Nonhistone Nuclear High Mobility Group Proteins 14 and 17 Stabilize Nucleosome Core Particles*

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Nucleosome core particles form well defined complexes with the nuclear nonhistone proteins HMG 14 or 17. The binding of HMG 14 or 17 to nucleosomes results in greater stability of the nucleosomal DNA as shown by circular dichroism and thermal denaturation. Under appropriate conditions the binding is cooperative, and cooperativity is ionic strength dependent. The specificity and cooperative transitions of high mobility group (HMG) binding are preserved in 1 M urea. Specificity is lost in 4 M urea. Thermal denaturation and circular dichroism show a dramatic reversal of the effects of urea on nucleosomes when HMG 14 or 17 is bound, indicating stabilization of the nucleosome by HMG proteins. Complexes formed between reconstructed nucleosomes containing purified inner histones plus poly(dA-dT) and HMG 14 or 17 demonstrate that the HMG binding site requires only DNA and histones. Electron microscopy reveals no major structural alterations in the nucleosome upon binding of HMG 14 or 17.

Cross-linking the nucleosome extensively with formaldehyde under cooperative HMG binding conditions does not prevent the ionic strength-dependent shift to noncooperative binding. This suggests mechanisms other than internal nucleosome conformational changes may be involved in cooperative HMG binding.

The nucleosome is well established as the subunit of chromatin in eukaryotic cells (1–3). The nucleosome has been studied widely as a model for chromatin structure and function (2–4).

The high mobility group proteins 14 and 17 are members of a class of nuclear nonhistone proteins characterized by low molecular weight, high charge density, and solubility in 2% trichloroacetic acid (5). HMG 14 and 17 are reported to be required for the DNase I sensitivity characteristic of active genes (6–9). In addition, HMG 14 and 17 are reported to bind preferentially to active chromatin (8, 10), and to co-isolate with fractions of chromatin enriched in active sequences (11–13).

The association of HMG 14 and 17 with active chromatin has resulted in several studies on nucleosome-HMG complexes. The results of studies on nucleosome-HMG complexes show that all nucleosomes have two discrete binding sites for HMG 14 or 17 near the ends of the nucleosomal DNA (14–16). Sandeen et al. (15) and Schrötter and Bode (17) have shown that HMG 14 and 17 can bind cooperatively at higher ionic strength. When added in excess, HMG 14 and 17 appear to stabilize the nucleosome against thermal denaturation (15). Sandeen et al. (15) also reported an enrichment for globin genes in the nucleosome fraction that bound HMG 14 and 17.

Previous studies have employed urea as a model perturbant to study the range of conformational states of the nucleosome (18–23). As the urea concentration is raised from 1 to 8 M the nucleosome unfolds into extended form with the denatured histones still bound to DNA.

In our study, urea was used as a model perturbant to determine if the stability of nucleosome-HMG protein complexes was sensitive to conformational changes, in analogy to the presumed transitions that may occur in actively transcribing chromatin.

In this paper we report that binding of HMG 14 or 17 to nucleosomes shifts from cooperative to noncooperative over a very narrow range of ionic strength. The binding is unchanged in low molar concentrations of urea. We further report that nucleosomes are stabilized against thermal denaturation and the effects of urea by HMG 14 or 17 at molar ratios of 1 or 2 HMGs per nucleosome. We also show that extensively cross-linked nucleosomes still shift from cooperative to noncooperative binding as a function of ionic strength. Electron micrographs show that nucleosome-HMG complexes are very similar in structure to nucleosome core particles. Possible mechanisms for stability and cooperativity are discussed and results are compared to related work by others.

