Characterization of the Interaction of Wheat HMGa with Linear and Four-Way Junction DNAs†

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Received February 18, 2003; Revised Manuscript Received April 6, 2003

ABSTRACT: Wheat HMGa protein is a typical member of the plant HMGA family. It has four AT hooks and a histone H1-like region. A panel of deletion mutants of HMGa was generated to study the role of different regions of HMGa in its binding to 4H (a synthetic DNA that mimics the *in vivo* structure of intermediates of homologous recombination and DNA repair) and linear DNAs. Although the histone H1-like region of HMGa does not bind to 4H or linear DNAs, it does enhance the binding. Mutants with any two adjacent AT hooks show specific binding to both 4H and linear P268 (and P31) with different binding affinities, which is partly due to the flanking regions between AT hooks. Conformational studies indicate that the α-helical content of HMGa increases significantly when it binds to 4H compared to that after binding to P31, linear DNA. In contrast, linear DNA, but not 4H, undergoes substantial conformational change when it binds to HMGa, indicating that linear DNA is relatively more flexible than 4H. A more significant difference in the affinities of binding of the mutants of HMGa to 4H was observed compared to their affinities of binding to linear DNA, P31. These differences could be due to the rigidity of the DNA and the characters of the AT hook regions in the mutants.

High-mobility group (HMG)[1] proteins are the most abundant of the nonchromosomal proteins, which are operationally defined as proteins that can be extracted from chromatin with 0.35 M NaCl and are soluble in 2% trichloroacetic acid or 2−5% perchloric acid (*1*). They are divided into three groups, namely, HMGA, HMGB, and HMGN groups, based on the amino acid sequences (*1*). Mammalian HMGA proteins have three AT hooks and an acidic carboxyl-terminal domain (*2*). They bind to DNA in the minor groove of AT-rich sequences through AT hooks (*3*−*5*). The three AT hooks of human HMGA1a appear to exhibit different binding properties. Only the second AT hook along with the six amino acid residues in the flanking segment specifically binds to the IFN-β enhancer (*5*−*7*). The role of the first and third AT hooks in the specific binding to DNA is not fully established. Some studies demonstrate that the first and second AT hooks play a major role in the specific binding to DNAs (*6*, *8*), whereas other reports show that the second and third AT hooks are in direct contact with DNAs (*5*, *6*, *9*). Similarly, the data regarding the role of the acidic tail are also controversial (*5*, *6*). Yie et al. (*6*) have shown that the acidic tail plays an important role in mediating the specific binding. In contrast, the acidic tail of HMGA1a does not affect the ability to recognize linear DNA (*6*, *7*). It is not clear whether these observed differences are due to the source of HMGA proteins and DNAs. In addition to binding to linear DNAs, animal HMGA proteins also bind to the deformed DNA structures. For example, they bind to the opposite arms across the center of the "open" form of four-way junction DNA (4H) (*9*−*11*). Structure−function studies show that the first two AT hooks and the flanking region between the second and the third AT hook of *Chironomus* HMGA are involved directly in the binding to 4H (*12*). In contrast, the second and third, but not the first, AT hooks of human HMGA protein are indicated to be in direct contact with 4H (*9*). Thus, although a significant amount of work has been done to understand structure−function relationships of animal HMGA proteins, some of these results are contradictory, and the sites involved in the recognition and binding of DNAs are not yet unambiguously characterized.

HMGA proteins have also been isolated from several plant species (*11*, *13*). They display distinct structural features compared to their animal counterparts despite their overall similarities. (a) Plant HMGA proteins are larger in size and have a molecular mass of ∼20 kDa, compared to a mass of ∼10 kDa in animals (*1*). (b) Plant HMGA proteins have a histone H1-like region at the N-terminus followed by three or four AT hooks (*2*, *14*). In contrast, animal HMGA proteins do not have the histone H1-like region, and have only three AT hooks. (c) The acidic carboxyl-terminal domain, which is found in animal HMGA proteins, is missing in the plant counterparts (*2*). (d) The sequences of the flanking regions between the AT hooks in the plant proteins also differ from those in the animal proteins in size as well as in the nature of amino acid residues. Thus, except for their AT hook regions, plant proteins differ significantly from animal HMGA proteins.

† This work is supported by academic research grants from the National University of Singapore.

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[1] Abbreviations: 4H, four-way junction DNA; BSA, bovine serum albumin; CD, circular dichroism; EMSA, electrophoretic mobility shift assay; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMG, high-mobility group; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SPR, surface plasmon resonance.

10.1021/bi034280h CCC: $25.00
Published on Web 05/08/2003

Like animal HMGA proteins, plant proteins interact with linear DNA containing AT-rich segments (*13*, *15*−*17*). Mutation studies have shown that the presence of the two N-terminal AT hooks is sufficient for the high-affinity binding of rice and maize HMGA proteins to the promoters containing AT tracks (*17*, *18*). The histone H1-like region alone shows no binding to the AT-rich tract (*17*, *18*). However, detailed information about the role of all AT hooks and their flanking regions in the binding of plant HMGA proteins to DNA is still unavailable. The interaction between plant HMGA and 4H was only studied recently (*11*). Accordingly, wheat HMGa binds to the "open" form of 4H. However, the sites involved in the recognition and binding to 4H are unknown.

Given the significant differences in the structure between plant and mammalian HMGA proteins, we were interested in determining the structural requirements in plant HMGA proteins for binding to DNA. In this work, a panel of deletion mutants of wheat HMGa protein was produced to explore the role of different structural motifs on the binding of HMGa to 4H and linear DNAs [P268, the region between nucleotides −444 and −176 of the *PetE* promoter (*19*), and P31, a region within P268 that was heavily protected and acts as an enhancer element (*16*, *20*)] through the use of an electrophoresis mobility shift assay (EMSA) and surface plasmon resonance (SPR). Our results show that mutants containing any two AT hooks specifically bind to both forms of DNAs with variable binding affinities. Here, we show that the first two AT hooks appear to play a more important role than the last two AT hooks. We have also employed circular dichroism to examine the conformational changes in the protein as well as DNAs resulting from the interaction.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. The wheat *HMGA* gene was cloned as described in detail previously (*11*). The HMGa and its deletion mutants were generated by PCR using the appropriate primers, and their integrity was verified by DNA sequencing. In each pair of primers, *Bam*HI and *Hin*dIII restriction sites were introduced. PCR products were digested with *Bam*HI and *Hin*dIII, purified from a gel (gel extraction kit, Qiagen), and then cloned in pET-32a (Novagen) vectors in frame with a fused part including the six-His moieties.

Protein Expression and Purification. The HMGa and its deletion mutants were expressed in BL21(DE3) pLysS and purified following the protocol of Qiaexpressionist (Qiagen), and appropriate controls have been used. HPLC-purified wheat HMGa was obtained as described previously (*11*, *15*).

Preparation of Four-Way Junction DNA (4H) and Linear DNAs, P268 and P31. Four-way junction DNA was prepared essentially as described by Bianchi (*21*). The oligonucleotides were synthesized and purified by HPLC (Operon). Labeled 4H was made by following the method of Hill and Reeves (*10*). P268 fragments were prepared by digesting the pPC-H268.7 plasmid containing the P268 insert at *Hin*dIII sites (*22*). After the fragments had been separated by electrophoresis, the insert was purified from the agarose gel. The DNA was labeled with [γ-^{32}P]ATP. After labeling had been carried out, the labeled p268 was separated from the unincorporated free [γ-^{32}P]ATP with the nucleotide removal kit (Qiagen). P31 (nucleotides −289 to −259 of the *PetE* gene promoter) has the sequence 5′-AATATACTAGTATTATTTACTAAAAAAAATC (*19*). P31 used for the electrophoretic mobility shift assay was prepared and labeled as described previously (*23*). Labeled P31 was purified using Nick columns (Pharmacia). 4H and P31 for surface plasmon resonance (SPR) analysis and circular dichroism (CD) experiments were prepared by mixing the matching strands (equal concentrations) in Milli-Q water, boiling for 5 min, and slowly cooling them.

