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Ku Antigen Binds to *Alu* Family DNA[1]

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The GC-rich segment containing GGAGGC (*Alu* core) is conserved within the RNA polymerase III (pol III) promoters of *Alu* family sequences. We have shown that the GGAGGC motif functions as a modulator of DNA replication as well as of transcription, and identified the proteins binding to the motif in human HeLa cells. In this study, the *Alu* core binding proteins were partially purified from human Raji cells by using an *Alu* core DNA affinity column. Both the proteins thus purified were implied to be subunits of Ku antigen based on the following criteria: The molecular weights of the proteins estimated on gel electrophoreses were 70 and 85 kDa, respectively, under denaturing conditions, while under non-denaturing conditions only one band was observed for the same sample at 150 kDa, probably representing hetero-dimer formed between the 70 and 85 kDa proteins. The sizes and the hetero-dimer formation are reminiscent of the 70 and 80 kDa subunits of Ku antigen (Ku-p70 and Ku-p80). Moreover, the purified proteins were immunoreactive with anti-Ku antibodies, and the specific DNA-protein complex on the *Alu* core element was cancelled by the anti-Ku antibodies. The nucleoprotein complex showed the same clipping patterns as those of the complex between the *Alu* core element and an authentically purified Ku antigen after proteolytic cleavage with trypsin and chymotrypsin.

Key words: *Alu* family DNA, Ku antigen.

Alu family sequences, dispersed throughout vertebrate genomes, are sometimes transposed in the vicinity of transcriptionally active genes, as follows and modulate their promoters positively or negatively: the rabbit β1 globin (*1*), human ε-globin (*2*), adenosine deaminase (*3*), CD8α (*4*), γ chain of Fc and T cell receptors (*5*), and keratin 18 (*6*) genes. In addition, *Alu* sequences have experimentally been shown to repress transcription (*7-10*). *Alu* repeats have also been suggested to provide *cis*-elements affecting chromatin structures (*16*), initiation sites of DNA replication (*12-15*), sites of recombination (*16-18*), stability of cytoplasmic RNA (*19, 20*), gene conversion (*21*), and mRNA processing (*19, 20*). Although many *Alu* family members contain internal promoters for RNA polymerase III (pol III) and are transcribed *in vitro* (*11, 21-23*), most of them are transcriptionally silent in HeLa cells (*24*). The pol III promoter region of *Alu* elements is basically composed of two regions, namely the A and B boxes. Beside the A and B boxes there is the GC-rich domain conserved among older and younger members. The GGAGGC motif (*Alu* core) in the GC rich domain is also present in the simian virus 40 (SV40) origin of DNA replication, and has been suggested to have functions in DNA replication (*14, 15, 25*). We and others previously identified the nuclear proteins specifically interacting with the GGAGGC motif (*9, 26-28*), and have shown the proteins are involved in the modulations of DNA replication and transcription (*9, 27*).

In this study, we have partially purified GGAGGC motif-binding proteins from human Raji cells. The purified proteins were indistinguishable from Ku antigen, a ubiquitous nuclear protein. The results suggest that Ku antigen binds to the *Alu* core elements.

MATERIALS AND METHODS

Cell Culture—Human Raji cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in a spinner flask.

Purification of Alu Core Binding Proteins—A nuclear extract was prepared from 9×10^9 Raji cells as described previously (*29*), with minor modifications (*30*). All the procedures were performed at 4°C. The extract was extensively dialyzed against buffer D comprising 20 mM Hepes-KOH (pH 7.9), 20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol (DTT), and then applied to a Heparin-Sepharose column (20 ml bed volume) (Pharmacia LKB Biotechnology) equilibrated with buffer D. After washing with 40 ml of buffer D, the proteins bound

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Abbreviations: *Alu* BP, *Alu* core sequence binding protein; pol III, polymerase III; Ku-p70, 70 kDa subunit of Ku antigen; Ku-p80, 80 kDa subunit of Ku antigen; DTT, dithiothreitol; DMS, dimethylsulfate; TBP, TATA box binding protein; DNA-PK, DNA-dependent protein kinase.

