ARTICLES

Characterization of Nuclear Factors Binding to AT-Rich Element in the Rat p53 Promoter

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Abstract   In this study, we identified AT-rich element located at positions −504 to −516 in the rat p53 promoter by DNase I foot printing assay. This region was previously identified as a positive regulatory element in the murine p53 promoter and designated as PBF1 (p53 binding factor 1) binding site. However, the proteins binding to this AT-rich element have not been identified yet. Therefore, we characterized the binding protein by various biochemical methods. First, we confirmed that by the oligonucleotide competition assay, nuclear factors bound to the AT-rich element in a sequence-specific manner. Two binding proteins were identified in southwestern blotting analysis and the molecular masses of the proteins were 60 and 40 kDa, respectively. The proteins were stable to denaturants or ionic strength. Treatment of chelators showed that the binding proteins did not require divalent cation for DNA-binding activity. In addition, the binding proteins were labile to protease treatment. This study showed that 60 and 40 kDa proteins bound to AT-rich element and the physico-chemical properties provided new insights into the binding proteins. J. Cell. Biochem. 80:580–588, 2001. © 2001 Wiley-Liss, Inc.

Key words: AT-rich element; *cis*-regulatory element; p53 tumor suppressor; promoter; transcription regulation

p53 is a well-known tumor suppressor protein. The mutations of the gene have been observed in over 50% of all human cancers [Hollstein et al., 1991; Levine et al., 1994; Ko and Prives, 1996]. p53 is a checkpoint at the G1-S phase transition [Levine, 1997]. In the case of DNA damage by DNA damaging agents such as UV irradiation at the prereplicative phase, it arrests cell cycle at G1 phase and tries to repair the damage [Levine, 1997]. It functions as a transcription factor and induces the expressions of the genes that have p53 responsive element in their promoter regions. These p53 responsive genes include p21$^{\text{Waf1/Cip1}}$[El-Deiry et al., 1993], GADD45 [Chin et al., 1997], and Bax [Miyashita and Reed, 1995], which are

related to cell cycle arrest, apoptosis, or DNA repair. Recently, it was suggested that p53 function as a checkpoint at G2-M phase transition [Cross et al., 1995].

The p53 promoter does not have TATA or CAAT box [Bienz-Tadmor et al., 1985; Lamb and Crawford, 1986]. Features of cellular TATA-box-less promoters include the presence of multiple repeats of the GC rich SP1 binding site [Reynolds et al., 1984; Calabi and Neuberger, 1985; Ishii et al., 1985]. The rat p53 promoter contains the structures analogous to those found in other TATA-box-less promoters. There are two repeats of the sequence CTTGCCC (positions −252 and −229) as well as a CCCGCC (position −265). It was suggested that these elements might play a role in determining the activity of the p53 promoter [Bienz-Tadmor et al., 1985]. However, there is no direct evidence that SP1, or indeed any factor, binds to these regions, although the sequences are conserved across the species. Instead, it was reported that the transcription of the p53 gene was regulated by several transcription factor-binding sites. These regulatory elements include NF1 (nuclear factor 1) [Ginsberg et al., 1990; Furlong et al., 1996; Lee et al., 1998], YY1 (Ying yang 1) [Furlong et al., 1996], bHLH

(basic helix-loop-helix) [Reisman and Rotter, 1993; Reisman et al., 1993; Roy et al., 1994; Hale and Braithwaite, 1995], PF1 (p53 factor 1) [Ginsberg et al., 1990], AP1 (activator protein 1) [Kirch et al., 1999], and PF2 (p53 factor 2) [Hale and Braithwaite, 1995] binding motifs. The sequences of the motifs are highly conserved across the mouse, rat, and human p53 promoters, which indicates that the motifs are common transcription regulatory elements across the species. However, tissue-specific or species-specific motifs have also been identified. In the rat p53 promoter, we found another NF1 binding motif that does not exist in the p53 promoters of other species [Lee et al., 1998]. NF-κB motif was involved in the transcription of the human p53 gene when the expression of the p53 gene was induced by DNA-damaging agent [Wu and Lozano, 1994]. Recently, it was also reported that mutation of the NF-κB motif abolished transcription from the human p53 promoter in HeLa, HepG2, and adenovirus type 5 E1-transformed 293 cells [Kirch et al., 1999]. Although the sequence of the NF-κB motif was highly conserved across the species, in the basal level transcription of the mouse and rat p53 genes, protein binding to NF-κB motif was not detected [Hale and Braithwaite, 1995; Lee et al., 1998]. In addition, in the transcription of the human p53 gene, NF1 or YY1 binds to the same region and regulates the transcription in a tissue-specific manner [Furlong et al., 1996]. However, our recent result showed that in the rat p53 transcription, NF1, not YY1, binds to the motif irrespective of tissue type [Lee et al., 1999]. These suggest that the transcriptional regulatory mechanism of the p53 gene may be different, depending on the species or the signal for the transcription of the p53 gene.