Electrophoretic Mobility Shift Assay (EMSA). EMSAs for 4H were performed as described by Hill and Reeves (*10*). Labeled 4H (10 fmol) was incubated with a target protein at room temperature in a total volume of 10 μL of protein binding buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 25 mM NaCl, 0.28 μg of sheared salmon sperm DNA, 2.8 μg of BSA, and 2% glycerol] for 10 min. EMSAs for linear DNAs were performed by incubating 0.3 ng of labeled p268 or 0.25 ng of labeled p31 with a target protein at room temperature in a total volume of 10 μL of binding buffer [25 mM Hepes/KOH (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 0.75 μg of poly(dI·dC)·poly(dI·dC) (Sigma), and 2% glycerol] for 10 min. The studies of the binding of two mutant proteins to P268 were carried out by incubating labeled P268 with the first protein for 10 min. Then the second protein was added and incubated for an additional 10 min. The samples were loaded onto a nondenaturing polyacrylamide gel (0.75 mm thick, comprised of 0.5× TBE and 6.5% polyacrylamide at a 29:1 bisacrylamide ratio). For P31, 10% gels were used. The competition binding of P268 with polynucleotides was done by incubating P268 with the protein for 10 min. Then polynucleotides were added, and the incubation was continued for an additional 10 min. Gels were prerun for 30 min at 90 V, and the electrode buffer was replaced before samples were loaded. Samples were electrophoresed at 90 V for 1−2 h at 4 °C, after which the gels were dried on DE81 paper (Whatman) in a gel dryer (SG210D SPEEDGEL Gel Drying System, Savant) at 80 °C for 15−20 min. The dried gels were covered with plastic wrap and exposed to X-OmatTM AR-5 film (Kodak) at −80 °C with an intensifying screen. The film was developed using an X-ray developer (M35 X-OMAT Processor, Kodak).

Surface Plasmon Resonance (SPR) Analysis. We examined the interaction between DNA (4H or P31) and recombinant HMGa (or its deletion mutants) using SPR analysis in a Biacore 3000 system (BIACORE Inc.). The activation and deactivation of the surface of sensor chip CM5 (BIACORE Inc.) followed the standard protocol provided by the manufacturer. The activated sensor chips were utilized to capture the appropriate amount (∼1000 RU) of the purified recombinant HMGa or its deletion mutants which were diluted with 20 mM sodium acetate buffer (pH 4.2). No target protein was immobilized in flow cell 1, which was used as the reference, while proteins were immobilized in the other three flow cells. The assay for 4H was carried out at 25 °C in running buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 25 mM NaCl, and 0.005% (v/v) Surfactant P20 (BIACORE Inc.)]. For P31, the running buffer contained 25 mM Hepes/KOH (pH 7.6), 40 mM KCl, 0.1 mM EDTA, and 0.005% (v/v) Surfactant P20. The DNAs were diluted to appropriate concentrations with the running buffer and injected over the flow cells at a flow rate of 30 μL/min. The bound DNAs were removed with a 30 s wash using 2 M NaCl. This wash did not affect the affinity of proteins for the CM5 chips.

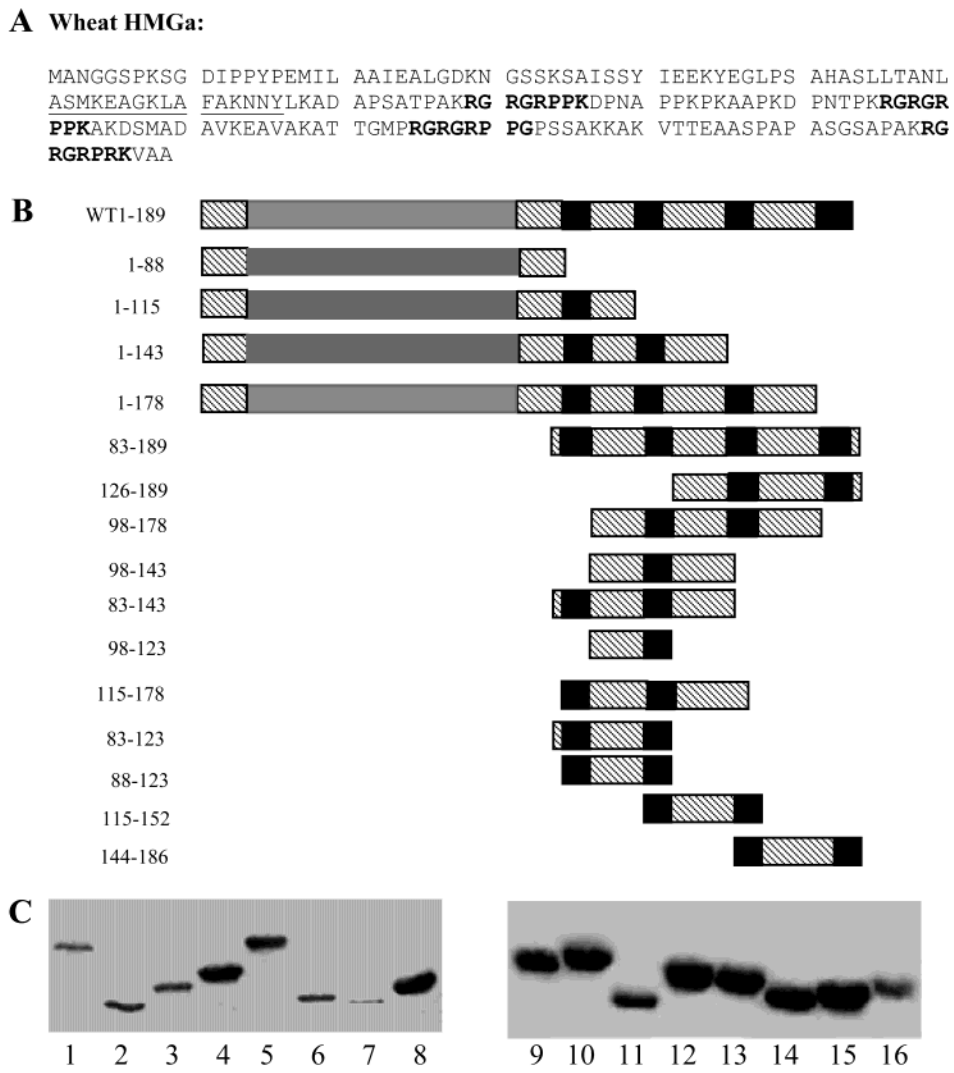

FIGURE 1: Structure of HMGa and its deletion mutants. (A) Amino acid sequence of wheat HMGa protein deduced from the HMGa cDNA. Underlined letters make up the histone H1-like region of HMGa, and bold letters represent four AT hooks. (B) Diagrammatic representation of the wheat HMGa and its deletion mutants used in the study. The four AT hooks are represented by black boxes. The boxes with slanted lines represent spacer amino acidic residues. The gray boxes are the regions containing the histone H1-like region. (C) Coomassie blue-stained SDS−PAGE gel containing the His-fused HMGa and its deletion mutants after they have been purified on Ni−NTA agarose bead columns: lane 1, HMGa (residues 1−189); lane 2, mutant 1−88; lane 3, mutant 1−115; lane 4, mutant 1−143; lane 5, mutant 1−178; lane 6, mutant 83−189; lane 7, mutant 124−189; lane 8, mutant 98−178; lane 9, mutant 98−143; lane 10, mutant 83−143; lane 11, mutant 98−123; lane 12, mutant 115−178; lane 13, mutant 83−123; lane 14, mutant 88−123; lane 15, mutant 115−152; and lane 16, mutant 144−186.

Data from flow cell 1 containing no protein were subtracted from the corresponding data from the protein-containing flow cells to correct for bulk refractive index changes. The data were analyzed using BIAevaluation version 3.1 (BIACORE Inc.).

Circular Dichroism (CD) Experiments. CD spectra of the recombinant HMGa, 4H, and P31 were obtained on a J-715 spectropolarimeter (Jasco) at room temperature. All spectra were recorded in a nitrogen atmosphere using a quartz cell with a path length of 1 mm (Jasco). To reduce random error and noise, each spectrum was recorded as an average of three scans. The following parameters were used for data acquisition: response, 2 s; scanning speed, 20 nm/min; bandwidth, 1.0 nm; sensitivity, 5 mdeg; and step resolution, 0.1 nm. HMGa, its deletion mutants, or 4H in 2 mM sodium phosphate buffer (pH 7.6) was titrated with 4H, P31, or HMGa protein, respectively, to investigate the conformational changes after binding. For the investigation of the conformational change of HMGa protein after its interaction with DNAs, the spectra of the sodium phosphate buffer and the buffer with 4H or P31 alone were subtracted to isolate the conformational change solely from protein. For the investigation of the conformational change of 4H or P31 after the interaction with HMGa protein and its deletions, the spectra of the sodium phosphate buffer and the buffer with proteins alone were subtracted to ensure the observed conformational changes were only from that of 4H or P31. The data were smoothed and expressed as mean residue ellipticity and mean base ellipticity for protein and DNA, respectively. Secondary structure analysis of the protein was carried out using the CCA program (*24*).

