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PROPERTIES OF A DNA-BINDING PROTEIN FROM RAT NUCLEAR SCAFFOLD FRACTION

Yasuhide Hibino, Shuichi Tsukada and Nobuhiko Sugano*

Cell Biology Division, Faculty of Pharmaceutical Sciences, Toyama Medical & Pharmaceutical University, Toyama 930-01, Japan

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Summary: Our previous work [Hibino et al. (1992) Biochem. Biophys. Res. Commun. 184, 853-858] has shown that a highly repetitive component in rat nuclear DNA forms a sequence-directed bend to have the binding affinity for the nuclear scaffold protein, P130. In the present experiment, the mobility shift DNA-binding assay suggested that the formation of the repetitive component-P130 complex is based on some cooperative mode of interaction. The DNase I footprint analysis revealed that the major binding region of this protein in the DNA is located near the center of the 370-bp *Xmn*I repeat which has a strongly bent overall structure. These results imply that a nuclear scaffold protein such as P130 binds to sequence-directed bend(s) in a highly repetitive DNA to play an important role in construction of a higher-order chromatin structure.

Highly repetitive DNA sequences in various mammalian genomes have been evidenced to be located at centromeres of metaphase chromosomes [1-3] and to be enriched in nuclear matrix/scaffold which may be competent for frameworks of metaphase chromosome and/or interphase nuclei [4-6]. Our recent work has predicted that direct interaction between a highly repetitive bent component (370-bp *Xmn*I fragment) from rat nuclear DNA and the nuclear scaffold protein, P130, results in the formation of a complex which consists of one molecule of the protein and two of the *Xmn*I fragment, alternatively, three molecules of the protein and one of the fragment [7]. Thus, to elucidate such a DNA-protein interaction in more detail, the present study is concerned with the mode of formation of the complex and with the binding sites of P130 in the repetitive component.

MATERIALS AND METHODS

Purification and isolation of nuclear scaffold protein: Rat-liver nuclei were isolated as described previously [7,8]. From the nuclei (400 A_{260}), the scaffold fraction was prepared

* To whom correspondence should be addressed.

Abbreviations: bp, base pairs; PMSF, phenylmethylsulfonyl fluoride; TE, 10 mM Tris-HCl (pH 8.0)/1 mM EDTA; TP, 25 mM Tris-HCl (pH 6.5)/0.1 mM PMSF.

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principally according to the method of Laemmli and his co-workers [7-10]. The fraction was dissolved in 4 ml of 25 mM Tris-HCl (pH 6.5)/5% glycerol/1% 2-mercaptoethanol/1% SDS. The solution was applied onto a column (1.6 x 36 cm) of Sephadex G-200, equilibrated, and eluted with the same buffer. The void fraction was dialyzed against 25 mM Tris-HCl (pH 6.5)/0.1 mM PMSF (TP). The dialyzate was mixed with a gel slurry of CM-Sephadex C-50 (appropriate volume) in TP. The mixture was gently stirred for 30 min and centrifuged. The precipitate was slurried again in TP containing 1.0 M NaCl and gently stirred for 1 hr. The stirred material was centrifuged and the supernatant (1.0 M NaCl-soluble fraction) was dialyzed against TP. The dialyzate was mixed with a gel slurry of DNA-Sepharose 4B (appropriate volume) in TP and treated in the same way. The resulting 1.0 M NaCl-soluble fraction from the gel was dialyzed against 1 mM Tris-HCl (pH 6.5)/1 mM PMSF. The dialyzate was applied onto a column (0.6 x 10 cm) of DNA-Sepharose 4B equilibrated with the same buffer. The elution was performed with the same buffer in a 0-1.0 M NaCl gradient. The protein fraction eluted with about 0.6 M NaCl was dialyzed against 1 mM Tris-HCl (pH 7.0) and lyophilized. The lyophilized material was taken as a DNA-binding protein and designated P130. The detail of the column chromatography has given in our recent report [7]. DNA-Sepharose 4B was prepared in a conventional way with calf thymus DNA (Worthington) which has an AT content of 57%.