In this study, we analyzed the rat p53 promoter by DNase I footprinting analysis, and found AT-rich element at the positions of −504 to −516 region. This element was previously reported as a positive regulatory element in the murine p53 promoter and designated as PBF1 (p53 binding factor 1) [Roy and Reisman, 1996]. The element had high contents of adenine and thymine and did not have homology to any known transcription factor-binding site. However, the binding proteins to this element have not been identified yet. Hence, we analyzed the binding proteins by various biochemical methods. Molecular masses of the binding proteins were determined by southwestern blotting analysis. The influence of ionic strength, denaturant, or heat on DNA-binding activities of the proteins was determined by EMSA (electrophoretic mobility shift assay). The effects of chelators or proteases were also evaluated. This study showed that two proteins bound to the AT-rich element in a sequence-specific manner and their physico-chemical properties provided new insights into the binding proteins.

METHODS

Preparation of Nuclear Extract

Female Wistar rats (Animal Breeding Center in Seoul National University, Korea) weighing 120–140 g were used for all experiments. The animals were kept in temperature-controlled rooms with 12 h alternating light and dark cycles. Nuclear extract was prepared by the method described previously [Lee et al., 1998]. Briefly, rat liver was homogenized in four volume of buffer A (10 mM Tris, pH 7.9, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 1 mM DTT (1,4-dithiothreitol), and 0.1 mM PMSF (phenylmethanesulfonyl fluoride)) containing 0.32 M sucrose. Homogenates were layered over buffer A containing 2 M sucrose and centrifuged for 50 min at 40,000 g. The nuclei were suspended in lysis buffer (15 mM Hepes (N-2-Hydroxyethylpeperazine-N'-ethanesulfonic acid), pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF), to which 0.1 volume of 4 M $(NH_4)_2SO_4$ was added and stirred at 4°C for 30 min. After the centrifugation for 1 h at 130,000 g, 0.3 g of $(NH_4)_2SO_4$ per ml of supernatant was added and stirred for 30 min. Nuclear extract was centrifuged for 15 min at 16,000 g, dissolved in dialysis buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 20% glycerol, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, and 0.5 mM PMSF), and dialyzed against dialysis buffer overnight at 4°C.

DNase I Footprinting Analysis

The 447 bp (−537 to −91) fragment of the 5′ end of the rat p53 gene was cloned by the method described previously [Lee et al., 1998]. The rat p53 promoter was numbered by the convention proposed by Tuck and Crawford [1989]. The 447 bp fragment was treated with CIP (Calf intestinal phosphatase, Promega Biotech, WI) and end-labeled, using [γ-^{32}P]ATP

(NEN Life Science, MA) and T4 polynucleotide kinase (New England Biolabs, Inc., MA). This ^{32}P-labeled DNA was digested with Hind-III and isolated on an 8% polyacrylamide gel. Binding reaction mixture, containing 40 μg of nuclear extract, 2 μg of poly(dI-dC) (Boehringer Mannheim Biochemicals, Germany) and ^{32}P-labeled DNA fragment (0.1 pM), was allowed to proceed for 20 min at room temperature after mixing. Then the mixtures were treated with 0.2–1 unit DNase I (Promega Biotech, WI) for 30 s, extracted with phenol/chloroform, and separated by electrophoresis through 8% acrylamide/7M urea gels. The gel was dried and subjected to autoradiography.

EMSA

The synthetic oligonucleotides containing the AT-rich element (oligo 1: 5′-AGACTCTG TTTCAAAAAGCCAAA-3′, 5′-TTTGGCTTTTT GAAACAGAGTCT-3′) were annealed and labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reaction mixtures contained 3 μg of nuclear extract, 2 μg of poly(dI-dC), binding buffer (1 × : 12 mM Hepes, pH 7.9, 60 mM KCl, 5 mM MgCl$_2$, 0.2 mM EDTA, 0.2 mM DTT, and 12% glycerol) in volume of 20 μl. For competition assay, 1 pM (10 × molar excess) or 10 pM (100 × molar excess) of oligonucleotide competitor (oligo 1, NF1 consensus (5′-TTTGGCACGGAGCCAAC-3′, 5′-GTTGGC TCCGTGCCAAA-3′), or CRE (cAMP responsive element, 5′-TGCTGACGTCAAC-3′, 5′-GTT TGACGTCAGCA-3′) was added to each reaction mixture. After the addition of the end-labeled oligo 1 (0.1 pM), the mixtures were incubated at room temperature for 20 min. Then they were electrophoresed through a 6% polyacrylamide gel in the presence of 0.25 × Tris-borate-EDTA. The gel was dried and subjected to autoradiography.

