Journal of Cellular Biochemistry 78:1–7 (2000)

ARTICLES

Biochemical Characterization of a Nuclear Factor That Binds to NF1-like Elements in the Rat p53 Promoter

Minhyung Lee, Sunhee Yu, and Jong-sang Park*

Department of Chemistry, Seoul National University, Seoul 151-742, Korea

Abstract We previously reported that two nuclear factor 1-like elements mediated the transcription of the rat p53 gene. A 40-kDa protein was shown to bind to these elements, which was different from common NF1 family proteins. In this study, the biochemical properties of the 40-kDa binding protein were investigated. The metal ion dependency of the protein was examined with various chelators; the protein was proved to require Mg^{2+} for maximum DNA-binding activity. The binding protein was highly resistant to ionic strength and denaturant. The protein-DNA complex was reduced at high NaCl concentration, but residual DNA-binding activity remained. Even 2 M urea did not completely eliminate the formation of protein-DNA complex. DNA-binding activity of the protein was also stable at high temperature. Treatment of the protein-DNA complex with increasing concentrations of proteinase K or trypsin demonstrated the existence of a protease-resistant DNA-bound core. These biochemical properties provide new insight into the 40-kDa NF1-like nuclear factor. J. Cell. Biochem. 78:1–7, 2000.

Key words: 40-kDa protein; biochemical characterization; NF1-like element; p53 tumor suppressor; transcription regulation

p53 is a phosphonuclear protein that regulates cell cycle progression. Under certain stress conditions, it preserves genomic integrity by cell cycle arrest, or apoptosis [Levine, 1997]. It has a very strong transcriptional activation domain near its N-terminus [Fields and Jang, 1990; Raycroft et al., 1990]. In response to DNA damage, p53 transiently accumulates and induces the transcription of several genes involved in apoptosis, or cell cycle arrest. These genes include WAF1 [El-Deiry et al., 1993], MDM2 [Barak et al., 1993], muscle creatine kinase [Weintraub et al., 1991; Tamir and Bengal, 1998], and SFN [Hermeking et al., 1997].

To define the elements involved in the transcription regulation of the p53 gene, the regulatory region of the p53 gene has been analyzed. Unlike many genes transcribed by RNA polymerase II, the p53 promoter lacks a remarkable TATA or CAAT box [Bienz-Tadmor et al., 1985; Lamb and Crawford, 1986]. Instead, several potential transcription factors binding sites have been suggested. These transcription factors include nuclear factor-κB (NF-κB) [Wu and Lozano, 1994; Kirch et al., 1999], upstream stimulating factor (USF) [Hale and Braithwaite, 1995], Myc/Max [Roy et al., 1994], nuclear factor 1 (NF1) [Ginsberg et al., 1990; Furlong et al., 1996; Lee et al., 1998b], ying yang 1 (YY1) [Furlong et al., 1996], activator protein 1 (AP1) [Kirch et al., 1999], PF1 [Ginsberg et al., 1990], and PF2 [Hale and Braithwaite, 1996]. These transcription factors may be involved in the p53 transcription tissue-specifically or species-specifically. For instance, NF-κB motif is well conserved across

Abbreviations used: AP1, activator protein 1; DTT, 1,4-dithiothreitol; EGTA, (ethylene-dioxy)diethyl-enedinitrilotetraacetic-acid; Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; NF1, nuclear factor 1; NF-κB, nuclear factor-κB; PF1, p53 factor 1; PF2, p53 factor 2; PMSF, phenylmethanesulfonyl fluoride; USF, upstream stimulating factor; YY1, ying yang 1.

Grant sponsor: Center for Molecular Catalysis; Grant sponsor: Korean Science and Engineering Foundation; Grant sponsor: Genetic Engineering Fund of Korea Research Foundation; Grant sponsor: Ministry of Education in Korea.

Minhyung Lee is currently at the Center for Controlled Chemical Delivery, University of Utah, Salt Lake City, UT 84112.