Labeling of restriction fragment: A highly repetitive component in rat nuclear DNA was isolated by *Hin*dIII digestion and cloned in pUC9 plasmid [11]. The resulting 370-bp cloned *Hin*dIII fragment was subcloned in pUC19 plasmid [12]. The clone containing a self-ligated tandem dimer of the 370-bp *Hin*dIII fragment was identified through a boiling minilysis procedure [13,14]. The plasmid DNA containing the dimer was isolated in a conventional way [14] and digested with *Xmn*I (NEB) which cleaves only once in the monomer sequence. The digest was extracted with an equal volume of 1 mM Tris-HCl (pH 8.0)/0.1 mM EDTA (0.1 x TE)-saturated phenol/chloroform (1:1, v/v) and further with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The final aqueous phase was subjected to ethanol precipitation. The precipitate was electrophoresed on a slab gel of 1.0% agarose/40 mM Tris-HCl (pH 7.2)/20 mM sodium acetate/1 mM EDTA. The resulting 370-bp *Xmn*I fragments were recovered from the gel with a Prep-A-Gene kit (Bio-Rad) and incubated with [α-^{32}P]dCTP (Bresatec) in a T4 DNA polymerase labeling system (BRL), according to the methods described in instruction manuals from the suppliers. The reaction mixture was extracted with an equal volume of 0.1 x TE-saturated phenol/chloroform (1:1, v/v). To remove unincorporated nucleotides, the aqueous phase was applied onto a column of Sephadex G-50, equilibrated, and eluted with 0.1 M NaCl/10 mM Tris-HCl (pH 8.0)/1 mM EDTA. The void fraction was subjected to ethanol precipitation. The precipitate was taken as the 370-bp [^{32}P]*Xmn*I fragments. In addition, the 370-bp *Xmn*I fragments were digested with *Taq*I (BRL). The resulting 285-bp *Taq*I fragments were recovered and labeled with ^{32}P, in the same way. The labeled DNA was taken as the 285-bp [^{32}P]*Taq*I fragments. The specific activities of these fragments were adjusted to be 2.5 x 10^7 cpm/μg DNA. On the other hand, the 370-bp cloned *Hin*dIII fragments were incubated with an alkaline phosphatase (Takara) at 37°C for 1 hr and extracted with an equal volume of 0.1 x TE-saturated phenol/chloroform (1:1, v/v). The aqueous phase was subjected to ethanol precipitation. The precipitate was incubated with a T4 polynucleotide kinase (Toyobo) and [γ-^{32}P]ATP, at 37°C for 1 hr. The incubated material was applied onto and eluted through a column of Sephadex G-50, as described above. The void fraction was subjected to ethanol precipitation. The precipitate was taken as the 370-bp [5'-^{32}P]*Hin*dIII fragments. All the digestions and incubations were performed according to the methods described in instruction manuals from the suppliers.

Mobility shift DNA-binding assay: The assay was performed according to the method of Singh et al. [15]. P130 was mixed with the 370-bp [^{32}P]*Xmn*I fragments (0.2 ng) in 20 μl of 20 mM potassium phosphate (pH 7.5)/5 mM 2-mercaptoethanol/0.1 mM EDTA/10% glycerol (binding buffer). The doses of P130 were 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 ng. All the mixtures were incubated at room temperature for 30 min. The incubated mixtures were directly electrophoresed on a slab gel of 4% polyacrylamide/7.6 mM Tris-HCl (pH 7.9)/3.3 mM sodium acetate/1 mM EDTA at 4°C for 2.5 hr and subjected to an autoradiographic assay. The autoradiograph was densitometrically assayed with a TLC scanner (Shimadzu CS-910). On the other hand, P130 (150 ng) was mixed with the 370-bp [^{32}P]*Xmn*I or 285-bp [^{32}P]*Taq*I fragments (0.2 ng) in 20 μl of the binding buffer. In addition, P130 (150 ng) was mixed with both the [^{32}P]*Xmn*I and [^{32}P]*Taq*I

fragments (0.2 ng of the total amount), in 20 μl of the binding buffer. The weight ratios of the *Taq*I/*Xmn*I fragments were 0.25, 0.67, 1.5 and 4.0. All the mixtures were incubated at room temperature for 30 min. The incubated mixtures were electrophoresed on a slab gel of 4% polyacrylamide as described above and subjected to an autoradiographic assay.