Southwestern Blotting Analysis

Nuclear extract (50 μg) from liver was subjected to electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel. For western blotting, proteins were transferred from a 10% acrylamide gel to a PVDF membrane for 60 min at 300 mA. The blot was gently washed in TNE-50 (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT) and blocked for 1 h at 4°C in blocking buffer (2.5% (w/v) dried milk powder, 25 mM Hepes, pH 8.0, 1 mM DTT, 10% glycerol, 50 mM NaCl, 0.05% Nonidet P-40, and 1 mM EDTA). Hybridization was performed in TNE-50 buffer containing radiolabeled oligo 1 (2 pM) for 3 h at 4°C. Poly(dI-dC) (5 μg/ml) was added as a nonspecific competitor. For the competition assays, 100 pM (50 × molar excess) of oligo 1 or CRE was added to the hybridization solution. The blot was washed three times with TNE-50 for 20 min and subjected to autoradiography.

Ionic Strength, Urea, and Temperature Sensitivity Assays

To study the effects of ionic strength or urea on DNA-protein complex formation, various amounts of NaCl or urea were added to the binding reaction mixtures. The binding reaction mixtures contained 3 μg of nuclear extract, radiolabeled oligo 1 (30,000 cpm), 2 μg of poly (dI-dC), and binding buffer. After binding reaction, the samples were analyzed by EMSA.

For the temperature sensitivity assay, aliquots of nuclear extract were incubated at the desired temperature for 5 min and placed on ice. The heat-treated nuclear extracts were used for binding reactions. Each binding reaction mixture contained 3 μg of nuclear extract, radiolabeled oligo 1 (30,000 cpm), 2 μg of poly (dI-dC), and binding buffer. After binding reaction, they were assayed by EMSA.

Treatment With Chelators

The divalent cation chelators used in EMSA were prepared as previously described [Hooft van Huijsduijnen et al., 1987]. Various amounts of the divalent cation chelators were added to the binding mixtures. The binding reaction mixtures contained 3 μg of nuclear extract, radiolabeled oligo 1 (30,000 cpm), 2 μg of poly(dI-dC), and binding buffer in volume of 20 μl. After 20 min of binding reaction, the mixtures were analyzed by EMSA.

Protease Clipping Assay

Protease clipping assay was performed as described previously [Lee et al., 2000]. The binding reactions were carried out at room temperature for 20 min before treatment of protease. The binding reaction mixtures contained 3 μg of nuclear extract, radiolabeled oligo 1 (30,000 cpm), 2 μg of poly(dI-dC), and binding buffer. Various amounts of proteinase K or trypsin were added to the binding reaction mixtures and the samples were incubated at room temperature for further 10 min. After the

protease reactions, the samples were immediately applied to polyacrylamide gel and analyzed.

RESULTS

Nuclear Factors Bound to AT-Rich Region Sequence-Specifically

We previously confirmed that the p53 gene was expressed in rat liver by northern blotting analysis [Lee et al., 1998]. Consequently, nuclear extract from rat liver was used to investigate the transcription factor-binding motifs of the rat p53 promoter. To identify the protein-binding element in the rat p53 promoter, DNase I footprinting assay was carried out with end-labeled probe. As a result, the region from position −504 to −516 was protected from DNase I (Fig. 1, lanes 1 and 2). This region has high adenine and thymine contents and partial palindromic sequence, CTGTTTCAAAAAG. In addition, at the center of the protected region, hypersensitive bases to DNase I were detected. The hypersensitive region usually appears at the boundary of a protein-binding site. Therefore, it is likely that two proteins bind to the protected region. To prove that nuclear factors bind to the element in sequence-specific manner, EMSA oligonucleotide competition assay was carried out with radiolabeled oligo 1 containing the specific binding sequence. NF1 consensus and CRE oligonucleotides were used as unrelated competitors. In Figure 2, two protein-DNA complexes were identified. This also suggests that more than two proteins are involved in the protein–DNA complexes on this AT-rich element. Complex II was much stronger than complex I. The protein–DNA complexes were decreased by the addition of $10 \times$ or $100 \times$ molar excess of self competitor (Fig. 2, lanes 2 and 3), but even $100 \times$ molar excess of NF1 consensus or CRE oligonucleotide did not show this effect (Fig. 2, lanes 4–7). Therefore, nuclear proteins bound to the AT-rich element in a sequence-specific manner.

