J. Biochem. 122, 586-594 (1997)

Difference in Affinity for DNA between HMG Proteins 1 and 2 Determined by Surface Plasmon Resonance Measurements[1]

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Received for publication, April 3, 1997

High mobility group (HMG) proteins 1 and 2 contain two similar but non-identical repeats of DNA-binding domains and an acidic C-terminal. The proposed functions of HMG proteins 1 and 2 imply a probable difference in their DNA-binding abilities. The primary studies by gel retardation assay showed that HMG2 has higher affinity than HMG1 for supercoiled and linear DNA. The DNA-binding of HMG2 appeared strong enough to allow exchange with HMG1 molecule already bound to DNA, while the DNA-binding region of HMG1 showed higher affinity than that of HMG2. In order to compare more quantitatively the affinities, surface plasmon resonance (SPR) measurements using a BIAcore instrument were conducted. The kinetic data indicated that the K_d for the complex of HMG2 with DNA is smaller than that of HMG1, in contrast to the situation for the DNA-binding region of these proteins. The sequence between the second DNA-binding domain and the acidic C-terminal of HMG proteins is required for tight DNA-binding. Also, the acidic C-terminal strongly modulates the DNA-binding ability of each protein. The usefulness of SPR measurement for quantitative analysis of affinity and regions involved in DNA-binding under conditions nearly identical to those in solution is discussed.

Key words: DNA-binding protein, HMG proteins, surface plasmon resonance.

High mobility group (HMG) proteins 1 and 2 are closely related nuclear non-histone chromosomal proteins which show highly conserved primary sequences in different mammalian species (reviewed in Refs. *1* and *2*). The proteins have characteristic structures consisting of two similar, but non-identical, repeats of DNA-binding domains containing about 76 amino acid residues and an acidic C-terminal region containing a continuous run of 30 acidic amino acids in HMG1 and 23 in HMG2 (*3, 4*). The DNA-binding domains mediate non-specific binding to DNA, as well as preferential interaction with non-B DNA conformations such as the four-way junction (*5*), cruciform (*6, 7*), and B-Z junction (*8*). They have also been discovered in several transcription factors where they are involved in sequence-specific recognition of promoter elements (*9*). The NMR structure of DNA-binding domain B of HMG1 has a characteristic L-shaped fold formed by three α-helices (*10, 11*). Precisely how the HMG proteins as well as their DNA-binding domains bind to DNA is not known, as no structure of a relevant protein-DNA complex has yet been determined.

The HMG proteins have been considered to be implicated in transcription, replication, and cellular differentiation (reviewed in Refs. *1, 12*, and *2*). Recent observations have suggested that HMG1 protein plays a direct role in transcriptional events on the genes and/or functions as a quasi-transcription factor (*13-15*; and references cited therein). We have assessed the transcriptional activation potential of HMG1 protein using cultured cell systems and shown that the acidic carboxyl terminus is essential for enhancement of gene expression in addition to elimination of the repression caused by DNA-binding (*14*). HMG1 protein, but not HMG2, may be a gene quasi-activator that modulates chromatin structure to orient the respective gene, thus ensuring that its activity as a template is expressed fully (*15*). In contrast, the function of HMG2 in living cells has not been definitely clarified. The level of HMG2 protein parallels the proliferation activity of several organs, suggesting that HMG2 plays a role in cell replication (*16*). The expression level of the HMG2 gene during the cell cycle is markedly enhanced in the post-S phase and reaches a maximum at the G2 phase. Progression of the cell cycle in COS-1 cells was repressed during G1 to S phase by expression of the antisense RNA for HMG2, resulting in a decrease of cell growth, suggesting that fluctuation of the HMG2 message during the cell cycle is not a consequence of, but a prerequisite for cell proliferation (*17*).

These proposed functions of HMG1 and 2 proteins predicted us a probable difference in their abilities to bind DNA. However, very few comparative studies of their DNA-binding ability have been reported, probably because the high degree of homology in the primary sequences between their DNA-binding regions was expected to result in no appreciable difference employing usual observation

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant from the Science Research Promotion Fund from Japan Private School Promotion Foundation.

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Abbreviations: bp, base pair(s); HMG, high mobility group; RU, response unit(s); SPR, surface plasmon resonance.

procedures. In this connection, Bustin and Soares (*18*) reported that HMG2 displayed a greater interaction than HMG1 with negatively supercoiled DNA using a nitrocellulose binding assay. However, spontaneous contamination of HMG1 or HMG2 with "HMG3" could have resulted in greater interactions with double-stranded DNA (*19*).

In the present study using a gel retardation assay, we obtained preliminary evidence that HMG2 may have higher affinity than HMG1 for negatively supercoiled as well as linear DNA. This was confirmed by quantitative measurements of surface plasmon resonance (SPR) using a BIAcore instrument. We also demonstrated a substantial involvement of the sequences adjacent to the DNA-binding regions and acidic carboxyl-terminal regions in the differing binding affinities of HMG1 and HMG2 proteins for DNA.

MATERIALS AND METHODS

Preparation of Proteins and Peptides—HMG1 and HMG2 were prepared from pig thymus chromatin as described previously (*20*)—Peptides AlBj (amino acid residues 1-181 of HMG1 and 2, respectively) with a truncated acidic carboxyl-terminal region and AlB (amino acid residues 1-164 of HMG1 and 2, respectively) were overexpressed in *Escherichia coli* BL21 cells transfected with the pGEM plasmids carrying the corresponding cDNA sequences downstream from the T7 promoter, and purified to homogeneity (Tanabe *et al.*, in preparation).

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 (*21*) and purified by CsCl-ethidium bromide centrifugation (*22*). Linearized DNA (form III DNA) was prepared by digestion with *Hin*dIII.

Gel Retardation Assay—Agarose gel electrophoresis of the complexes of plasmid pBR322 DNA with HMG or the peptides was performed as a function of the HMG/DNA molar ratio. An aliquot of HMG protein 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 dithiothreitol, 10% glycerol, and 40 μg of bovine serum albumin. The solution of total volume 18 μl was incubated at 25°C for 60 min and electrophoresed on a 0.7% agarose gel in 40 mM Tris-acetate containing 1 mM EDTA. After electrophoresis, the gel was stained with ethidium bromide.