RESULTS

Preparation of HMGa and Its Deletion Mutants. Wheat HMGa protein has four AT hooks and a histone H1-like region (Figure 1A). To investigate the structural basis of the HMGa−DNA binding, a series of deletion mutants of HMGa (Figure 1B) was generated and expressed in *Escherichia coli*

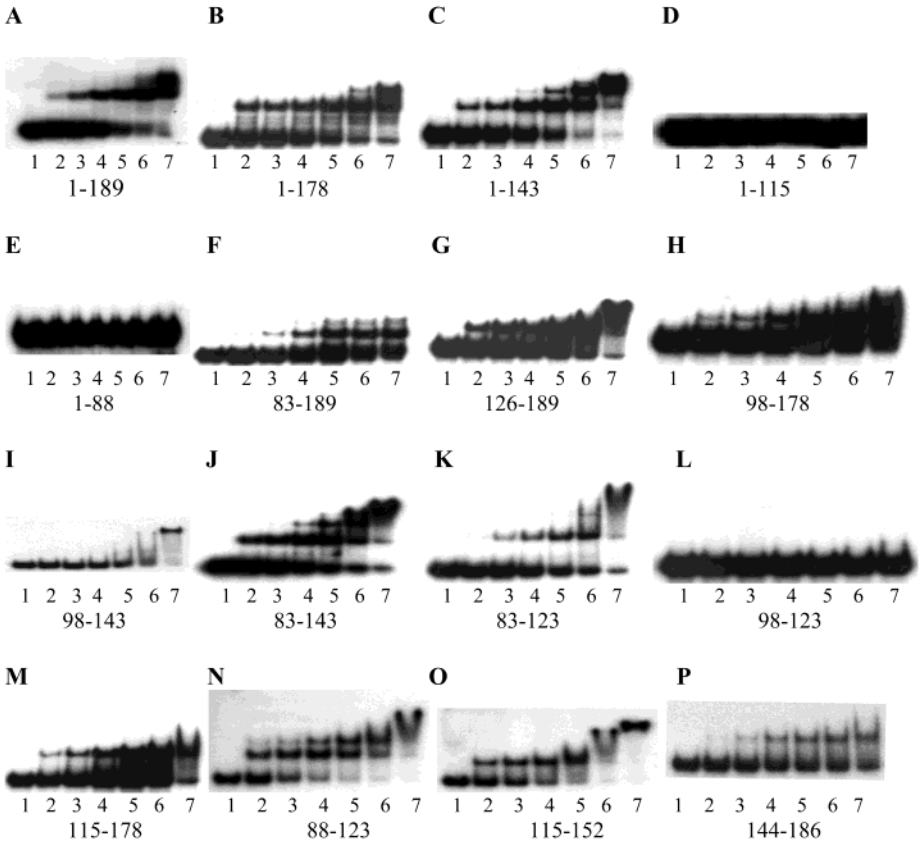

FIGURE 2: Binding of HMGa and its deletion mutants to 4H. EMSA using 10 fmol of ^{32}P-labeled 4H titrated with increasing concentrations of recombinant HMGa and its deletion mutants. The protein concentrations were 0, 20, 40, 80, 160, 320, and 640 nM in lanes 1−7, respectively.

and purified to near homogeneity. A representative Coomassie blue-stained SDS−polyacrylamide gel containing the recombinant and deletion mutants is shown in Figure 1C. The recombinant HMGa and its deletion mutants matched the expected molecular mass as determined by SDS−PAGE. These proteins were used to test their binding to 4H, P268, and P31.

Binding of HMGa and Its Deletion Mutants to 4H. The interactions between HMGa and its deletion mutants with 4H were studied with an EMSA. ^{32}P-labeled 4H was titrated with increasing amounts of HMGa or its deletion mutants to examine the function of different parts of HMGa in the interaction with 4H. Figure 2 shows that recombinant HMGa (residues 1−189) and mutants 1−178 and 1−143 specifically bind to 4H. Plant HMGA possesses a histone H1-like region at its N-terminus (*14*). Histone H1 protein and its globular domain exhibit specific binding to 4H (*11*, *25*). The lack of detectable binding of mutants 1−88 and 1−115 to 4H, together with the difference between the binding of mutant 1−189 or 83−189 to 4H, may indicate that the H1-like region of plant HMGA alone is not sufficient for the binding to 4H, but may slightly enhance the binding of HMGa to 4H. All mutants possessing two or more AT hooks bind to 4H with high affinity (Figure 2). Thus, any two AT hooks are sufficient to bind to 4H (see below).

Mutant 98−143 containing the second AT hook and the flanking regions binds to 4H, but mutant 98−123, which lacks the C-terminal flanking segment following the second AT hook, does not (Figure 2). This indicates the importance of the amino acid residues following the second AT hook in the specific binding of HMGa to 4H. Among mutants 88−123, 83−123, and 83−143, only mutant 88−123 does not have the amino acid residues beyond the first two AT hooks (Figure 1A), but the bindings of these mutants to 4H are similar (Figure 2). Similar binding was also observed with mutants 126−189 and 144−186 (Figure 2). Both these mutants have AT hooks 3 and 4, but mutant 126−189 has the additional flanking segment residues at the amino-terminal side of the third AT hook (Figure 1A). These results indicate that the amino acid residues following the second AT hook are not necessary for the specific binding to 4H, when the mutants possess two AT hooks, instead of one as in the case of mutant 98−123. The interactions of mutants 98−178, 115−178, and 115−152 with 4H show no apparent binding differences (Figure 2). These results indicate that AT hooks 2 and 3 are also sufficient to bind to 4H. We compared the binding characteristics of all three mutants (88−123, 115−152, and 144−186) which contain successive AT hooks. All these mutants show specific binding to 4H (Figure 2), indicating the presence of any two AT hooks is sufficient for the high-affinity binding to 4H. When the first of the first two AT hooks is deleted, the mutant fails to bind to 4H (compare the binding characteristics of mutants 88−123 and 98−123) (Figure 2).

Binding of HMGa and Its Deletion Mutants to Linear DNA P268. The interactions between HMGa or its deletion mutants and P268 were studied with an EMSA. ^{32}P-labeled P268 was titrated with increasing amounts of HMGa or its deletion mutants to demonstrate the possible roles of different parts of HMGa in the binding between HMGa protein and P268. Recombinant HMGa specifically binds to P268 (Figure 3A). The similar binding of the native and recombinant

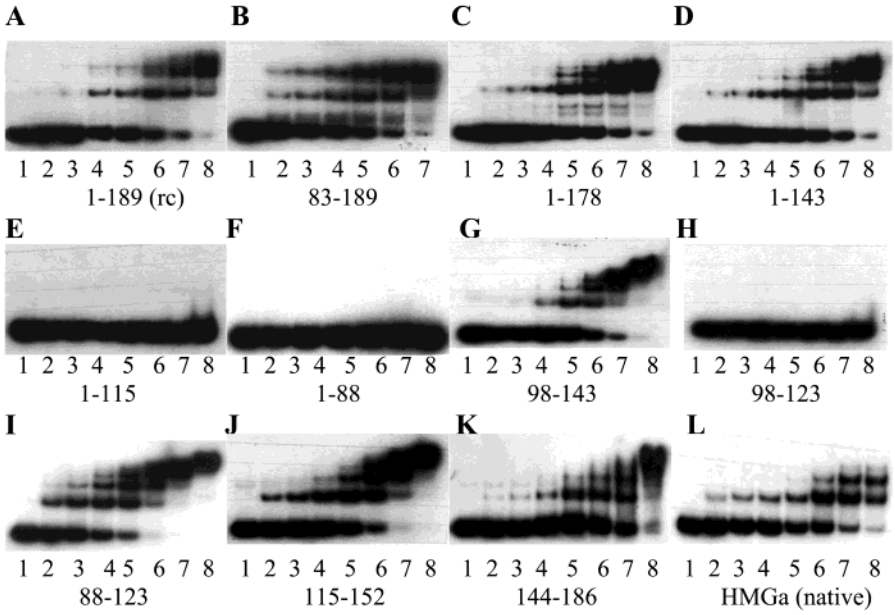

FIGURE 3: Binding of HMGa and its deletion mutants to P268. EMSA using 0.3 ng of ^{32}P-labeled P268 titrated with increasing concentrations of recombinant HMGa and its deletion mutants. The protein concentrations were 0, 20, 40, 80, 160, 320, and 640 nM in lanes 1−7, respectively, or 0, 10, 20, 40, 80, 160, 320, and 640 nM in lanes 1−8, respectively.