Molecular Masses of the Binding Proteins

To determine molecular masses of the binding proteins, southwestern blotting analysis was carried out with the radiolabeled oligo 1. As a result, three bands were identified whose molecular masses were 91 kDa, 60 kDa, and 40 kDa, respectively (Fig. 3, lane 1). To identify sequence-specific binding proteins, oligonucleo-
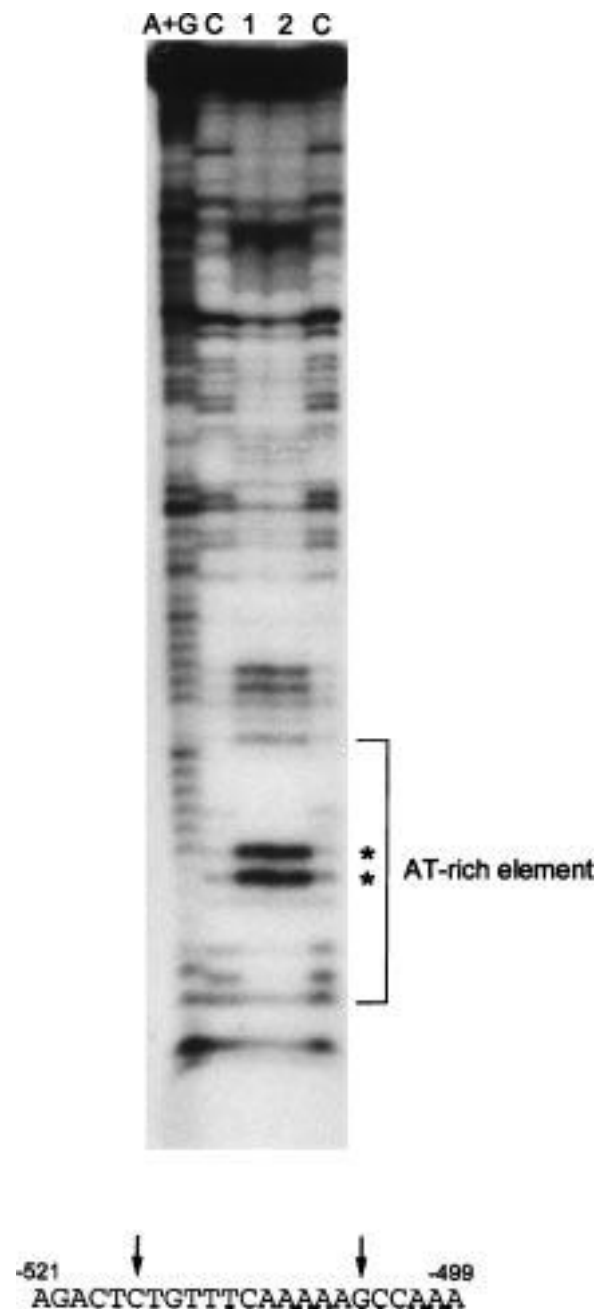

Fig. 1. DNase I footprinting assay. The ^{32}P-labeled coding strand DNA of p53 promoter fragment, containing −91 and −537 region, was assayed for digestion by DNase I in the absence (lane C) or presence of the nuclear proteins (lanes 1 and 2). The lane indicated A+G is the free probe digested at adenine and guanine residue. The sequence around the protected region was shown below the autoradiogram. The boundaries of the protected region were indicated by arrows. The hypersensitive bases to DNase I were indicated by asterisks.

tide competition assays were performed. CRE oligonucleotide was used as an unrelated competitor. As a result, the three bands were abolished by the addition of 100 pM of self
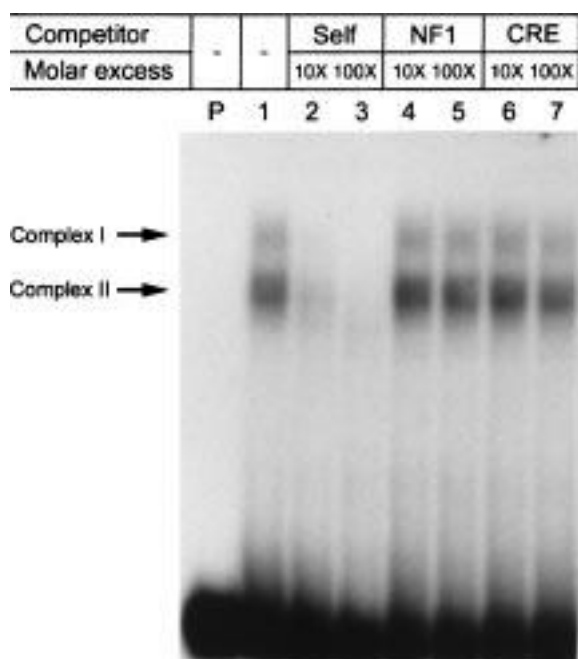
Fig. 2. EMSA oligonucleotide competition assay. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1 without competitor (lane 1) or in the presence of the competitor: oligo 1 (Lanes 2 and 3); NFI consensus (lanes 4 and 5); CRE (lanes 6 and 7). P indicates EMSA without nuclear extract.