Surface Plasmon Resonance (SPR) Measurement—The BIAcore biosensor (Pharmacia) has the potential to measure the real-time interaction of a protein molecule with DNA. The protein injected in a flow of buffer solution interacts with the DNA immobilized on a dextran matrix. The binding event is monitored using SPR detection (*23*), and the resulting binding curve and the dissociation curve with successive injection of the buffer alone can be used to determine the kinetic parameters of the interaction. BIAcore sensor chip SA (Pharmacia Biosensor) surfaces with streptavidin pre-immobilized to dextran were used. A continuous flow of TME (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM EDTA) containing 100 mM NaCl was maintained at 5 μl/min. A 30-bp DNA with one strand biotinylated at the 5′-end of the sequence 5′-TGTATGAAATCTAACAATGCGCTCATCGTC-3′ was obtained from Sawady Technology (Tokyo). DNA was diluted to 0.1 μg/ml 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 between 800 and 1,000 response units (RU) of the synthetic DNA. The HMG (or the peptides derived from it) was diluted at various concentrations in TME containing 100 mM NaCl. To measure the association in TME containing 100 mM NaCl, an injection command was used which allowed consecutive injection of the sample with no intermediate delay. The dissociation was measured by injecting TME containing 100 mM NaCl but no HMG (or the peptides). At the end of each experiment, 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 values were recorded at 0.2-s intervals.

Data were analyzed using the BIAevaluation software package, which was supplied with the BIAcore. A treatment of the molecular binding kinetics for BIAcore has been presented previously (*23, 24*) and used for the recent kinetic analysis of lactose repressor-operator DNA interaction (*25*) and the action of the co-repressor in the *E. coli* methionine repressor-operator complex (*26*). Similar treatments were used for the present analysis. Briefly (*26*), when HMG (or the peptide) is injected over the sensor chip surface, the solution in contact with the DNA is constantly being replenished and the protein concentration can therefore be thought of as constant and equal to the concentration of HMG in the solution being injected. The reaction between immobilized DNA and HMG is therefore assumed to follow pseudo-first-order kinetics described by the equation

$$dR/dt = k_{ass}C(R_{max} - R) - k_{diss}R \quad (1)$$

Where dR/dt is the rate of complex formation, k_{ass} is the apparent association rate constant of DNA-HMG (or the peptide), k_{diss} is the apparent dissociation rate constant, R is the amount of bound HMG, R_{max} is the maximum HMG binding capacity of the surface, and C is the HMG concen-

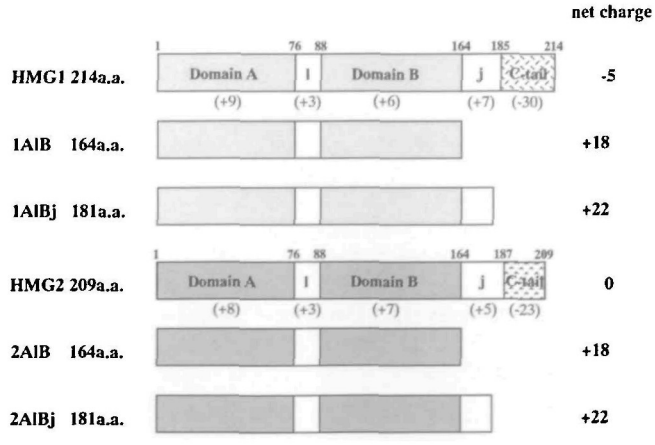

Fig. 1. **Schematic representation of HMG1, HMG2, and their peptides containing the DNA-binding domains expressed in *E. coli*.** Above, the structures of the intact HMG1 and HMG2 proteins are shown with domain A, domain B, l (linker)-region, j (joiner)-region, and C (carboxyl)-tail. Below, the structures of peptides AlB of HMG1 (1AlB) and HMG2 (2AlB), and peptides AlBj of HMG1 (1AlBj) and HMG2 (2AlBj). The positive and negative numbers in brackets are the total net charge of the respective domains or regions of HMG proteins and their peptides. Amino acid residues are abbreviated as a.a. in this figure.