MATERIALS AND METHODS

Preparation of Nucleosomes—Nucleosome core particles from chicken erythrocytes were isolated by the method of Lotter (24), with modifications. Briefly, the published procedure is followed until the step separating nucleosomes from nucleosome dimers. For this step, Sephacryl S-300 is used and the sodium chloride concentration in the column buffer is increased to 60 mm. Pooled fractions of nucleosome

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1 The abbreviations used are: HMG, high mobility group; PIPES, 1,4-piperazinediethanesulfonic acid; TES, N-tris(hydroxymethyl) methyl-2-amino-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N′,N′,N′-tetraacetic acid; SDS, sodium dodecyl sulfate.
monomers are then dialyzed against 100 mM KCl, 12 mM MgCl₂, 1 mM PIPES, pH 7.2, to precipitate any residual dimer contamination. Precipitate is removed by centrifugation at 8000 × g for 20 min. The supernatant is exhaustively dialyzed against a storage buffer (5 mM Tris, 20 mM NaCl, 0.2 mM EDTA, 3 mM Na azide, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and concentrated in collidin bags to 20 mg/ml.

**Reconstructed Nucleosomes**—Nucleosome core particles were reconstituted from purified chicken inner histones and poly(dA-dT) according to Bryan et al. (25). Purified chicken inner histones were obtained from chicken erythrocyte nuclei by the method of Butler et al. (26). Synthetic poly(dA-dT) was purchased from P-L Biochemicals, checked for purity by buoyant density centrifugation, and used without further purification.

**Preparation of HMG 14 and 17**—HMG 14 and 17 were prepared in quantity as described by Uberbacher et al. (27). Stock solutions of HMG 14 or 17 were prepared by dissolving lyophilized HMG 14 or 17 in glass distilled water. These stocks were stored at −20 °C until used. Purity and stability were monitored by SDS-microslab gel electrophoresis.

**Electrophoresis**—Nucleosome-HMG complexes were analyzed on nondenaturing gels containing 6% acrylamide, 0.2% bisacrylamide (28). Gels contained various dilutions of Tris-borate-EDTA (TBE) buffer (29). Higher ionic strength gels had additional 5x buffer replacing some of the water. Samples were mixed with sample buffer containing TBE at the same concentration as the gel, plus glycerol (or Ficoll) and bromphenol blue. Urea used in some gels was deionized during preparation and monitored for pH and conductivity before each use. Concentration was monitored by refractive index measurements (18).

**Urea**—Samples were stained for protein with Coomassie blue. Gels stained for DNA with toluidine blue or ethidium bromide gave identical patterns.

**Thermal Denaturation**—Thermal denaturation was done in a Gilford model 2000 equipped with a thermal programmer, base-line correction, and manual offsets. Samples were heated by Gilford chamber heating blocks that were connected to a water/glycerol bath controlled by the thermal programmer. Heating rate was approximately 1 °C per min. Absorbance was monitored at 260 nm. Samples of 300 μl were degassed under vacuum before the run. Data were collected and analyzed by computer.

**Circular Dichroism**—Circular dichroism was monitored with a Jasco J-40A spectropolarimeter. Samples of 500 μl were placed into standard round cells. All spectra were recorded at 20 °C and were collected and analyzed by computer. Resolution of the data collected was 10 points/nm. Buffer blank spectra were collected separately for each sample and subtracted during analysis.

**Electron Microscopy**—Samples of nucleosomes or nucleosome-HMG 14 complexes, at an A₂₆₀ of 1.0, were prepared for electron microscopy by application to a freshly glow carbon-coated grid. The sample was allowed to stand on the grid for 30-60 s and then was drained. Photo-Flo (Eastman Kodak) at pH 7, diluted 3 drops in 50 ml of distilled water, was applied, blotted, and the grid was air dried. The grids were stained for 30 s with a solution of 0.1% w/v uranyl formate in water and air dried. Samples were examined in a Siemens 102 under tilted beam darkfield conditions.

**Cross-linking**—Nucleosome core particles were cross-linked using a modification of the procedure of Burch and Martinson (31). Nucleosomes at a concentration of 150 μg/ml were dialyzed exhaustively against 2.5 mM Na phosphate, 20 mM NaCl, 0.1 mM EDTA, pH 6.7. After equilibration, the dialysis buffer was changed to the same buffer containing a final concentration of 6% or 8% formaldehyde. Formaldehyde (37% aqueous), containing less than 12.5% methanol as preservative, was obtained from commercial suppliers and used directly. The sample was allowed to equilibrate for 16 h at 4 °C, and then the sample was exhaustively dialyzed against several changes of the dialysis buffer without formaldehyde. Removal of residual formaldehyde was checked initially using an extremely sensitive and rapid colorimetric test for formaldehyde (32). Extent of cross-linking was monitored on SDS-polyacrylamide gels. Satisfactory cross-linking was represented by a single band, composed of both DNA and protein, migrating at M₈ = 200,000 with no residual free histone. Higher concentrations of formaldehyde produced high molecular weight oligomers and were avoided.

