0.4 M KCl-TM and 0.6 M KCl-TM buffer. The eluted fractions were assayed for their binding activities to the U5RE DNA probe in the gel mobility shift assay. The DNA-binding activity was enriched in fractions eluted with 0.4 M KCl-TM buffer, named Hep (0.4 M). This fraction was either dialyzed against 0.1 M KCl-TM buffer or diluted to about 0.1 M KCl by adding TM buffer, and applied to the U5RE DNA-Sepharose column equilibrated with ice-cold 0.1 M KCl-TM buffer. After incubation for 1 h at 4°C, the column was washed with 4 bed volumes of 0.1 M KCl-TM buffer, and stepwise eluted with 0.2 M KCl-TM, 0.4 M KCl-TM and 0.6 M KCl TM buffer. The DNA-binding activity was enriched in 0.4 M KCl-TM buffer, named U5(0.4M), and kept in aliquots at −80°C.

2.8. Amino acid sequencing

The amino-terminal amino acid sequence of the 110-, 80-, and 70-kDa proteins was determined by the method of Matsudaira [32] with minor modifications. A large-scale U5(0.4M) fraction of Jurkat nuclear proteins was electrophoresed and then electrotransferred to a PVDF membrane (Trans-Blot Transfer Medium; BIO-RAD Lab., CA, USA). After being stained with 0.1% Amido black 10B, the membrane pieces with 110-, 80-, and 70-kDa proteins were excised and analyzed with an amino acid sequencer (model PSQ-1; Shimadz Corporation, Kyoto, Japan).

3. Results

3.1. Characterization of the U5 region of HTLV-I LTR

The U5 sequence in the HTLV-I LTR was previously suggested to contain a repressive element for viral gene expression when transactivated with *tax* protein at a post-transcriptional level [17].To further examine this repression mechanism, we prepared three CAT expression plasmids derived from the LTR promoter with the deletion mutants within the U5 region as shown in Fig. 1A: pBLTR-1, -2 and -9, in which the CAT gene was under the control of the U3-R-U5 (−325/+316), (−325/+276), and (−325/+246), region of the LTR, respectively. We observed that pBLTR-1 expressed a week CAT activity when transfected into HeLa or Jurkat cells in the absence of viral transactivator *tax* protein (Fig. 1B). The same level of the CAT activity was observed in transfectants with the pCHL4 plasmid (data not shown), in which the CAT gene is under the