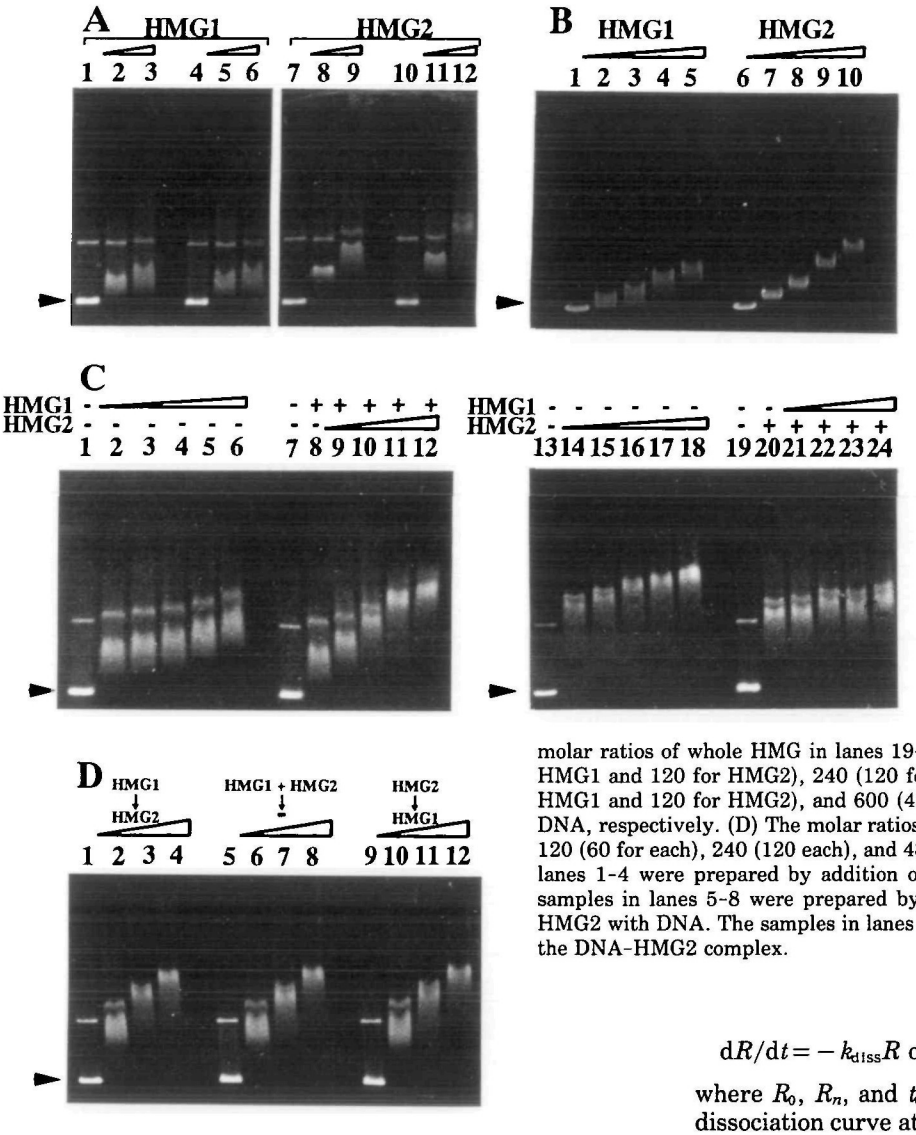

Fig. 2. **Gel retardation of DNA by HMG1, HMG2, and a mixture of the two proteins.** HMG1, HMG2, and or both were complexed with form I (negatively supercoiled) and form III (linearized) pBR322 DNA at different molar ratios of protein to DNA, and separated by agarose gel electrophoresis. (A) The molar ratios of HMG1 in lanes 1-3 and 4-6, and HMG2 in lanes 7-9 and 10-12 to form I DNA (marked by an arrow) were 0, 60, and 120, respectively. The HMG1 proteins in lanes 1-3 and 4-6, and HMG2 in lanes 7-9 and 10-12 were prepared independently. (B) The molar ratios of HMG1 in lanes 1-5 and HMG2 in lanes 6-10 to form III DNA (marked by an arrow) were 0, 60, 120, 240, and 480, respectively. (C) The molar ratios of HMG1 in lanes 1-6 were 0, 120, 180, 240, 360, and 600 to form I DNA (marked by an arrow). The molar ratios of whole HMG in lanes 7-12 were 0, 120 (HMG1 alone), 180 (120 for HMG1 and 60 for HMG2), 240 (120 for HMG1 and 120 for HMG2), 360 (120 for HMG1 and 240 for HMG2), and 600 (120 for HMG1 and 480 for HMG2) to form I DNA, respectively. The molar ratios of HMG2 in lanes 13-18 were 0, 120, 180, 240, 360, and 600 to form I DNA (marked by an arrow). The molar ratios of whole HMG in lanes 19-24 were 0, 120 (HMG2 alone), 180 (60 for HMG1 and 120 for HMG2), 240 (120 for HMG1 and 120 for HMG2), 360 (240 for HMG1 and 120 for HMG2), and 600 (480 for HMG1 and 120 for HMG2) to form I DNA, respectively. (D) The molar ratios of HMG in lanes 1-4, 5-8, and 9-12 were 0, 120 (60 for each), 240 (120 each), and 480 (240 each) to form I DNA. The samples in lanes 1-4 were prepared by addition of HMG2 to the DNA-HMG1 complex. The samples in lanes 5-8 were prepared by mixing the solution containing HMG1 and HMG2 with DNA. The samples in lanes 9-12 were prepared by addition of HMG1 to the DNA-HMG2 complex.

tration in the solution. Rearranging Eq. 1 shows that the derivative of the binding curve is a linear function of the response.

$$dR/dt = k_{ass}CR_{max} - (k_{ass}C + k_{diss})R \quad (2)$$

In theory, k_{ass} and k_{diss} can be calculated by plotting dR/dt *vs.* R. However, determination of R_{max} requires prohibitively high concentrations of protein. To circumvent this problem, plots of dR/dt are made for a range of protein concentrations. The slopes k_s, from each of these lines are plotted against C where

$$-k_s = k_{ass}C + k_{diss} \quad (3)$$

The slope of this line yields the apparent association rate constant and the y-intercept the apparent association rate constant. However, for low values of k_{diss} the intercept is too close to the origin to allow accurate determination. 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. 1 becomes

$$dR/dt = -k_{diss}R \text{ or } \ln(R_0/R_n) = K_{diss}(t_n - t_0) \quad (4)$$

where R_0, R_n, and t_0 and t_n are values obtained along the dissociation curve at times 0 and n. The apparent dissociation rate constant can be obtained from the slope of the line $\ln(R_0/R_n)$ *vs.* $(t_n - t_0)$. The ratio of the apparent rate constants allows the apparent equilibrium constant to be calculated, $K_d = k_{diss}/k_{ass}$.

RESULTS

Larger Gel Retardation of DNA by HMG2 than by HMG1—Pig HMG1 and HMG2 show 79% homology in their primary structures (*3, 4*) and are considered to be sister proteins with similar characteristics. As shown in Fig. 1, these proteins contain two repeated DNA-binding domains with non-identical amino acid sequences (domain A: residues 1-76, and domain B: residues 88-164) connected by a short linker (l) region (residues 77-87), respectively. In addition, a unique carboxyl terminal region (C- tail) consisting of a continuous run of acidic amino acids (30 residues for HMG1 and 23 for HMG2) is connected to the DNA-binding domain B through a joiner (j) region.

In order to determine whether any difference exists in the DNA-binding properties of the two proteins, gel retardation analyses were conducted using HMG proteins extracted from pig thymus and plasmid pBR322 DNA. Figure

2 shows representative electrophoretic patterns on agarose gel of complexes of HMG1 and HMG2 with forms I (negatively supercoiled) and III (linearized) DNA. HMG proteins complexed with DNA showed band retardation as the amount of protein increased. The gel retardation of form I DNA by HMG2 was considerably larger than that by HMG1 when compared at an equimolar ratio of HMG to DNA (Fig. 2A). Similar results were obtained for form III DNA, as shown in Fig. 2B. In order to examine whether the degree of gel retardation by one HMG is influenced by the presence of the other HMG, a sample containing both proteins was subjected to the gel retardation assay. When compared at equimolar ratios of HMG to DNA, the gel retardation of form I DNA (Fig. 2C, left) shown by samples containing HMG1 alone (lanes 3-6) was smaller than that of samples containing HMG2 (lanes 9-12). In contrast, the gel retardation shown by samples containing only HMG2 (Fig. 2C, right, lanes 15-18) was markedly larger than that of samples containing HMG1 (lanes 21-24). Furthermore, the profiles of gel retardation, which were unchanged upon reversed order of mixing of the two HMG proteins with form I DNA (Fig. 2D), were similar to those for HMG2 (Fig. 2C, lanes 13-18). The molecular weights of the proteins did not seem to have an effect on the gel retardation. Therefore, these results suggest the following possibilities: (1) HMG2 has a higher affinity than HMG1 for DNA. (2) The amount of HMG2 tightly bound to DNA is larger than that of HMG1. In addition, the HMG1 molecule, once bound to DNA, is exchangeable with HMG2, which has a higher affinity for DNA. (3) The DNA conformations produced by binding with HMG1 and HMG2 are sufficiently different to be distinguishable in the gel retardation assay. (4) The differences in total net charges between HMG1 and 2 affect the relative mobility of the DNA-HMG complex on agarose gel.