DNase I footprint analysis: The analysis was performed according to the method of Galas and Schmitz [16]. The 370-bp [5'-^{32}P]*Hin*dIII fragments were digested with *Taq*I and electrophoresed on a slab gel of 1.5% agarose/40 mM Tris-HCl (pH 7.2)/20 mM sodium acetate/1 mM EDTA. The resulting 290-bp [5'-^{32}P]*Taq*I fragments were recovered from the gel with a Mermaid kit (BIO 101) according to the method described in an instruction manual from the supplier. The recovered fragments (0.4 ng) were mixed with P130 (1 or 10 ng) in 40 μl of 12 mM Hepes-KOH (pH 7.9)/60 mM KCl/4 mM $MgCl_2$/1 mM dithiothreitol/1 mM EDTA/0.5 mM PMSF/12% glycerol/polydI•dC (100 μg/ml, Sigma). The mixture was incubated at 4°C for 10 min and then at 20°C for 2 min. To the incubated material, DNase I (3 units/ml, Takara) was added and the mixture was further incubated at 20°C for 1 min. The reaction was terminated by the addition of 100 μl of 0.3 M NaCl/20 mM EDTA (pH 8.0)/0.2% SDS/tRNA (2 μg/ml, BMY). The incubated material was extracted with an equal volume of 0.1 x TE-saturated phenol/chloroform (1:1, v/v) and further with chloroform/isoamyl alcohol (24:1, v/v). The final aqueous phase was subjected to ethanol precipitation. The precipitate was dissolved in 4 μl of formamide/25 mM EDTA (pH 8.0) containing 2.5% xylene cyanol and 2.5% bromophenol blue (24:1, v/v), and heated at 90°C for 3 min. The heated material was electrophoresed on a slab gel of 8% polyacrylamide/8 M urea and subjected to an autoradiographic assay. The sequences protected against DNase I were determined on the basis of the sequence ladder resulting from the A + G reaction [17] and of the sequence of the 370-bp *Hin*dIII fragment [8]. The same analysis was performed with 0.3 or 0.6 μM distamycin A.

Others: The DNA content was estimated as 50 $\mu g/A_{260}$. The protein content was determined according to the method of Lowry et al. [18].

RESULTS AND DISCUSSION

A complex formed between the 370-bp *Xmn*I fragment from rat nuclear DNA and the nuclear scaffold protein, P130, has been shown to have an apparent molecular weight of 600,000 [7]. Such a result has predicted that this complex consists of one molecule of P130 (apparent molecular weight, 120,000) and two of the *Xmn*I fragment (molecular weight, 240,000 x 2), alternatively, three molecules of the protein and one of the fragment [7]. In the present experiment, the mixture of P130 and the 370-bp *Xmn*I fragment was subjected to the mobility shift DNA-binding assay (Fig. 1). With increasing dose of the protein, the mixture exhibited two bands, α and β, in the range of 40-100 ng, whereas band β disappeared in the presence of more than 100 ng (Fig. 1A). The densitometric assay suggested that the formation of the complex corresponding to band α is based on some cooperative mode of interaction (Fig. 1B). The appearance of band β still remains unexplained although the result is reproducible. Moreover, the mixture of P130 and the 370 -bp [^{32}P]*Xmn*I or 285-bp [^{32}P]*Taq*I fragment was subjected to the same assay (Fig. 2). The electrophoretic mobility of the *Xmn*I fragment-P130 complex (band A) was lower than that of the *Taq*I fragment-P130 complex (band C). The mixture of P130, the *Xmn*I and *Taq*I fragments was also assayed in the same way (lanes 3-6). With increasing weight ratio of the *Taq*I/*Xmn*I fragments, the mixture exhibited another band, B, between bands, A and C. Such a result might be explained by supposing that the complex corresponding to band B consists of one molecule of P130 and one each of the *Xmn*I and *Taq*I fragments. Indeed, on the basis of the electrophoretic mobilities of DNA size marker

