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The interaction of high mobility proteins HMG14 and 17 with nucleosomes

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ABSTRACT

The interaction of the high mobility group proteins, HMG14 and HMG17, with nucleosome core particles has been studied. The results show that two molecules of HMG14/17 can be bound tightly but reversibly to each core particle and that their affinity for core particles is greater than their affinity for histone-free DNA of core size. Thermal denaturation and nuclease digestion studies suggest that major sites of interaction are located near the ends of the nucleosome core DNA. When nucleosome preparations from chicken erythrocyte nuclei stripped of HMG proteins are partially titrated with HMG14/17, the nucleosome-HMG complex fraction is enriched in β-globin gene sequences.

INTRODUCTION

The binding of HMG proteins, and particularly HMG14 and 17, has been associated with various manifestations of transcriptional activity in the eukaryotic genome. HMG14 and 17 can be found associated with nucleosome core particles isolated from nuclei (1). Levy et al. (2) have shown that H6 (the equivalent in trout testis of HMG14 or 17) co-fractionates with nucleosomes enriched for transcribed genes. Weintraub and his collaborators (3,4) have documented in detail the role of HMG14/17 in conferring upon transcriptionally active genes sensitivity to pancreatic DNase (DNase I).

In this paper, we study the binding of HMG14 and 17 to nucleosome core particles previously stripped of these proteins. Over a considerable range of salt concentrations two molecules of HMG protein can be bound strongly, but reversibly, to each nucleosome. Although HMG14 and 17 also bind histone-free DNA, the affinity of HMG protein for nucleosomes is greater than that for DNA.

We have also studied the sedimentation, thermal denaturation, and DNase I digestion of nucleosome core-HMG complexes. The results suggest that major sites of binding are located near the termini of the core particle DNA.

Finally, in order to assay possible gene specificity of nucleosome-HMG

protein interaction, we have developed a method for the isolation of a radioactive single-strand probe from a cloned globin cDNA sequence. This probe permits quantitation of the sequence of interest by a probe excess titration. By this procedure, we show that HMG14/17 preferentially bind to chicken erythrocyte nucleosomes containing the β-globin genes under conditions where binding to bulk nucleosomes is reversible.

METHODS

Nuclei were prepared from chicken erythrocytes as follows. After washing, the erythrocytes were lysed in 0.01 M NaCl, 3 mM $MgCl_2$, 0.01 M Tris pH7.4, 0.5% Triton X-100, and the nuclei washed three times in this solvent by gentle centrifugation and resuspension. They were then washed three times in the same buffer with Triton omitted.

HMG proteins were prepared from these nuclei by a variety of methods similar to those described by Johns (5). In one preparation of HMG17, a cold perchloric acid extract of nuclei was partially fractionated by addition of cold trichloroacetic acid (see below) and chromatographed on CM-Sephadex (6,7). Other preparations were made by direct perchloric acid extraction of washed chicken red blood cells, followed by fractionation by precipitation with acetone (5). A third preparative method for HMG14/17 made use of fractionation with trichloroacetic acid (TCA). Purified nuclei derived from 40 ml of adult chicken blood were washed with 20 ml of 0.35 M NaCl, 5 mM potassium phosphate buffer pH7.0, 5 mM sodium butyrate, 0.1 mM phenyl methyl sulfonyl fluoride. The nuclei were pelleted, washed successively with 20 ml and 30 ml of the same solvent, and all supernates pooled. The extract was then adjusted to a final concentration of 2% TCA by dropwise addition of cold 100% TCA. After 5 min. at ice temperature, the mixture was centrifuged to remove precipitated protein, and the procedure repeated to bring the total TCA concentration successively to 10%, 15%, and 25%. At each step precipitated fractions were collected by centrifugation. The last fraction contained HMG14/17.

Protein components were identified by gel electrophoresis using the method of Laemmli (8), and staining with a mixture of Coomassie blue and Buffalo black. HMG protein preparations referred to as 'HMG14/17' in this paper had approximately equal concentrations of HMG14 and 17, as judged by staining. HMG17 had some contamination (20% or less) with HMG14, but all preparations were free of other proteins.