*Correspondence to: Jong-sang Park, Department of Chemistry, Seoul National University, San 56-1, Shilim-dong, Kwanak-gu, Seoul 151-742, Korea. E-mail: pfjspark@plaza.snu.ac.kr

Received 29 July 1999; Accepted 16 November 1999

This article published online in Wiley InterScience, April 2000.

the species, but the protein binding to the motif was identified only in the human p53 promoter, and not in mouse and rat p53 promoters. AP1 plays a critical role in human p53 transcription [Kirch et al., 1999], but another nuclear factor, not AP1, binds to this motif in the induction of the mouse p53 gene by phorbol ester or in the induction of the rat p53 gene after partial hepatectomy [Ginsberg et al., 1990; Lee et al., 1998a]. In addition, in the human p53 promoter, NF1 or YY1 binds to the NF1 recognition element and regulates the expression of the p53 gene in a tissue-specific manner [Furlong et al., 1996]. However, our recent results showed that in the rat p53 promoter, an NF1-like protein bound to the motif irrespective of tissue types [Lee et al., 1999].

Studies on the regulation of the rat p53 gene showed that another NF1 recognition element was located at −298 to −312 in the rat p53 promoter [Lee et al., 1998b]. The two NF1 recognition elements are essential to transcription of the rat p53 gene. The NF1 recognition elements, which were designated NF1-like element 1 (−296 to −312) and NF1-like element 2 (−195 to −219), have the sequence of TGGC (Fig. 1). This sequence is inverse-repeated in NF1-like element 2, which is well conserved across the species. However, in NF1-like element 1, the sequence is not inverse-repeated and exists only in the rat p53 promoter. In a previous report, the protein binding to the two NF1-like elements of the rat p53 promoter had a 40-kDa molecular mass, which was not compatible with those of common NF1 family proteins (52–66 kDa) or NF1-L (liver, 31 kDa). In addition, the binding protein to the NF1-like elements was not recognized by anti-NF1 antibody [Lee et al., 1998b]. These results suggested that the binding protein might not be a common type of NF1 family protein. Therefore, in this study we characterized the binding protein to the NF1-like elements by various biochemical methods. This study has provided new insights of the 40-kDa NF1-like protein and should be useful in attempts to verify the identity of purified preparations of the 40-kDa NF1-like protein.

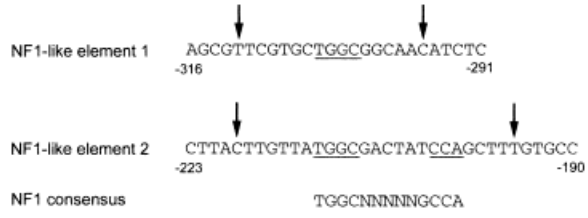

Fig. 1. Sequence around the two NF1-like elements in the rat p53 promoter. Sequences around the two NF1-like elements are presented; the NF1 consensus sequence is underlined. Arrows, protection boundaries in DNase I footprinting assay [Lee et al., 1998b].

MATERIALS AND METHODS

Animals and Preparation of Nuclear Extract

Female Wistar rats (Animal Breeding Center in Seoul National University) weighing 120–140 g were used for all experiments. Nuclear extracts were prepared by the method described previously [Lee et al., 1997]. Briefly, rat liver was homogenized in 4 vol of buffer A (10 mM Tris, pH 7.9, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 1 mM 1,4-dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) 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, 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 being centrifuged 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.

Electrophoretic Mobility Shift Assay (EMSA)

The synthetic oligonucleotides containing NF1-like element 2 (oligo 1: 5'-TTGTTATGGCGACTATCCAGCTTTG-3',5'-CAAAGCTGGATAGTCGCCATAACAA-3') were annealed and labeled 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 (5 ×: 60 mM Hepes, pH 7.9, 300 mM KCl, 25 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 60% glycerol) in 20 μl vol. After the addition of end-labeled oligonucleotides (30,000 cpm), the mixtures were incubated at room temperature for 20 min. They were then electrophoresed through a 6% poly-

acrylamide gel in the presence of 0.25 × Tris-borate-EDTA. The gel was dried and subjected to autoradiography.

Treatment With Chelators

The divalent cation chelators used in EMSA were prepared as previously described [Hooft van Huijsduijnen et al., 1987]. The divalent cation chelators were added to the binding reaction mixtures as indicated in Figure 2. After 20 min of binding reaction, the mixtures were analyzed by EMSA. To identify the required divalent cation for protein binding, various divalent cations (Mg^{2+}, Zn^{2+}, Ca^{2+}, and Cu^{2+}) were added to the reaction mixtures in the presence of 8 mM EDTA. After a 20-min binding reaction, the samples were analyzed by EMSA.

