Differences in DNA Recognition and Conformational Change Activity between Boxes A and B in HMG2 Protein

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ABSTRACT: High mobility group (HMG) 2 is a sequence-nonspecific DNA-binding protein consisting of a repeat of DNA-binding domains called HMG1/2 boxes A and B and an acidic C-terminal. To understand the mode of HMG2 interaction with DNA, we expressed various HMG2 peptides containing HMG1/2 box(es) in *Escherichia coli* cells and purified them. Gel retardation and DNA supercoiling assay indicated that the region essential for the preferential binding of HMG2 with negatively supercoiled DNA and DNA unwinding activity is located in box B, but not sufficient alone. The flanking C-terminal basic region or box A linked by a linker region is necessary to express activities. The SPR measurements certified that the intrinsic DNA binding affinity of box B is weaker (K_d = 170 μM), and these adjoining regions largely strengthen the affinity ($K_d \leq 1.2$ μM). In contrast, box A, even in the presence of the adjoining basic linker region, showed no such activities, indicating that boxes A and B are different in their DNA recognition mode. The computer modeling suggested that the side chain of Phe-102 in box B is inserted into the base stack to cause DNA conformational changes, while the side chain of Ala-16 in box A is too small to intercalate. These represent that boxes A and B have similar tertiary structures but their activities for DNA conformational changes obviously differ. Box B is the main region for DNA recognition and conformational changes, and box A must play an assistant to increase its DNA recognition.

High mobility group (HMG)[1] proteins 1 and 2 constitute a highly abundant class of nonhistone chromosomal proteins. They have closely related and highly conserved primary sequences in different mammalian species (*1*−*4*). The proteins have been considered to have tripartite structures: two DNA binding domains called HMG1/2 boxes A and B each of which contains about 75 amino acid residues, and an acidic C-terminal region consists of a continuous run of 30 acidic amino acids in HMG1 and 23 in HMG2 (*3, 5*−*7*).

The biological function of HMG1 and 2 is only poorly understood, but their apparent roles in replication, transcription and cellular differentiation have been suggested (*1, 8*). In addition, HMG1, but not HMG2, may be a gene quasi-activator which modulates chromatin structure to orient the respective gene, thus ensuring that its activity as a template is expressed fully (*9*). HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation (*10*). HMG1 and 2 form the V(D)J recombination complexes with RAG1 and RAG2 (*11*). The function of HMG2 in living cells has also not been definitely confirmed. The level of HMG2 parallels the proliferation activity of several organs, suggesting that HMG2 is involved in cell replication (*12*). Progression of the cell cycle in COS-1 cells is repressed during the G1 to S phase by expression of the antisense RNA for HMG2, resulting in a decrease of cell growth. This suggests that fluctuation of the HMG2 message during the cell cycle is not a consequence of, but a prerequisite for, cell proliferation (*13*).

The structures of HMG1/2 boxes A and B of HMG1 have been analyzed using the NMR technique and show a characteristic L-shaped fold formed by three α-helices (*14*−*16*). The structures of the complex between the HMG box in SRY or LEF-1 and its target site show that binding occurs exclusively in the minor groove and induces a large conformational change in DNA (*17, 18*). The structures of the HMG1/2 boxes in HMG2 as well as their complexes with DNA have not yet been determined. HMG1 and 2 mediate nonspecific binding with DNA, preferential interaction with four-way junctions (*19*), supercoiled and cruciform (*20, 21*) and B−Z junction DNA (*22*). HMG1 and 2 also mediate DNA unwinding (*23*) and DNA bending (*24*). Similar DNA unwinding by a peptide containing two HMG1/2 boxes of HMG1 and 2 (*25, 26*) and DNA bending by the peptide containing two HMG1/2 boxes or box B with its flanking region of HMG1 (*27*) were observed. The characteristic binding with four-way junction DNA reappeared by not only boxes A and B in HMG1 but also the HMG1/2 box in SRY (*28*). These results motivated us further to solve the problems of whether or not both of two HMG1/2 boxes in HMG1 and 2 are essential for DNA unwinding and preferential binding with negatively supercoiled DNA whether the preferential binding with negatively supercoiled DNA and the four-way junctions, DNA bending, and DNA unwinding have inter-

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[1] Abbreviations: HMG, high mobility group; SRY, sex-determining region Y; LEF-1, lymphoid enhance-binding factor 1; PCR, polymerase chain reaction; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); SPR, surface plasmon resonance; RU, response unit(s).

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related mechanism of the reactions, why single box B cannot express the bending activity by itself in HMG1 (*27*), how its flanking regions and box A take part in the DNA-bending activity, does box A with its flanking region express the activity, and are the results analyzed for HMG1 common to HMG2.

To address these issues, we expressed various HMG2 peptides containing HMG1/2 box(es) in *Escherichia coli* cells and purified them. We analyzed the functional region for DNA unwinding and the region necessary for preferential binding with supercoiled DNA. Our observations indicated that the box B flanked by the adjoining region plays the lead in DNA recognition and that the modes of DNA recognition and the activities for DNA conformational changes of boxes A and B are different despite their similar tertiary structures.