to the column were eluted with a linear gradient from 50 to 500 mM, of KCl in a total 100 ml of buffer D at the flow rate of 8 ml/h. The column was finally washed with 40 ml of buffer D containing 1 M KCl. The proteins in the fractions were examined for *Alu* core binding activity by bandshift assay, and the active fractions, 31 to 43 (26 ml in total), were pooled. The pooled fractions were then dialyzed against buffer D and applied to a DNA affinity column containing *Alu* core oligonucleotides (2×2 cm; 8 ml bed volume) (the *Alu* DNA affinity column) prepared by the standard method. Briefly, approximately 100 nmol (600 μg) each of oligonucleotides corresponding to either the upper or lower strand of the *Alu* core sequence were annealed and then phosphorylated at their 5′-ends with T4 polynucleotide kinase. The oligonucleotides were coupled with Sepharose CL-2B (Pharmacia LKB Biotechnology; 10 ml packed volume) with cyanogen bromide, and the resin was suspended in buffer D. After washing with buffer D containing 50 mM KCl, the proteins bound to the *Alu* DNA affinity column were eluted with a linear gradient, from 50 to 500 mM, of KCl in 60 ml of buffer D at the flow rate of 8 ml/h, and then washed with 40 ml of buffer D containing 1 M KCl. The proteins in the fractions were examined for *Alu* core binding activity by bandshift assay and the active fractions, 24 to 30 (totally 7 ml), were pooled and dialyzed against buffer D. The purified proteins were stored at −80°C in aliquots.

Bandshift Assay—The nucleotide sequences of the oligonucleotides used as probes and competitors were as follows, where capital letters indicate the sequences corresponding to the *Alu* element and small letters represent artificial sequences of the *Bam*HI recognition site: upper strand of wild type (*Alu*A), 5′-gatccGGAGGCTGAGGCAg; lower strand of *Alu*A, 5′-gatccTGCCTCAGCCTCCg; upper strand of mutant 1,5′-gatccGGCTACTGAGGCAg; lower strand of mutant 1,5′-gatccTGCCTCAGTAGCCg; upper strand of mutant 2,5′-gatccGGGGGCTGGGACAg; and lower strand of mutant 2,5′-gatccTGTCCCAGCCCCCg. Probes were labeled as described previously (*27*). Briefly, 1 μg of annealed oligonucleotides was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, purified by gel filtration on Sephadex G50, separated on a 10% polyacrylamide gel, and then eluted from the gel. Binding reactions were carried out by incubating 0.2-1 pmol of a labeled probe (10,000 cpm) with 2-3 μg of proteins and 2 μg of poly(dI-dC) in a buffer comprising 50 mM KCl, 1 mM EDTA, 4% Ficoll 400, 1 mM DTT, and 4 mg/ml of bovine serum albumin, in a volume of 15 μl. In the experiments involving the proteins eluted from the Heparin-Sepharose or *Alu* DNA affinity column, 0.2 μg/μl of poly(dI-dC) were included in the reactions. After incubation at room temperature for 10 min, the reaction mixtures were electrophoresed at 10 V/cm on a 4% polyacrylamide (19:1) gel in 0.25×TBE buffer. For competition experiments, 10-100-fold excess amounts of non-labeled double-stranded oligonucleotides were added to the reaction mixtures prior to the addition of proteins. For supershift experiments, 1 μl of human serum containing anti-Ku antibodies (serum KuT and OM) (*31, 32*) or IgG was incubated with the purified *Alu* binding proteins or the purified Ku antigen as a standard (*31, 32*) for 60 min at 0°C. The mixtures were then subjected to binding reactions with the *Alu* core probe as above.

Proteolytic Clipping Bandshift Assay—All the procedures followed the methods described previously (*33*). Briefly, binding reactions of the purified *Alu* binding proteins or the standard Ku antigen with the *Alu* core probe was carried out as above. After the reaction, the mixtures were further incubated with various concentrations of trypsin or chymotrypsin for 10 min at room temperature and then subjected to gel electrophoresis.

Western Blotting—The purified *Alu* core binding proteins and the standard Ku protein were separated in a 10% polyacrylamide gel containing SDS, and then transferred to a nitrocellulose filter and reacted with two types of antisera containing different anti-Ku antibodies (serum KuT and OM) (*31, 32*). The immunoreacted proteins were visualized with an alkaline phosphatase detection system or on ECL kit (Amersham).