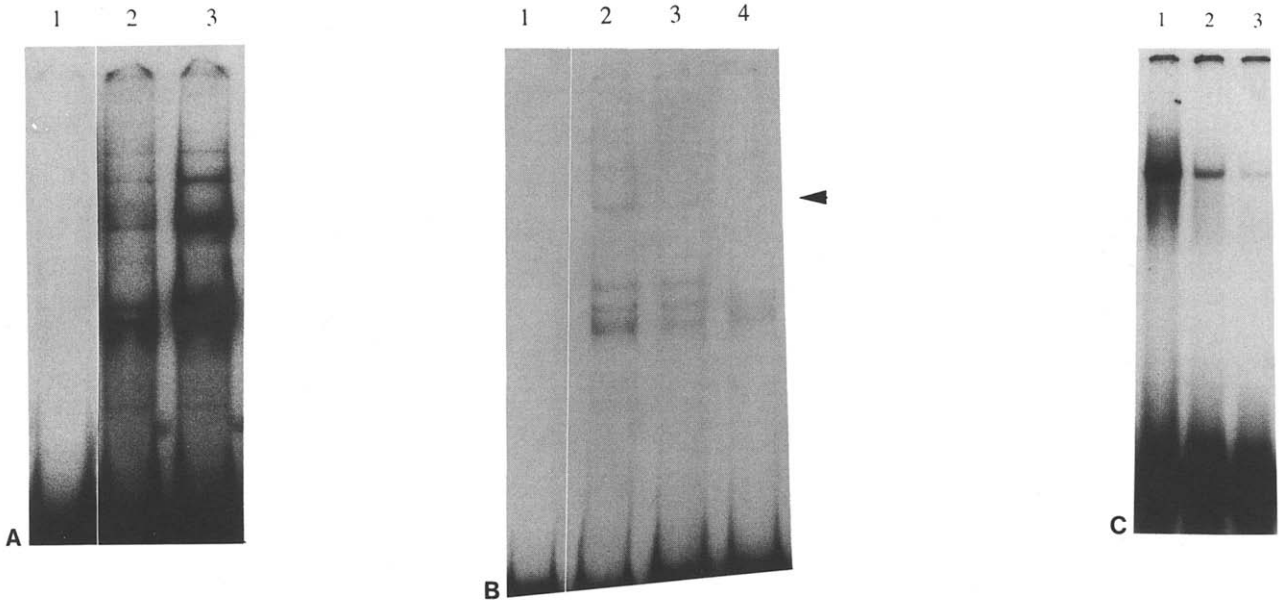

Fig. 2. Gel mobility shift assays of the Jurkat nuclear factors. Gel mobility shift assays were performed with 10 ng of a ^{32}P-labeled R-U5 DNA (A and B) or U5RE DNA probe (C) and approximately 2 μg of Jurkat crude nuclear extracts as described in section 2. (A) Reactions were performed in the absence (lane 3) or in the presence of a 100-fold molar excess of competitors the R-U5 DNA (179-bp, +128 to +306) (lane 1), and the pX region DNA (159-bp, +7,358 to +7,516) (lane 2). (B) Reactions were performed in the absence (lane 2) or in the presence of a 100- or 500-fold (lane 3 or 4, respectively) molar excess of the U5RE DNA as a competitor. Lane 1 shows a reaction without Jurkat nuclear extracts. An arrowhead indicates a specific competition band with the U5RE DNA. (C) Reactions were performed in the absence (lane 1) or in the presence of a 100- or 500-fold (lanes 2 and 3, respectively) molar excess of a specific competitor U5RE DNA. An arrowhead indicates a specific band binding to the U5RE DNA.

control of the U3-R-U5 (−325/+306), region of LTR [16]. In contrast, pBLTR-2 showed about 5- or 2-fold increases of CAT activities in HeLa or Jurkat cells, respectively. The similar augmentation of CAT activities was observed in transfectants with pBLTR-9 (Fig. 1B). Moreover, as shown in Fig. 1C, significant differences in the promoter activity of pBLTR-1 compared with those of pBLTR-2 and -9 were confirmed by at least three independent experiments, indicating that the U5 region containing the sequence from +277 to +306 exerts a repressive effect on the LTR-directed expression in the absence of *tax* protein. Thus, these results suggest another possibility that the U5 region (+277 to +306) is responsible for repressing the basal HTLV-I gene expression.

Table 1
In vivo competition experiments[a]

Reporter	Competitor		Relative CAT activity
Experiment 1			
pCHL4 (4.2 μg)	poly(dI-dC)/poly(dI-dC)	(3 μg)	1.0
	U5RE DNA	(3 μg)	2.7
Experiment 2			
pBLTR-1 (2 μg)	Non-spe DNA	(3 μg)	1.0
	U5RE DNA	(3 μg)	2.9
Experiment 3			
pBLTR-1 (2 μg)	Non-spe DNA	(3 μg)	1.0
	U5RE DNA	(3 μg)	4.3
pRSV-CAT (2 μg)	Non-spe DNA	(3 μg)	1.0
	U5RE DNA	(3 μg)	1.2

[a]Cotransfection of each of the reporter plasmids with a specific or a non-specific competitor into Jurkat cells was performed, and the percent CAT conversion determined as described in the text. The results were normalized to the percent CAT conversion of the reporter with a non-specific competitor. A specific competitor, U5RE DNA. Non-specific competitors, poly(dI-dC)/poly(dI-dC) and Non-specific DNA were as described in sector 2.

As the U5 segment (+266 to +415) that contains our +277/+306 sequence was shown to exert its repressive effect when transactivated with *tax* protein [17], we then examined whether *tax* protein affects this U5-mediated repression. When cotransfected with the *tax* protein expression plasmid pMAXneo [9], pBLTR-2 showed about 2-fold augmentation of CAT activities in HeLa or Jurkat cells compared with pBLTR-1, although the two constructs, pBLTR-1 and -2, showed strong promoter activities (Fig. 1D). The result might suggest that this U5-mediated repression is independent of *tax* protein.

3.2. *Detection of U5RE-binding proteins in Jurkat nuclear extracts*

Cellular proteins interacting with the U5 segment might be possible to fulfil the repression of gene expression from the LTR. We then prepared crude nuclear extracts from Jurkat cells and looked at nuclear proteins binding to the U5 segment by using a gel mobility shift assay. First, six major shift bands were detectable with the ^{32}P-labeled R-U5 DNA probe (*SacI–BglI* fragment: 179-bp, from +128 to +306), which contains the +247/+306 sequence in the LTR U5 region. Of these bands, all disappeared in the presence of a cold R-U5 DNA competitor but not in the presence of a cold pX DNA competitor (Fig. 2A), while only one was significantly reduced in the presence of a cold U5RE DNA probe (+270/+296) as a competitor (Fig. 2B), suggesting that the Jurkat nuclear extracts contain proteins binding to the U5RE DNA. Then, in gel mobility shift assays with the ^{32}P-labeled U5RE DNA probe (Fig. 2C), one major

shift band was detectable. The cold U5RE DNA competitor inhibited this shift band formation, whereas a non-specific cold DNA fragment had no effect at all. These results indicate that Jurkat nuclear extracts contain proteins that specifically interact with U5RE within HTLV-I LTR.