EXPERIMENTAL PROCEDURES

Preparation of HMG2 Protein and Its Peptides Containing HMG1/2 Boxes. HMG2 protein was prepared from pig thymus chromatin as described previously (*29*). To express the various HMG2 peptides containing HMG1/2 boxes in *E. coli*, an expression vector, pGEM-KI, was constructed by *Xba*I digestion of the plasmid pGEM-EX1 (Promega) to remove of the SD sequence and the T7 gene 10, followed by intramolecular ligation. The resultant plasmid contains the SP6 promoter in a reverse direction, the T7 terminator, the F1 ori, the β-lactamase gene, and the pBR322 ori downstream from the T7 promoter. DNA fragments encoding HMG1/2 box A (1−76 amino acid residues in HMG2), Al (1−87), B (88−164), Bj (84−181), AlB (1−164), and AlBj (1−181) were amplified by polymerase chain reaction (PCR) and ligated into the region downstream from the T7 promoter. After confirming their nucleotide sequences, they were introduced into *E. coli* BL21 (DE3) containing the T7 RNA polymerase gene under control of the *lac* promoter inductive with isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were grown in LB medium containing ampicillin at 28 °C and induced with 0.5 mM IPTG.

Purification of Peptides Containing HMG1/2 Boxes. The purification procedure for peptide B is described here as an example of peptide purification. The packed cells (pGEM-B/*E. coli* BL21) were suspended in buffer A (10 mL/1 g of packed cells) containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 2 mM dithiothreitol (DTT) and sonicated with a Sonifer 250 (Branson). The sonicate was then centrifuged at 12000*g* for 15 min. DEAE-cellulose (1 g/10 mL of the supernatant) equilibrated with buffer A was added to the supernatant and stirred for 2 h. The supernatant of the centrifugation at 5000*g* for 10 min was mixed with solid ammonium sulfate to a final saturation of 70% (w/v), and the resulting suspension was centrifuged at 12000*g* for 30 min. The supernatant was applied to an alkyl-Superose (Pharmacia-LKB) column equilibrated with buffer B (50 mM sodium phosphate, pH 7.0) containing 2.7 M ammonium sulfate and eluted with a linear descending gradient of 2.40 to 2.25 M ammonium sulfate in buffer B. The fraction (P3 in Figure 2) of peptide B eluted at an ammonium sulfate concentration of 2.34−2.28 M was dialyzed against water and then lyophilized. The purification procedures for the various HMG2 peptides are summarized as a flowchart in Figure 2c.

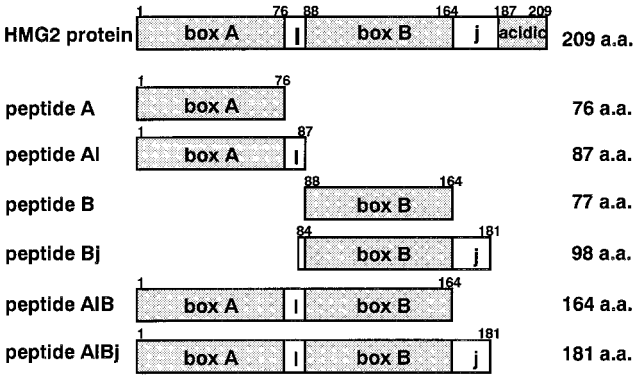

FIGURE 1: Schematic representation of HMG2 and their peptides containing HMG1/2 box(es) produced in *E. coli* cells. (Top) The intact HMG2 protein contains box A, box B, l (linker)-region, j (joiner)-region and acidic tail. (Bottoms) The peptides A (1−76), Al (1−87), B (88−164), Bj (84−181), AlB (1−164) and AlBj (1−181) were produced. Amino acid residues are abbreviated as a. a.

Preparation of Plasmid DNA. The plasmid pBR322 was propagated in *E. coli* HB101. The negatively supercoiled DNA (form I DNA) was prepared by the alkaline lysis method (*30*) and purified by CsCl-ethidium bromide centrifugation (*31*). Linearized DNA (form III DNA) was prepared by digestion with *Eco*RV. Relaxed closed-circular DNA (form Ir DNA) was prepared by incubation with topoisomerase I.

Gel Retardation Assay. Agarose gel electrophoresis of the complexes of plasmid pBR322 DNA with HMG2 or the peptides was performed as a function of the HMG2 (or the peptide)/DNA molar ratio. An aliquot of HMG2 or the peptide solution was mixed with 0.5 μg of DNA in a reaction buffer containing 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 40 μg bovine serum albumin. The solution (total volume, 20 μL) was incubated at 25 °C for 60 min and electrophoresed on a 1.2% agarose gel in 40 mM Tris-acetate, pH 7.8, containing 1 mM EDTA. After electrophoresis, the gel was stained with ethidium bromide.

DNA Supercoiling Assay. HMG2 or the peptides were complexed with DNA under the same condition with that of the gel retardation assay described above. The solution of a total volume of 20 μL was incubated with 1 unit of topoisomerase I at 37 °C for 60 min. DNA samples were deproteinized and analyzed by electrophoresis as described for the gel retardation assay.