Difference in Dissociation Constant (K_d) Obtained by Gel Retardation Assay of DNA-HMG Complexes—To compare more precisely the DNA gel retardation produced by HMG

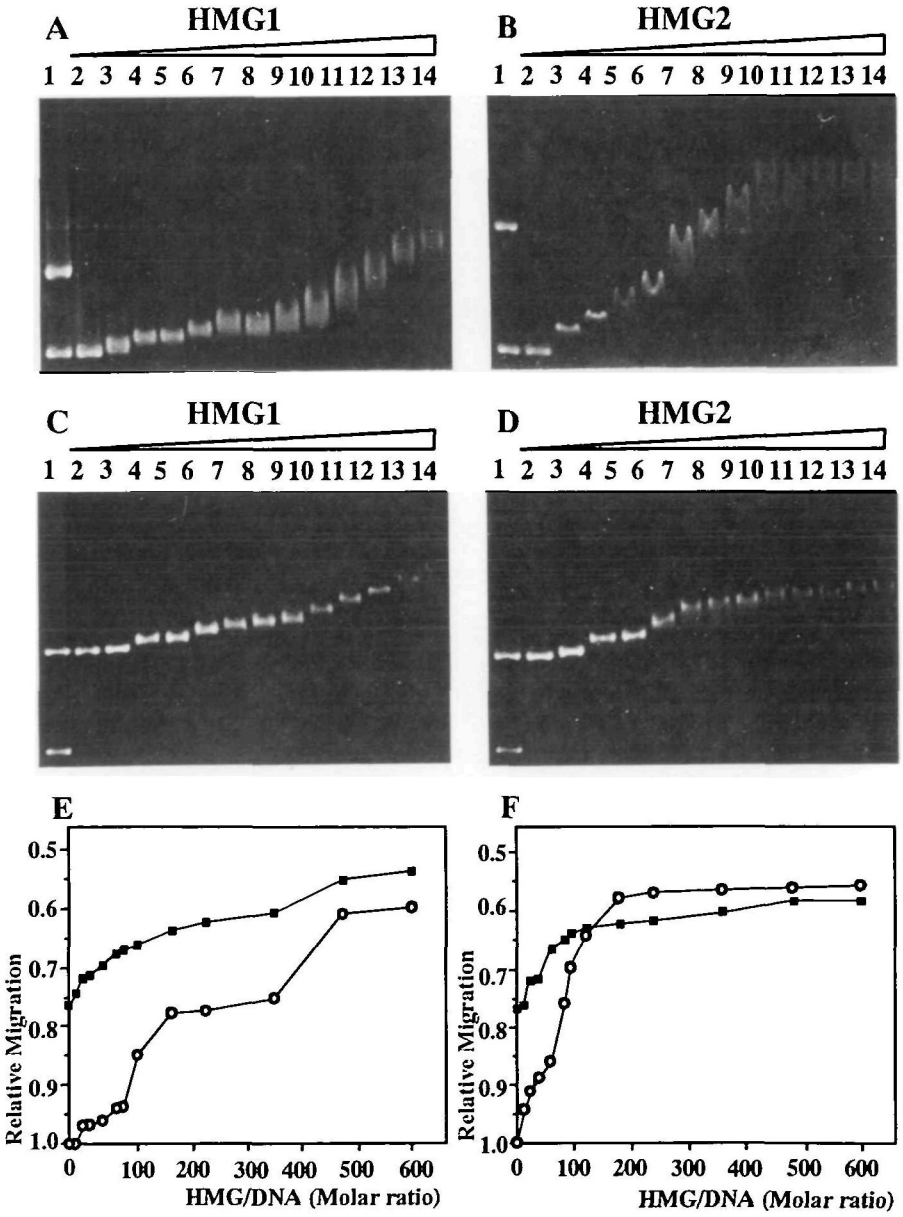

Fig. 3. **Gel retardation of DNA by HMG1 and HMG2 proteins.** HMG1 and HMG2 were complexed with form I (negatively supercoiled) and form III (linearized) pBR322 DNA at different molar ratios of protein to DNA, and separated by agarose gel electrophoresis. The molar ratios of HMG to DNA in lanes 2-14 were 0, 12, 24, 36, 60, 84, 96, 120, 180, 240, 360, 480, and 600, respectively. Lane 1 contained marker DNAs of forms I and III. (A) The complexes of form I DNA with HMG1. (B) The complexes of form I DNA with HMG2. (C) The complexes of form III DNA and HMG1. (D) The complexes of form III DNA and HMG2. (E and F) The relative migrations of DNA-HMG1 and DNA-HMG2 were plotted against the molar ratios of HMG to DNA, respectively. Clear circles show the complexes of form I DNA, and solid squares the complexes of form III DNA.

proteins, DNAs complexed with serial amounts of HMG were analyzed as shown in Fig. 3. With increasing amounts of HMG1 and HMG2, the DNA bands became progressively more retarded (Fig. 3, A–D). The retardation of form I DNA (Fig. 3, A and B) was greater than that of form III DNA (Fig. 3, C and D) at equimolar ratios of HMG to DNA. The relative migration of the bands was plotted against the amount of HMG added to the reaction mixture for comparison. The relative migration of form I DNA reached maximum at 480 for HMG1 and 180 for HMG2 in terms of HMG/DNA molar ratio. The K_d calculated from the midpoints of the ratio to give the maximum was $1.5\pm0.3\times10^{-6}$ M for HMG1 and $7.8\pm0.4\times10^{-7}$ M for HMG2, suggesting that HMG2 had higher affinity than HMG1 for form I DNA. However, the relative migrations appeared biphasic for both HMGs. The K_d values for HMG2 in the first phase ($1.1\pm0.2\times10^{-6}$ M for HMG1 and $1.8\pm0.3\times10^{-7}$ M for HMG2) as well as the second phase ($3.7\pm0.5\times10^{-6}$ M for HMG1 and $1.0\pm0.3\times10^{-6}$ M for HMG2) were smaller than those for HMG1. The K_d value of HMG2 for form III DNA was also smaller than that of HMG1. These results suggest that HMG2 has a higher affinity than HMG1 for DNA.