Nucleosomes were prepared from chicken erythrocyte nuclei by methods

described previously (9). Monomer fractions isolated from sucrose gradient centrifugation of nuclear digests were typically dialyzed into 0.1 M NaCl, 1 mM or 10 mM Tris pH7.5, 0.1 mM EDTA. The precipitate was removed by centrifugation, leaving in the supernate a nucleosome core preparation free of H1 and H5. This was dialyzed into 0.34 M NaCl, 1 mM Tris pH7.5, and centrifuged in a 5-20% sucrose gradient (Beckman SW-27 rotor, 26 Krpm, 20 hr) containing the same solvent. The monomer fraction was collected. This procedure removed HMG proteins. Similar results were obtained if the 0.1 M NaCl precipitation step was omitted. In that case, the starting monomer fractions were dialyzed directly into 0.35 M NaCl and the precipitate removed before performing the last sucrose gradient sedimentation. All solvents used in nucleosome preparation also contained 5 mM sodium butyrate, and usually 0.1 mM phenyl methyl sulfonyl fluoride. Full size nucleosomes, stripped of both H1/H5 and HMG proteins, were prepared from monomer fractions by dialysis into 0.63 M NaCl, 1 mM Tris, and sucrose gradient sedimentation in this solvent, under conditions like those described above. Nucleosome concentrations in this paper are expressed in terms of weight of DNA present. Some core preparations contained only DNA of average size 145 base pairs; others contained some 165 base pair DNA as well. Results with either kind of preparation were always the same.

Gel electrophoresis of nucleoprotein was performed at 4° in 3 mm thick 4% polyacrylamide gels (acrylamide: bisacrylamide ratio - 20:1), in the buffers shown in the figures. Tris-borate-EDTA (TBE) buffer is 0.089 M Tris, 0.089 M boric acid, and 2.5 mM Na_2EDTA.

Protein concentrations were determined by the method of Lowry. Standard curves for this assay were determined by quantitative measurements of amino acid concentration on HMG protein hydrolysates, using the published amino acid compositions of chicken erythrocyte HMG14 and 17 (10-12).

Analytical ultracentrifugation was carried out in the Beckman Model E ultracentrifuge, with absorbance optics and a Hewlett-Packard 2100 computer for data acquisition and reduction. Thermal denaturation was carried out in a Beckman Acta III spectrophotometer also under the control of this computer.

Labelling of nucleosome cores with ^{32}P at the 5' terminus was carried out by methods previously described (13,14). To complex HMG proteins with these core particles, 37 µg of nucleosomes cores were mixed with 17 µg of HMG14/17, in 0.33 M NaCl, 0.17 M Tris pH7.4, 3.3 mM $CaCl_2$, 3.3 mM $MgCl_2$. The mixture was diluted by dropwise stirring with 5 mM Tris pH7.4 to a final solvent concentration of 0.068 M NaCl, 0.034 M Tris, 0.68 mM $CaCl_2$ and $MgCl_2$,

in a total volume of 1.1 ml. 0.5 units of DNase I were added and digestion carried out at 37° for the times shown in Fig. 9. The reaction was stopped by addition of excess EDTA, the samples deproteinized by methods described below, and samples denatured and run on acrylamide gels containing 7 M urea.

Preparation of materials for measurement of interactions with globin-specific sequences were carried out as follows. Nucleosome cores, freed of HMG proteins as described above, were mixed with HMG14/17 in 0.05 M NaCl, 0.0125 M Na phosphate, pH7.15, and electrophoresed in that solvent in a 6 mm thick polyacrylamide gel similar to that described above for nucleoproteins. Typically, a total of about 0.5 mg of nucleosomes were loaded, in four slots on a 12 cm wide gel. After electrophoresis, gels were stained with ethidium bromide and examined with a long-wavelength ultraviolet illuminator. The bands corresponding to unperturbed monomer and monomer-HMG complex were excised, and the nucleoprotein isolated by electrophoresis (in 0.1 M Tris-acetate buffer, pH7.9) into hydroxylapatite. After elution with 0.5 M sodium phosphate buffer, pH7.15, and dialysis into 0.5 M NaCl, the nucleoprotein was digested at 37° for 2 hr with 20 μg/ml of Proteinase K, then $NaDodSo_4$ was added to a concentration of 0.2% and digestion continued overnight. The digest was extracted twice with phenol and then with chloroform-isoamyl alcohol (20:1), ethanol precipitated, redissolved in a small volume of 0.3 N NaOH and incubated at 37° overnight. The solution was neutralized, again ethanol precipitated, and redissolved in a small volume of water.