**RESULTS**

Nucleosome core particles from chicken erythrocyte chromatin were mixed with HMG 14, at a molar ratio of 0, 1, 2, or 3 HMG 14 per nucleosome, in a range of TBE buffer concentrations. The resulting complexes were electrophoresed on gels containing TBE concentrations identical with that of the samples. The results are shown in Fig. 1. The four gels clearly show a sharp transition from the noncooperative HMG binding pattern to the cooperative pattern between 0.2 × and 0.25 × TBE. This transition to cooperative binding is marked by the disappearance of the middle band corresponding to nucleosomes with only one HMG 14 bound (14–17). At TBE concentrations above 0.25 × only two bands are seen on the gel, the lower one corresponding to nucleosomes alone, and the upper band corresponding to nucleosomes with 2 HMG 14 proteins bound. The sharp transition from noncooperative to cooperative binding occurs over a very narrow range of ionic strength. The conductivity of TBE dilutions compared to standard concentrations of potassium chloride indicates a very low effective ionic strength for TBEH buffers. The comparison shows that the transition to cooperative HMG binding occurs at an equivalent potassium chloride concentration of 2.2–2.6 mM (data not shown). This range of ionic strength is close to where the nucleosome is known to undergo an ionic strength-dependent conformational change (33–35).

**Electrophoresis in Urea**—We asked whether unfolding the nucleosome with urea would alter the specificity or cooperativity of HMG protein binding. Nucleosome core particles were combined with HMG 14 at molar ratios of 0, 1, 2, and 3 HMG 14 per nucleosome. The resulting complexes were electrophoresed on 5% polyacrylamide gels in 0.1 × or 0.3 × TBE containing 1 M urea. The results are shown in Fig. 2. Fig. 2A shows the typical noncooperative HMG binding pattern also seen on 0.1 × TBE gels without urea. Fig. 2B shows the
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Fig. 2. Electrophoresis of nucleosome-HMG 14 complexes in the presence of urea. A, 0.1 × TBE, 1 M urea. B, 0.3 × TBE, 1 M urea. Both left to right, samples are nucleosomes with 0, 1, 2, or 3 mol of HMG 14 per mol of nucleosomes.

Fig. 3. Electrophoresis of nucleosome-HMG 14 complexes in the presence of 4 M urea. A, 0.1 × TBE, 4 M urea. B, 0.3 × TBE, 4 M urea. Both gels samples, left to right, nucleosomes with 0, 1, 2, or 3 mol of HMG 14 per mol of nucleosomes. The faint rapidly migrating bands, seen most clearly in B, probably represent dissociated subnucleosomal fragments.

Typical cooperative HMG binding pattern also seen on 0.3 × TBE gels without urea. Clearly, in both cases, HMG binding is specific and discrete. The mild unfolding of the nucleosome induced by 1 M urea has no obvious effect on nucleosome-HMG complex formation. The sites of HMG binding remain intact and accessible. Control experiments in which urea was added to the nucleosome core particles prior to HMG binding showed identical results (data not shown).

In the next set of experiments, nucleosome-HMG 14 complexes were formed and run on 5% polyacrylamide gels containing 0.1 × or 0.3 × TBE and 4 M urea. This concentration of urea is known to unfold the nucleosome extensively and to disrupt the majority of histone α-helix (18). The results are shown in Fig. 3.

Fig. 3A shows the results of electrophoresis in the presence of 0.1 × TBE and 4 M urea. The nucleosomes without added HMG migrate as discrete bands. As HMG 14 is added, the lanes show diffuse staining in the region normally occupied by discrete bands of nucleosome-HMG complexes. This diffuse staining is interpreted as non-specific binding of HMG 14 (14).