DNA-Binding Region of HMG1 Shows Higher Affinity than That of HMG2 for DNA by Gel Retardation Assay—Each of the HMG proteins has two DNA-binding domains. The net charges of these DNA-binding regions are similar, as shown in Fig. 1. Therefore, differences in the net charge of the j-region and the length of the acidic C-tail may affect the gel retardation of the complexes. In order to examine this possibility, we overexpressed and isolated a peptide AlB (amino acid residues 1–164), which has truncated j-region and C-tail, and another peptide AlBj (1–181) which has a truncated C-tail. DNA complexed with serial amounts of the peptides was subjected to gel retardation analysis, as shown in Fig. 4. Surprisingly, with increasing amounts of the peptides, the band shifts of complexes with peptides AlB and AlBj (Fig. 4, A, D, B, and E) were increased in comparison with those of the full-length HMGs (Fig. 4, C and F). These results suggested that the acidic C-tail modulates the ability of HMG to bind DNA. In addition, the gel retardation of forms I and III DNA complexed with peptides AlBj (Fig. 4, B and E) was more marked than that with peptides AlB (Fig. 4, A and D), indicating that the j-region functions to strengthen the DNA-binding. The larger gel retardation of the complexes with peptides 1AlB than those with 2AlB (Fig. 4, A and C) suggests that the DNA-binding region of HMG1 has higher affinity than that of HMG2, in contrast to the whole HMG molecules. In addition, greater gel retardation of the complexes with peptides 1AlBj than those with 2AlBj was observed (Fig. 4, B and E). However, the complexes formed at higher ratios of peptide AlBj to DNA migrated in another direction from the slots in which the samples were applied. These results

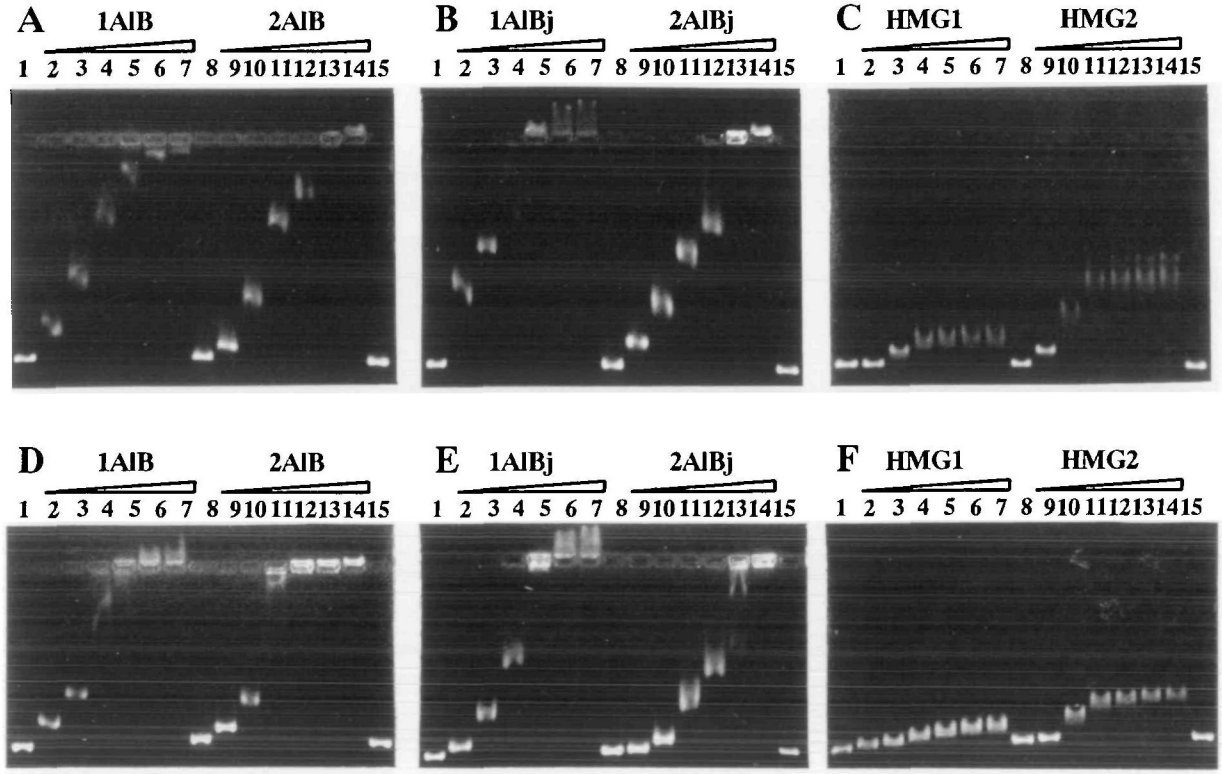

Fig. 4. **Gel retardation of DNA by peptides AlBj and AlB.** Peptides AlBj and AlB were complexed with form I (negatively supercoiled) and form III (linearized) pBR322 DNA at different molar ratios of peptide (or protein) to DNA, and separated by agarose gel electrophoresis. The molar ratios of peptide (or HMG protein) to DNA in lanes 1–7 and 8–14 were 0, 100, 200, 400, 600, 800, and 1,000, respectively. Lane 15 contained marker DNAs of forms I or III. (A and D) Lanes 1–7 and 8–14 contained the complexes of peptides 1AlB and 2AlB, respectively. (B and E) Lanes 1–7 and 8–14 contained the complexes of peptides 1AlBj and 2AlBj, respectively. (C and F) Lanes 1–7 and 8–14 contained the complexes of HMG1 and HMG2, respectively. Form I DNA was used for gel retardation in (A), (B), and (C), and form III in (D), (E), and (F).

strongly indicated that the differences in net charge of HMG proteins and their peptides affect the migration of their complexes, resulting in incorrect determination of their binding abilities.