To examine whether the U5RE-binding proteins are responsible for the U5-mediated repression, we next carried out in vivo competition experiments. As shown in Table 1, only when the U5RE DNA was used as a competitor, the repression effect of pBLTR-1 disappeared, whereas the CAT activities of the control pRSV-CAT were not affected, indicating that U5RE contains a functional repressive element. In addition, the results suggest the possibility for involvement of the U5RE-binding proteins in the U5-mediated repression.

3.3. *Purification of U5RE-binding proteins*

U5RE-binding proteins were purified by several chromatography purification steps including passage through heparin-agarose and the U5RE DNA-Sepharose columns as described in section 2. Jurkat crude nuclear extracts were applied to the heparin-agarose column in 0.1 M KCl-TM buffer, and eluted stepwise by increasing the concentration of KCl. The U5RE DNA-binding activity was monitored by the gel mobility shift assay, and was found to be enriched in fractions eluted with 0.4 M KCl-TM buffer (Fig. 3A). These fractions, named Hep(0.4M), were applied to the U5RE DNA-Sepharose column. The binding activity was again retained in 0.2 M KCl-TM buffer and eluted with 0.4 M KCl-TM buffer, designated U5(0.4M) (Fig. 3B). The profiles of the shift bands in these purified fractions are shown to be exactly the same as in the crude extracts. The specificity of the gel shift complexes is also demonstrated by competition with specific but not with non-specific competitors (Fig. 3A). These purified fractions were SDS-polyacrylamide gel electrophoresed and visualized by silver staining (Fig. 4). The molecules of about 70, 80, and 110 kDA were apparently enriched after the stepwise column purification, indicating that the U5RE-binding proteins contain at least these three proteins, termed p70, p80 and p110, respectively.

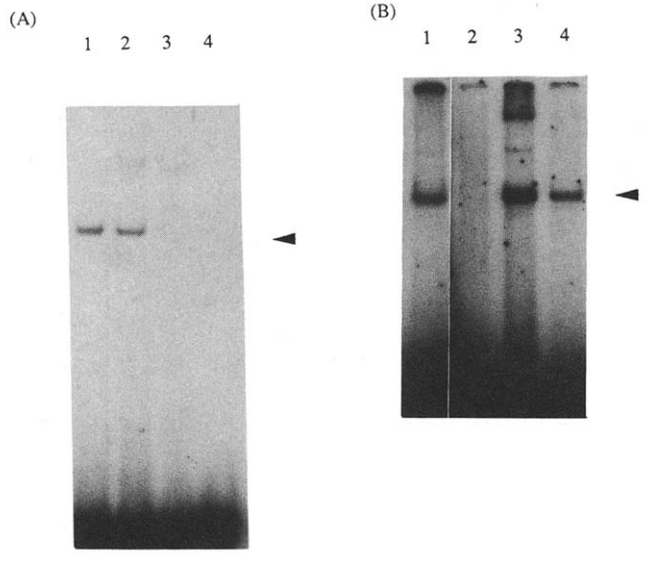

Fig. 3. Gel mobility shift patterns of purified nuclear proteins with a ^{32}P-labeled U5RE DNA as a probe. The fractions of the Jurkat nuclear proteins purified as described in section 2 were used. (A) Using the 0.4 M KCl fractions (Hep(0.4M)) of the heparin column (lanes 1–3), the specificity of the major gel shift complexes is demonstrated by competition with specific U5RE (lane 3) and non-specific DNA competitors (lane 2) at a 100-fold molar excess. Lane 4, no nuclear protein control. (B) Hep(0.4M) (lane 1) and the 0.2 KCl fractions (U5(0.2M)) (lane 2) and the first and second tubes of the 0.4 M KCl fractions (U5(0.4M)-1 and -2 respectively) (lanes 3 and 4, respectively) of the DNA affinity column were used for the assay. An arrowhead indicates a specific band binding to the U5RE DNA.