Fig. 3. Southwestern blotting analysis. Nuclear extracts measuring 50 µg were separated by electrophoresis, transferred to the PVDF membrane, and assayed by incubation with the radiolabeled oligo 1 without competitor (lane 1) or in the presence of the competitor: 100 pM oligo 1 (Lane 2); 100 pM CRE (lane 3).

competitor (Fig. 3, lane 2), but 100 pM of CRE oligonucleotide reduced only the band of 91 kDa (Fig. 3, lane 3). Therefore, the 91 kDa protein was a nonspecific binding protein; and the 60 and 40 kDa proteins bound to the AT-rich motif in a sequence-specific manner.

Effects of Urea, NaCl, and Heat

To determine the sensitivity of the binding proteins to ionic strength, the indicated quantity of NaCl was added to the binding reaction mixtures (Fig. 4A). The protein–DNA complexes were highly stable at high concentrations of NaCl. Although the level of the protein–DNA complex was decreased significantly in the presence of low concentrations, the residual protein–DNA complexes still remained at 0.8 M NaCl (Fig. 4A, lane 11). At 1.0 M NaCl concentration, the complexes were completely abolished (Fig. 4A, lane 13). The sensitivity of the binding proteins to denaturant was determined by EMSA with increasing concentrations of urea (Fig. 4B). This assay showed that the binding proteins were highly resistant to denaturant. At even 3.0 M of urea,
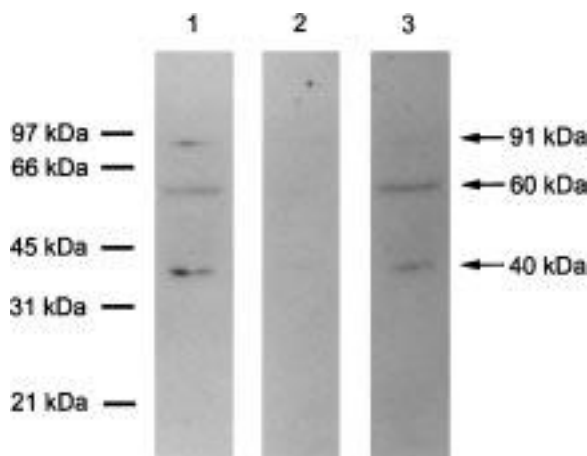
the DNA–protein complexes were still formed. (Fig. 4B, lane 11). To determine the heat sensitivities of the binding proteins, aliquots of the nuclear extracts were heated at the indicated temperature for 5 min, before assaying for DNA-binding activity of the protein (Fig. 4C). The DNA–protein complexes were stable at high temperature. The levels of the protein–DNA complexes were not decreased up to 60°C. At 65°C, the complexes disappeared. These results showed that the binding proteins were stable to denaturants or ionic strength.