HMGa proteins (Figure 3A,L) indicates that the carrier protein and the His tag have no significant effect on the binding. Both mutant 1−178 (without the fourth AT hook) and mutant 1−143 (without the third and fourth AT hooks) show similar binding to P268 compared to that of recombinant HMGa (in Figure 3, compare panels C and D with panel A). But deletion of last three AT hooks (mutant 1−115) abolishes the binding of HMGa to P268 (Figure 3E). Mutant 1−88 containing the histone H1-like region (Figure 1B) shows no binding to P268 (Figure 3F). The similar binding pattern of mutant 83−189 (containing all four AT hooks) compared to that of native or recombinant HMGa protein shows that the histone H1-like region may not be directly involved in the specific binding of HMGa to P268 (Figure 3A,B). Similarly, the H1-like region of rice and maize HMGA protein does not contribute their binding to the linear DNA (*17*, *18*). Interestingly, histone H1 specifically binds to P268 (data not shown).

Mutants possessing any two adjacent two AT hooks (88−123, 115−152, and 144−186) specifically bind to P268 (Figure 3B,J,K). Mutant 98−123 containing the second AT hook and the N-terminal flanking region does not bind to P268 (Figure 3H). In contrast, mutant 98−143, with the addition of the C-terminal flanking region, specifically binds to P268 (Figure 3G). These results demonstrate the importance of the flanking region in the specific binding to P268 of the mutant possessing the second AT hook. Together with the sequence identity of the first two AT hooks (Figure 1A), the different binding characteristics of mutants 1−115 and 98−143 support a functional role for the flanking regions (Figure 3E,G). Mutant 88−123 (possessing the first and second AT hooks) specifically binds to P268, but mutant 98−123 (with the first AT hook missing) shows no binding, which indicates the importance of the simultaneous binding of two AT hooks for the specific binding to P268 (Figure 3H,I). When the binding characteristics of mutants 1−143, 1−115, and 88−123 are compared, it is also evident that the addition of a second AT hook leads to the specific binding to P268 (Figure 3D,E,I). The multiple complexes formed between HMGa or its mutants (Figure 3) indicate multiple binding sites of HMGa on P268, which is consistent with the results from the footprinting (*26*).

Two DNA-Binding Sites on HMGa. We further studied the role of AT hooks in the binding of HMGa to P268. In this experiment, we added enough first protein so that no "free" DNA can be detected in an effort to examine whether the second protein replaces the first protein or binds simultaneously. HMGa, mutant 88−123 (containing first two AT hooks), and mutant 144−186 (containing the third and fourth AT hooks) are able to bind to P268 (Figure 3A,I,K,L). These results indicate that one HMGa molecule has at least two potential binding sites for P268; the first site may be made up of the first two AT hooks, and the second site may be made up of the last two AT hooks. The addition of native HMGa displaced mutant 88−123 or 144−186 from P268 and decreased the size of the mutant 88−123−P268 or mutant 144−186−P268 complex because the molecular mass of native HMGa was lower than that of mutant 88−123 or 144−186, as the pET-32a vector introduces 165 amino acid residues into the expressed proteins (Figure 4A,C). Panels E and F of Figure 4 show that mutants 88−123 (containing only the first and second AT hooks) and 144−186 (containing only the third and fourth AT hooks) bind to P268 simultaneously. Thus, there are two DNA binding sites on HMGa, and P268 contains at least two independent protein binding sites for one HMGa molecule. Mutant 88−123 was replaced by the addition of 160 nM native HMGa (Figure 4A, lane 6). Similarly, native HMGa was replaced by the addition of 160 nM mutant 88−123 (Figure 4B, lane 6). These observations indicate similar binding affinities of native HMGa and mutant 88−123 for P268. On the other hand, native HMGa displaced mutant 144−186 from P268 and formed new complexes that were smaller in size (compare lane 2 with lanes 6−8 in Figure 4C). However, mutant 144−186 does not displace native HMGa or mutant 88−123 (Figure 4D), indicating the binding affinity for P268

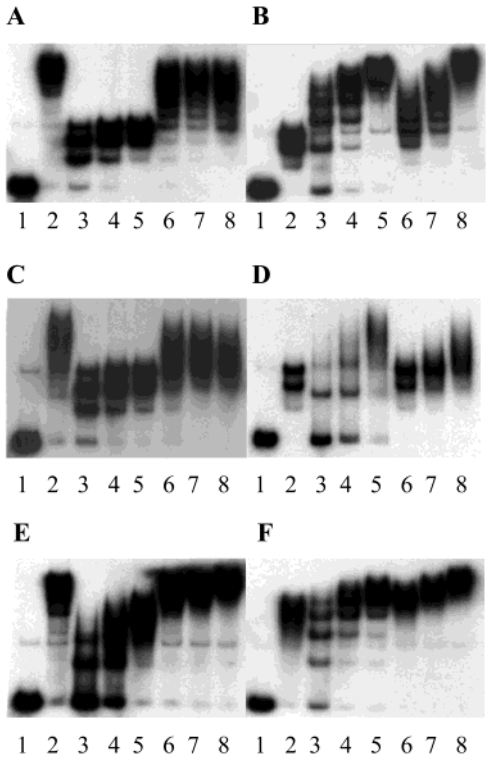

FIGURE 4: Competition between binding of native HMGa and two mutants to P268. All assay mixtures containing 0.2 ng of ^{32}P-labeled P268. Lane 1 of each panel (A−F) does not have tested proteins. (A) Mutant 88−123 (520 nM) was added to lane 2. EMSAs were performed with increasing amounts of native HMGa (160, 320, and 640 nM) in the absence (lanes 3−5) or presence (lanes 6−8) of mutant 88−123 (520 nM). (B) HMGa (520 nM) was added to lane 2. P268 was incubated with increasing amounts of mutant 88−123 (160, 320, and 640 nM) in the absence (lanes 3−5) or presence (lanes 6−8) of HMGa (520 nM). (C) Mutant 144−186 (640 nM) was added to lane 2. P268 was incubated with increasing amounts of HMGa (160, 320, and 520 nM) in the absence (lanes 3−5) or presence (lanes 6−8) of mutant 144−186 (640 nM). (D) HMGa (520 nM) was added to lane 2. EMSAs were performed with increasing amounts of mutant 144−186 (160, 320, and 640 nM) in the absence (lanes 3−5) or presence (lanes 6−8) of HMGa (520 nM). (E) Mutant 88−123 (520 nM) was added to lane 2. EMSAs were performed with increasing amounts of mutant 144−186 (160, 320, and 640 nM) in the absence (lanes 3−5) or presence (lanes 6−8) of mutant 88−123 (520 nM). (F) Mutant 144−186 (640 nM) was added to lane 2. EMSAs were performed with increasing amounts of mutant 88−123 (160, 320, and 520 nM) in the absence (lanes 3−5) or presence (lanes 6−8) of mutant 144−186 (640 nM).

of mutant 144−186 is lower than that of native HMGa and mutant 88−123.

Specificity of the Interaction of HMGa with Linear DNA. We also examined the specificity of the interaction of the wheat HMGa with linear DNA molecules of different sequences. Three distinct complexes were formed between 80 nM recombinant HMGa and 0.3 ng of labeled P268. The addition of 100× , 500×, and 1000× nonlabeled poly(dA)·poly(dT) or poly(dAdT)·poly(dAdT) completely abolished all P268−HMGa complexes (data not shown), indicating the specific binding of HMGa to AT-rich DNA. Poly(dA)·poly(dT) has a higher affinity for HMGa than poly(dAdT)·poly(dAdT) does. However, poly(dG)·poly(dC) and poly(dgdC)·poly(dGdC) failed to abolish the P268−HMGa complexes (data not shown), suggesting that these two polymers do not bind to HMGa. Similar specific binding results were also observed for deletion mutants 1−178, 1−143, 83−189, 88−123, and 144−186 (data not shown). These results indicate that HMGa and its mutants specifically bind to AT-rich DNA and not to GC-rich DNA.

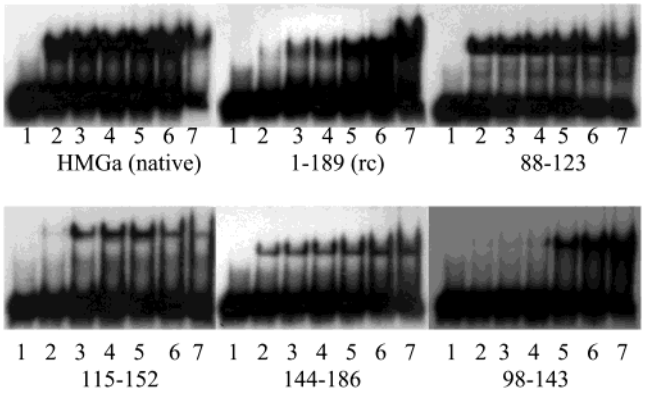

FIGURE 5: Binding of recombinant (rc) HMGa and its deletion mutants to P31. EMSAs using 0.25 ng of labeled P31 titrated with increasing concentrations of recombinant HMGa and its deletion mutants. The protein concentrations were 0, 20, 40, 80, 160, 320, and 640 nM in lanes 1−7, respectively.