Methylation Interference—Oligonucleotides corresponding to either strand of the *Alu* sequence, *Alu*A and *Alu*B, respectively, were end-labeled using T4 polynucleotide kinase and then annealed with unlabeled complementary oligonucleotides. The probes were partially methylated for 2 min at 20°C using dimethylsulfate (DMS) (*34*), and then subjected to scaled-up band shift assays. The probes at both shifted (bound) and free positions were excised and eluted from the gel, and purified by DEAE 52 column chromatography. After ethanol precipitation, each fraction of the recovered probes was resuspended in 100 μl of 1 M piperidine, incubated at 90°C for 30 min (*35*), and then analyzed with a 15% polyacrylamide gel containing 8.3 M urea.

RESULTS

Identification and Purification of the Alu Core Binding Proteins in Human Raji Cells—We previously identified the proteins which specifically recognize GGAGGC, the *Alu* core sequence present in *Alu* family members, in human cervical carcinoma HeLa cells (*9, 27*). We prepared a nuclear extract of human Burkitt lymphoma Raji cells and examined the extract for GGAGGC-binding proteins by bandshift assays with a ^{32}P-labeled *Alu* core probe as described (*27*). One major band and another faint band exhibiting more rapid migration were observed (Fig. 1). The bands disappeared in the presence of an excess amount of non-labeled oligonucleotides with the same sequence as the probe, but remained in the presence of oligonucleotides with mutated sequences, despite the weak specificity (data not shown). The results indicated that proteins which specifically recognize the *Alu* core sequence exist in Raji cells as well as in HeLa cells.

A Raji nuclear extract was prepared from approximately 10^{10} cells, applied to a Heparin-Sepharose column and then separated with a linear gradient of KCl. The fractions were examined for *Alu* core binding activity by bandshift assays as described under "MATERIALS AND METHODS," and the active fractions (fraction numbers 31 to 43) were pooled (Fig. 1A) and further applied to a DNA affinity column containing *Alu* core oligonucleotides (*Alu* DNA affinity column). The proteins bound to the column were eluted with a KCl gradient and collected (Fig. 1B). In the fractions obtained on Heparin-Sepharose column chromatography, two bands exhibiting different migration were observed in the bandshift assays, as in the case of the crude extract described above. We focused on the nucleoprotein complex