Effects of Chelators or Proteases

Since many transcription factors require the presence of metal ions for maximum DNA binding activity, various metal ion chelators such as EDTA, 8-hydroxyquinoline, EGTA, and o-phenanthroline were added to the binding reactions as indicated in Figure 5. Each chelator shows different specificity for metal ion. For example, EGTA has high affinity to Ca^{2+} and o-phenanthroline has been used to demonstrate the presence of Zn^{2+} in the catalytic center of Zn^{2+} metallo-enzymes [Posorske et al., 1979; Hooft van Huijsduijnen et al., 1987; Collins et al., 1998]. However, the chelators did not have an influence on the level of the protein–DNA complexes. It suggests that the binding protein does not require divalent cation for maximum DNA-binding activity.
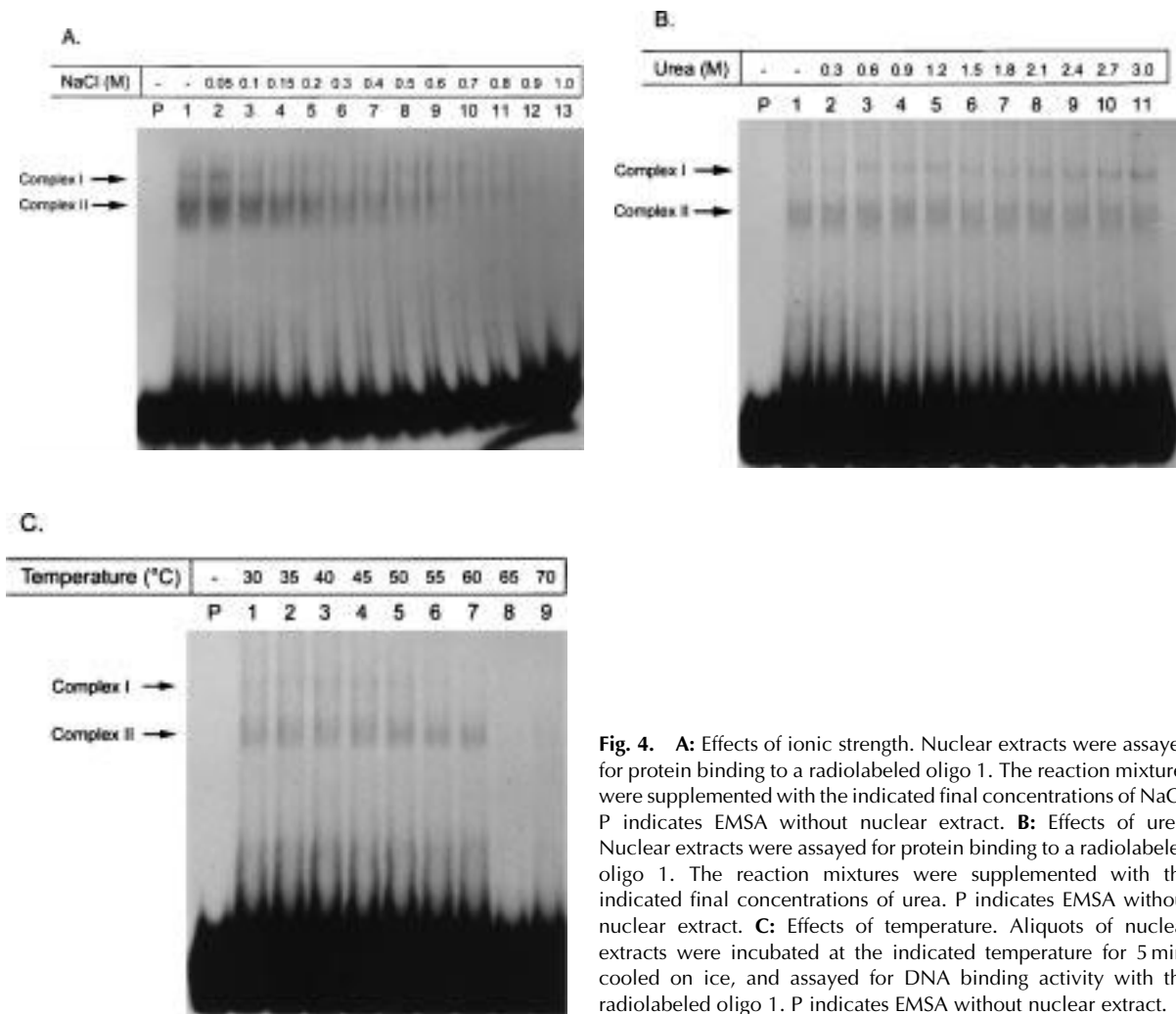
A.

NaCl (M)	-	-	0.05	0.1	0.15	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
	P	1	2	3	4	5	6	7	8	9	10	11	12	13

Complex I →
Complex II →

B.

Urea (M)	-	-	0.3	0.6	0.9	1.2	1.5	1.8	2.1	2.4	2.7	3.0
	P	1	2	3	4	5	6	7	8	9	10	11

Complex I →
Complex II →

C.

Temperature (°C)	-	30	35	40	45	50	55	60	65	70
	P	1	2	3	4	5	6	7	8	9

Complex I →
Complex II →

Fig. 4. **A:** Effects of ionic strength. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1. The reaction mixtures were supplemented with the indicated final concentrations of NaCl. P indicates EMSA without nuclear extract. **B:** Effects of urea. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1. The reaction mixtures were supplemented with the indicated final concentrations of urea. P indicates EMSA without nuclear extract. **C:** Effects of temperature. Aliquots of nuclear extracts were incubated at the indicated temperature for 5 min, cooled on ice, and assayed for DNA binding activity with the radiolabeled oligo 1. P indicates EMSA without nuclear extract.

To determine whether the binding protein has a protease-resistant DNA-binding core domain, protease such as trypsin or proteinase K was added to the binding mixtures (Fig. 6A and 6B). However, any faster migrating protein–DNA complex than complexes I and II was not detected in this assay. Instead, the intensities of the protein–DNA complexes were decreased and eliminated with increasing amount of proteinase K or trypsin (Fig. 6A or 6B). Therefore, these results suggest that the proteins do not have structurally distinct DNA-binding domain and that the proteins are unstable to protease treatment.