Binding of HMGa and Its Mutants with P31. P31 (nucleotides −289 to −259) is a region within P268 that acts as an enhancer element when multimerized (*20*). This P31 region was heavily footprinted by HMG proteins (*26*). To simplify the studies using SPR and CD (described below), we chose P31 instead of P268 as the DNA substrate. Both recombinant and native HMGa show similar binding to P31 (Figure 5). Mutants 98−143, 88−123, 115−152, and 144−186 were selected to represent the regions that play an important role in the binding of HMGa to P268. These mutants also specifically bind to P31. HMGa and all its mutants formed one major complex with P31 (Figure 5).

Binding Studies Using SPR. To determine the effect of deletion on the interaction of HMGa with 4H and the affinity of their interactions, we measured the affinities of recombinant HMGa and its deletion mutants for 4H and P31 using SPR. Initial experiments were carried out with biotinylated 4H immobilized on a CM5, SA, or B1 chip. Wheat HMGa proteins were injected first over an underivatized control flow cell, and then over the DNA-containing flow cell. The HMGa proteins bind equally well to the control surface without DNA and to the DNA-containing surface even when a high concentration of sperm DNA is added to prevent the possible nonspecific binding (data not shown). This phenomenon was also observed when other HMGA and HMGB proteins were injected over a SA sensor chip coupled with P31 (*27*). Therefore, HMGa or its deletion mutants, instead of 4H or P31, were immobilized on the CM5 sensor chips in this study.

Binding Affinity of HMGa and Its Mutants for 4H. As shown in Figure 6, recombinant HMGa and the deletion mutants bind to 4H in a concentration-dependent manner. The affinity of recombinant HMGa was calculated with the equilibrium dissociation constant ($K_d = k_{diss}/k_{ass}$) to be 0.83 nM (Table 1). This value is comparable to the value of 6.5 nM obtained for human HMGA protein using an EMSA (*10*). The apparent K_d values of deletion mutants 83−189, 1−178, 1−143, 88−123, and 115−152 were comparable to those of recombinant HMGa (Table 1). The slightly different binding affinities of HMGa and mutant 83−189 may indicate the minor contribution of the H1-like region of HMGa to its

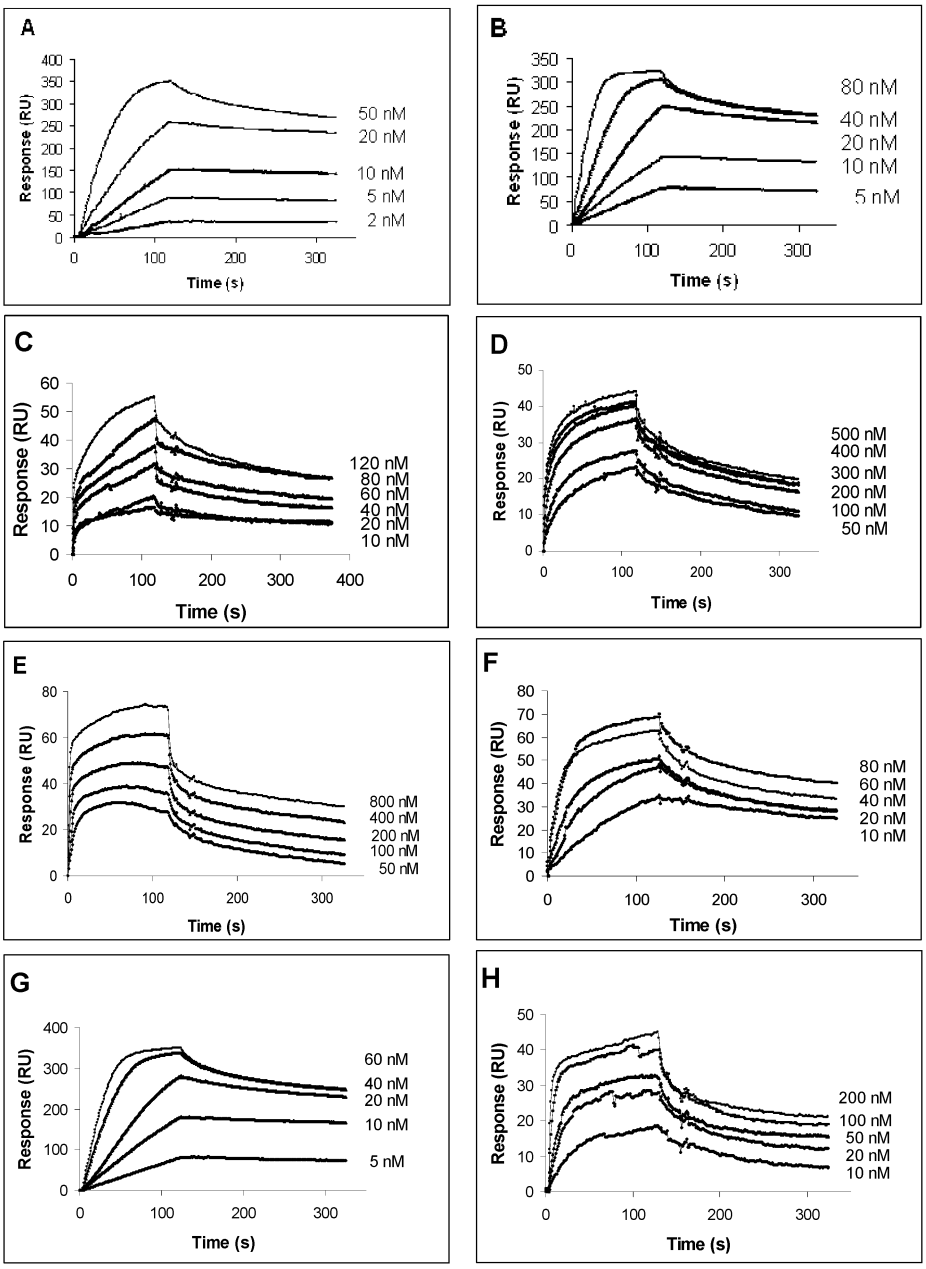

FIGURE 6: SPR analysis of the interaction between HMGa or its deletion mutants and 4H. The analysis of the interactions is described in detail in Experimental Procedures. Five or six concentration series were used in a single experiment to obtain the association and dissociation rate constants. The concentrations of 4H that were injected are indicated: (A) recombinant HMGa (residues 1−189), (B) mutant 88−123, (C) mutant 115−152, (D) mutant 144−186, (E) mutant 98−143, (F) mutant 83−189, (G) mutant 1−178, and (H) mutant 1−143.

Table 1: Binding Constants of Recombinant HMGa and Its Deletion Mutants with 4H

sample	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_d (M)
HMGa (residues 1−189)	$(4.18 \pm 0.01) \times 10^5$	$(3.47 \pm 0.05) \times 10^{-4}$	$(8.30 \pm 0.18) \times 10^{-10}$
mutant 83−189	$(1.02 \pm 0.02) \times 10^6$	$(1.67 \pm 0.05) \times 10^{-3}$	$(1.64 \pm 0.31) \times 10^{-9}$
mutant 1−178	$(7.43 \pm 0.07) \times 10^5$	$(1.28 \pm 0.07) \times 10^{-3}$	$(1.72 \pm 0.50) \times 10^{-9}$
mutant 1−143	$(4.75 \pm 0.07) \times 10^5$	$(1.81 \pm 0.06) \times 10^{-3}$	$(3.82 \pm 0.16) \times 10^{-9}$
mutant 88−123	$(3.01 \pm 0.02) \times 10^5$	$(3.91 \pm 0.05) \times 10^{-4}$	$(1.31 \pm 0.10) \times 10^{-9}$
mutant 115−152	$(2.18 \pm 0.03) \times 10^5$	$(1.08 \pm 0.05) \times 10^{-3}$	$(4.95 \pm 0.31) \times 10^{-9}$
mutant 144−186	$(1.34 \pm 0.02) \times 10^5$	$(2.49 \pm 0.03) \times 10^{-3}$	$(1.85 \pm 0.18) \times 10^{-8}$
mutant 98−143	$(5.64 \pm 0.10) \times 10^4$	$(2.19 \pm 0.10) \times 10^{-3}$	$(3.88 \pm 0.33) \times 10^{-8}$

binding to 4H, which is consistent with the EMSA results (Figure 2). SPR studies show that the binding affinities of mutants 83−189 and 1−178 are similar, whereas EMSA studies indicate a better binding of mutant 1−178 than of mutant 83−189. This inconsistency may be due to either the potential effects of immobilizing the proteins rather than DNA (*27*) or the size of the DNAs used in these experiments. The apparent K_d values of the mutants containing the two successive AT hooks (88−123, 115−152, and 144−186) were 1.31, 4.95, and 18.5 nM, respectively (Table 1). Thus, the mutant with the first and second AT hooks has an affinity for 4H similar to that of recombinant HMGa, whereas the

mutant with the third and fourth AT hooks has an affinity for 4H that is 1 order of magnitude lower. These results indicate that the first two (or maybe the third) AT hooks may play a major role in the binding of HMGa to 4H. The affinity of mutant 98−143 (containing only one AT hook) was 38.8 nM, indicating that at least two AT hooks are required for higher-affinity binding. This requirement demonstrates the importance of simultaneous binding of two or more AT hooks.