Ionic Strength, Urea, and Temperature-Sensitivity Assays

To study the effect of ionic strength or urea on DNA-protein complex formation, NaCl or urea was added to the final concentration indicated in Figure 4A and 4B, respectively. After binding reaction, the samples were analyzed by EMSA.

For the temperature sensitivity assay, aliquots of nuclear extract were incubated at the desired temperature as indicated in Figure 5 for 5 min and placed on ice. After binding reaction, they were assayed by EMSA.

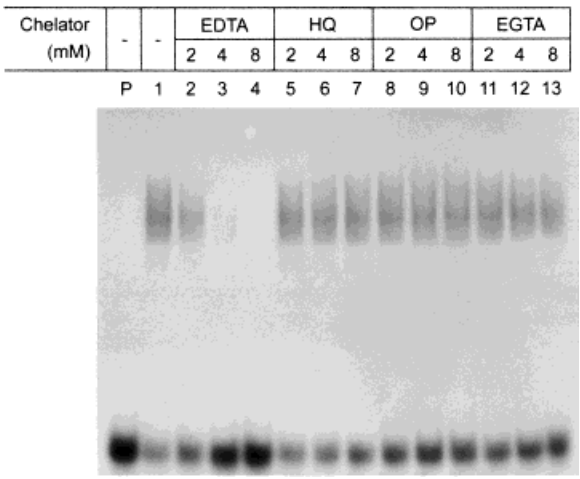

Fig. 2. Effects of chelators on DNA-protein complex. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1 without chelator (**lane 1**) or in the presence of chelator. **Lanes 2–4**, EDTA; **lanes 5–7**, 8-hydroxyquinoline (HQ); **lanes 8–10**, orthophenanthroline (OP); **lanes 11–13**, EGTA. P, EMSA without nuclear extract.

Protease Clipping Assay

The binding reactions were carried out at room temperature for 20 min, followed by protease treatment. The indicated quantity of proteinase K or trypsin (see Fig. 6A,B) was added to the binding reaction mixture, and the reaction proceeded for a further 10 min for protease reaction. After the reaction, the samples were immediately applied to polyacrylamide gel and analyzed. For the competition assay, 1 pmole (10 × molar excess) or 10 pmole (100 × molar excess) of oligonucleotide competitors (oligo 1, NF1 consensus (5'-TTTGGCACGGAGCCAAC-3', 5'-GTTGGCTCCGTGCCAAA-3'), YY1 consensus (5'-AGCGGCCATCTTGGCTG-3', 5'-CAGCCAAGATGGCCGCT-3'), or CRE (5'-TGCTGACGTCAAC-3',5'-GTTTGACGTCAGCA-3')) were added to the reaction mixtures after protease treatment. The mixtures were incubated at room temperature for 10 min and immediately applied to polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography.

RESULTS

Mg^{2+} Is Required for Protein Binding to NF1-like Elements

EMSA with radiolabeled oligo 1 showed that the DNA-protein complex was formed on an NF1-like element 2 (Fig. 2, lane 1). Since many transcription factors require the presence of metal ions for maximum DNA binding activity, various metal ion chelators, such as EDTA, 8-hydroxyquinoline, ortho-phenanthroline, and EGTA, were added to the binding reaction mixtures, as indicated in Figure 2. Of the chelators, only EDTA reduced the formation of the protein-DNA complex (Fig. 2, lanes 2–4). At 4 mM EDTA, the complex was definitely decreased and completely abolished at 8 mM EDTA. However, other chelators did not show this effect. This result demonstrated that the binding protein requires divalent cations for maximum binding to its target sequence. To determine the divalent cation required for protein binding, the ability of Mg^{2+}, Zn^{2+}, Ca^{2+}, or Cu^{2+} to restore the complex formation was examined in the presence of 8 mM EDTA (Fig. 3A,B). As a result, Mg^{2+} restored the formation of the protein-DNA complex, suggesting that