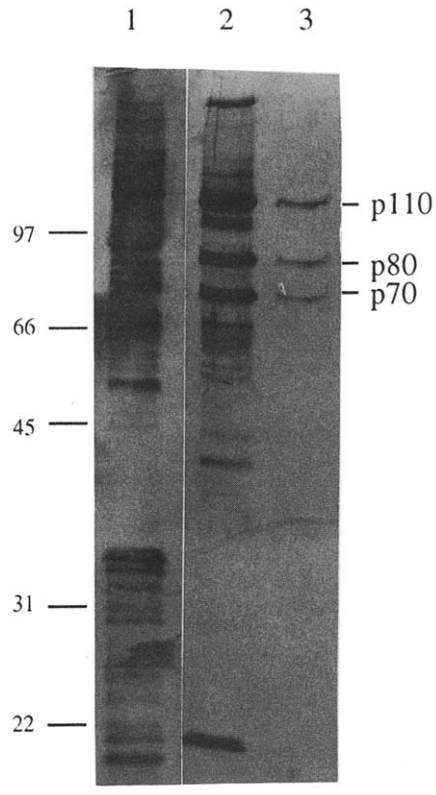

Fig. 4. SDS-gel electrophoresis of the purified U5RE DNA binding proteins. The Jurkat crude nuclear extracts were applied to the heparin column and subsequently to the DNA-affinity column as described in section 2. Proteins of the heparin column purified fraction, Hep(0.4M) (lane 1) and the DNA-affinity purified fractions, U5(0.4M)-1 and -2 fractions (the first and second tubes were applied on lanes 2 and 3, respectively) were separated on a SDS-polyacrylamide gel and visualized by silver staining. The molecular standards of the markers (kDa; prestained SDS-PAGE standards, BIO-RAD) are indicated to the left.

3.4. *p70 and p80 proteins are human autoantigen Ku-related proteins*

We attempted to determine the partial amino-terminal amino acid sequence of the three proteins, p70, p80, and p110, as described in section 2. Although the N-terminal amino acids of p70 and p110 failed to be determined, probably due to the blocking of the N-terminal amino acid, 21 amino acid residues at the amino terminus of p80 were determined: Val-Arg-Ser-Gly-Asn-Lys-Ala-Ala-Met-Val-Leu-X-Met-Asp-Val-Gly-Phe-X-Met-Ser-Asn (X = not determined). The computer search of this amino acid sequence against the Protein Identification Resource sequence bank revealed over 85% homology to the amino-terminal sequence of the human autoantigen Ku80 protein [33]: Val-Arg-Ser-Gly-Asn-Lys-Ala-Ala-Val-Val-Leu-Cys-

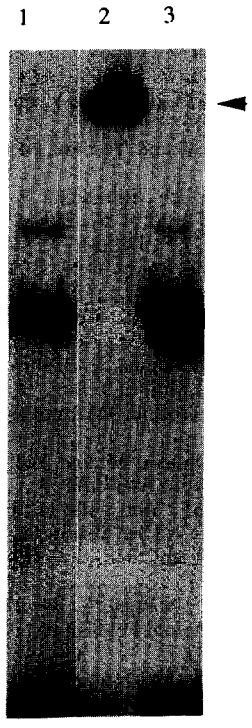

Fig. 5. Gel mobility shift assays using Jurkat purified nuclear proteins (Hep(0.4M) fraction) and ^{32}P-labeled U5RE oligonucleotide as a probe followed by incubation with a control normal human serum (lane 3), an anti-Ku(p80/p70) serum (lane 2) and saline (lane 1). An arrowhead on the right; position of a super shifted complex seen with the anti-Ku serum (lane 2).

Met-Asp-Val-Gly-Phe-Thr-Met-Ser-Asn. As Ku is known to be a heterodimer composed of two subunits with molecular weights of 70 and 80 kDa, we next examined whether the p70 and p80 proteins are immunologically Ku-related proteins. The result demonstrated that the p70 and p80 proteins were identified as the Ku or Ku-related proteins by Western blotting using anti-Ku(p80/p70) sera from patients with scleroderma-polymyositis overlap syndrome (data not shown). Furthermore, the gel shift induced by the nuclear extract can further shift following incubation with an anti-Ku(p80/p70) patient serum but not by a control serum (Fig. 5). These data demonstrated that the Ku or Ku-related proteins are involved in the U5RE-binding protein complex, suggesting the possible involvement of Ku in the U5-mediated repression.