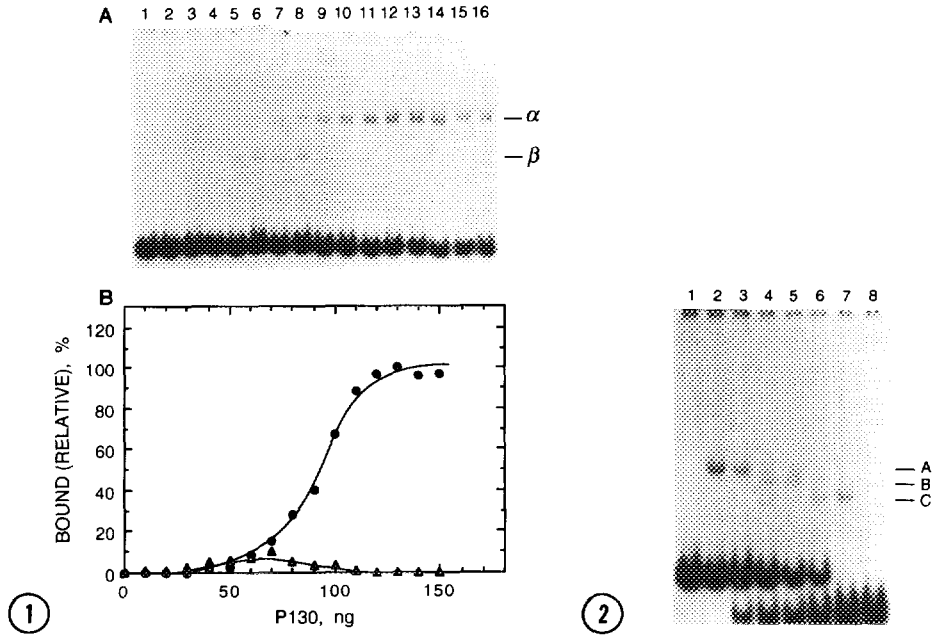

Fig. 1. Mobility shift DNA-binding assay for *Xmn*I fragment-P130 interaction. P130 was incubated with the 370-bp [^{32}P]*Xmn*I fragments (0.2 ng). The doses of P130 were 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 ng, from left to right. The incubated materials were electrophoresed on 4% polyacrylamide gel at 4°C and subjected to the autoradiographic assay. The autoradiograph was densitometrically assayed. **A**, autoradiograph. **B**, densitometric assay; closed circle, band α; closed triangle, band β. The result is represented as % of the density of band α in lane 14.

Fig. 2. Mobility shift DNA-binding assay for *Xmn*I or *Taq*I fragment-P130 interaction. P130 (150 ng) was incubated with the 370-bp [^{32}P]*Xmn*I or 285-bp [^{32}P]*Taq*I fragments (0.2 ng). In addition, P130 (150 ng) was incubated with both the *Xmn*I and *Taq*I fragments (0.2 ng of the total amount). All the incubated materials was electrophoresed on 4% polyacrylamide gel at 4°C and subjected to the autoradiographic assay. Lane 1, *Xmn*I fragment; lane 2, P130 and *Xmn*I fragment; lanes 3-6, P130, *Xmn*I and *Taq*I fragments. The weight ratios of the *Taq*I/*Xmn*I fragments were 0.25 (lane 3), 0.67 (lane 4), 1.5 (lane 5) and 4.0 (lane 6). Lane 7, P130 and *Taq*I fragment; lane 8, *Taq*I fragment.