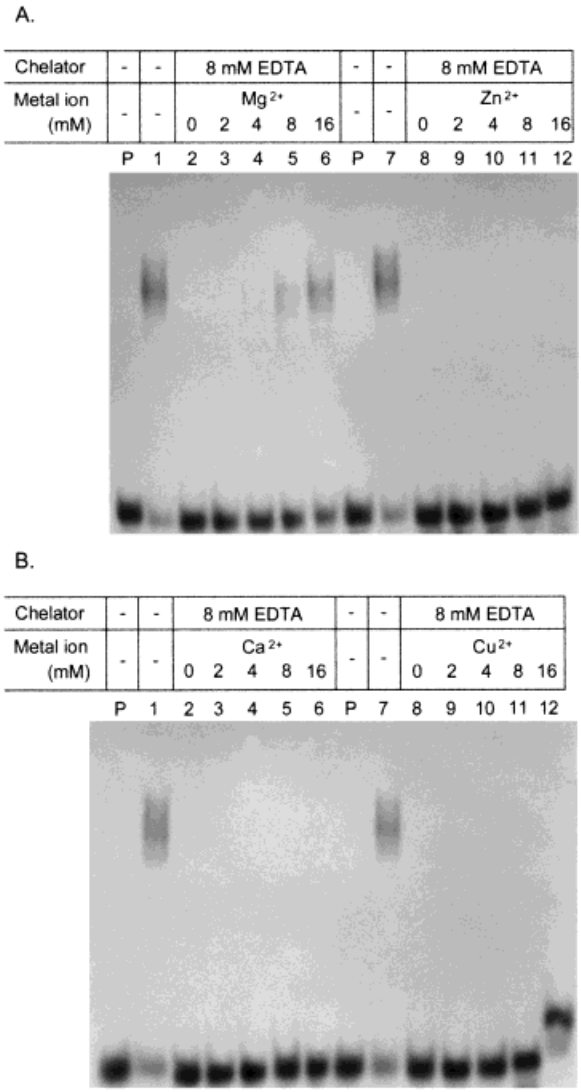

Fig. 3. Mg^{2+} requirement for DNA-protein complex formation. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1 without (**lanes 1,7**) or in the presence of 8 mM EDTA (**lanes 2–6,8–12**). The reaction mixtures were supplemented with the indicated final concentrations of divalent cations. **A: Lanes 2–6**, Mg^{2+}; **lanes 8–12**, Zn^{2+}. **B: Lanes 2–6**, Ca^{2+}; **lanes 8–12**, Cu^{2+}.

the binding protein requires Mg^{2+} (Fig. 3A, lanes 2–6). Other divalent cations were unable to restore the formation of the complex.

Sensitivity of the Binding Protein to Ionic Strength and Denaturants

The sensitivity of the binding protein to ionic strength or denaturant was determined by EMSA. NaCl was added to the final concentration indicated in Figure 4A. Although there was a significant decrease in the protein-DNA complex formation with increasing concentrations of NaCl, residual protein-DNA complex still remained at 0.7 M NaCl (Fig. 4A). An increasing concentration of urea also reduced the protein-DNA complex formation, but most of the protein-DNA complex remained at 2.0 M urea (Fig. 4B). These results showed that the binding protein to NF1-like elements was highly resistant to ionic strength and denaturant.

To determine heat stability of the binding protein, small aliquots of undiluted crude extract were heated at the indicated temperature