Determination of the β-globin gene abundance in these samples was performed by annealing an excess of ^{32}P labelled β-globin single-strand probe (see below) to an aliquot of the DNA sample in 50 mM NaHEPES, pH7.5, 0.3 M NaCl, 1 mM EDTA. Each mixture contained a total of 12 μl of solution, and varying amounts of the DNA sample, but the probe was always in at least 10-fold sequence excess over the sample. The mixtures included calf thymus DNA so that the total DNA concentration was maintained at about 0.7 g/l. Mixtures were sealed in capillaries, denatured by heating at 107°, and incubated at 68° for 23 hrs. The contents were expelled into 288 μl of 31.25 mM Na acetate buffer pH4.5, 104 mM NaCl, 1.04 mM $ZnSO_4$, 1.04 g/l sonicated, native calf thymus DNA, and digested with 30 units of S1 nuclease (P-L Biochemicals) for 30 min. at 45°. The reaction was terminated by the addition of 150 μl of 300 g/l TCA, 30 mM $Na_4P_2O_7$, and the precipitate counted on Whatman GF/C filters.

Single-strand β-globin probe was synthesized from the cDNA plasmid pHb1001 (15). Forty microliters of a solution containing 250 mM Tris HCl

buffer pH7.4, 35 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 10 μM dCTP, 250 μM each dATP, dGTP and dTTP were added to 1 mCi of α-^{32}P-dCTP, 500 kCi/mol (New England Nuclear) which had been dried in a conical centrifuge tube. A limited number of nicks were introduced into 2 μg of the DNA by the addition of 20 μl of 20 μg/l DNase I. DNase I (Worthington DPFF) was stored at a concentration of 1 g/l at -20° in 1.25 mM HCl, 50% glycerol and diluted with 0.5 g/l bovine serum albumin. The reaction was incubated at 21° for 5 min. and terminated by the addition of 20 μl of EGTA, pH8, which selectively binds the calcium required for DNase I activity. The amount of DNase I required was determined empirically based on the strand separating gel described below. Thirty microliters of DNA polymerase I (New England BioLabs, 8 units/μl) were then added and nick-translation carried out for about five hr at 15°. The reaction was terminated by the addition of EDTA to 20 mM, yeast tRNA to 2 g/l, $NaDodSO_4$ to 1 g/l, and Proteinase K to 0.1 g/l. After digestion at 37° for 30 min, the DNA was phenol and chloroform extracted and isolated as the void volume fraction from a Bio-Gel P-10 column. The specific activity of the DNA was about 150-200 cpm/pg. The DNA was ethanol precipitated and digested with the restriction endonuclease Msp I (New England BioLabs), as described by the manufacturer, in a volume of 200 μl. Msp I generates a 450 base pair fragment of pHbl001 that contains about 90% of the β-globin cDNA insert (15). The DNA was ethanol precipitated, dissolved in 0.2 M NaOH and electrophoresed at room temperature on a 4% polyacrylamide gel in TBE buffer. The globin strands were separated from each other and from all other restriction fragments arising from the parent plasmid. The bands were visualized by ethidium bromide staining. They were excised, eluted by maceration and extraction in 0.5 M ammonium acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% $NaDodSO_4$, 10 mg/ml tRNA (16), ethanol precitated, and redissolved in water. Residual self hybridizing material was removed by hydroxylapatite chromatography at 60°.

RESULTS

Gel Electrophoresis

The interaction of HMG14/17 with nucleosome cores can be detected by gel electrophoresis. Except where noted, the cores previously had been stripped of HMG proteins. In typical experiments, increasing amounts of HMG17 or mixtures of HMG14 and HMG17 were added to fixed amounts of core particles, and the samples electrophoresed in 4% polyacrylamide gels. The first experiments were carried out at low ionic strength; the results are shown in Fig. 1. The nucleosome core itself migrates as a sharp band. With the addition of small

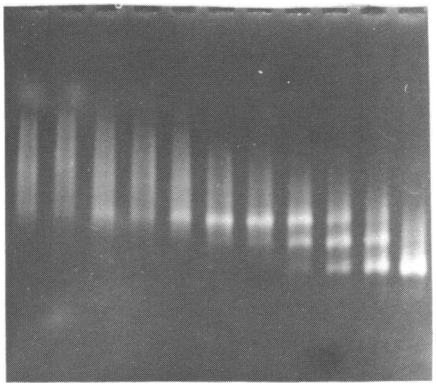


Figure 1. Electrophoresis on 4% acrylamide gel showing titration of nucleosome core particles with HMG17 at low ionic strength (0.1x TBE buffer). Each well has 1 μg of nucleosome cores (concentration expressed as DNA) and increasing amounts of HMG17 as follows: from right to left, 0 to 10 μl, in 1 μl steps, of a 42 μg/ml solution of HMG17. Direction of electrophoresis is downward. The fifth channel from the right, where titration is nearly complete, has about 0.17 μg of HMG17.

amounts of HMG17, a new band, with decreased mobility, is observed. With increasing amounts of HMG protein, a third band, of still lower mobility, appears. At the "end point", all of the nucleoprotein migrates in the position of the third band. At this low ionic strength, HMG17 added beyond this point is bound, but no discrete complexes are visible.