DISCUSSION

In this study, we analyzed the rat p53 promoter by DNase I footprinting assay. As a result, we found an AT-rich element located at positions of −504 to −516 region. This region was previously identified as a positive regulatory element in the murine p53 promoter and designated as PBF1 binding motif [Roy and Reisman, 1996]. The sequence of the element is conserved between the rat and mouse p53 promoters, but not in the human p53 promoter. Previously, we found an NF1-like element located at positions of −296 to −312 region [Lee et al., 1998]. This NF1-like element was rat-specific and did not exist in the p53 promoters of other species. Like this NF1-like element, the AT-rich element may be a species-specific element. The AT-rich element had high adenine and thymine contents and did not have remarkable homology to any known transcription factor-binding site. The AT-rich element was known as a positive cis-regulatory element, but the binding proteins to this element have not been identified yet. Therefore, we
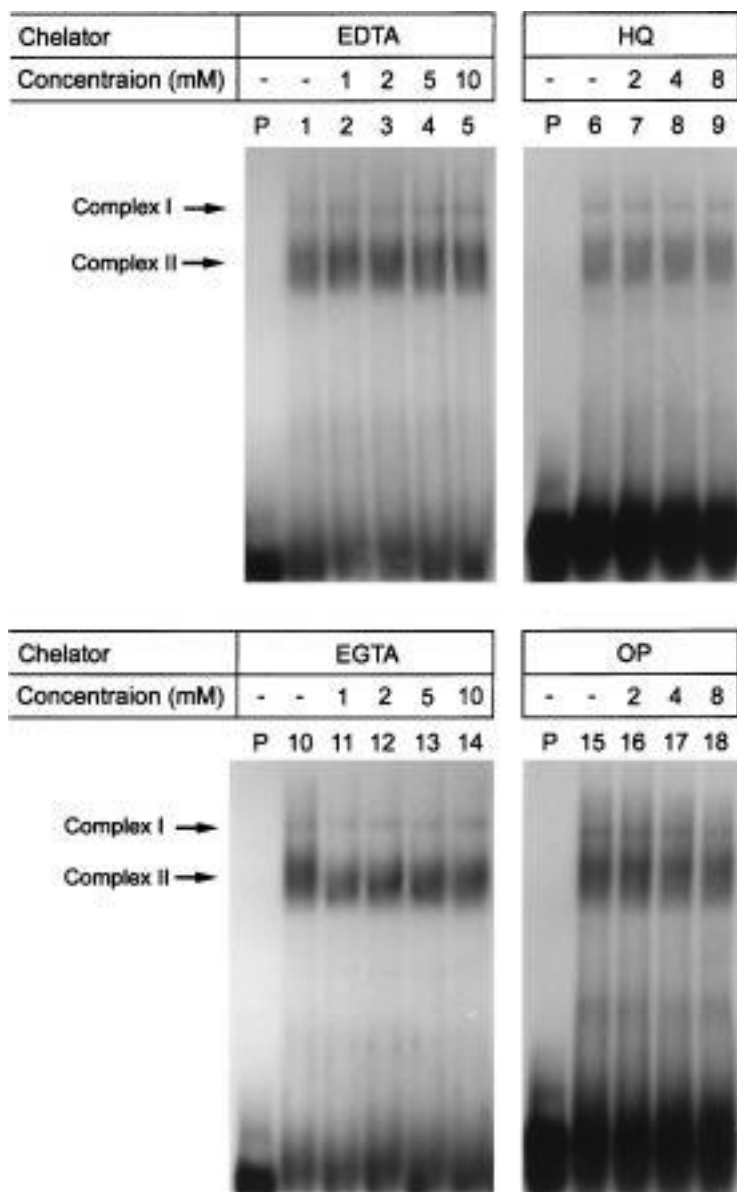
Fig. 5. The effects of chelators on DNA-protein complex. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1 without chelator or in the presence of chelator. EDTA (Lanes 1–5); 8-hydroxyquinoline (HQ) (lanes 6–9); EGTA (lanes 10–14); ortho-phenanthroline (OP) (lanes 15–18). P indicates EMSA without nuclear extract.

characterized the protein using various biochemical methods. First, we confirmed that by EMSA oligonucleotide competition assay, nuclear factors bound, to the AT-rich in element, in a sequence-specific manner. In Figure 2, we identified two protein–DNA complexes, suggesting that more than two proteins bind to the AT-rich element. Actually, we identified two sequence-specific binding proteins that had different molecular masses, 60 and 40 kDa respectively in southwestern blotting analysis (Fig. 3). These two complexes were decreased by the addition of self-competitor, not by other unrelated competitors. This result showed that 60 kDa and 40 kDa of nuclear factors

bind to AT-rich element in a sequence-specific manner.