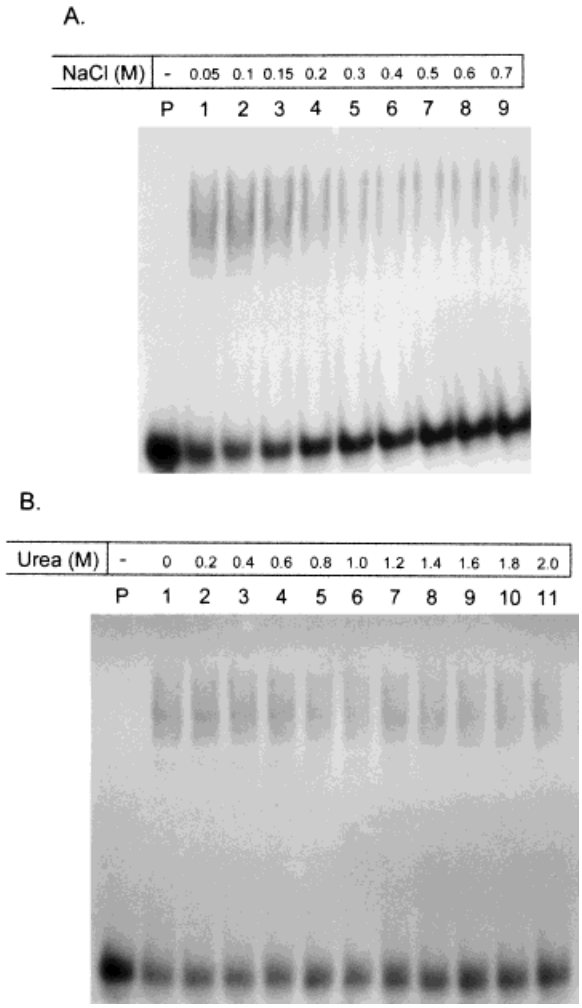

Fig. 4. **A:** Effects of ionic strength. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1. The reaction mixtures were supplemented with the indicated final concentrations of NaCl. P, EMSA without nuclear extract. **B:** Effects of urea. Nuclear extracts were assayed for protein binding to a radiolabeled oligo 1. The reaction mixtures were supplemented with the indicated final concentrations of urea. P, EMSA without nuclear extract.

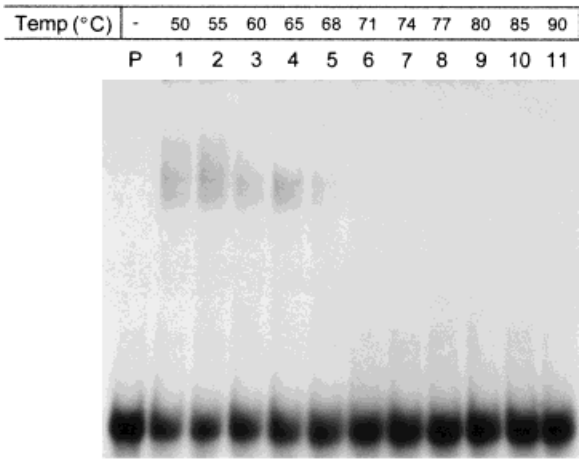

Fig. 5. Effects of temperature. Aliquots of nuclear extracts were incubated at the indicated temperature for 5 min, cooled on ice and assayed for DNA binding activity with the radiolabeled oligo 1. P, EMSA without nuclear extract.

(Fig. 5) for 5 min. The heat-treated extracts were used in a standard binding reaction. The formation of protein-DNA complexes was not decreased up to 65°C. However, at 71°C, the protein-DNA complex was completely abolished (Fig. 5). The abruptness of the active/inactive transition is quite striking.

Protease Clipping Assay

To prove the existence of the core DNA binding domain of the binding protein, protease clipping assay was carried out with proteinase K or trypsin (Fig. 6A,B). Thus, the DNA-protein complex was reduced to a faster-migrating band. The limit digest was remarkably stable, persisting over a wide range of proteinase K or trypsin concentrations. Trypsin or proteinase K treatment shows an almost same migrated pattern. However, unlike proteinase K treatment, treatment of trypsin made more migrating band, as the amount of trypsin was increased (Fig. 6B). While proteinase K is a nonspecific endopeptidase, trypsin is serine protease of narrow specificity. Therefore, digestion of the binding protein with trypsin at an intermediate concentration may make the partial digestion products.

NF1-like element 2 was also reported as a YY1 binding motif in the human p53 promoter [Furlong et al., 1996]. In addition, NF1-like elements 1 and 2 contain the regions that have partial homology to CRE (NF1-like element 1: TGGCGGCA, NF1-like element 2: TGGCGACT, CRE: TGACGTCA). Therefore, there was a possibility that the proteolyzed protein-DNA complex was derived from another protein, not the NF1-like protein. To prove that the peptide was derived from the NF1-like protein, an oligonucleotide competition assay was carried out after protease treatment (Fig. 6C,D). The oligonucleotides containing YY1, NF1, or CRE consensus motifs were used as competitors. Figure 6C,D shows that the proteolyzed protein-DNA complex was decreased by the addition of oligo 1 and NF1 consensus oligonucleotide (Fig. 6C, lanes 3, 4, 7, 8; Fig. 6D, lanes 3, 4, 7, 8). YY1 and CRE consensus oligonucleotide did not decrease the formation of the protein-DNA complex. This suggests that the reduced binding peptide was derived from the NF1-like protein.