Surface Plasmon Resonance (SPR) Measurement. The BIAcore biosensor (BIAcore Inc.) was employed to measure the real-time interaction of protein molecules with DNA. The protein injected in a flow of buffer solution interacts with DNA immobilized on a dextran matrix. The binding event is monitored using SPR detection (*32*), and the resulting binding curve and dissociation one with successive injection of the buffer alone can be used to determine the kinetic parameters of the interaction. A BIAcore sensor chip SA surface with streptavidin preimmobilized on dextran was used. A continuous flow of TME (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 1 mM EDTA) containing 25 mM NaCl was maintained at 5 μL/min. A 30 bp DNA with one strand biotinylated at the 5′-end of the sequence 5′-TGTAT-GAAATCTAACAATGCGCTCATCGTC-3′ was obtained from Sawady Technology (Tokyo). DNA was diluted to 0.1

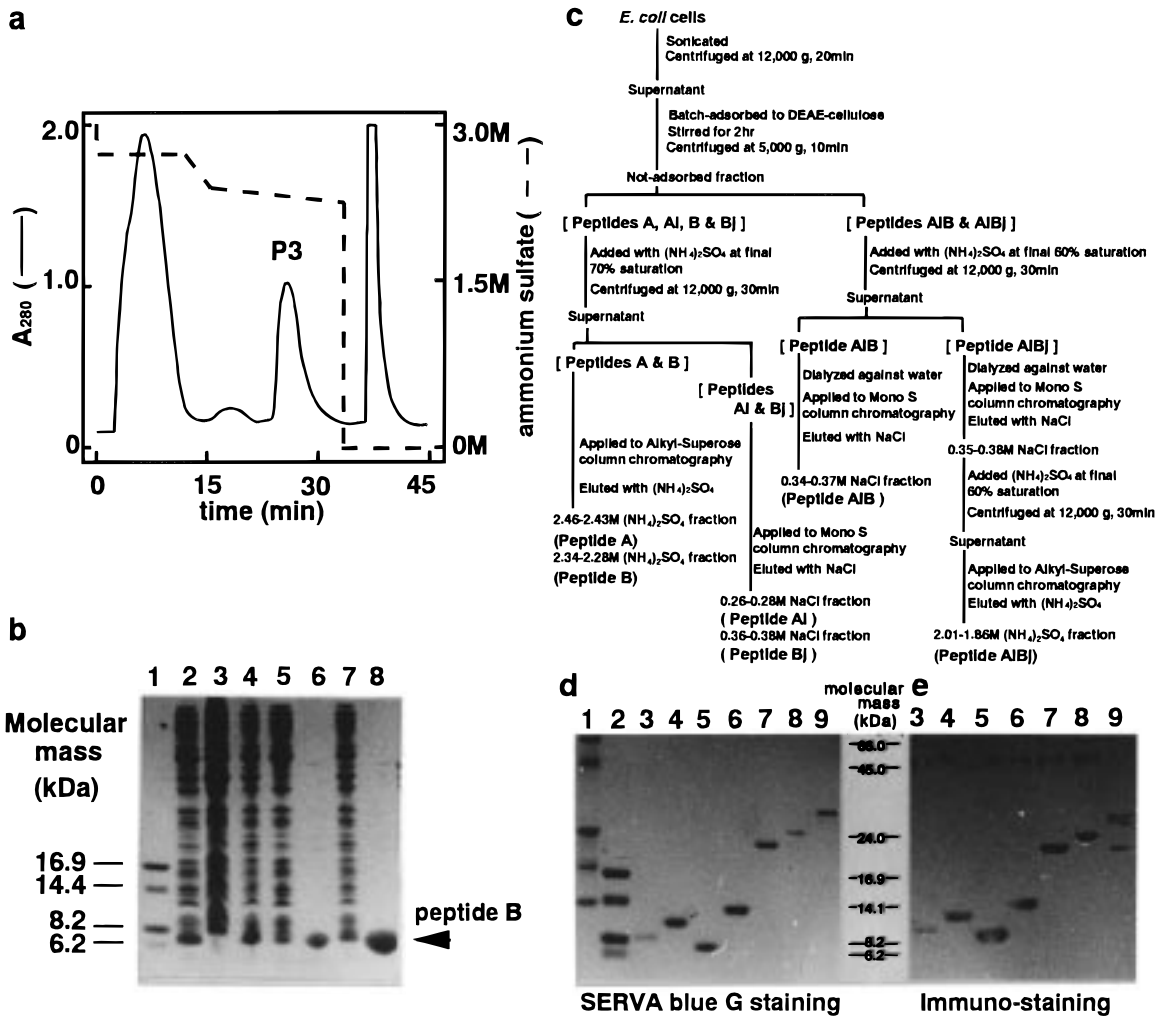

FIGURE 2: Purification of HMG2 peptides. (a) Elution profile of peptide B from an alkyl-Superose column. The supernatant obtained after 70% ammonium sulfate precipitation was applied to an alkyl-Superose column and eluted with a linear descending gradient of 2.40 to 2.25 M ammonium sulfate. Peptide B was eluted in a single peak fraction indicated as P3. (b) SDS−polyacrylamide electrophoretic gel profile of the respective fraction; supernatant of centrifugation after sonication (lane 2), precipitate of centrifugation after sonication (lane 3), fraction unadsorbed to DEAE-cellulose (lane 4), fraction batch-adsorbed to DEAE-cellulose (lane 5), supernatant obtained after 70% ammonium sulfate precipitation (lane 6), precipitate obtained after 70% ammonium sulfate (lane 7) and P3 fraction eluted from the alkyl-Superose column (lane 8). Molecular weight markers are also presented (lane 1). (c) Summary of the purification procedures for HMG2 peptides from *E. coli* cells. (d and e) SDS−polyacrylamide gel electrophoretic profile of the peptides stained with SERVA blue G (d) and immunostained with antiserum against HMG2 after westernblotting (e). Peptide A (lane 3), peptide Al (lane 4), peptide B (lane 5), peptide Bj (lane 6), peptide AlB (lane 7), peptide AlBj (lane 8), intact HMG2 (lane 9), high-molecular-weight markers (lane 1), and low-molecular-weight markers (lane 2).

μg/μL in TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.3 M NaCl) and applied to the sensor chip surface for a contact time of 5 min, resulting in capture of the synthetic DNA between 800 and 1000 response units (RU). The HMG2 or the peptides was diluted at various concentrations in TME containing 25 mM NaCl. To measure the association in TME containing 25 mM NaCl, an injection command, which allowed consecutive injection of the sample with no intermediate delay, was used. The dissociation was measured by injecting TME containing 25 mM NaCl, but no HMG or the peptides. At the end of each measurement, the DNA surface was regenerated by injecting 8 μL of 2 M NaCl. A flow rate of 2 μL/min was used, and the RU value was recorded at 0.2 s intervals.

Data were analyzed using the BIAevaluation software package, which was supplied with BIAcore. The reaction between immobilized DNA and HMG is therefore assumed to follow a simple one step reaction in eq 1:

$$\mathrm{HMG} + \mathrm{D} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\leftrightarrow}} \mathrm{HMG\text{-}D} \qquad (1)$$

where HMG is HMG protein or the peptides in the solution, D is the immobilized 30 bp DNA, HMG-D is the HMG-DNA complex, k_{on} is the apparent association rate constant, and k_{off} is the apparent dissociation rate constant. The binding of HMG is described by the equation

$$dR/dt = k_{on}C(R_{max} - R) - k_{off}R \qquad (2)$$

where dR/dt is the rate of the complex formation, R is the amount of bound HMG, R_{max} is the maximum HMG binding capacity of the surface, and C is the HMG concentration in the solution. During the dissociation phase, when buffer containing HMG has been replaced with buffer alone, the concentration of HMG effectively drops to zero if there is no significant rebinding and then eq 2 becomes

$$dR/dt = -k_{off}R \quad (3)$$

The ratio of the apparent rate constant to be calculated is $K_d = k_{off}/k_{on}$.

Model Building of the Complexes of HMG2 Boxes A and B with DNA. The PDB (Protein Data Bank of Brookhaven National Laboratory) data of HMG1 boxes A (1aab) (*14*) and B (1hme) (*16*) and LEF-1−DNA complex (1lef) (*18*) were applied for the modeling. The calculation was performed using insight II/Discover3 (Molecular Simulations Inc.), using amber force field. A 9.5 Å cutoff for van der Waals interaction and cell multipole mode for coulomb interaction with a distance-dependent dielectric function of $\epsilon = 1 \times r$, where r is the distance in angstroms between interacting atoms, were used. The boxes A and B solvated in a 6 Å layer of water were subjected to 500 steps of energy minimization and 50 ps of molecular dynamic simulation at 298 K. The dynamics step size was 1 fs, and structures were saved along the trajectory every 0.5 ps. Then, simulated HMG2 boxes A and B were superimposed to that of LEF-1 on DNA. Successively, box A− and box B−DNA complexes solvated in a 6 Å layer of water were subjected to 500 steps of energy minimization and 70 ps molecular dynamic simulation at 298 K.

RESULTS

Structural Characteristics of HMG2. HMG2 as well as HMG1 is the most typical representative of proteins containing a novel DNA binding motif called the HMG1/2-box. As shown in Figure 1, HMG2 contains repeated DNA-binding motifs with nonidentical amino acid sequences called box A (1−76 amino acid residues) and box B (88−164), respectively. These boxes are connected by a short linker (l) region (77−87). In addition, a unique carboxyl terminal region (C-tail) consisting of a continuous run of 23 acidic amino acids (187−209) is connected to the box B through a joiner (j) region (165−186) (*7*).

Expression of HMG2 Peptides in E. coli Cells and Their Purification. To analyze the DNA binding activity of HMG1/2 box(es) and the effect of their flanking regions on the activity, we tried to overproduce in *E. coli* cells the respective single HMG1/2 boxes (peptides A and B), peptides containing the respective flanking regions (peptides Al and Bj), a peptide containing the two HMG1/2 boxes connected by the l-region (peptide AlB) and a peptide containing the two HMG1/2 boxes flanked by the j-region (peptide AlBj), as shown in Figure 1.