To determine the sensitivities of the binding proteins to ionic strength or denaturant, the binding reactions were performed in the presence of NaCl or urea. The binding proteins were stable to denaturants or ionic strength. There is no significant decrease of DNA-binding activity of the protein in the presence of even 3.0 urea (Fig. 4B, lane 11). 0.8 M NaCl did not abolish the complex formation completely (Fig. 4A, lane 11). In addition, DNA-binding activities of the proteins are stable at high temperature (Fig. 4C). These results suggest that the binding proteins have stable
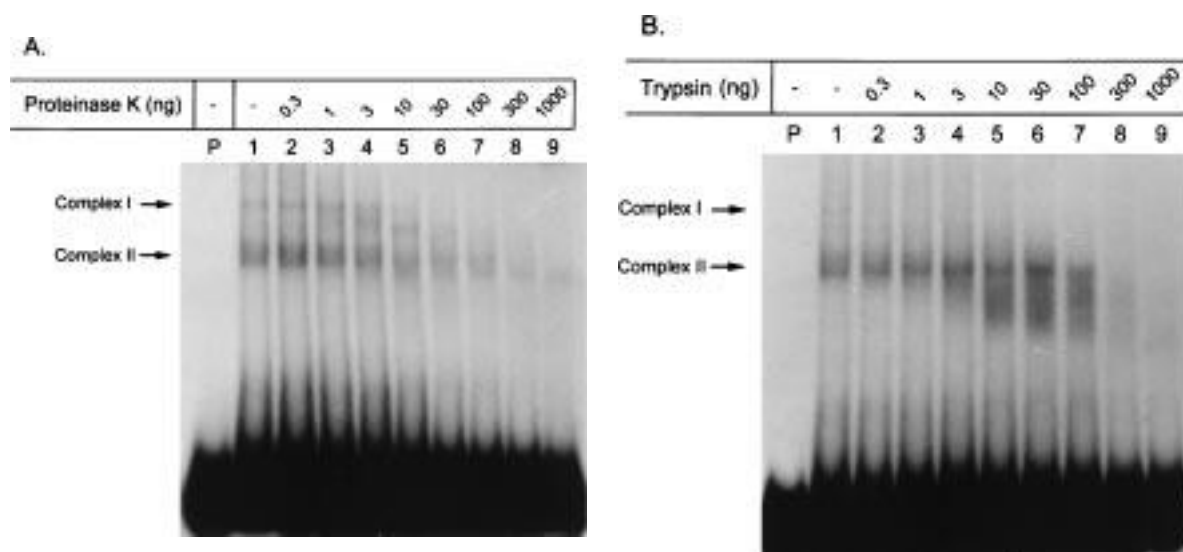
Fig. 6. Protease clipping assay. Reactions were set up as usual with 3 µg of nuclear extracts and the radiolabeled oligo 1. After binding reaction at room temperature for 20 min, proteinase K (**A**) or trypsin (**B**) was added. After further 10 min reaction, the samples were analyzed by EMSA.

structure and high affinity to their target DNA sequence.

In southwestern blotting analysis, we identified two proteins that bound to AT-rich element in a sequence-specific manner. It is unclear how these two binding proteins are related to each other. The two proteins may be two distinct transcription factors that bind to the AT-rich element or they may contain a common DNA-binding domain. One example of the former is NF1/YY1 in the transcription of the human p53 gene [Furlong et al., 1996]. While YY1 binds to the NF1/YY1 motif and regulates the transcription of the p53 gene in spleen and testis, NF1 replaces the role of YY1 in liver and kidney. The two complexes in this study showed almost same sensitivities to urea, ionic strength, or temperature (Fig. 4). Therefore, it is likely that the two proteins contain a common DNA-binding domain.

To identify the effect of divalent cation to DNA-binding activity, various chelators were added to DNA-binding mixtures. However, any change in DNA-binding activity was not detected by chelator treatment (Fig. 5). Therefore, the binding proteins do not require divalent cation for maximum DNA-binding activity. In protease clipping assay, we could not identify proteolyzed peptide–DNA complexes. Instead, the levels of the complexes were decreased with increasing amount of proteases. Treatment of trypsin showed that protein–DNA complexes were degraded to a broad range band below complex II at the intermediate concentrations (Fig. 6B, lanes 5–7). Trypsin is serine protease of narrower specificity than proteinase K. Therefore, digestion of the binding proteins with trypsin at intermediate concentration may make the partial digested products. However, the partial digested products were also completely abolished in the presence of 0.3 µg of trypsin or higher (Fig. 6B, lanes 8 and 9). Therefore, it is likely that the intactness of the binding protein should be conserved for maximum DNA-binding activity.

In summary, two nuclear factors, whose molecular masses are 60 and 40 kDa respectively, bind to the AT-rich element and may be involved in the transcriptional regulation of the rat p53 gene. These two proteins have unusual stability to denaturants and ionic strength. Divalent cation is not required and the intactness of the protein should be conserved for DNA-binding activity. These physico-chemical properties of the binding proteins provided new insights into the proteins and should be useful to identify the proteins.

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