(Gibco/BRL), the *Xmn*I and *Taq*I fragments, the complex has been estimated to have a molecular weight of about 540,000 which is equal to the sum of the molecular weights of one molecule of P130 and one each of the *Xmn*I and *Taq*I fragments (data not shown here). In this calculation, the molecular weight of the *Taq*I fragment was estimated to be 180,000.

Binding of P130 to a bent region in the repetitive component was analyzed by the DNase I footprinting with the 290-bp [5'-^{32}P]*Taq*I fragment which was prepared from the 370-bp [5'-^{32}P]*Hin*dIII fragment (Fig. 3A). This 370-bp fragment has been suggested to have a strongly bent structure in the sequence ranging from position around 270 (*Dra*I site) to the 3'-terminus [12]. The sequences of the binding regions are shown in the 370-bp *Hin*dIII repeat and emphasized by the capitals (Fig. 4). The major region was shown to consist of the 43-bp sequence at positions 293-335 in the strongly bent structure. Another region was located at positions around 250-287. Figure 5 shows that the 43-bp sequence is located near the center of

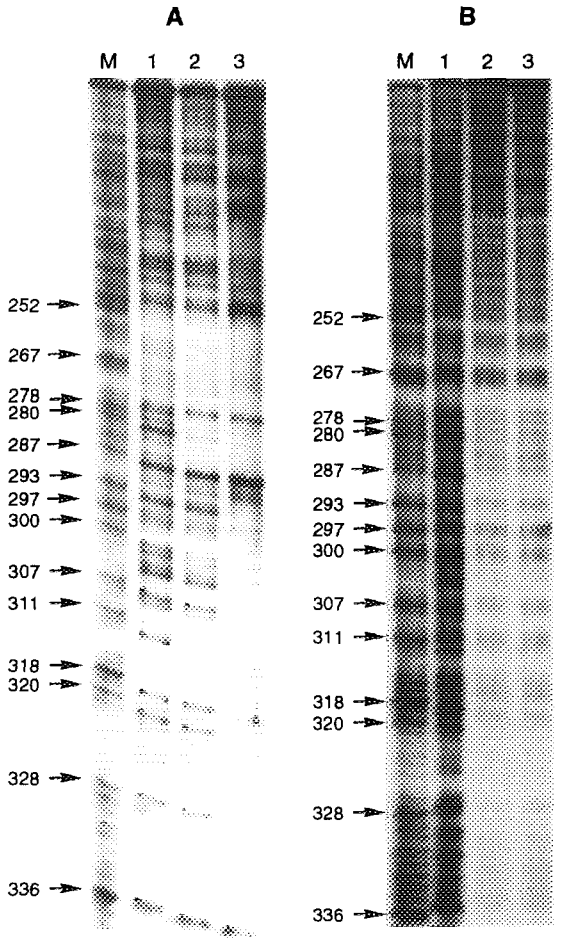

Fig. 3. Binding of P130 or distamycin A to *Hin*dIII fragment. The 370-bp [5'-^{32}P]*Hin*dIII fragments were digested with *Taq*I. The resulting 290-bp [5'-^{32}P]*Taq*I fragments were incubated with P130 or distamycin A as described in the text. The incubated material was subjected to the DNase I footprint analysis. Lane M, A + G reaction; lanes 1-3 in **A**, 0, 1 and 10 ng of P130, respectively; lanes 1-3 in **B**, 0, 0.3 and 0.6 μM distamycin A, respectively. The number is the distance in base pairs from the 5'-terminus of the 370-bp *Hin*dIII fragment to the site of guanine in the complementary fragment.

the 370-bp *Xmn*I repeat which has a strongly bent overall structure [7,12]. The same assay was performed with an antibiotic, distamycin A, which binds preferentially to AT-rich DNA (Fig. 3B and Fig. 4). The binding sites are underlined (Fig. 4). It is noteworthy that the sites are interspersed throughout the major binding region of P130. These results might support our previous findings that distamycin A removes the bend in the 370-bp *Xmn*I fragment and inhibits binding of P130 to the fragment [7]. Thus, these results imply that a nuclear scaffold protein such as P130 binds to sequence-directed bend(s) in a highly repetitive DNA to play an important role in construction of a higher-order chromatin structure.