The purification procedure for peptide B was described in the Experimental Procedures as an example of peptide purification. Figure 2a shows an elution profile of peptide B from the alkyl-Superose column using an FPLC system. The P3 fraction eluted at 2.34−2.28 M of ammonium sulfate was shown as a single band on the SDS−polyacrylamide electrophoretic gel (Figure 2b), together with the profiles of the respective fractions during the process of purification. The purification procedures for the various HMG2 peptides are summarized in Figure 2c. All of the purified peptides gave a single band with dye-staining on the SDS−polyacrylamide electrophoretic gel as well as by immunostaining after western blotting of the gel (Figure 2, panels d and e). The amino acid sequences of the peptides were confirmed using a protein sequencer (data not shown).

Requisite for Preferential Binding of HMG2 with Form I DNA. To estimate the structural relationships between the two HMG1/2 boxes, DNA complexes with a varied amount of peptides were analyzed by DNA gel retardation (Figure 3). With increasing amount of HMG2 peptides, DNA bands were progressively retarded. The gel retardation of forms I and III DNA by peptides Al and Bj was larger than that by peptides A and B (Figure 3, panels a and b), suggesting that l- and j-regions increase DNA-binding ability of the respective HMG1/2 box. The gel retardation of forms I and III DNA by peptides AlB and AlBj was larger than that by peptides containing a single HMG1/2 box. In addition, the gel retardation of forms I and III DNA by peptide AlB was larger than that by an equimolar mixture of peptides A and B or of peptides Al and B (not shown). Together, a tandem array of two HMG1/2 boxes linked by the l-region may be required for effective binding with plasmid DNA. The gel retardation of forms I and III DNA by HMG2 was markedly smaller than those by peptides AlB and AlBj, showing that the acidic C-tail largely weakens the binding ability of HMG2 with plasmid DNA.

To determine the region in HMG2 necessary for preferential binding with form I DNA, a gel retardation assay at low peptide/DNA ratios was carried out using the mixture of forms I and III DNA (Figure 3c). When the relative gel retardation of form I DNA was compared with that of form III DNA at equimolar ratios of peptide to DNA, peptides Bj, AlB, AlBj and HMG2 preferentially interacted with form I DNA (lane 23 for peptide Bj, lane 30 for peptide AlB, lane 37 for peptide AlBj, and lane 46 for HMG2). On the other hand, form I DNA was retarded by peptides A, Al, and B to extents similar to form III DNA (lanes 2−7 for peptide A, lanes 9−14 for peptide Al, and lanes 16−21 for peptide B). These results indicated that the essential region for preferential binding with form I DNA is box B, but not sufficient alone. The j-region or box A linked by the l-region was needed for the preferential binding with form I DNA.

DNA-Unwinding Activities of the HMG2 Peptides. To identify the functional region for DNA unwinding, DNA-supercoiling assay was employed (Figure 3e). Peptides Bj, AlB, and AlBj produced topoisomers induced by their DNA-unwinding activities, while no changes in linking number were observed for peptides A, Al, and B. These topoisomers were certified to be negatively supercoiled DNA (not shown). Gel retardation assay using form Ir (relaxed closed-circular) of pBR322 was conducted for further conformation. Peptides A, Al, and B showed simple dose-dependent gel retardation, as shown in Figure 3d. Peptides Bj, AlB, and AlBj complexes at low protein/DNA ratios migrated faster than DNA probe alone, while migration was decreased at the higher ratios. A similar change was observed for HMG2. These results demonstrated that essential region for the DNA unwinding is box B, but insufficient alone. The j-region or box A flanked by the l-region was needed for the activity as well as the preferential binding of HMG2 with form I DNA.

DNA Binding Affinity of HMG2 and the Peptides Analyzed Using Surface Plasmon Resonance. It is also considered that the above gel retardation profiles were affected not only by the affinity of each respective peptide with DNA but also by the net charge of the peptide complexed with DNA. For example, the bands of form I DNA complexed with peptide AlBj (Figure 3, panels a and c) moved in a reverse direction