Binding Affinity of HMGa and Its Mutants for P31. The recombinant HMGa and the deletion mutants also show specific binding to P31 in a concentration-dependent manner (Figure 7). The K_d for interaction of recombinant HMGa was found to be 4.9 nM (Table 2). This value is comparable to the K_d value of 3.1 nM between HMGA from pea and P31 by SPR (*27*). The apparent K_d values of mutants 88−123 and 115−152 were similar to those of native HMGa (Table 2). However, mutant 144−186 exhibited ∼2−3-fold lower affinity than the recombinant HMGa and mutant 88−123. Thus, mutant 88−123 binds to P31 with higher affinity than mutant 144−186, consistent with the EMSA results (Figure 4). Mutant 98−143, containing the second AT hook and its flanking regions, binds to P31 with a K_d of 18 nM, similar to that of mutant 144−186 (Table 2).

Conformational Changes in the HMGa−4H Complex. We used CD studies to monitor the conformational changes in protein and DNA after the binding interaction between recombinant HMGa and 4H. When HMGa was free in 2 mM sodium phosphate buffer (pH 7.6), the CD spectrum of HMGa shows a negative band at 222 and 208 nm and a positive band between 190 and 195 nm (Figure 8A), which indicate an α-helical structure (23%) and random coil (77%) (*14*). With the increase in the ratios of 4H to HMGa, the protein mean residue ellipticity of HMGa increased, indicating an increase in the α-helix content (Figure 8A). The α-helical content was increased to 33% at a 1:1 HMGa:4H ratio, indicating that the HMGa protein undergoes a significant conformational change upon binding to 4H. The observed conformational changes are mostly due to the HMGa and not due to the fusion protein as the mutants which failed to bind 4H did not show any conformational changes upon addition of DNA (data not shown), despite containing the same fusion protein.

To monitor the conformational change in 4H after it interacts with HMGa, 4H (2.5 μM) was titrated with varying concentrations of HMGa. No changes in ellipticity of 4H were detected after its binding to HMGa protein (Figure 8B). Similar results were obtained when the EMSA binding buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 25 mM NaCl] was used (data not shown). Thus, no conformational change in 4H is observed when it binds to HMGa.

Conformational Changes in the HMGa−P31 Complex. The mean residue ellipticity of HMGa increased with the addition of P31, indicating a slight increase in the α-helical structure content. At a 1:1 HMGa:P31 ratio, there is 26% α-helical structure and 74% random coil (Figure 9A). When the ratio of P31 to HMGa was increased from 1:1 to 2:1, almost no change in ellipticity was observed (Figure 9A).

We also examined the conformation of P31 after it binds to HMGa. Figure 9B shows the mean base pair ellipticity change of P31 at different recombinant HMGa:P31 ratios. When the HMGa:P31 ratios were increased, the ellipticity

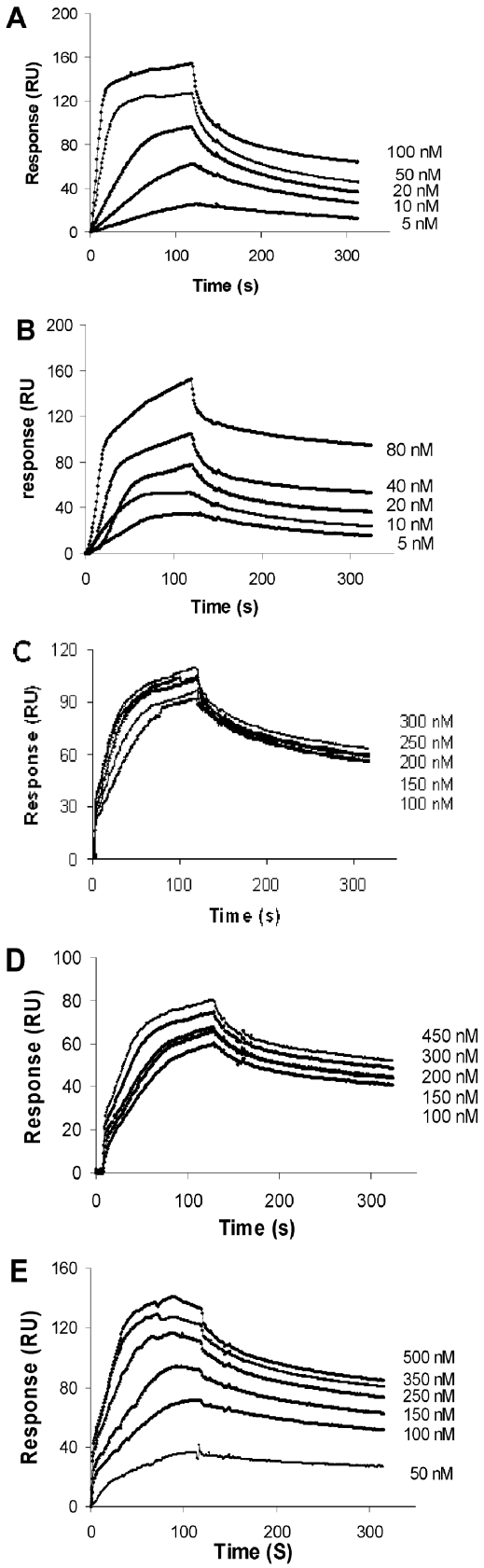

FIGURE 7: SPR analysis of the interaction between HMGa or its deletion mutants and P31. The analysis of the interactions is described in detail in Experimental Procedures. Five or six concentration series were used in a single experiment to obtain the association and dissociation rate constants. The concentrations of the P31 injected are indicated: (A) recombinant HMGa (residues 1−189), (B) mutant 88−123, (C) mutant 115−152, (D) mutant 144−186, and (E) mutant 98−143.

Table 2: Binding Constants of Recombinant HMGa and Its Deletion Mutants with P31

sample	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K_d (M)
HMGa (residues 1−189)	$(7.28 \pm 0.10) \times 10^5$	$(3.57 \pm 0.09) \times 10^{-3}$	$(4.90 \pm 0.31) \times 10^{-9}$
mutant 88−123	$(2.29 \pm 0.05) \times 10^5$	$(1.41 \pm 0.12) \times 10^{-3}$	$(6.33 \pm 0.42) \times 10^{-9}$
mutant 115−152	$(2.06 \pm 0.02) \times 10^5$	$(1.92 \pm 0.05) \times 10^{-3}$	$(9.30 \pm 0.35) \times 10^{-9}$
mutant 144−186	$(1.22 \pm 0.01) \times 10^5$	$(1.53 \pm 0.04) \times 10^{-3}$	$(1.26 \pm 0.26) \times 10^{-8}$
mutant 98−143	$(8.22 \pm 0.06) \times 10^4$	$(1.48 \pm 0.04) \times 10^{-3}$	$(1.80 \pm 0.30) \times 10^{-8}$