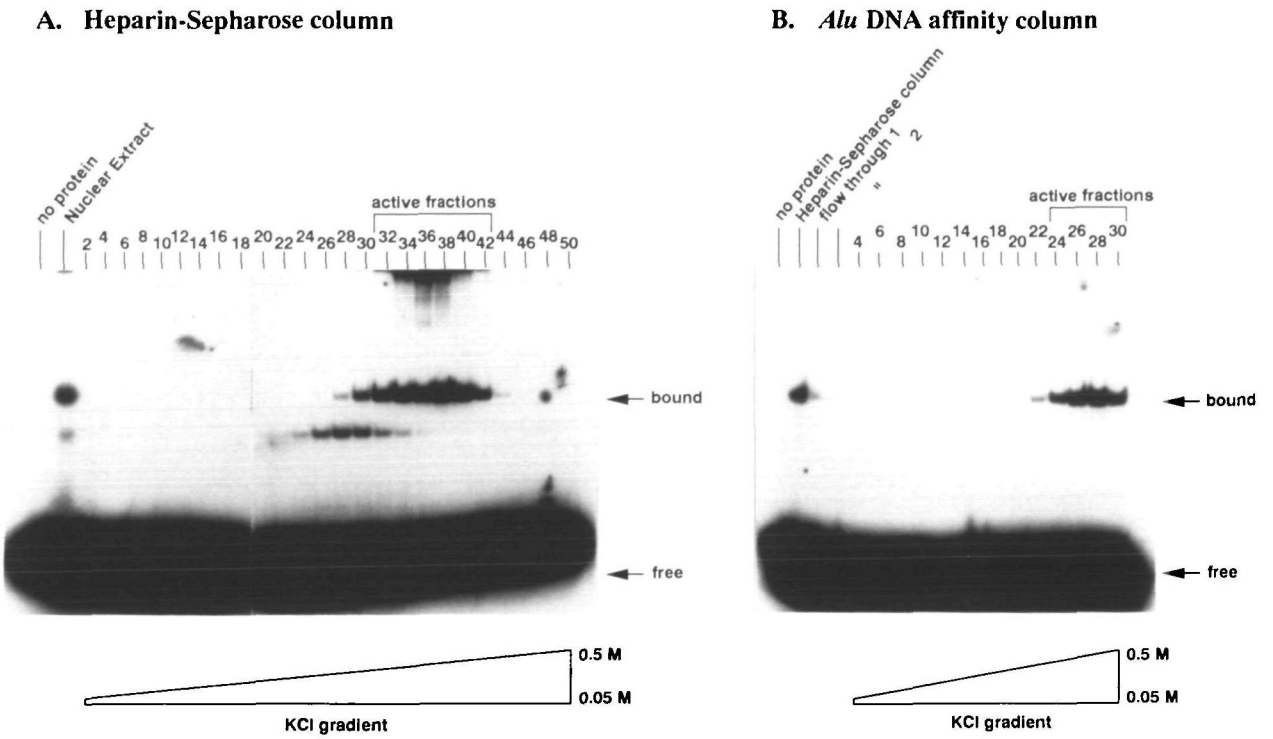


Fig. 1. **Purification of the *Alu* core binding proteins by two steps of column chromatography.** Nuclear extracts prepared from Raji cells were applied to a Heparin-Sepharose column (A) and the bound proteins were eluted with a linear gradient of increasing KCl concen-tration. The fractions active as to *Alu* core binding in bandshift assay were pooled and applied to an affinity column containing *Alu* core oligonucleotides (*Alu* DNA affinity column) (B). The results of the bandshift assays are shown. Arrows indicate positions of a specific DNA-protein complex (bound) and the free probe (free).

TABLE I. **Purification of Ku antigen/*Alu* core binding proteins from human Raji cells.**

Fractions	Total proteins (mg)	Total activity (unit)	Specific activity (unit/μg)	Purification (fold)	Yield (%)
Nuclear extract	13	52,000	4	1	100
Heparin-Sepharose	5	40,000	8	2	77
Alu DNA affinity	0.0007	2,800	4,000	1,000	5

One unit of binding activity was defined as the amount of protein that retarded 5 fmol of end-labeled probe of the wild-type *Alu* core sequence.

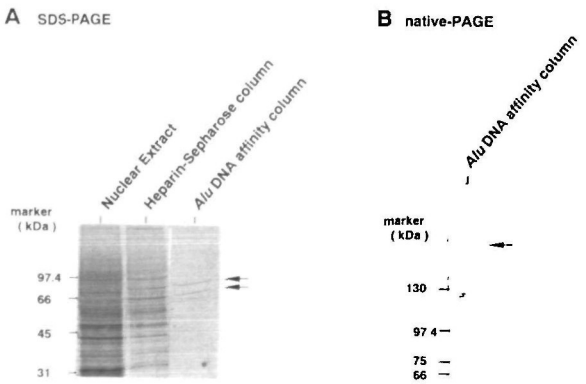
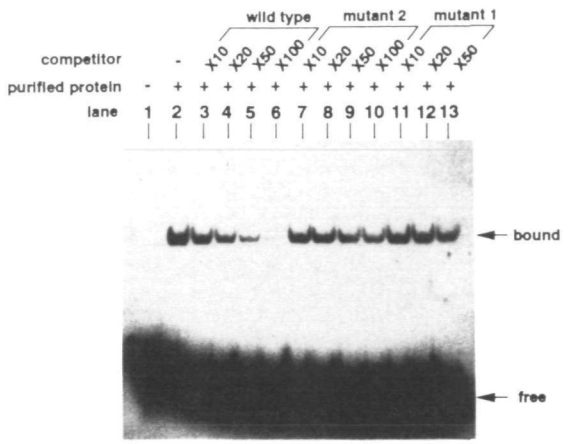


Fig. 2. **Silver staining of the proteins eluted from the *Alu* DNA affinity column.** Proteins in the crude nuclear extract as well as in the active fractions eluted from the Heparin-Sepharose and *Alu* DNA affinity columns were separated on a 10% polyacrylamide gel containing SDS (A) or a 7.5% native polyacrylamide gel (B), and then silver-stained according to the standard procedure. The major bands observed for the eluate from the *Alu* affinity column are indicated by arrows. Molecular weight markers are indicated on the left.