Fig. 3B shows the results of electrophoresis in 0.3 × TBE and 4 M urea. These results show that extensive unfolding of the nucleosome in 4 M urea prevents specific cooperative binding of HMG 14 normally seen at this ionic strength.

Reconstructed Nucleosomes—Nucleosomes reconstructed from purified chicken inner histones and synthetic poly(dA-dT) were titrated with HMG 14. The resulting complexes were run on gels containing 1 × TBE. The results are shown in Fig. 4. The cooperative binding pattern is observed and is very similar to that seen for chicken nucleosomes at this ionic strength. Samples run on gels in 0.1 × TBE are very similar, if not identical, to chicken nucleosomes-HMG 14 complexes and exhibit the noncooperative pattern (data not shown).

The results with reconstructed nucleosomes are important because the presence of synthetic DNA and of purified inner histones in the reconstructed nucleosomes eliminates the possibility that undetected DNA-binding proteins are affecting the results. Such proteins could remain bound to DNA in native nucleosomes and influence the binding of HMG 14 or 17 to the nucleosome. In addition, it demonstrates that only DNA and histone protein are required to create the HMG-binding sites on the nucleosome core particle.

Thermal Denaturation—Chicken erythrocyte nucleosomes were mixed with HMG 14 or 17, in 0.1 × TBE buffer, at a ratio of 2 mol of HMG protein per mol of nucleosomes. The resulting complexes were melted and compared to chicken erythrocyte nucleosomes alone. The results are given in Fig. 5.

The thermal denaturation profiles shown are first derivative plots. Nucleosomes alone show two distinct melting transitions. The first, around 62 °C in the indicated buffer, is the early melting region and corresponds to the denaturation of DNA ends on the nucleosome (25, 36, 37). The curves corresponding to nucleosomes with bound HMG 14 or 17 reveal a marked suppression in this region, indicating greater stability of the DNA ends in the presence of HMG 14 or 17. The main
the DNA signal from the nucleosomes with bound HMG 14, compared to nucleosomes alone. The suppression of the DNA signal is analogous to that seen when free DNA is complexed with histones to form chromatin (25) and is interpreted as representing conformational stabilization of the DNA resulting from its association with the histones. Our results indicate that additional stability is conferred upon nucleosomal DNA when HMG 14 is bound.

The region of the spectrum from 265–230 nm represents the sum of signals derived both from protein secondary structure and from DNA structure. The large negative signal in this region results from the high α-helix content of the histone proteins (38). In the presence of HMG 14, the signal is slightly reduced (i.e., less negative). This is most likely the result of HMG 14-induced changes in histone α-helix or HMG 14 interactions with DNA since HMG 14 has no secondary structure in solution (5, 39). Similar CD spectra were obtained on complexes containing HMG 17 and on complexes in 0.3 X TBE or 0.2 mM EDTA (data not shown).

Circular Dichroism in Urea—Urea is known to alter nucleosome structure by destabilizing histone-histone contacts and histone α-helix. The presence of urea also reverses the characteristic suppression of the signal from DNA in the 260–300 nm region of the spectrum.