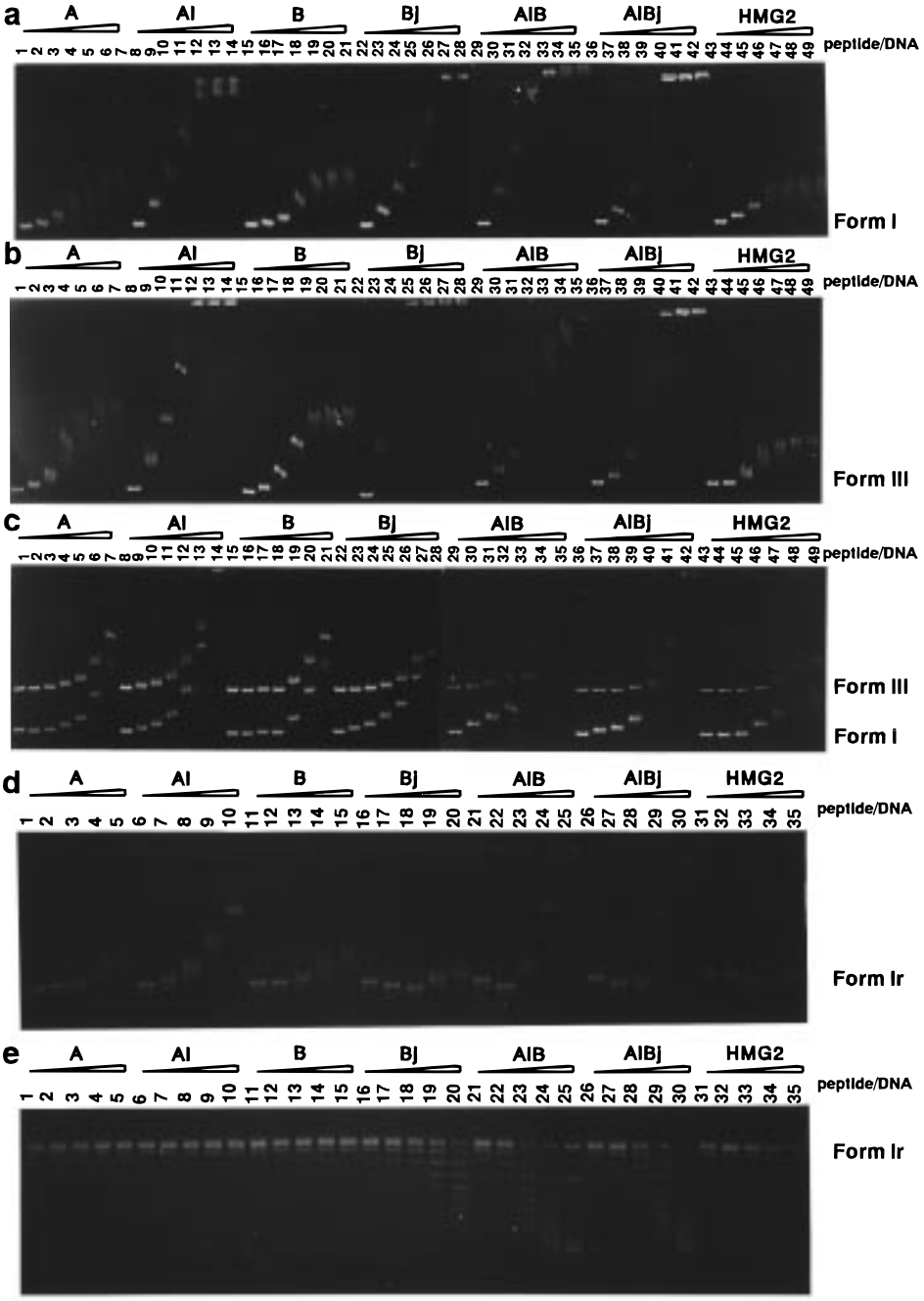

FIGURE 3: Gel retardation assay and unwinding assay of DNA using HMG2 peptides. HMG2 peptides at different molar ratios of the peptide (or protein) to DNA were complexed with (a) form I, (b) form III, (c) the equimolar mixture of forms I and III, or (d) form Ir DNA, and separated by agarose gel electrophoresis. (e) HMG2 peptides at different molar ratios of the peptide (or protein) to DNA were incubated with topoisomerase I and analyzed by agarose gel electrophoresis. (a−c) Lanes 1−7, 8−14, 15−21, 22−28, 29−35, 36−42, and 43−49 were contained the complexes of peptides A, Al, B, Bj, AlB, AlBj, and HMG2, respectively. (a and b) Molar ratios of peptide (or HMG2) to DNA in lanes 1−7, 8−14, 15−21, 22−28, 29−35, 36−42, and 43−49 were 0, 100, 200, 400, 600, 800, and 1000, respectively. (c) Molar ratios of peptide (or HMG2) to DNA (the mixture of forms I and III at a ratio of 1:1) in lanes 1−7, 8−14, 15−21, 22−28, 29−35, 36−42, and 43−49 were 0, 12.5, 25, 50, 100, 200, and 400, respectively. (d and e) Lanes 1−5, 6−10, 11−15, 16−20, 21−25, 26−30, and 31−35 were contained the complexes of peptides A, Al, B, Bj, AlB, AlBj, and HMG2, respectively. Molar ratios of peptide (or HMG2) to DNA in lanes 1−5, 6−10, 11−15, 16−20, 21−25, 26−30, and 31−35 were 0, 40, 80, 160, and 360, respectively.

upon electrophoresis on agarose gel from the slots in which the complexes were applied. In addition, the preferential binding of HMG2 to form I DNA may have partly originated from binding to unusual structures on form I DNA such as cruciform, B−Z junction, and bent and partially unwound regions (*20*−*22*). The same peptides showing unwinding and preferential binding with the form I DNA in the present study bend DNA (Nakamura et al., manuscript in preparation). Therefore, more precise measurements of the affinity of peptides with DNA were considered to be important for elucidating the mechanism of DNA recognition by HMG2. For this purpose, real-time DNA-binding measurement with a BIAcore Biosensor system (BIAcore Inc.) using surface plasmon resonance (SPR) as the detection system was applied. To obtain kinetic data under conditions of effector-dependent binding, a 30 bp DNA fragment bound to the sensor chip surface was prepared from complementary annealed oligonucleotides. Multiple sensorgrams were col-