If the titration and gel electrophoresis are carried out at higher ionic strength, a somewhat different result is obtained. Only one slower moving component is observed when HMG17 is added (Fig. 2). The mobility of this component relative to uncomplexed core particles equals that of the slowest moving band (band 3) in Fig. 1. Little HMG17 is bound beyond this point.

The end point of the titration can be estimated by examination of Figs. 1 and 2. In each case, all of the nucleoprotein has been displaced from its usual position on the gel to the position of band 3 when about 0.21 to 0.23 g of HMG17 have been added per gram of nucleosome DNA. This corresponds to the addition of two molecules of HMG protein per nucleosome.

The titration data in Figs. 1 and 2 suggest that HMG17 can bind tightly to the nucleosome core. To confirm this, the experiment of Fig. 2 contained HMG protein labelled with ^{125}I Bolton-Hunter reagent, and nucleosome cores labelled with ^{32}P at the 5' termini of the DNA. The gel was stained with ethidium bromide, the bands excised, and both DNA and HMG protein content

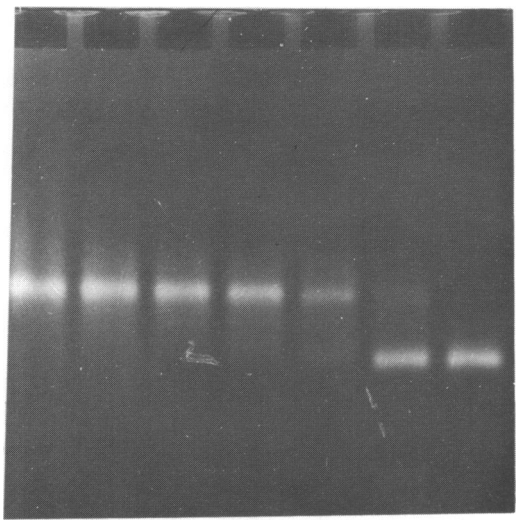


Figure 2. Electrophoresis on 4% acrylamide gel showing titration of core particles with HMG17 at higher ionic strength (TBE buffer). Each well has 1 μg of nucleosome cores and increasing amounts of HMG 17 as follows: from right to left, 0, 2, 4, 6, 8, 10 and 15 μl of a 38 μg/ml solution of HMG17.

determined. As shown in Fig. 3, the addition of HMG17 results in transfer of nucleosome core DNA from the unreacted position to the core-HMG complex position on the gel. The amount of HMG17 found in the complex position increases linearly with HMG17 added; the ratio of HMG protein to DNA in the complex is essentially constant throughout the titration. The endpoint of the titration again corresponds approximately to a 2:1 molar ratio of HMG protein to nucleosomes. It is not possible to carry out a similar experiment under the conditions described in Fig. 1; under such low ionic strength conditions, core particles are non-specifically adsorbed throughout the upper part of the gel, in such a way as to make precise measurement difficult.

When mixtures of HMG14 and 17 are used in titrations similar to that described in Fig. 2, the results obtained are indistinguishable from those shown for HMG17 alone, as seen in Fig. 4. As will be shown below, both HMG14 and HMG17 bind to nucleosome core particles, and appear to behave interchangeably under the conditions of our experiments.

In contrast to the behavior of HMG14/17, the two other HMG proteins, HMG1 and HMG2, do not bind to nucleosome core particles in 0.1x TBE, as

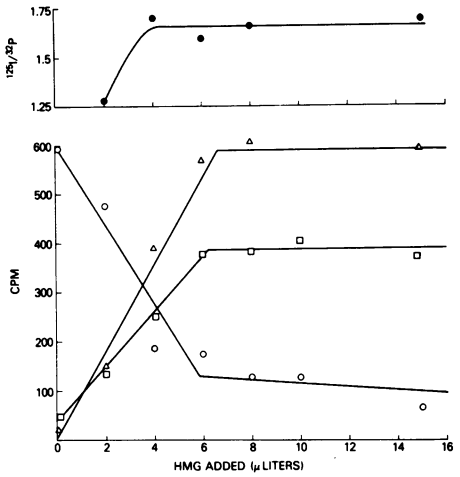


Figure 3. Excised bands from the gel in Figure 2 were counted for ^{32}P (Cerenkov) or ^{125}I (gamma). Lower graph: ○, ^{32}P in the lower band (negligible ^{125}I detected). □, ^{32}P in the upper band. △, ^{125}I in the upper band. Upper graph: ratio of ^{125}I to ^{32}P in the upper band. Molar ratio at endpoint is HMG:nucleosomes = 2.4:1 (approximately).

judged by the criterion of alteration in electrophoretic mobility (data not shown; see also Ref. 22).