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Buffer*</th>
<th>Early peak°C</th>
<th>Main peak°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleosomes</td>
<td>0.5 mM cacodylate, pH 7.0</td>
<td>—</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>1.0 mM cacodylate, pH 7.0</td>
<td>—</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>0.5 mM phosphate, pH 7.0</td>
<td>—</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>1.0 mM phosphate, pH 7.0</td>
<td>—</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>0.5 mM acetate, pH 4.5</td>
<td>—</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>0.2 mM EDTA, pH 7.0</td>
<td>—</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>0.2 mM EDTA, pH 7.0</td>
<td>55°C</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>0.1 X TBE, pH 8.3</td>
<td>62°C</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>0.3 X TBE, pH 8.3</td>
<td>Shoulder, 65°C</td>
<td>77</td>
</tr>
<tr>
<td>Nucleosomes +</td>
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<td>Shoulder, 67°C</td>
<td>78</td>
</tr>
<tr>
<td>1 HMG 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleosomes +</td>
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<td>58°C</td>
<td>72</td>
</tr>
<tr>
<td>1 HMG 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleosomes +</td>
<td>0.2 mM EDTA, pH 7.0</td>
<td>Shoulder, 65°C</td>
<td>79</td>
</tr>
<tr>
<td>2 HMG 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 X TBE, pH 8.3</td>
<td>Shoulder, 65°C</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>0.3 X TBE, pH 8.3</td>
<td>Shoulder, 67°C</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>0.5 mM acetate, pH 4.5</td>
<td>—</td>
<td>70</td>
</tr>
<tr>
<td>Nucleosomes +</td>
<td>0.2 mM EDTA, pH 7.0</td>
<td>Shoulder, 58°C</td>
<td>74</td>
</tr>
<tr>
<td>2 HMG 17</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.2 X TBE, pH 8.3</td>
<td>Shoulder, 64°C</td>
<td>76</td>
</tr>
<tr>
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<td>0.3 X TBE, pH 8.3</td>
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<tr>
<td></td>
<td>0.5 mM acetate, pH 4.5</td>
<td>—</td>
<td>70</td>
</tr>
<tr>
<td>146-base pair</td>
<td>0.2 mM EDTA, pH 7.0</td>
<td>—</td>
<td>39</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.1 X TBE, pH 8.3</td>
<td>—</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>0.3 X TBE, pH 8.3</td>
<td>—</td>
<td>68</td>
</tr>
</tbody>
</table>

* Buffer compositions: cacodylate, sodium cacodylate/cacodylic acid; phosphate/disodium phosphate/monosodium phosphate; acetate, sodium acetate/acetic acid; 0.1 X TBE, 9 mM Tris base, 9 mM boric acid, 0.25 mM EDTA.

A dash (—) means that no transition was detected.
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The effects of bound HMG on the stability of nucleosomes in the presence of urea were also studied. Fig. 8 shows the spectra of nucleosomes, with and without bound HMG 14, in the presence of 0.1 x TBE and 1 or 4 M urea. The spectra are presented as a composite. Spectra taken above 262 nm are shown at a higher sensitivity than data below 262 nm, to clearly show the changes in DNA signal strength. From 262 to 300 nm the results show the expected increase in DNA signal as urea concentration is increased. The results also show that the presence of 2 mol of HMG 14 per mol of nucleosomes substantially reverses the effects of urea. This strongly indicates stabilization of the nucleosome against the unfolding induced by urea. Results of experiments in 0.3 x TBE and 1 or 4 M urea gave identical results, as did experiments with HMG 17 (data not shown).

Cross-linking—Cross-linking of nucleosome core particles with formaldehyde was done to determine if the shift from cooperative to noncooperative HMG binding required a major conformational change in the nucleosome. Nucleosome core particles were cross-linked in 6% formaldehyde and complexes were formed with HMG 14. Nucleosome-HMG 14 complexes were electrophoresed on a 6% polyacrylamide gel containing 0.1 x TBE and 1 M urea. Urea was included as an additional check on the extent of cross-linking. The results are shown in Fig. 9. Cross-linked nucleosomes and nucleosome-HMG 14 complexes show migration identical with uncross-linked controls. These results show that extensive cross-linking of the nucleosome does not prevent discrete specific binding of HMG 14 to the nucleosome shown by the typical three band pattern seen at this ionic strength.

The next cross-linking experiment was done to determine if a nucleosome conformational change was required for the shift from cooperative to noncooperative binding of HMG proteins. Nucleosome core particles were cross-linked extensively in 8% formaldehyde at high ionic strength (20 mM NaCl, 2.5 mM Na phosphate, 0.1 mM EDTA). The particles were purified and complexed with HMG 14 at a molar ratio of 1 or 2 HMG 14 per nucleosome. Uncross-linked controls were mixed with HMG 14 at high ionic strength. The resulting complexes were diluted to low ionic strength (0.1 x TBE) and electrophoresed on a 6% polyacrylamide gel containing 0.1 x TBE. The results are shown in Fig. 10. The figure shows the cross-linked nucleosome-HMG 14 complex in lane 3 giving results essentially identical with the cross-linked complexes in lane 6. These results show that nucleosomes extensively cross-linked at high ionic strength are still able to bind HMG protein noncooperatively as a function of ionic strength.