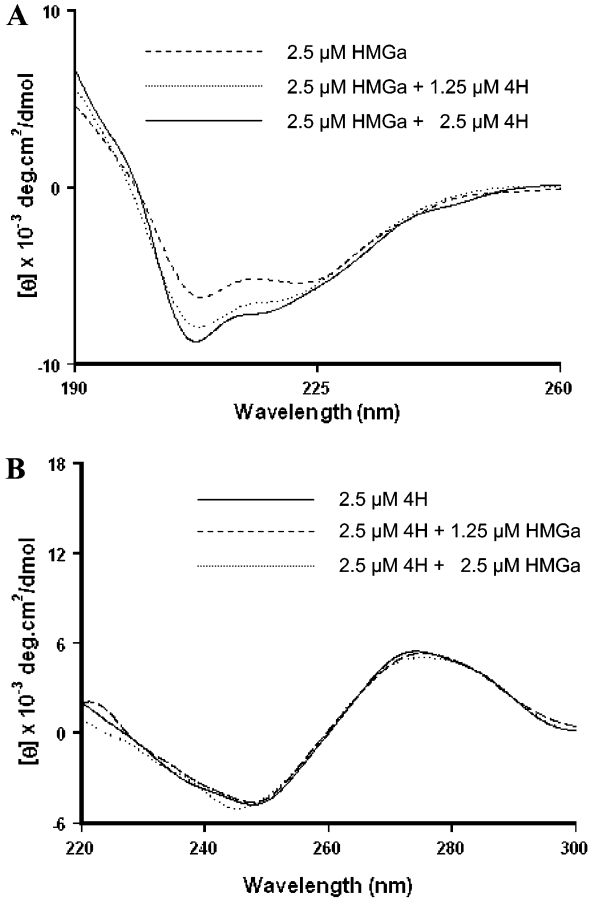

FIGURE 8: Circular dichroism (CD) analysis of the conformation of the recombinant HMGa and 4H after their interaction. (A) Conformational changes of recombinant HMGa after its binding to 4H. The concentrations of HMGa and 4H were indicated. θ refers to the protein mean residue ellipticity. (B) Conformational changes of 4H after its interaction with different concentrations of recombinant HMGa protein. The tested concentrations of 4H and HMGa are indicated. θ refers to the DNA mean base ellipticity.

of P31 decreased between 260 and 285 nm (Figure 9B). Mutant 98−143 also induced similar conformational changes in P31 (data not shown). The decrease in intensity at 275 nm suggests the number of base pairs per turn decreases after the addition of HMGa which may indicate the change in the conformation of DNA from B-form to A-form. Thus, linear DNA, P31, undergoes a conformational change upon binding to HMGa, in contrast to 4H which does not exhibit a significant change in conformation (Figure 8B).

We also studied the conformational changes in P31 that occur upon interaction with deletion mutants of HMGa. Studies were carried out at 2:1 (protein:DNA) ratios. The mean base pair ellipticities of P31 and mutant 1−115 were almost the same as that of free P31 (Figure 9C), which indicates no binding or conformational change. This result is consistent with the gel shift studies (Figure 3). When recombinant HMGa was incubated with P31, the biggest change in ellipticity was observed (Figure 9C). The mean base pair ellipticities of P31 were similar after incubation with mutants 98−143, 88−123, 115−152, and 144−186 (Figure 9C), indicating similar conformational changes in P31. Similar results were also obtained using the binding buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 25 mM NaCl] that was used in EMSA studies (data not shown).

DISCUSSION

Structure−Function Relationships of HMGa. Wheat HMGa has a histone H1-like region at the N-terminus and four AT hooks (Figure 1). The histone H1-like region of wheat HMGa alone does not bind to 4H and P268 (Figures 2 and 3), although both chicken erythrocyte histone H1 and its globular domain bind to 4H with high affinity (*25*) and wheat histone H1 binds to P268 (W. Zhang, unpublished observations). Thus, the overall homology in this region is not sufficient for interaction, but the specific sequence is important for the recognition of 4H and P268.

The binding of deletion mutants to 4H (Figure 2 and Table 1) indicates that mutants with two or more AT hooks bind to the DNAs specifically with different affinities. Thus, any two AT hooks appear to be sufficient for the specific binding to 4H. The SPR binding studies indicate that the affinity of the mutant containing the third and fourth AT hooks (mutant 144−186) is slightly more than 20 times lower than that of recombinant HMGa (Table 1). However, the K_d of mutants containing the first and second (mutant 88−123) or the second and third (mutant 115−152) AT hooks is similar to that of recombinant HMGa (Table 1). These results suggest that the binding of HMGa to 4H is mainly through the first two (or may be three AT hooks) motifs and the role of the fourth (or may be third) AT hook is less important. This proposal is also supported by the small difference in the K_d values of recombinant HMGa (1−189) and mutants 1−178 (without the fourth AT hook) and 1−143 (without the third and fourth AT hooks). The footprinting studies of binding of *Chironomus* HMGA on 4H show that both the first and second AT hooks are protected, but the third AT hook remains unprotected (*12*). In human HMGA protein, however, the second and third, but not the first, AT hooks appear to be involved in binding to 4H (*9*).

For the binding of HMGa to linear DNA, P268, the deletion of the last one or two AT hooks has little effect on the binding pattern (Figure 3C,D). Similarly, the deletion of the fourth AT hook of maize HMGA does not affect its binding to the γ-zein AT-rich tract (*17*). The presence of the two N-terminal AT hooks is sufficient for the binding of rice HMGA to the PE1 region of the promoter of the phytochrome A gene (*18*). However, it is not clear from their results whether the removal of one or both of the remaining N-terminal AT hooks would reduce the affinity. Here we show that mutants containing any two adjacent AT hooks

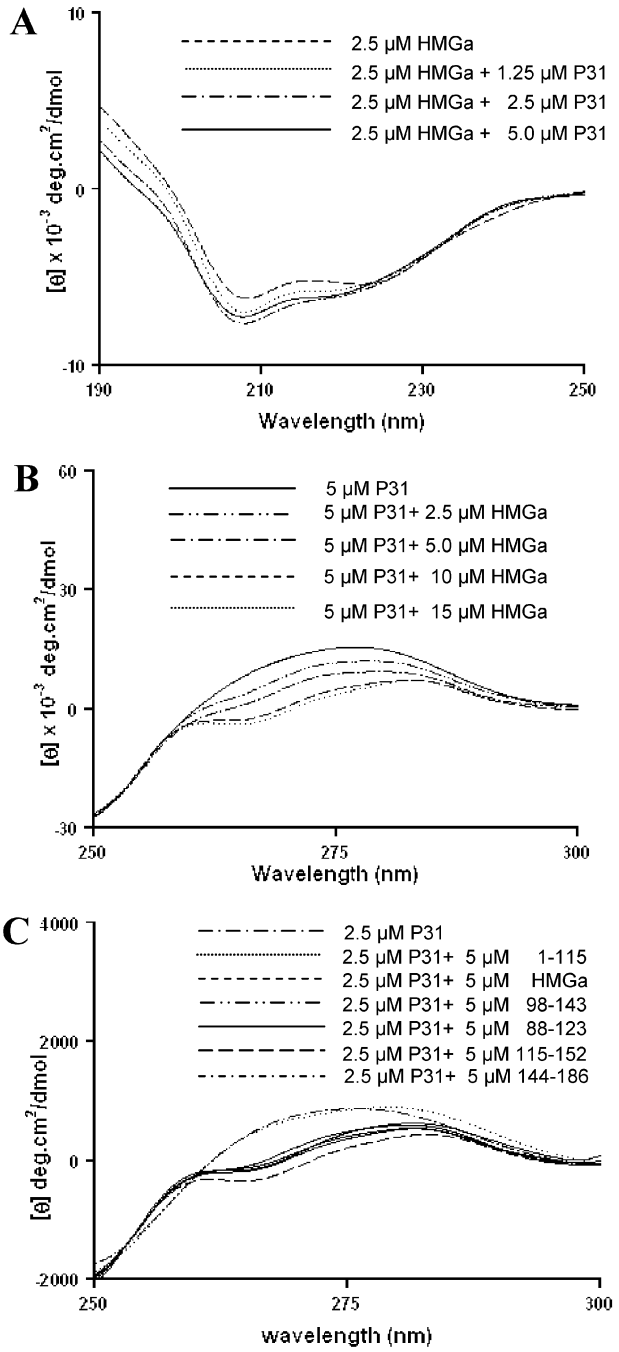

FIGURE 9: Circular dichroism (CD) analysis of the conformational changes of HMGa and P31 after their interaction. (A) Conformational changes of recombinant HMGa after its binding to P31. The concentrations of HMGa and P31 are indicated. (B) Conformational changes of P31 after its interaction with different concentrations of recombinant HMGa protein. The tested concentrations of P31 and HMGa are indicated. (C) CD analysis of the conformational changes of P31 after its interaction with the same concentration of recombinant HMGa protein or its deletion mutants. The tested concentrations of P31 and proteins are indicated. θ refers to protein mean residue ellipticity in panel A and DNA mean base pair ellipticity in panels B and C.

specifically bind to P268 (and P31) (Figures 3I−K and 5). The mutant containing the first two AT hooks shows the greatest binding affinity, and the mutant containing the last two AT hooks shows the lowest binding affinity; these differences are less than 3-fold (Table 2). The mutants containing the first two and last two AT hooks (mutants 88−123 and 144−186) bind simultaneously to different sites on P268 (Figure 4). Consistent with the SPR results, EMSA results also show that the first two AT hooks have greater affinity for P268 (and P31) than the last two AT hooks (Figure 4 and Table 2). Thus, the first two AT hooks may play a major role in the binding of HMGa to P268 (or P31). The similar affinity of mutant 115−152 (containing the second and third AT hooks) and mutant 88−123 for P31 (Table 2) seems to indicate that the third AT hook could also be important for the binding of HMGa to DNA. Thus, the first two (maybe the third) AT hooks play a major role in the binding of HMGa to 4H and P268. Considering the involvement of the third and fourth AT hooks in binding to HMGB proteins as in the case of pea HMG proteins (*23*), the third and fourth AT hooks may have some other functions besides strengthening the binding of HMGa to DNAs.