exhibiting the slower migration (yielding the upper band) for further purification. On *Alu* DNA affinity column chromatography, proteins specifically recognizing the *Alu* core sequence were purified 1,000-fold, as compared to those in the crude nuclear extract (Table I). The purified proteins were analyzed by gel electrophoresis, followed by silver staining (Fig. 2). On *Alu* DNA affinity column, two bands due to 70 and 85 kDa proteins were observed in a polyacrylamide gel containing SDS (Fig. 2A). Under the native conditions without SDS, on the other hand, a single band was detected at approximately 150 kDa, but not around 70–80 kDa (Fig. 2B). The results suggest that the 70 and 85 kDa proteins exist in dimeric forms in the absence of denaturing reagents. The binding specificity of the purified proteins was then verified by competition experiments (Fig. 3). Inhibition of the DNA-protein binding was observed in the presence of lower amounts of the wild type oligonucleotides homologous to the probes, than of the oligonucleotides with mutated sequences. The proteins purified from Raji cells are hence suggested to recognize the *Alu* core sequence specifically.

To confirm that the purified protein directly binds to the oligonucleotides, a methylation interference assay was carried out (Fig. 4). Binding of the proteins to four G and one A residue in the *Alu*A oligonucleotide were interfered



competitors

Alu A wild type	5'- gatcc GGAGGC TGAGGCA g g CCTCCG ACTCCGT cctag -5'
Alu A mutant 2	5'- gatcc GGGGGC TGGGACA g g CCCCCG ACCCTGT cctag -5'
Alu A mutant 1	5'- gatcc GGCTAC TGAGGCA g g CCGATG ACTCCGT cctag -5'

Fig. 3. **Binding specificity of the purified *Alu* core binding proteins.** Bandshift assays were performed using the proteins purified on the *Alu* DNA affinity column. The proteins were incubated with a labeled *Alu* core probe in the presence or absence of non-labeled competitor DNAs at the indicated molar ratios to the probe. The sequences of the oligonucleotides used as competitors are shown in lower panel. Arrows indicate the positions of a specific DNA-protein complex (bound) and the free probe (free).

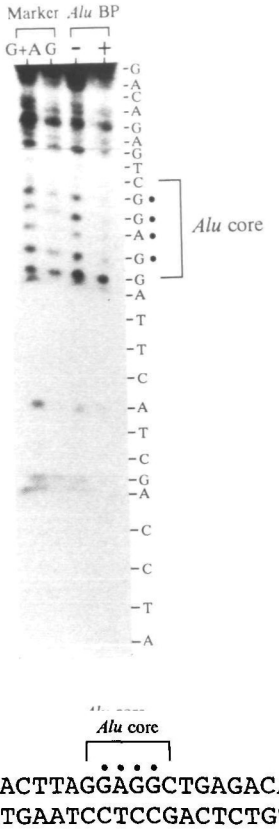


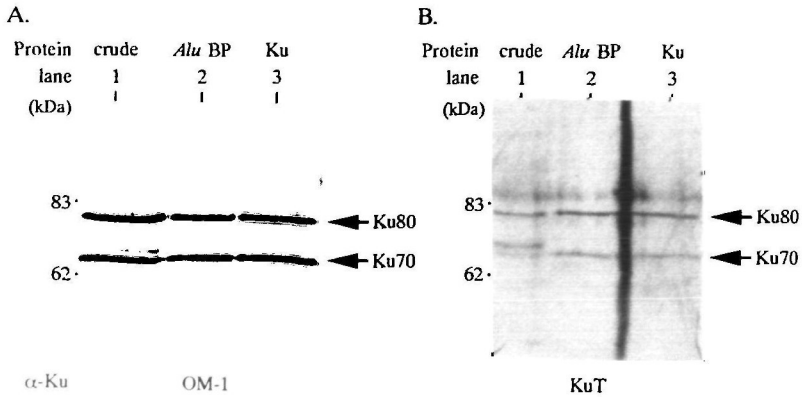
Fig. 4. **Methylation interference assay of the proteins binding to *Alu*A and *Alu*B oligonucleotides.** *Alu*A and *Alu*B oligonucleotides were end-labeled and annealed with the complementary oligonucleotides. The labeled oligonucleotides were methylated with DMS and then bandshift assays with the purified proteins were carried out. The probes at the free and protein-bound positions were eluted from the gel, reacted with piperidine, and then separated in a denaturing polyacrylamide gel. G+A residues, or G alone, of the same oligonucleotides were labeled by the Maxam and Gilbert method (*34*), and run in the same gels as markers. Lanes *Alu*BP+ and − indicate the reaction of the probes in the protein-bound and free band, respectively. The *Alu* core sequence and the nucleotides interacting with proteins are indicated by a bracket and dots, respectively.