Electron Microscopy—Nucleosome-HMG 14 complexes were examined in the electron microscope to determine whether large conformational changes were taking place in the nucleosome upon binding HMG 14 protein. The results are shown in Fig. 11. Clearly nucleosomes with bound HMG 14 protein closely resemble nucleosomes alone. Preliminary measurements on 50–75 nucleosomes from each sample show
**Fig. 9.** Electrophoresis of cross-linked nucleosomes and nucleosome-HMG 14 complexes in 0.1 × TBE and 1 M urea. Samples left to right: nucleosomes, nucleosomes with 0.75 mol of HMG 14 per mol of nucleosomes (lanes 1 and 2), cross-linked nucleosomes and cross-linked nucleosomes with 1 mol of HMG 14 per mol of nucleosomes (lanes 3 and 4).

**Fig. 10.** Electrophoresis of cross-linked nucleosomes and nucleosome-HMG 14 complexes in 0.1 × TBE. Samples left to right: nucleosomes with 0, 0.75, 1.5 HMG 14 per nucleosome (lanes 1–3); cross-linked nucleosomes with 0, 2, or 1 HMG 14 per nucleosome (lanes 4–6).

**Fig. 11.** Darkfield electron micrographs of nucleosomes and nucleosome HMG 14 complexes. A, nucleosomes in 0.2 mM EDTA, pH 7.0. B, nucleosome-HMG 14 complexes, same buffer as A. C, nucleosomes in 0.3 × TBE, pH 8.3. D, nucleosome-HMG 14 complexes in same buffer as C.
no significant differences, in diameter or shape, between nucleosomes and nucleosome-HMG 14 complexes.

DISCUSSION

The results of electrophoresis in varying ionic strength show a transition from noncooperative HMG binding to cooperative HMG binding. This transition occurs over a narrow range of ionic strength near that at which the nucleosome itself is known to change conformation (33–35). The cooperativity of HMG binding as defined by the patterns seen on gel electrophoresis also implies a conformational change in the nucleosome upon binding of HMG protein (17). However, initial calculations assigning the entire mobility difference to a change in axial ratio required ratios on the order of 16 to 1. Ratios of this magnitude are clearly ruled out by the results of neutron scattering (27), sedimentation (15), and our electron micrographs. The mobility shift could also be caused by added mass or charge or both (14). HMG 14 from chicken erythrocytes has a molecular weight of 12,950, calculated from sequence data (40). With both binding sites occupied, the nucleosome-HMG complex has a mass only 12.7% greater than the nucleosome itself. This alone is insufficient to account for the observed mobility shift. HMG 14 and 17 are highly charged molecules with 56 and 45% charged residues, respectively. Each has significant potential to alter the overall charge of the nucleosome. However, the nucleosome has an overwhelming negative charge due to the phosphate backbone of the DNA and is reported to have a minimum of 108 negative charges available (41). Using the example of 2 HMG 14s contributing 74 positive charges and 62 negative charges, a net charge of +12 would be added. This would result in the loss of 11% of the nucleosomal negative charge. This is still insufficient to account for the total mobility shift observed for nucleosome-HMG complexes. However, added mass and charge together could account for the ~20% mobility change seen for the nucleosome after HMG binding. These simple calculations assume a negligible change in the frictional coefficient of nucleosomes with two HMGs bound and further incorporate the assumptions about histone-DNA interactions made by Mirzabekov and Rich (41). The estimate of 108 negative charges for the nucleosome may be a minimum number. These observations suggest a variety of possible mechanisms through which the binding of the first HMG facilitates the binding of the second at higher ionic strengths, where cooperativity is seen. These mechanisms would include an alteration of the nucleosome surface including DNA-histone contacts to create greater affinity for HMG protein at the second HMG-binding site. An alternate explanation is the restoration of a compact nucleosome structure, in higher ionic strength, that facilitates binding of a second HMG protein. It is also conceivable that subtle changes might occur in HMG protein structure that would alter their affinity for nucleosomes as a function of ionic strength. HMG proteins also contain a wide variety of postsynthetic modifications including acetylation (42), methylation (43), ADP-ribosylation (44, 45), phosphorylation (46–48), and glycosylation (45). Some of these modifications could alter the response of HMG protein structure to ionic strength.