Difference in K_d Values for Complexes of HMG and Their Peptides with DNA Measured by Surface Plasmon Resonance (SPR)—The gel retardation assay has been generally considered a useful procedure for analyzing the binding of proteins with DNA. However, the above results indicated that the binding profiles must be influenced by factors such as the net charge of protein bound to DNA and possible alterations in the conformation of the DNA-protein complex. In order to compare quantitatively the affinities of HMG or its peptides for DNA and to analyze the binding mechanism more precisely under conditions that are nearly identical, measurements of surface plasmon resonance (SPR) using the recently developed Pharmacia BIAcore instrument system are useful. To obtain kinetic data under conditions of effector-dependent binding, it was first necessary to establish the concentrations of the different effectors. In these experiments, a linear, 30-bp DNA fragment homologous with nucleotides 83-112 of pBR322 DNA, immobilized on the sensor chip surface, was prepared from complementary, annealed oligonucleotides. Multiple sensorgrams were collected, keeping the DNA concentration constant at 800 RU, and the concentration of effector (*i.e.* HMG or truncated peptides) was varied between 1 and 10 μM. Typical sensorgrams of HMG1 and HMG2, corrected for changes in bulk solution refractive index, are shown in Fig. 5. The absence of significant differences in the shape of the real-time binding curves for interaction of HMG1 and HMG2 with 30-bp DNA fragment suggested that the association reactions follow the same kinetics. For fitting an appropriate model to provide reasonable insight into the

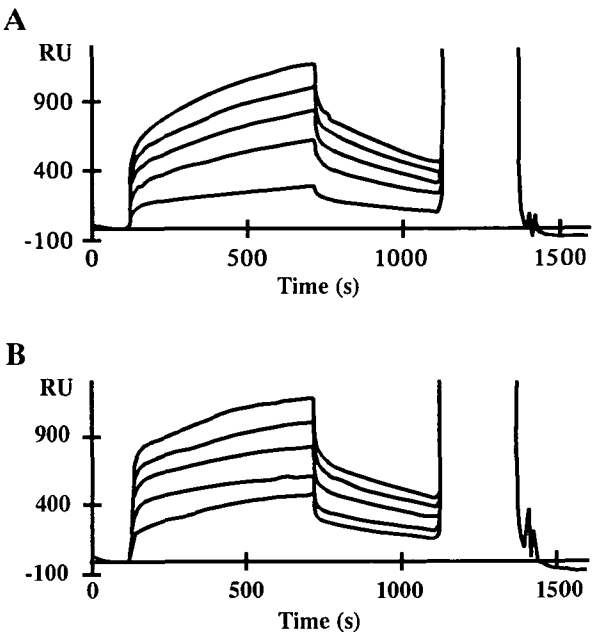

Fig. 5. **Superimposed sensorgrams obtained at five concentrations of HMG1 (A) and HMG2 (B) interacting with a 30-bp DNA fragment immobilized on the sensor chip surface.** After initial equilibration of the sensor chip surface with TME containing 100 mM NaCl, HMG1 (A) at 5 different concentrations of 1.5, 3.0, 4.5, 6.0, and 7.5 mM (bottom to upper lines) and HMG2 (B) at 1.0, 2.0, 3.0, 4.0, and 5.0 mM in the same solution were injected at 170 s. The sample pulse was replaced by TME containing 100 mM NaCl at 720 s, and the dissociation of bound peptides was followed until about 1,100 s. Thereafter, the surface was regenerated with 8 μl of 2 M NaCl.

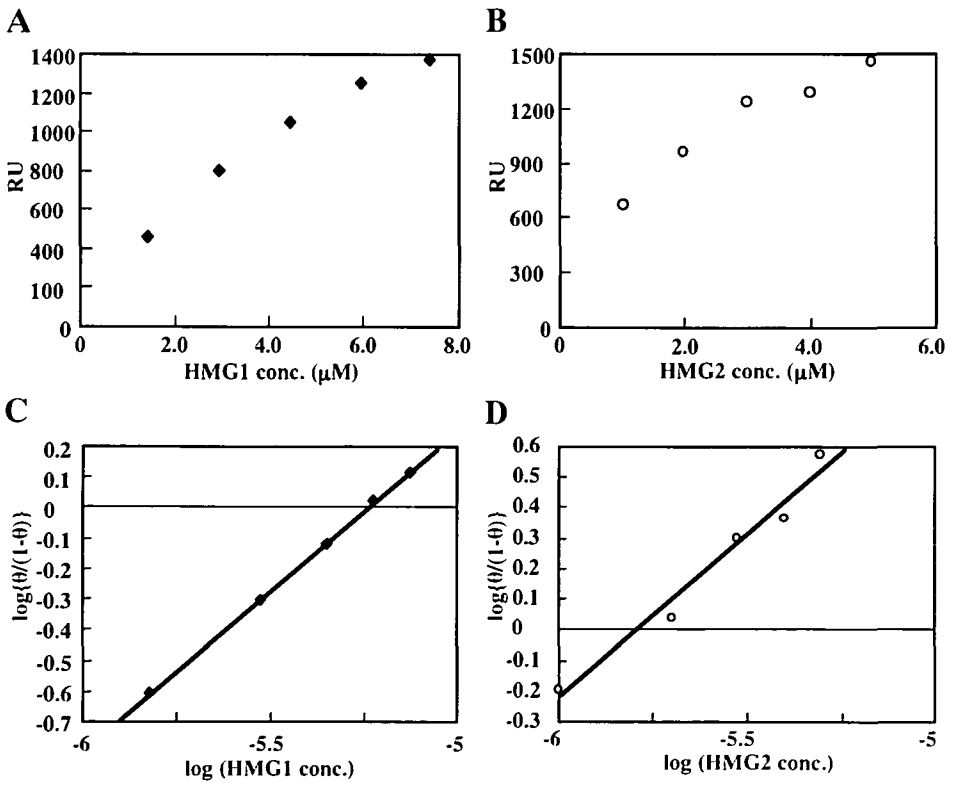

Fig. 6. **Determination of the Hill coefficient for interaction of HMG1 or HMG2 with 30-bp DNA fragment.** The extrapolated responses (RU) at infinite time of sensorgrams in Fig. 5 were plotted against HMG1 (panel A) or HMG2 (panel B) concentration. Hill plots of the binding data for HMG1 (panel C) and HMG2 (panel D), where q is the fractional saturation of the DNA $\{q=1-[\text{free DNA}]/[\text{total DNA}]\}$. The straight lines have slopes of 1.01 for HMG1 (panel C) and 1.05 for HMG2 (panel D).