DISCUSSION

NF1-like element in the rat p53 promoter has different properties from that of the human or mouse p53 promoter. First, unlike the human or mouse p53 promoter, the rat p53 promoter has two NF1-like elements [Lee et al., 1998b]. NF1-like element 1 has imperfect NF1 recognition motif, while NF1-like element 2 has inverted repeat sequence for full recognition of NF1. Second, NF1-like elements in the rat p53 promoter did not show tissue-specificity for the binding protein. NF1-like element 2 was reported as a YY1 binding motif in the human p53 promoter [Furlong et al., 1996]. In testis and spleen, YY1 binds to the motif and regulates the transcription, while NF1 replaces the role of YY1 in liver and kidney. However, NF1-like elements in the rat p53 promoter bound to an NF1-like protein irrespective of tissue type [Lee et al., 1999]. Third, the binding protein was partially purified from rat liver and the molecular mass of the protein was determined at 40 kDa. Interestingly, the 40-kDa binding protein was not recognized by the anti-NF1 antibody [Lee et al., 1998b], suggesting that the binding protein was not a common NF1 family protein. These results prompted us to characterize the 40-kDa binding protein.

The most interesting result of this study was that the binding activity of the protein was dependent on Mg^{2+} concentration. DNA-binding activity of the protein was decreased by the addition of EDTA, which can chelate Mg^{2+} effectively (Fig. 2). The increasing level of Mg^{2+} restored the complex formation in the