from the result of the methylation, indicating that three G and one A residue in, and another G beside, the *Alu* core sequence were directly bound by the purified proteins. Since the *Alu*B oligonucleotide, the complementary strand of *Alu*A, is composed mainly of C and T, the methylation interference pattern gave little result. The binding to the terminal G residues in both *Alu*A and *Alu*B was not interfered with, suggesting that proteins do not bind to the ends of the oligonucleotides.

Identification of the Alu Core Binding Proteins as Ku Antigen—The molecular weights and possible dimer formation of the purified *Alu* core binding proteins are reminiscent of those of Ku antigen. The purified *Alu* core binding proteins were therefore blotted onto a nitrocellulose filter and then reacted with two different anti-Ku antigen antisera, OM and KuT (Fig. 5). The Ku antigen purified from HeLa cells using an affinity column of a patient's serum was used as a standard (*31, 32*). Both the standard Ku antigen and the *Alu* core binding proteins gave bands at 70 and 85 kDa with either antiserum. The results indicated that the purified *Alu* core binding proteins of 70 and 85 kDa were immunologically indistinguishable from the p70 and p80 subunits of Ku antigen. On the blotting using serum OM-2, a different lot from the same patient as OM-1, the intensity of the band was much stronger for Ku-p80 than for Ku-p70 (Fig. 5A, lower panel), while both proteins were comparably detected in the blots for the same samples using OM-1 or KuT serum. Lot OM-2 was used as an anti-Ku antigen antiserum for the further experiments described below.

The purified *Alu* binding proteins and the standard Ku antigen were reacted with anti-Ku antibodies and subjected to the bandshift assay with the labeled *Alu* core oligonucleotide (Fig. 6). The standard Ku antigen gave rise to a single band exhibiting the similar migration to that of the nucleoprotein complex of the purified *Alu* binding proteins (Fig. 6, lanes 2 and 6). The complexes of both the standard Ku and the *Alu* binding proteins were cancelled by pretreatment with either anti-Ku antiserum (Fig. 6, lanes 4, 5, 8, and 9). Pretreatment of the proteins with non-immune serum, on the other hand, affected neither the complex formation nor the migration at all (Fig. 6, lanes 3 and 7). The results indicated that the proteins responsible for *Alu* core binding are, or contain, Ku antigen.

To address this possibility, the proteolytic clipping bandshift assay (*33*) was carried out. The purified *Alu* core binding proteins and the standard Ku antigen were incubated with a labeled *Alu* core probe as in usual bandshift assays. The reaction mixtures were further treated with various concentrations of either trypsin or chymotrypsin before separation in polyacrylamide gels (Fig. 7). Both the



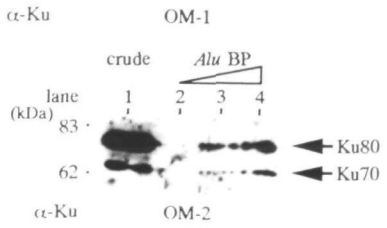


Fig. 5. **Identification of the *Alu* core binding proteins as Ku antigen.** Fifteen micrograms of the Raji nuclear proteins (crude), 25 ng of the purified *Alu* core binding proteins (*Alu*BP), and 25 ng of the purified Ku antigen (Ku) were separated on a 10% polyacrylamide gel containing SDS, transferred to a nitrocellulose filter, and then reacted with two different antisera, OM-1 (A) and KuT (B), containing anti-Ku antibodies. The blotting of the Raji crude extract (crude; 2 μg) and the purified *Alu* core binding proteins (*Alu*BP; 2, 5, and 10 ng in lanes 2–4, respectively) with OM-2, a different lot of serum from the same patient as for OM-1, is also shown (lower panel). The reacted proteins were visualized by the alkaline phosphatase detection method, for OM-1 and KuT, or with an ECL kit, for OM-2. The bands due to the authentic 70 and 80 kDa subunits of Ku antigen (Ku70 and Ku80) are indicated by arrows. Molecular weight markers are shown on the left of the panels.