Competition with DNA

We have studied the competition between nucleosome cores and histone-free DNA for HMG14/17. This has been done both by an extension of the gel electrophoretic methods described above, and by sedimentation in a sucrose gradient. In both cases, HMG proteins were added to a mixture of core particles and DNA of approximately core size. A variety of ionic conditions have been employed in these studies. Two typical electrophoretic patterns are shown in Fig. 5. When DNA alone is used (Fig. 5b, lanes 8,9), HMG proteins bind to it and alter its mobility. The binding of HMG17 to DNA has been described in some detail (17). In the Tris-borate buffer used in Fig. 5b, the addition of between 1 and 2 molecules of HMG per DNA molecule is sufficient to displace all the DNA from its uncomplexed position. In 0.05 M NaCl, 0.0125 M Na phosphate buffer pH7, a larger excess of HMG protein is required (Fig. 5a). In both buffers, when core particles and DNA are present together, the HMG proteins are distributed between them, (see Fig. 5b) but it is clear that the end point of the core particle titration is not greatly displaced to higher HMG protein concentrations by the presence of DNA. Note that the complex of

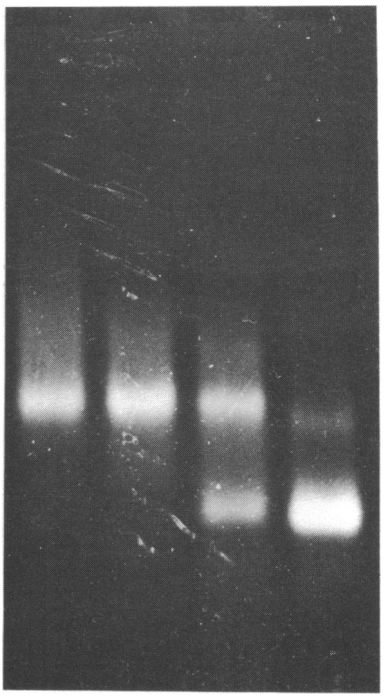
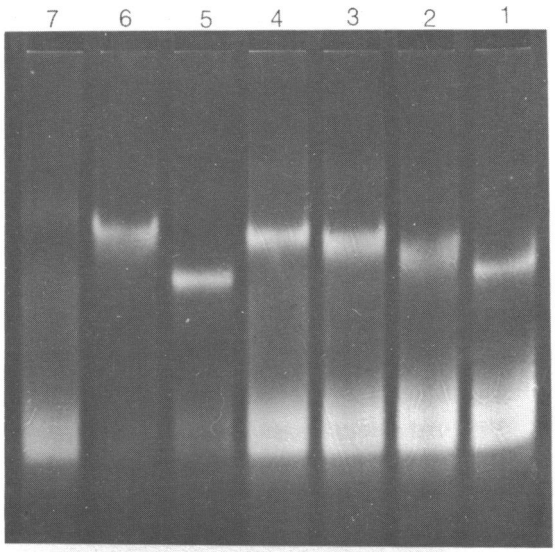


Figure 4. Electrophoresis on 4% polyacrylamide gel showing titration of nucleosome cores with HMG14/17 in 2x TBE buffer. 0.72 μg nucleosome core (expressed as DNA) in each lane. From right to left, 0, 6, 10 and 15 μl of a 22 μg/ml solution of HMG14/17.