Table 1: Kinetic Data Calculated from the SPR-Binding Measurements for HMG2 Protein and the Peptides

HMG2 and peptides	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (M)[a]
A	$(3.2 \pm 1.2) \times 10^3$	$(4.8 \pm 0.3) \times 10^{-1}$	$(1.5 \pm 0.3) \times 10^{-4}$
Al	$(1.2 \pm 0.3) \times 10^4$	$(3.8 \pm 0.1) \times 10^{-1}$	$(3.2 \pm 0.5) \times 10^{-5}$
B	$(1.4 \pm 0.6) \times 10^3$	$(2.4 \pm 0.1) \times 10^{-1}$	$(1.7 \pm 0.4) \times 10^{-4}$
Bj	$(6.4 \pm 0.8) \times 10^4$	$(7.7 \pm 0.9) \times 10^{-2}$	$(1.2 \pm 0.1) \times 10^{-6}$
AlB	$(7.3 \pm 0.9) \times 10^4$	$(3.5 \pm 0.4) \times 10^{-2}$	$(4.8 \pm 0.6) \times 10^{-7}$
AlBj	$(5.0 \pm 0.9) \times 10^4$	$(2.4 \pm 0.4) \times 10^{-2}$	$(4.7 \pm 0.9) \times 10^{-7}$
HMG2	$(1.2 \pm 0.5) \times 10^4$	$(7.3 \pm 0.3) \times 10^{-3}$	$(6.1 \pm 1.5) \times 10^{-7}$

[a] K_d is defined as $K_d = k_{off}/k_{on}$.

lected, keeping the amount of the DNA fragment constant at 800−1000 RU, and the concentration of HMG2 or the peptides was varied between 0.1 and 25 μM. The Hill coefficients (*33*), calculated from the extrapolated responses at infinite time for various protein concentrations, were close to 1, indicating that HMG2 and the peptides form 1:1 complex with 30 bp DNA and that the binding is noncooperative in a simple one-step reaction shown as eq 1 (*34*). The calculated kinetic data for all of the peptides are listed in Table 1.

For interaction of the 30 bp DNA with a single HMG1/2 box, the close K_d values of 1.5×10^{-4} (M) for peptide A and 1.7×10^{-4} (M) for peptide B (Table 1) suggested their similar DNA-binding affinity. The flanking regions of the HMG1/2 boxes increased the k_{on} values 4-fold (l-region for box A) and 50-fold (j-region for box B). However, the K_d values of 3.2×10^{-5} (M) for peptide Al and 1.2×10^{-6} (M) for peptide Bj (Table 1) showed that the j-region for box B is more effective in stable DNA binding than the l-region for box A. The k_{on} values for peptides AlB and AlBj were similar to that for peptide Bj, whereas the k_{off} values for peptides AlB and AlBj were reduced 2−3-fold compared with that for peptide Bj, resulting in the reduced K_d values for peptides AlB and AlBj compared with peptide Bj. These stable complexes were considered to be formed by a tandem array of two boxes through the l-region. It is noticeable that the K_d values for HMG2 and the peptides which show DNA unwinding activity and preferential binding with supercoiled DNA in the gel shift assay (Figure 3, panels c−e) were low, less than micromolar order (Table 1).

DISCUSSION

In the present paper, we have described the preferential binding of HMG2 with negatively supercoiled DNA and the peptide regions involved in the preference, together with their DNA-binding reaction mode. The preferential binding of HMG2 with supercoiled DNA rather than linear DNA was reexpressed in peptides Bj, AlB, and AlBj (Figure 3c). This result indicated that box B must play a main role and the j-region or box A linked by the l-region also need for the preferential binding. Similar result was shown by DNA unwinding analysis (Figure 3, panels d and e). The box B with flanking j-region or the HMG1/2 boxes linked by the l-region were necessary for the DNA-unwinding activities of HMG2 and the regions were also in need for the preferential binding of HMG2 with form I DNA (Figure 3). The results which showed that peptide AlB most strongly induced negatively supercoiled topoisomers (Figure 3e) demonstrated that the box A flanked by the l-region is more effective for DNA unwinding than the j-region. The same regions were necessary for strong bending of the DNA in HMG2 (Nakamura et al., manuscript in preparation) as well as HMG1 (*27*). These results suggest that preferential binding with negatively supercoiled DNA, DNA unwinding, and the strong bending of the DNA have mutual relationships. However, the specific binding for four-way junction DNA was reexpressed box A as well as box B (*28*), suggesting that the mode of the specific recognition for four-way junction DNA is different from the preferential binding with negatively supercoiled DNA and not relationed with DNA unwinding activities.