The loss of HMG binding specificity in the presence of high urea concentrations is probably due to nucleosome unfolding. Urea is known to cause swelling of the nucleosome and to loosen interactions between histones (18–23). Histone secondary structure is also denatured, causing the loss of α-helix. Any one of these effects could be sufficient to prevent specific HMG binding. It is known that HMG 14 and 17 bind to the outer surface of the nucleosome at two specific sites (14–16). Based on the neutron-scattering studies of Uberbacher et al. (27) and known physical properties of HMG 14 and 17 (5, 39), it is probable that HMG 14 and 17 bind in their extended form. If multiple DNA and histone contact sites are required for specific binding, as previously proposed (14), any movement of histones or DNA could destroy the binding site. HMG binding to DNA alone would not be subject to catastrophic alteration upon minor swelling of the nucleosome. The results presented here support models that suggest HMG 14 and 17 bind to both DNA and histone. It is unlikely that the loss of HMG-binding specificity is due to urea effects on HMG 14 or 17. HMG 14 and 17 have no secondary structure and are highly charged proteins known to bind chromatin ionically (5). In addition, control experiments in which nucleosome-HMG complexes were sedimented on sucrose gradients in 6 M urea showed no loss of HMG protein from the nucleosomes (data not shown).

Binding of HMG 14 to nucleosomes reconstructed from poly(dA-dT) and the inner histones reveals two main points. First, the two binding sites on each nucleosome are an inherent structural property of the nucleosome. When normal nucleosome structure exists, so do two binding sites for HMG 14. Second, the binding of HMG 14 and 17 does not require accessory proteins or additional macromolecular factors. DNA and inner histones are the only structural components required.

Thermal denaturation of nucleosome-HMG complexes reveals stabilization of the DNA ends as well as the entire nucleosome, confirming results first reported by Sandeen et al. (15). Thermal denaturation in a variety of buffers, at ratios of 1 or 2 HMGs per nucleosome also shows stabilization of nucleosomes by HMG 14 or 17. The thermal denaturation results show that nucleosomes appear to have an early melting peak only in buffers containing EDTA. Extensive controls to eliminate contaminating metal ions, as well as atomic absorption analysis, failed to indicate chelation of residual stabilizing metal ions as the cause for this phenomenon (49).

Circular dichroism of nucleosome-HMG complexes provides additional support for stabilization of nucleosomes by HMG 14 or 17. The DNA region of the spectrum is reduced upon binding of HMG proteins indicating greater protein-DNA interaction and structural organization. This interaction results in greater stability of the complex as shown by experiments in the presence of urea.

The partial unfolding and destabilization of nucleosomes by urea results in lower melting temperatures or changes in circular dichroism of both DNA and protein. These effects are reduced or prevented if HMG 14 or 17 is bound to the nucleosome. In the case of thermal denaturation, two kinds of stabilization occur. First, the melting of the DNA ends, causing the early melting peak, is suppressed almost entirely by the presence of HMG protein. The DNA ends may be directly stabilized by ionic interactions between DNA phosphate and HMG basic amino acids. These interactions could effectively counter the disruption of hydrogen bonds by increased temperature or the weakening of hydrophilic interactions by urea. Second, the stabilization of the main melting peak results from the stabilization of both DNA and histones by HMG protein. These results are consistent with models describing HMG proteins spanning a large distance on the nucleosome surface rather than more localized interactions.

The results of circular dichroism on nucleosome-HMG complexes in urea also reveal stabilization of the nucleosome by HMG protein. The increase in signal from the DNA region of the spectrum caused by urea is suppressed or reduced when HMG 14 or 17 is bound to the nucleosome. This DNA signal suppression is analogous to that seen when histones are complexed with DNA to form the nucleosome, stabilizing and
organizing the DNA. Addition of urea also causes reduction of the large negative peak in the α-helical region of the protein spectrum. The presence of HMG protein reverses this change, restoring all or part of the α-helix lost due to urea-induced unfolding.