Role of Flanking Segments. Mutant 98−123 possessing only the second AT hook with its N-terminal part flanking region shows no binding to 4H and P268 (or P31). However, with the addition of the C-terminal flanking amino acids of the second AT hook, mutant 98−143 specifically binds to 4H and P268 (or P31) (Figures 3 and 6). In contrast, another mutant containing the first AT hook and its flanking segments on either side (mutant 1−115) fails to bind to 4H and P268 (Figures 2 and 3). Since the first and second AT hooks are identical (Figure 1A), the distinct binding properties can be attributed to the flanking amino acid residues, although the AT hook is essential for the specific binding. Furthermore, considering the high level of similarity or identity among the four AT hooks of HMGa, the difference in the flanking regions between the two AT hooks may be at least partially responsible for the different affinities of mutants 144−186, 115−152, and 88−123 for 4H and P31 (Figures 2 and 6 and Tables 1 and 2). In human HMGA1a, the second AT hook exhibits a high affinity for a linear DNA when six additional amino acid residues in the flanking segments are included (*4*, *6*). In the footprinting studies of *Chironomus* HMGA binding to 4H, the residues between the second and third AT hooks were protected (*12*).

The similar affinities of recombinant HMGa and mutant 88−123 for 4H demonstrate that the deletion of the N-terminal flanking region of the first AT hook and the C-terminal flanking region of the second AT hook have no apparent effect on the affinity (Table 1). In contrast, the truncated human HMGA1a with only the first and second AT hooks shows dramatic reduction in its affinity for IFN-β (*28*).

Simultaneous Binding and Intramolecular Cooperativity of AT Hooks. Mutant 88−123 possessing two AT hooks shows a high affinity for 4H, whereas mutant 98−123, with the missing first AT hook, shows no binding to 4H (Figure 2). Furthermore, the K_d values of mutants containing any two adjacent AT hooks (except mutant 144−186) are almost 10 times lower than that of mutant 98−143 (Table 1), which only has one AT hook and flanking regions on either side. Thus, the simultaneous binding of AT hooks appears to contribute to the high-affinity specific binding to 4H. The binding of mutant 144−186 to 4H, although with a lower affinity, may also be due to the simultaneous binding and intramolecular cooperativity of AT hooks. Footprinting studies have shown that the two AT hooks of HMGA protein bind to the open conformation of 4H and to the arms on opposite sides of the branch point of 4H (*9*). A similar

binding mechanism could explain the simultaneous binding of AT hooks of wheat HMGa. The distinct properties of mutants 98−123 and 88−123 binding to P268 (Figure 3H,I) also show that the specific binding is due to the cooperativity in the interaction among two AT hooks with AT tracts. The high affinity of HMGA proteins is thought to be due to the simultaneous binding of their AT hooks to properly positioned multiple AT tracts in linear DNAs (*4*, *29*).

Conformational Changes in HMGa and DNAs. Mammalian native HMGA1a (including the AT hooks) has little, if any, detectable secondary structure in solution (*2*). Upon binding to B-form duplex DNA, the AT hook assumes a planar, crescent-shaped configuration dictated by the shape of the narrow minor groove of the DNA substrate (*5*). However, the conformational changes in HMGA proteins upon interaction with 4H have not been studied. The free recombinant HMGa has 23% α-helical structure, and the remaining part is mostly random coil (Figure 8A); the binding of 4H increased its α-helical content by 10%. Once binding to P31 takes place, the α-helix content increases slightly (by ∼3%). In contrast, in mammalian HMGA proteins, the *β*-turn content increases upon binding to the DNA (*2*). Thus, DNA binding induces different conformational changes in plant and mammalian HMGA proteins. These differences could be due to the structural differences, such as the presence of the H1-like region of plant HMGA protein and the difference in the flanking segments of AT hooks, between these two groups of proteins.

The binding of HMGA proteins to linear DNA induces bending, straightening, and unwinding or loop formation (*2*). The HMGA protein also changes topological conformations and introduces supercoils into the relaxed plasmid DNAs *in vitro* (*2*). Therefore, we studied the conformational change in P31 after it binds to HMGa (Figure 9B). Recombinant HMGa and mutants that bind to P31 induce conformational changes in the DNA by unwinding the DNA. The full-length protein induces more conformational changes than its deletion mutants (Figure 9C), indicating more unwinding of the linear DNA is induced by a larger number of AT hooks. The conformational changes in relatively short fragments containing multiple AT-rich binding sites are probably due to asymmetric neutralization of charges on the phosphodiester backbone of the DNA by multiple AT hooks (*2*). Such DNA conformational changes induced by the binding of HMGA may have biological implications. For example, HMGA proteins promote gene activation by acting as architectural cofactors to facilitate formation of enhanceosomes on the promoter and/or enhancer regions. Human HMGA induces conformational changes within the IFN-*β* enhancer and recruits transcription factors (*30*). Rice HMGA protein, probably through bending the DNA, enhances the specific DNA binding of GT-2, a transcriptional activator of the phytochrome A gene (*PHYA*), to the GT-box element (*31*). The binding of HMGa induces the conformational change in P31 by decreasing the number of base pairs per turn of DNA and relaxing the P31 (Figure 9B,C). Webster et al. (*23*) shows that HMGA and HMGB cannot bind simultaneously to P31 and HMGA interacts with HMGB through its C-terminal AT hooks. Our results show the major role of the first two AT hooks on the binding to the DNAs. This may mean different AT hooks of HMGA protein have different functions. The unwinding of P31 by the binding of HMGa mainly through its N-terminal AT hooks may allow other transcription factors to be recruited to bind to the DNA.

Interestingly, there is no change in the conformation of 4H after it binds to HMGa (Figure 8B). HMGA proteins bind to the open conformation of 4H (*11*, *25*). The rigidity of the open form structure of 4H may explain the lack of conformational changes in 4H as well as greater conformational changes in HMGa upon binding. The flexible conformation of the linear DNA allows the protein to undergo a smaller conformational change.

The difference in the strength of the binding of HMGa and its three mutants containing two adjacent AT hooks to P31 is less than 3-fold (Table 2). However, their binding affinities for 4H vary significantly (as much as almost 20-fold, Table 1). The significant differences in the affinity change of HMGa and its mutants upon their binding to 4H and P31 could also be correlated to the rigidity and/or flexibility of the DNAs and the distance between the AT hooks. In the case of 4H, the less flexible of the two DNAs, the distance between the binding sites on DNA may be most suited for AT hooks separated by 19 amino acid residues. As the number of residues between the AT hooks increases, the affinities for 4H change significantly. The rigidity of 4H may not allow a significant change in the distances between the binding sites. On the other hand, P31 is relatively more flexible, and hence, the changes in the distance between the AT hooks are easily accommodated by minor changes in the conformation in the DNA. Upon binding to HMGa and its mutants, P31 DNA undergoes conformational changes and exhibits unwinding. Thus, the flexibility of the DNAs along with the spacing of AT hooks compared to binding sites on DNA molecules may play a crucial role in recognition and interaction between wheat HMGa and DNA.

The truncation of HMGa protein produced the same relative effects on its binding to both 4H and linear DNA. The different binding characteristics displayed in the binding of HMGa and its truncated forms to 4H and linear DNA may be due to the distinct properties of the DNAs.

ACKNOWLEDGMENT

We thank Dr. Seow Teck Keong and Mr. Lee Yeow Fong for their help in BIACORE experiments.

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BI034280H