4. Discussion

Recently, it has been shown that cellular nuclear factors such as Ets-1, Ets-2, SP1, TIF-1, Myb and YB-1 are activators of HTLV-I LTR-mediated transcription in the absence of *tax* protein [12–15,19,20]. Both Myb and Ets proteins are preferentially expressed at high levels in lymphoid tissues, particularly in the CD4$^+$ T cells that are natural targets for HTLV-I infection. Small amounts of YB-1 mRNA were also found in the lymphoid tissues. Therefore, it is reasonable to believe that these nuclear factors may play a crucial role in the expression of viral RNA in T cells in vivo. However, several reports have shown that the expression of HTLV-I viral mRNA as a proportion of total RNA in fresh uncultured peripheral blood mononuclear cells (PBMCs) from patients with ATL, TSP/HAM, or asymptomatic HTLV-I carriers is very low, and that some viral antigens are expressed only in a small number of circulating blood cells in vivo [34–38]. This discrepancy predicts that the HTLV-I gene expression in PBMCs in vivo is suppressed. So far, little is known of the suppression mechanisms in vivo. Analysis of the repressive element in the LTR seems to provide some important information about the mechanisms.

Seiki et al. have characterized a *cis*-acting repressive element in the LTR U5 segment [17]. When trans-activated with *tax* protein, some constructs of the LTR promoter with the deletion mutants within the U5 region showed strong promoter activities, while others did not. The differences suggested that the negative regulatory element that may function as an RNA element is located between +266 and +347. Moreover, they showed that the U5 segment exerts its repressive effect only when linked to the R region of the LTR. Recently, Xu et al. reported that HTLV-I LTR suppression is associated with cyclic- AMP responsive element binding protein (CREB) binding in the R region (+205 to +227) [21].

In this study, our findings that the sequences in the U5 region exert a repressive effect on the HTLV-I LTR-directed expression in the absence of *tax*-protein suggested the possibility that the U5 region controls basal HTLV-I transcription in initiation of viral gene expression. We showed that nuclear extracts from Jurkat T cells contain factors that bind to the U5 DNA segment (+269 to +295; U5RE) of the LTR. In vivo competition experiments showed that U5RE contains a functional repressive element. The binding of the nuclear factors to U5RE is specific in that they do not form shift bands with non-specific DNA.

To examine distribution of the U5RE-binding proteins in various kinds of cells, crude nuclear extracts were prepared from 15 human cell lines (Jurkat, Molt-4, CEM, MT-2, MT-4, TL-Om1, TL-SU, H582, TOM-1, Daudi, K562, U937, HeLa, U251 and NB9) and were analyzed U5RE-binding activities by the gel mobility shift assay (data not shown). The binding activities were detectable in all of the human cell lines examined, which include HTLV-I-infected and -uninfected T-cell lines and non-T-cell lines. Nuclear extracts from fresh preparations of human PBMCs also contained such binding activities. These findings indicate that the U5RE-binding proteins are ubiquitous proteins.

Our demonstration that the Ku or Ku-related proteins are involved in U5RE-binding will aid in examining the mechanism of the LTR U5-mediated repression. Ku, originally identified as an antigen recognized by sera from various autoimmune patients, is an abundant nuclear protein, and is a heterodimeric protein complex present in various mammalian systems [24,25]. Although the precise function of the Ku is still unclear, a role in transcriptional regulation has been proposed. Ku co-purifies with DNA-dependent protein kinase that phosphorylates RNA polymerase II and many transcriptional factors [39,40]. Ku also binds a critical motif in the T-cell receptor β enhancer [26] and the transcriptional control element of the human transferrin receptor gene [27]. Moreover, Ku was suggested to be involved in repression-mediated down regulation of mouse ribosomal gene transcription [41]. Thus, these results may suggest the possible involvement of Ku in the U5-mediated repression. On the other hand, Shindler et al. have reported that Ku also co-purifies as contaminants by binding to the termini of DNA fragments [42]. In our case, however, it seems unlikely that Ku

binds just DNA termini of the U5RE fragment, because the non-specific DNA competitor did not inhibit the shift band formation in gel mobility shift assays with the ^{32}P-labeled U5RE DNA probe (Fig. 3A).

We have not yet been successful in sequencing of another U5RE-binding protein p110, probably due to the blocking of the N-terminal amino acid. Alternatively, we have tried to isolate cDNA clones encoding p110 by using a South-Western screening method with the ^{32}P-labeled U5RE DNA probe. To date, we have isolated a clone encoding a novel protein that has an ability to bind the U5RE segment. We are now examining whether this cDNA-encoding protein corresponds to the P110 protein (will be published elsewhere).

Although the biological significances of these Ku-related proteins and the p110 protein remain to be determined, this study indicates the possibility that the U5RE-binding proteins play a role in the U5RE-mediated repression primarily at the transcriptional level.

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