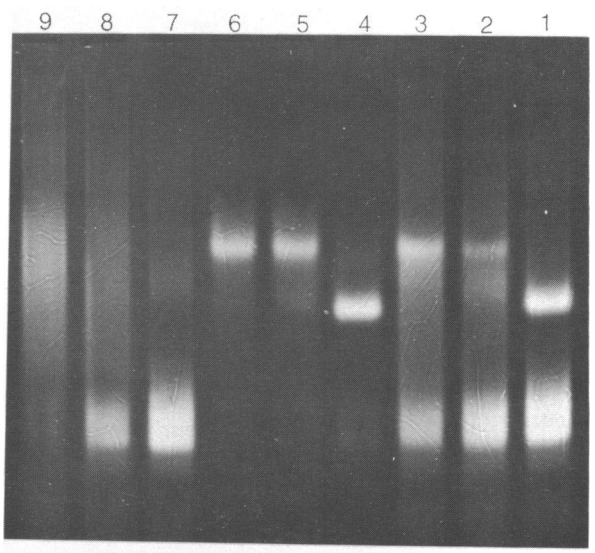
core-size DNA and HMG protein has a mobility like that of HMG-nucleosome complexes. This is fortuitous: the sedimentation properties of the DNA-HMG complex are those of an unfolded structure.

We have repeated the titration in a series of solvents containing 0.015 M Na phosphate buffer (pH7) and NaCl concentrations varying between 0.025 M and 0.075 M. The results are all similar to those shown in Fig. 5a. At NaCl concentrations above about 0.1 M, no binding to either core particle or DNA is observed.

Competition experiments have also been carried out by sucrose gradient sedimentation in a number of solvents. A typical result is shown in Fig. 6, in which ^{125}I labelled HMG proteins have been used. The result confirms that these proteins bind to both nucleosome core particles and DNA. Under conditions of DNA and nucleosome excess, we have never observed a distribu-



a



b

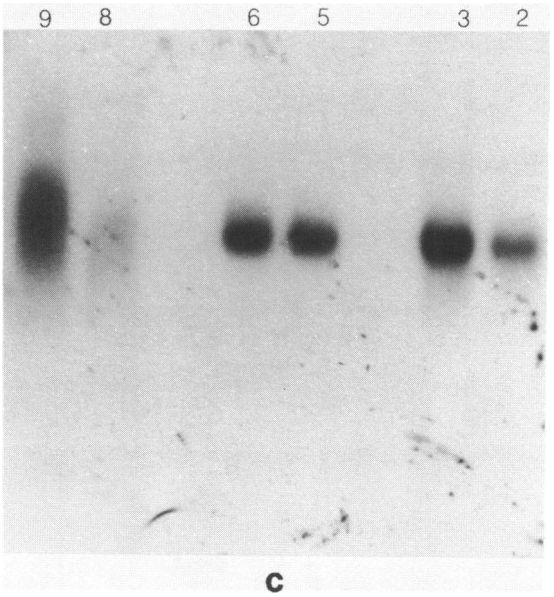
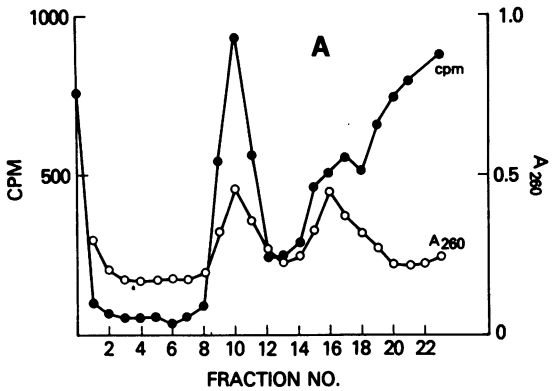


Figure 5. Electrophoresis on 4% polyacrylamide gels showing competition between DNA and nucleosome cores for HMG. Lanes contained 0.68 μg of nucleosome monomer, 0.73 μg of DNA 140 to 160 base pairs long, or a mixture of both. HMG14/17 at a concentration of 11 μg/ml and containing ^{125}I-labelled HMG protein was added as indicated. a) Solvent 0.05 M NaCl, 0.0125 M Na phosphate buffer, pH7.8. Lanes 1-4: 0, 4, 8 and 12 μl HMG added to cores + DNA. Lanes 5,6: 0, 12 μl HMG added to cores alone. Lane 7: 12 μl HMG added to DNA alone. b) A similar experiment in 2x TBE buffer, 5 mM sodium butyrate. Lanes 1-3: 0, 8 and 12 μl HMG added to cores + DNA. Lanes 4-6: 0, 8 and 12 μl HMG added to cores alone. Lanes 7-9: 0, 12 and 25 μl HMG added to DNA alone. c) Autoradiogram of gel in (b). Lanes are numbered correspondingly. In the original photograph, dark bands in 2, 3, 5 and 6 align with upper bands of those lanes in the stained gel (b).

tion of label in which the DNA binds more HMG protein than do the core particles. We estimate from these gradient and electrophoretic experiments that the relative binding affinities for HMG proteins are between 2- and 10-fold greater for core particles than for DNA.