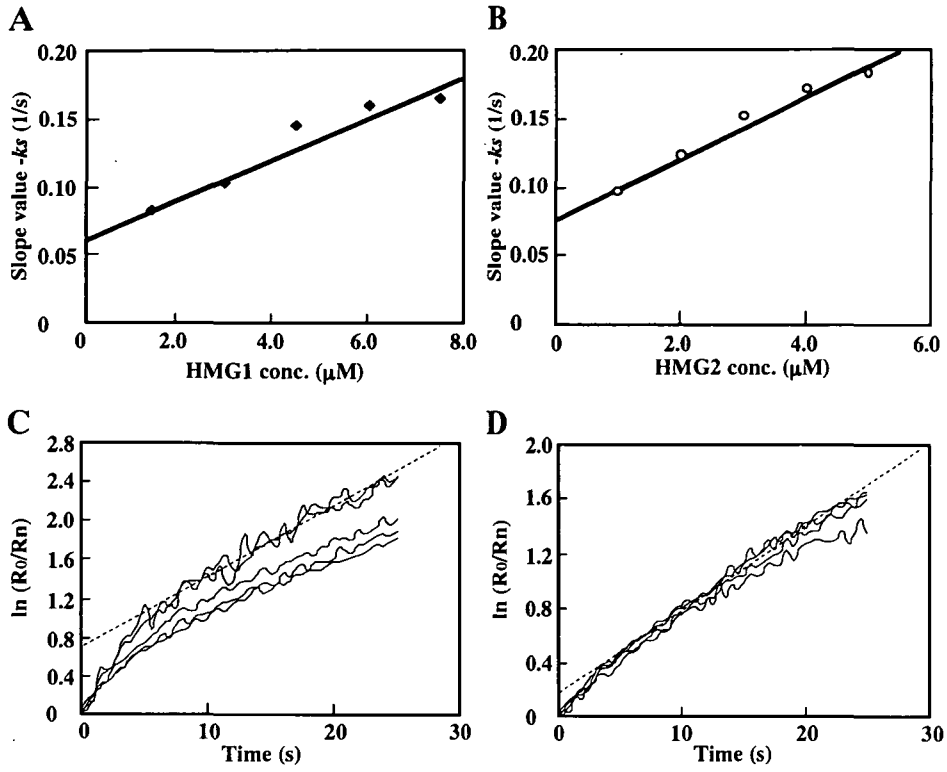

Fig. 7. **Panels A and B: Association rate constants of the complex of HMG1 (panel A) or HMG2 (panel B) with 30-bp DNA.** The binding rate (dR/dt) as a function of the response (R) for each HMG concentration was plotted. The slopes (k_s) of the resulting straight lines were then calculated. By plotting the slope values (k_s) *vs.* the HMG concentrations at which they were determined, k_{ass} was obtained as the slope of the resulting straight line. **Panels C and D: Dissociation rate constants of the complex of HMG1 (panel C) or HMG2 (panel D) with 30-bp DNA.** The amount of dissociated HMG was determined, starting at 720 s in Fig. 5. Data from each HMG concentration were used for calculation of $\ln(R_o/R_n)$. For determination of k_{dlss} the concentration-independent parts of the curves were selected.

TABLE I. **Affinity and kinetic data calculated from the SPR binding data for HMG proteins and their peptides.**

Protein or peptide	k_{ass} ($M^{-1}\cdot s^{-1}$)	k_{dlss} (s^{-1})	K_d (M)
1AlB	$2.9\pm0.2\times10^4$	$2.2\pm0.4\times10^{-2}$	$7.5\pm1.7\times10^{-7}$
2AlB	$1.2\pm0.1\times10^4$	$1.3\pm0.2\times10^{-2}$	$1.1\pm0.1\times10^{-6}$
1AlBj	$2.0\pm0.8\times10^4$	$2.0\pm1.0\times10^{-2}$	$9.5\pm3.7\times10^{-7}$
2AlBj	$1.5\pm0.1\times10^4$	$1.4\pm0.1\times10^{-2}$	$9.0\pm0.7\times10^{-7}$
HMG1	$1.6\pm0.2\times10^4$	$6.8\pm0.3\times10^{-2}$	$4.3\pm0.5\times10^{-6}$
HMG2	$2.0\pm0.1\times10^4$	$5.9\pm0.5\times10^{-2}$	$3.0\pm0.2\times10^{-6}$

nature of a binding reaction, it was necessary to obtain the stoichiometry of the protein-DNA interaction. Each binding to the DNA fragment did not reach equilibrium within the time studied (Fig. 5). Then, the dRU/dt values were plotted. The extrapolated responses at infinite time in various protein concentrations were obtained, as shown in Fig. 6, A and B. The Hill coefficients (*27*) calculated from the data were 1.01 for HMG1 (Fig. 6C), 1.05 for HMG2 (Fig. 6D), 1.01 for peptide 1AlB, 1.05 for peptide 2AlB, 0.98 for peptide 1AlBj, and 1.05 for peptide 2AlBj. These coefficients, close to 1, indicated that the interaction of HMG and the peptides with 30-bp DNA is noncooperative (*28, 29*). Therefore, we used the linear kinetic analysis package to obtain values for k_{ass} and k_{dlss} as described in "MATERIALS AND METHODS" (*24, 26*). By plotting the derivative of the binding curves *vs.* the response for each HMG concentration (Eq. 1) a set of straight lines was obtained (not shown). According to Eq. 2, there should be a linear relationship between the slopes of these lines and the concentrations at which they were obtained, as indicated in Fig. 7, A and B. From the slope of the fitted line in this plot the k_{ass} was calculated to be $1.6\pm0.2\times10^4\ M^{-1}\cdot s^{-1}$ for HMG1 and $2.0\pm0.1\times10^4\ M^{-1}\cdot s^{-1}$ for HMG2. From the sensorgrams shown in Fig. 5, it can be seen that the dissociation, starting at about 720 s, after a transient phase of rapid dissociation proceeds at steady rate. By plotting $\ln(R_0/R_n)$ *vs.* time as in Fig. 7, C and D, the biphasic dissociation is made more obvious. After about 10 s the contribution from the faster-dissociating fraction has become negligible. From the second half of the curves, k_{dlss} was calculated to be $6.8\pm0.3\times10^{-2}\ s^{-1}$ for HMG1 and $5.9\pm0.5\times10^{-2}\ s^{-1}$ for HMG2. The dissociation constants, calculated as the ratio between association and dissociation rate constants are listed in Table I. The K_d value of peptide 1AlB was apparently smaller than that of 2AlB, suggesting that the former has higher affinity for DNA than the latter. The K_d values of peptides 1AlBj and 2AlBj were similar. Finally, the K_d value for the whole HMG2 molecule was smaller than that of HMG1, and the values for both were larger than those of the respective peptides AlB and AlBj. Taken as a whole, these quantitative results support the contention that the processes occurring in solution and on the sensor chip surface are similar. In addition, the quantitative measurements obtained with SPR clearly confirmed that the whole HMG2 molecule has a higher affinity than HMG1 for DNA, in contrast to the situation for the main DNA-binding region in these proteins.