The stabilization revealed by CD spectra also support the idea of HMG protein interactions over a large span of the nucleosome including both DNA and histone for stabilization of the entire particle. The high charge density and asymmetric charge distribution of both HMG 14 and 17 argue for binding contributions from both acidic and basic residues on the HMG proteins (14, 49).

Cross-linking of nucleosomes with formaldehyde fixes the nucleosome with covalent bonds at sites of close proximity (50). Cross-linking extensive enough to prevent unfolding of the nucleosome in high concentrations of urea, or the separation of DNA and histones in SDS, should prevent major structural alteration. The results of adding HMG 14 or 17 to cross-linked nucleosomes shows major structural alterations are not required for HMG binding. Specifically, the ionic strength-induced shift from cooperative to noncooperative HMG binding still occurs with extensively cross-linked nucleosomes. While minor structural changes are still possible, the cross-linking results support the idea that cooperativity of HMG binding may be an ionic event acting only on HMG structure or charge density interactions between HMGs and nucleosomes. Electron microscopy shows that nucleosome-HMG complexes closely resemble nucleosomes, and that HMG binding does not induce major structural changes in the nucleosome.

Some recent reports on HMG-nucleosome and HMG-chromatin interaction are related to our work. With regard to nucleosomes, Schröter and Bode (17) reported a change in cooperativity over a broader range of ionic strength than we report here. Scans of our gels do not show a gradual disappearance of the central band corresponding to 1 bound HMG per nucleosome. Comparison of our nucleosome preparation methods reveals no significant differences except that nucleosomes used by Schröter and Bode may have been stored at −70 °C in dimethyl sulfoxide. We can find no other explanation for the apparent difference in our results. Our results and those of others (15, 27, 51) are also consistent with an open or unfolding upon HMG binding as proposed (17).

Swerdlow and Varshavsky (52) reported a 100-fold increase in affinity of HMG 17 for nucleosomes with DNA longer than 3-5 additional base pairs compared to core nucleosomes. Our results are not inconsistent with theirs and we find it plausible that the DNA portion of the HMG binding site may, in vivo, include 3-5 additional base pairs of DNA. However, this increase in affinity was not seen by Schröter and Bode (17).

Studies by McGhee et al. (53) on the interaction of HMG 14 or 17 with isolated chromatin show that HMG 14 and 17 do not prevent the salt-dependent compaction of long chromatin from either active or inactive regions. Their results suggest, as do ours, that the effects of HMG binding are confined to the level of the nucleosome. Sasi et al. (54) recently reported that HMG 17 condensed chromatin fragments of 100 nucleosomes when bound at ratios less than 1 per nucleosome. The changes in circular dichroic spectra reported by Sasi et al. (54) are identical with those we report here for nucleosome core particles. Thus the effects seen for binding of HMG 17 to long chromatin may be due to effects on the signal contributed by the nucleosomes. In addition, the reported binding of HMG 17 to linker DNA in long chromatin as interpreted from thermal denaturation studies of Sasi et al. (54) is in contrast to the two specific nucleosomal binding sites reported by us and others (14-16). In thermal denaturation studies we report here, the fraction of DNA corresponding to the DNA ends or near the DNA ends was dramatically stabilized by HMG 14 or 17 at ratios of 1 or 2 HMGs per nucleosome. We support their conclusion that HMG 17 stabilizes chromatin but find a linker binding site difficult to reconcile with studies on nucleosome core particles.

The results we report here make four main points. First, nucleosomes are stabilized against unfolding by bound HMG 14 or 17. Second, HMG 14 or 17 cannot bind specifically to swollen or partially unfolded nucleosomes. Third, the binding sites for HMG 14 or 17 are an inherent structural property of the nucleosome requiring only DNA and histones. Fourth, the ionic strength-dependent shift from noncooperative to cooperative HMG binding does not produce or require a major conformational change in the nucleosome.

The apparent paradox of HMG proteins stabilizing chromatin and being constituents of transcriptionally active chromatin may be resolved by further comparative biophysical studies on chromatin from active and inactive regions.

After this paper was submitted, Yau et al. (55) reported the suppression of early melting transitions of monomer and oligomer nucleosomes by HMG 17. Their results are entirely consistent with those we report here.

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