In order to conclude that these results reflect differences in equilibrium binding properties, it is necessary to show that the binding is reversible. We have therefore repeated the sucrose gradient sedimentation studies (in 2x TBE buffer) while varying the order of addition of the components. As shown in Fig. 6, the distribution of ^{125}I-HMG14/17 is similar whether the order of addition is DNA-HMG-core particles or core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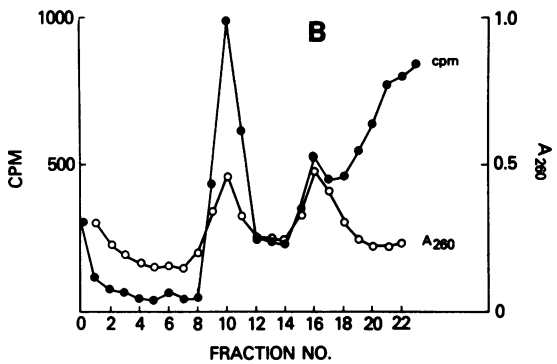
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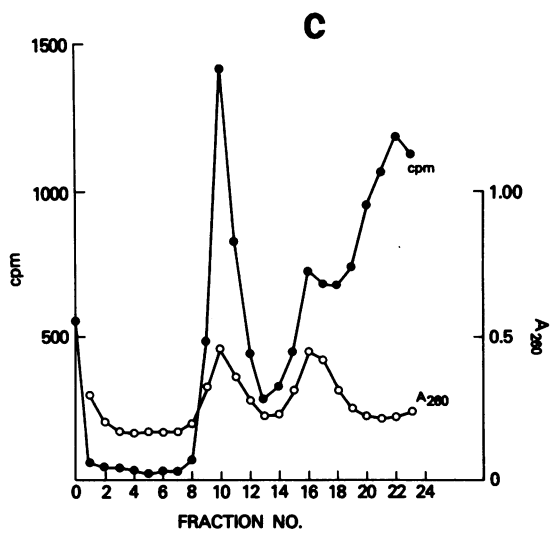
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particles-HMG-DNA. Thus, under these conditions, the binding is reversible.

In other sucrose gradient sedimentation experiments, we have examined by $NaDodSO_4$ gel electrophoresis the proteins bound to the nucleosome core and DNA peaks, using autoradiography to identify the ^{125}I containing components. Both HMG14 and HMG17 are bound to core particles in 50 mM NaCl, 10 mM Tris pH7.5. Experiments carried out with labelled HMG1 and 2 reveal no binding to nucleosome cores (data not shown).

Other binding experiments

We have also performed gel electrophoresis experiments designed to test further the reversibility of HMG14/17 binding. In one experiment a titration was carried out by mixing nucleosome core particles and DNA with HMG proteins in 0.35 M NaCl, a salt concentration at which the protein is not bound. The mixtures were then diluted by dropwise addition of buffer to a final concentration of 0.05 M NaCl, 25 mM Tris pH7.9. Gel electrophoresis at this final salt concentration gave results identical with those obtained when protein, DNA and core particles were mixed directly in the final solvent. A similar experiment in which the initial buffer was TBE, and the mixture was then diluted with 1/1000 TBE to a final concentration of 1/10 TBE, gave a result identical to that in Fig. 1, with two distinct slower-moving bands.

Although most experiments were carried out with nucleosome cores, some studies were also carried out with particles containing longer DNA (as large as 200 base pairs), but stripped of histones H1 and H5. The binding properties of these particles were quite similar to those of nucleosome cores.

Sedimentation properties

The sedimentation properties of nucleosome cores are not significantly altered by the binding of HMG14/17. Fig. 7 shows a sedimentation coefficient distribution analysis of nucleosome cores carrying a saturating quantity of HMG protein.. The pattern is similar to that of nucleosome cores themselves. There is a very small reduction in the sedimentation rate when HMG proteins are bound, even in the presence of large excess of protein.

Thermal denaturation

It is known that under certain conditions, HMG1 and 2 lower the melting temperature of DNA (18). As shown in Fig. 8a, this is not the case for

Figure 6. Sucrose gradient sedimentation of mixtures of nucleosomes cores, DNA of core size, (about 40 µg each) and ^{125}I labelled HMG14/17 (1.4 µg). ○ A_{260}; ● cpm in ^{125}I. The order of addition was: a) Nucleosomes, DNA, HMG. b) DNA, HMG, nucleosomes. c) Nucleosomes, HMG, DNA. The solvent was 2x TBE buffer. Nucleosome peak = Fraction 10. DNA = Fraction 16.