DISCUSSION

The measurement of binding affinity between DNA and the effector in the sequence non-specific, cooperative binding of multiple molecules must be influenced by various factors. In the present study, pBR322 DNA containing multiple sequences was employed considering the non-specific DNA-binding behavior of HMG proteins. HMG2 produced more marked gel retardation of form III as well as form I

DNA than HMG1 (Figs. 2 and 3). The binding ability of HMG2 must be strong enough to permit its preferential binding to DNA, and allow it to replace HMG1 molecules that have already bound (Fig. 2, C and D). This apparent preferential DNA-binding of HMG2 was confirmed by SPR measurements (Table I), suggesting also that the processes occurring in solution are similar to those observed on the sensor chip surface.

The primary sequences, molecular weights and net charges except for the C-tails (Fig. 1) are largely homologous in the two HMG proteins (*3, 4*). The multiple sequence data (*2*) and tertiary structures determined by NMR (*10, 11, 30*) the DNA-binding domain of HMG and proteins containing this motif suggest that the maximum amounts of HMG1 and 2 protein capable of binding with naked DNA are similar. In addition, neither of the proteins have any sequence specificity for binding distinct DNA regions. These features suggest that the difference in DNA-binding affinity between HMG1 and 2 is due to the differences in their primary sequences. The major differences between the two proteins lie in the j-region in addition to the length of the C-tail. Therefore, we focused on the DNA-binding abilities of peptide AlB with a truncated j-region and C-tail, and peptide AlBj bearing a truncated C-tail. It was noticeable that peptide 1AlB had higher affinity for DNA than peptide 2AlB, in contrast to the intact molecules (Fig. 4). In order to confirm this, the SPR measurement method was applied (Fig. 5). Previously, the presence of steric cooperativity has been shown in the binding of single-stranded DNA binding protein with polynucleotide by real-time BIAcore measurements (*31*). Then, the extrapolated responses at infinite time in various peptide concentrations were plotted (Fig. 6, A and B). The Hill coefficients close to 1 (Fig. 6, C and D) indicated that HMG or the peptide forms a 1:1 complex with 30-bp DNA and that the binding is noncooperative. The noncooperativity of HMG binding with short DNA fragments was also indicated by Wisniewski and Schulze (*28*). The kinetic data thus obtained by SPR measurements (Table I) may indicate that the binding activities of various DNA-binding domains containing homologous, but non-identical primary structures may differ from each other. However, the binding activities of 1AlBj and 2AlBj were found to be similar in terms of SPR. The larger gel retardation observed for peptide 1AlBj in comparison with 2AlBj (Fig. 4, B and D) might have resulted partly from the difference in their net charges, and not their DNA-binding activity. The binding domains in HMG1 and 2 have been deduced mainly from the homology of their primary sequences and similar hydrophobicity patterns. The present data, however, suggest that the sequence adjacent to the so-called HMG motif is important for the substantial binding of the proteins to DNA. The higher affinity of HMG2 for DNA as well as the weaker binding in comparison with the respective peptide AlBj was supported by SPR measurements, indicating that the acidic C-tails of the two proteins were responsible for the difference in DNA-binding and its weakening. The two C-tails differ in both sequence and length. It seems that the longer acidic C-tail of the HMG1 molecule weakens the binding in comparison with that of HMG2. The acidic tails may not form an ordered secondary structure at neutral pH (*32*). Therefore, the C-tails may fold back to interact with the adjacent j-region and/or l-region, which may lie close together in the tertiary structure of the whole HMG molecule. The binding of the C-tail with basic amino acid residues in the j- and l-regions may weaken the binding to DNA. The shorter acidic tail of HMG2 may be less effective than that of HMG1 for this reaction, resulting in higher affinity. Direct interactions of the acidic C-tail with the DNA-binding domains could also be considered as a possibility. The modulation of DNA-binding by the intrinsic acidic C-tail of HMG protein is consistent with our previous finding that removal of the tail leads to loss of stimulation of gene transcription in cell culture (*14*) and also with a recent report indicating that the recombinant A/B domains have a greater binding activity than the intact protein (*33*).

The dissociation of HMG1 and 2 from the 30-bp DNA fragment seemed to be biphasic of rapid and steady rates (Figs. 5 and 7). About 30% of the proteins bound with the DNA fragment captured on the surface of the sensor chip was dissociated rapidly after injecting TME containing 100 mM NaCl (Fig. 5). Most of this faster-dissociative protein is considered to be loosely bound with the 30-bp DNA fragment through one of the two DNA-binding domains in the respective molecule, because of their sequence nonspecific characteristics for DNA-binding. The binding affinity of peptides containing a single DNA-binding domain is lower than that of HMG proteins containing two domains (in preparation). HMG proteins tightly bound with DNA through two DNA-binding domains would dissociate at steady rate.

The homologous primary sequences, containing two internal repeats of DNA-binding domains and an acidic C-terminal, which are highly conserved in different mammalian species, and the similarity of the various chemical and physicochemical properties between HMG1 and 2 have predicted that they may have similar functions in transcription, DNA replication and cellular differentiation, although this has not yet been proved (for reviews, Refs. *1, 12*, and *2*). Recent observations showing that HMG1 proteins play a direct role in transcriptional events on genes and/or function as quasi-transcription factors (*13-15* and references cited therein) and that HMG2 may function in cell proliferation (*16, 17*, and references cited therein) predict that HMG1 and 2 proteins might have certain differences in their binding abilities or modulation. The present quantitative results obtained by SPR measurements, revealing differences in the DNA-binding activity of the two proteins consistent with the differences observed in gel retardation assay (Figs. 2-4) or primary filter binding assay (*18*), may be important for elucidating the functional difference between the proteins. In addition, the SPR data suggest that the DNA-binding abilities of individual domains of similar, but non-identical primary structures differ from each other and are modulated by the sequences adjacent to them. A similar mechanism may also apply in various regulatory proteins containing the HMG DNA-binding motif.

SPR measurements have been used to analyze the binding of the *lac* repressor (*25*) and the eukaryotic transcription factor ETS1 (*29*) to their respective target DNA sites, the binding of *E. coli* single-stranded DNA-binding protein to polynucleotide (*31*), and the binding of the co-repressor in the *E. coli* methionine repressor to the operator (*26*). The present data extend the applications of this technique to allow a detailed study of the roles of the

respective domains and their adjacent sequences in DNA-binding.

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