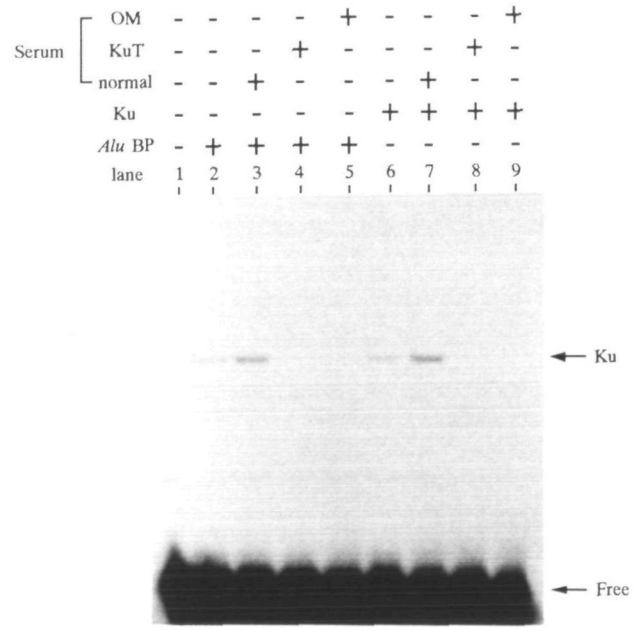
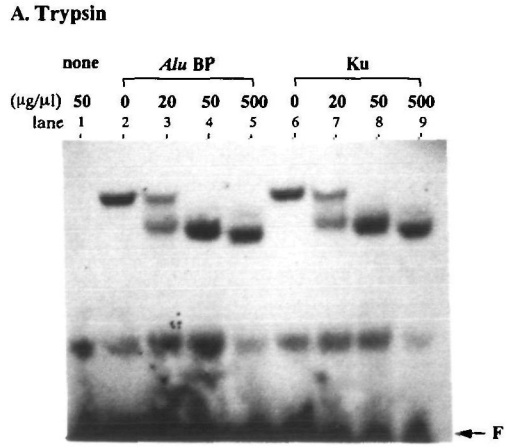


Fig. 6. **Inhibition of complex formation between the *Alu* core sequence and Ku antigen by an anti-Ku antibody.** Twenty-five nanograms of the purified *Alu* core binding protein (*Alu*BP), or the purified Ku antigen (*36, 37*) (Ku), were mixed with two different antisera containing anti-Ku antibodies (KuT and OM-2) and incubated at 0°C for 60 min, and then subjected to bandshift assays with a labeled *Alu* core sequence as a probe. Arrows "Ku" and "Free" indicate the positions of the specific DNA-protein complex and free probe, respectively.



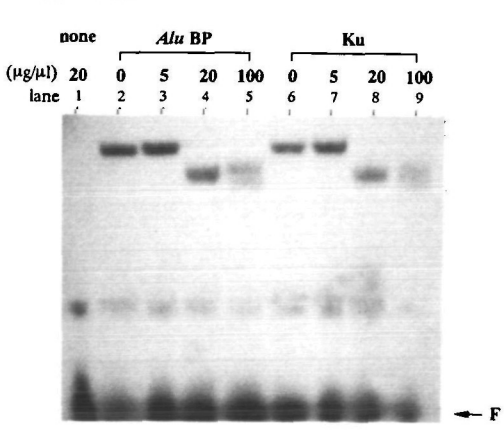


Fig. 7. **Proteolytic clipping bandshift assays of the *Alu* core binding proteins.** The binding reaction mixtures of the labeled *Alu* core oligonucleotide with the purified *Alu* core binding protein (*Alu*BP) or the purified Ku antigen (Ku) were treated with various concentrations of trypsin (A) or chymotrypsin (B) for 10 min at room temperature, and then analyzed by polyacrylamide gel electrophoresis as for normal bandshift assays. Concentrations of trypsin or chymotrypsin used are shown above the lanes. Arrow "F" indicates the position of the free probe.

protein samples yielded the same proteolytic cleavage patterns of DNA-protein complexes after the incubation with either proteinase. These results strongly suggest that the proteins purified as *Alu* core binding proteins were Ku antigen.