Nonhistone nuclear proteins have been demonstrated to have the binding affinities for nuclear matrix/scaffold attachment DNA regions (MARs/SARs). Of these proteins, a nuclear matrix

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        10         20         30         40         50
agcttattacatgcgaatcctattgggaacctactcaattcaacatgata
HindIII

        60         70         80         90        100
cttagattcccttccttaaaatgttgctcgatattgaaaagcaaactcat
                              TaqI

       110        120        130        140        150
gcaagcatgtcccattgggaactcactgaattcgcctagaaaatttgatt

       160        170        180        190        200
ccgttcgtgaaaatttttcctatatcccgaacagtccacttattactagtg
        XmnI

       210        220        230        240        250
cggcctattgggaactaaccgaattcaccatgttactcagtttcggctca

       260        270        280        290        300
CCAAATTTTTATAAATCTTTAAAAGtACACATATTACaagagCAGgCTAC
                  DraI

       310        320        330        340        350
TAGGAACTAACTGAATTCACAAAGAAACAGTGTTTcagtgcgttaaaacg

       360        370
ttgctctatcttgaataaca
                  HindIII
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Fig. 4. Binding sites of P130 and distamycin A, in 370-bp *Hin*dIII repeat. The sequence of the repeat has been determined by us [11]. Capital, P130; underlined, distamycin A; italic, restriction site; *Dra*I, *TTT*↓*AAA*; *Hin*dIII, *a*↓*agctt*; *Taq*I, *t*↓*cga*; *Xmn*I, *gaaaa*↓*ttttc*.

protein from chicken, ARBP (attachment region binding protein), has been suggested to bind as a homo-oligomer to MARs from several eukaryotic genes in a cooperative fashion [19]. Moreover, a nuclear scaffold protein from HeLa cells, SAF-A (scaffold attachment factor A), which has an apparent molecular weight of 120,000, has been shown to bind to several SARs from vertebrate cells and to form large aggregates which mediate the formation of looped DNA structures [20]. In contrast, P130 molecules did not interact with each other although the sedimentation analysis revealed that this nuclear scaffold protein also has an apparent molecular weight of 120,000 [7]. Okazaki and her co-workers have reported that a centromere protein

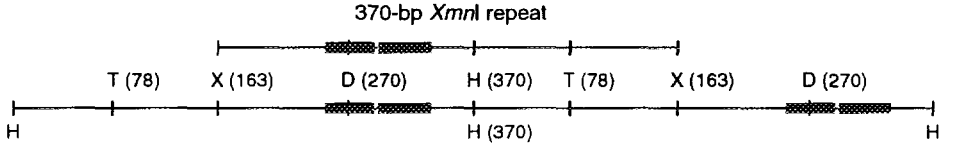

Fig. 5. Binding region of P130 in 370-bp *Xmn*I repeat or tandem dimer of 370-bp *Hin*dIII repeat. D, H, T and X indicate *Dra*I, *Hin*dIII, *Taq*I and *Xmn*I sites, respectively. Solid box is the binding region. The number in parentheses is the distance in base pairs from the 5'-terminus to the restriction site in the 370-bp *Hin*dIII fragment.

(CENP-B) binds to the 17-bp sequence in a human centromeric α-satellite DNA [21,22] and tentatively proposed that the major complex formed between the CENP-B and the DNA consists of the dimer of the CENP-B molecule and two DNA molecules [22]. Tsutsui et al. have recently evidenced that a nuclear scaffold protein from rat brain, SP120, binds selectively to a mouse MAR and suggested that protein-protein interaction is important to stabilize the DNA-protein complex [23]. This protein has properties similar to P130, in an apparent molecular weight, formation of the DNA-protein complex in a cooperative fashion and salt dissociation profile of the complex, etc. However, as described above, direct interaction between P130 molecules was not observed in the absence of the DNA [7]. Therefore, further studies would be necessary to elucidate whether or not P130 forms aggregates in contact with the DNA.

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