Real-time DNA-binding analyses were carried out using a BIAcore instrument, permitting reasonable models to be derived which might offer insight into the nature of the DNA-binding reaction. Assumption of a stationary state (at infinite time) showed that the Hill coefficients for all HMG2 peptides are close to 1. This result indicated that interactions of HMG2 and the peptides with 30 bp DNA are noncooperative at 1:1. The peptides A and B, composed of folded domain structures in the complete HMG1/2 box motif (*14*−*16*), exhibited only moderate affinity for DNA (Table 1). The presence of the basic region flanking the respective box yielded DNA-binding affinity 4-fold stronger for box A and 50-fold stronger for box B, indicating simultaneous participation of the basic flanking regions in the interaction with DNA. However, the presence of the flanking j-region to peptide AlB (peptide AlBj) had no additional effect on the DNA binding (Table 1). This suggested that the N-terminal basic region of box A, the l-region, the N-terminal basic region of box B, and the j-region close together in the tertiary structure of HMG2 protein (*35*) create competition in their DNA-binding effects. The K_d value for peptide Bj was twice the value of peptide AlB. Thus, the j-region seems not to be essentially concerned in the DNA binding of HMG2 protein.

As described above, the region essential for preferential binding with negatively supercoiled DNA and DNA-unwinding activity is located in box B, but not sufficient alone. The real-time DNA-binding measurements showed that the K_d value for peptide B was 1.7×10^{-4} (M), while those for all peptides showed preferential binding with form I DNA, and DNA-unwinding activity was more than 100-fold less in K_d values ($\leq 1.2 \times 10^{-6}$ M). These data show that DNA-binding affinity of box B is weak and that the j-region and box A strengthen the DNA-binding affinity of box B to express the full activity necessary for preferential binding to supercoiled DNA and DNA unwinding. In contrast, peptide Al showed no preferential binding with supercoiled DNA and DNA-unwinding activity, indicating that boxes A and B are different not only in their DNA recognition modes but also in the activity of DNA conformation changes.

NMR studies on boxes A and B in HMG1 show a characteristic L-shaped fold formed by three α-helices (*14*−*16*). These results were applied for computer molecular modeling to generate the models of DNA-free boxes A and B in HMG2 (Figure 4, panels a and b). The tertiary structures similar to those in HMG1 were obtained. The complex structure of HMG1/2 box in LEF-1 with the target site, which was determined by NMR spectroscopy (*18*), enabled us to model the complexes of HMG2 boxes A and B with DNA (Figure 4, panels c and d). Met-10 in HMG1/2 box in LEF-1 partially intercalates into the base stack between A23 and

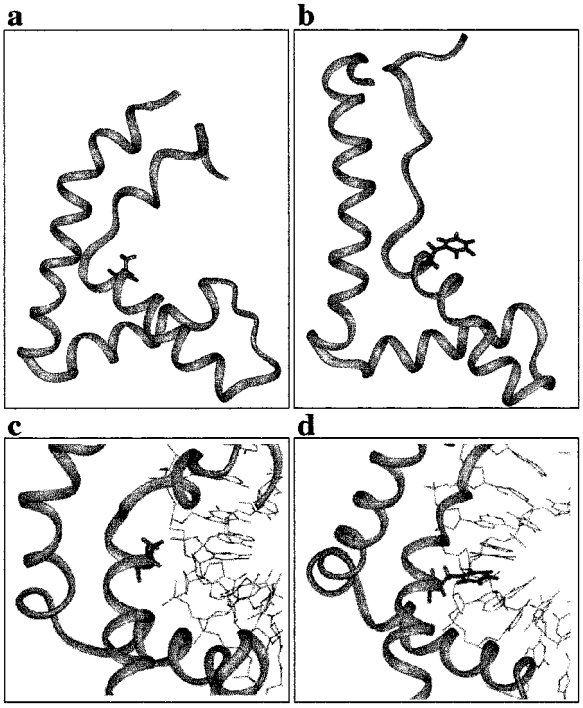

FIGURE 4: Computer molecular modeling of the complexes of single HMG1/2 boxes in HMG2 with DNA. (a) A view of HMG2 box A. The side chain of Ala-38 is represented by stick. (b) A view of HMG2 box B. The side chain of Phe-102 is represented by stick. (c) A close-up view around the site of Ala-38 in HMG2 box A complexed with DNA. (d) A close-up view around the site of Phe-102 in HMG2 box B complexed with DNA. The Phe-102 is inserted into the base stack.

A24 in the target DNA, resulting in a large local roll angle at this base pair step, followed by both DNA unwinding and bending (*18*). Similar results have been reported for the binding of HMG1/2 box in SRY (*17*). Ala-16 in HMG2 box A and Phe-102 in HMG2 box B correspond to the intercalating residues in LEF-1 and hSRY basing on their sequence alignment and their models (Figure 4, panels a and b). The computer modeling strongly suggested that the side chain of Phe-102 in box B is inserted into the base stack to cause DNA conformational changes, while the side chain of Ala-16 in box A is small not enough to intercalate (Figure 4, panels c and d).

Boxes A and B in HMG2 are similar tertiary structures (Figure 4, panels a and b), but their modes of DNA recognition (Figure 3c) and activities for DNA conformational changes obviously differ as shown in this paper (Figures 3d and 4, panels c and d). Box B must be the main region for DNA recognition, and box A must play as an assistant to increase DNA-binding affinity for expressing preferential binding to supercoiled DNA and activity for DNA conformational changes by HMG2. HMG1 may be a gene quasi-activator which modulates chromatin structure to orient the respective gene, thus ensuring that its activity as a template is expressed fully (*9*). Here we reported that DNA conformational changes and the preferential binding observed in the present investigation may be important for such the chromatin modulation.

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