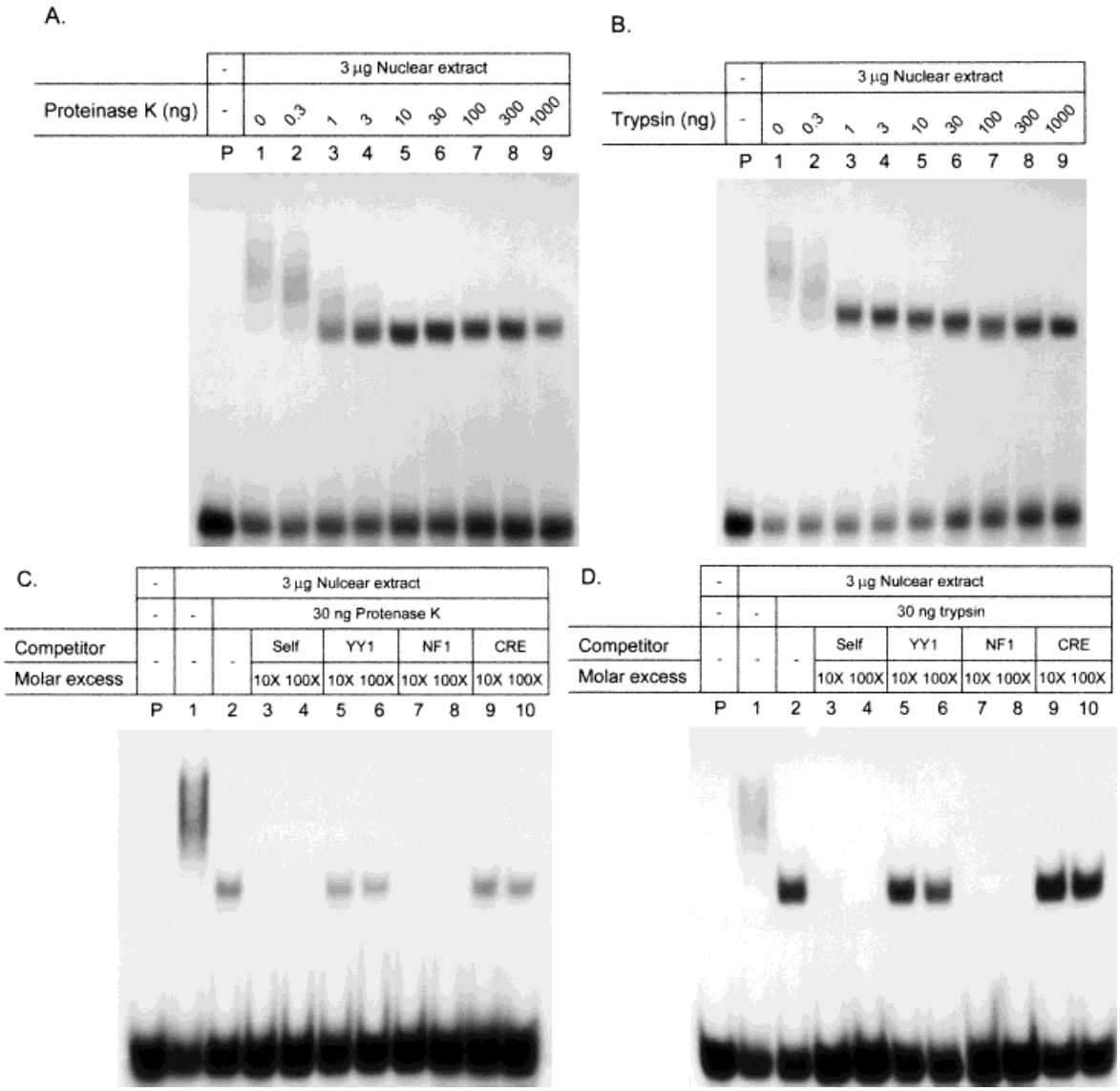

Fig. 6. Protease clipping assay. Reactions were set up as usual with nuclear extracts and the radiolabeled oligo 1. After binding reaction at room temperature for 20 min, proteinase K (**A**) or trypsin (**B**) was added. After further 10 min reaction, the samples were analyzed in EMSA. For competition assay, oligonucleotide competitors were added to the reaction mixtures after proteinase K (**C**) or trypsin (**D**) treatment. **Lanes 3,4**, oligo 1; **lanes 5,6**, YY1 consensus; **lanes 7,8**, NF1 consensus; **lanes 9,10**, CRE. P, EMSA without nuclear extract.

presence of 8 mM EDTA (Fig. 3A). Each chelator shows different specificity for the metal ion. For example, EGTA has high affinity to Ca^{2+} and orthophenanthroline has been used to demonstrate the presence of Zn^{2+} in the catalytic center of Zn^{2+} metalloenzymes. EGTA, 8-hydroxyqunoline, and orthophenanthroline have lower affinity to Mg^{2+} than does EDTA and did not show chelation effect in this study. We tried to determine the optimum concentration of Mg^{2+} by the addition of extra Mg^{2+} to the standard binding reaction without EDTA. However, in this case, the level of complex formation was not changed with increasing concentrations of Mg^{2+} (data not shown). Therefore, the residual level of Mg^{2+} in nuclear extract may be enough for maximum DNA-binding activity of the protein.

The binding protein was remarkably stable to denaturant and ionic strength. Even 2.0 M of urea failed to abolish DNA-binding activity of the protein. In addition, a high concentration of

NaCl failed to eliminate the complex completely. In Figure 5, DNA-protein complex was formed up to 68°C and was comparatively stable at high temperature. The binding protein also has a protease-resistant core polypeptide. The polypeptide was remarkably stable over a wide range of protease. The intact binding protein appeared as the broad range complex containing at least two bands in EMSA, but the bands seem to include the same DNA binding protein. First, the bands in EMSA showed almost the same sensitivity to urea, NaCl, or temperature (Figs. 4A,B, 5). Second, we previously identified one enriched binding protein that had a 40-kDa molecular mass after purification [Lee et al., 1998b]. Third, only the 40-kDa protein was detected in the Southwestern blotting assay [Lee et al., 1998b]. Fourth, in the protease clipping assay (Fig. 6A,B), only one protease-resistant core polypeptide was identified after protease treatment. Therefore, it is likely that the 40-kDa protein may bind to NF1-like elements as diverse multimeric forms with itself or other proteins, which produce the broad range complex. These multimers may be dissociated during the course of the protease treatment, and a DNA-binding core remained after protease treatment.

As described above, the 40-kDa protein, previously identified as the protein binding to the NF1-like elements, has unusual biochemical properties. Mg^{2+} was required for maximum DNA-binding activity of the protein. The protein was highly resistant to ionic strength and denaturants. In addition, the protein had a protease-resistant DNA-binding domain. These biochemical properties provide new insights of the 40 kDa binding protein and will be helpful to identify the type of the protein.

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