DISCUSSION

This report describes the purification and functional analyses of the *Alu* family DNA binding proteins present in human Raji cells. Two proteins of 70 and 85 kDa, were purified by a DNA affinity column containing the GGAGGC

motif. From the profiles of the purified proteins on polyacrylamide gels under denaturing and non-denaturing conditions, the 70 and 85 kDa proteins were suggested to comprise heterodimers. In addition to the heterodimer formation, the proteins were immunologically indistinguishable from Ku antigen, and the interaction of the proteins with the *Alu* core sequence was inhibited by the addition of anti-Ku antibodies. Furthermore, no difference was seen in the proteolytic cleavage patterns of the DNA-protein complex on the *Alu* core sequence between the purified *Alu* core binding proteins and the standard Ku antigen.

Ku antigen is abundantly present in cell nuclei and is known to be an autoantigen protein with DNA binding activity. The cDNAs for its subunits, Ku-p70 and Ku-p80, have been cloned (*36-39*). Functions of Ku antigen have been suggested in transcription, DNA replication, recombination, and repair, especially in excision-repair (reviews see Refs. *40* and *41*). Mutations of the hamster genes *XRCC5* and *XRCC6*, corresponding to the human Ku-p80 and Ku-p70 genes, respectively, resulted in defects of both double-stranded DNA break repair and the V(D)J recombination (*40-43*). Knockout mice with deletion of the Ku-p70 or Ku-p80 gene also showed the same defects (*44*). In these cases, Ku antigen is suggested to bind to the DNA ends produced in the reactions. In addition to binding to the ends of double-stranded DNA with less sequence-specificity (*32, 45-47*), various transcription factors, including PSE, TREF, CTCBF, and CHBF, are considered to be identical or related to Ku antigen. The factors recognize the promoters of the snRNA, transferrin receptor, collagen IV, heat shock protein 70, and HTLV-1 genes, respectively, in a sequence-specific manner to regulate the transcription of the genes of interest (*48-51*). E1BF, another protein related to Ku antigen, specifically recognizes the pol I-dependent promoters to regulate the transcription (*52*). Ku antigen was also reported to bind to the major breakpoint region of *bcl2* in sequence- and cell cycle-specific manners (*53*). Recent reports suggested that Ku antigen is identical to human DNA helicase II (*54*) and associates with the catalytic subunit of DNA-PK, which phosphorylates various proteins including transcription factors (*55-57*). As a transcription factor to recruit DNA-PK directly to specific DNA sequences, Ku antigen represses glucocorticoid-induced transcription from the mouse mammary tumor virus (*58*). Although we cannot completely exclude the possibility that Ku antigen bound to the ends of the DNA in this study, the results of the competition bandshift assays and the methylation interference assays indicate that Ku antigen, *i.e.* the *Alu* core binding protein purified here, specifically recognizes the *Alu* core sequence, but not the ends of the oligonucleotides. Little similarity, however, seems to exist among the sequences reported so far to be recognized by Ku antigen, including the *Alu* core element we identified here. One possible explanation for the inconsistency on the binding specificity is that different sequences may be recognized by Ku antigen in complexes with different proteins (*48, 59*). Preliminary results showed that the fraction purified here as *Alu* core binding proteins contained TBP, which binds to at least Ku-p80 to enhance the *Alu* core-Ku antigen complex formation (data not shown). The association of TBP with Ku antigen has also been reported for the collagen IV enhancer (*48*), where the element 5′-CCCTCC-3′ is recognized by Ku antigen/TBP. The complementary sequence of the element, 5′-GGA-GGG-3′, is very similar to the *Alu* core sequence. Since the methylation interference experiment in this report showed that GAGG at the center of the *Alu* core sequence is important for Ku antigen binding, Ku antigen may recognize 5′-GGAGGG-3′ in the collagen IV gene in association with TBP.

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