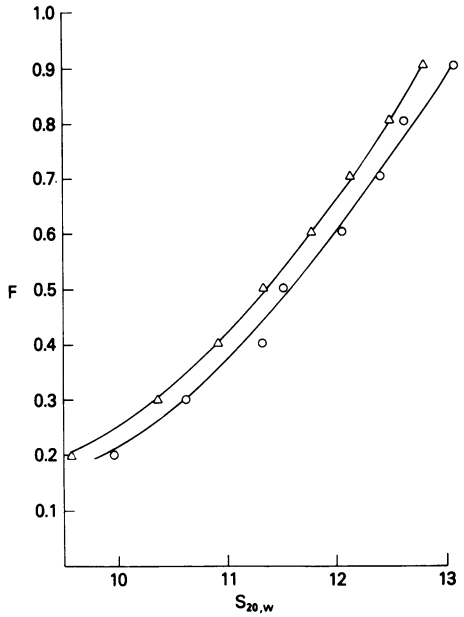

Figure 7. Integral sedimentation coefficient distribution of nucleosome cores without (○) and with (△) HMG17 added. F is the fraction of material absorbing light at 260 nm having a value of $s_{20,w}$ less than that shown. Nucleosome concentration (as mass of DNA) 17.5 µg/ml. HMG17 concentration 18 µg/ml. Solvent was TBE buffer.

HMG14/17, which stabilize DNA against denaturation. A stabilizing effect is also observed when HMG14/17 binds to nucleosomes (Fig. 8b). The stabilization of the nucleosome core results in elimination of the early-melting region of the denaturation curve, but has little effect on the main portion of the melting.

DNase I cutting pattern

We have studied the effect of HMG14/17 on the pattern of cuts generated in nucleosome cores by DNase I (pancreatic DNase). Nucleosome cores, labelled with ^{32}P at the 5' termini of DNA, were digested partially with DNase I, the DNA denatured and electrophoresed on polyacrylamide gels, and the gel autoradiographed. The well-known characteristic pattern of fragments was obtained (Fig. 9a) (13,19). The fragments are roughly integral multiples of 10.4 nucleotides in length (20); some fragments in the series are missing or weak, while others are quite intense. When the same digestion experiment is repeated using HMG-nucleosome core complexes, the same general pattern is obtained. However, at all stages in the digestion, the bands at about 11

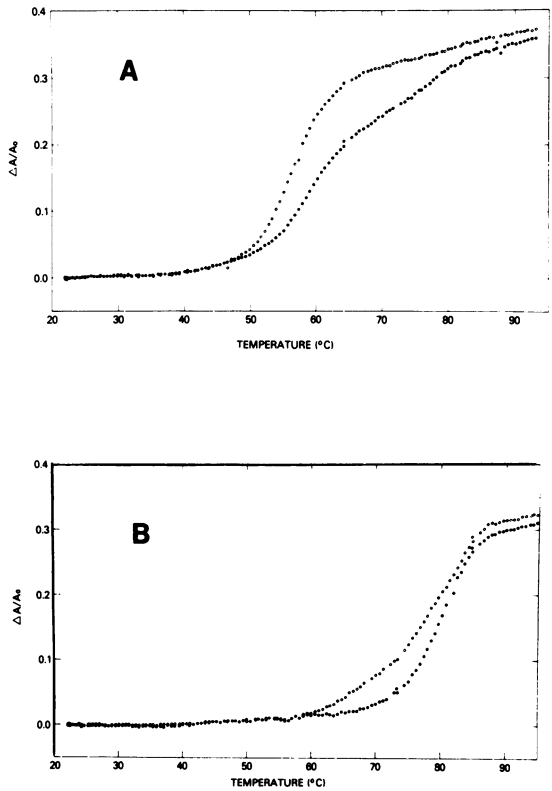

Figure 8. Effect of HMG14/17 on thermal denaturation of DNA and nucleosomes. (A) DNA without (O) and with (●) HMG14/17. DNA concentration 34 μg/ml. HMG concentration 11 μg/ml. Solvent, 0.025 M Na phosphate buffer, pH7.15, 0.02 mM EDTA. (B) nucleosome cores without (O) and with (●) HMG14/17. (In both A and B, the lower curve on the temperature scale corresponds to no HMG.) Nucleosome concentration (as DNA) 31 μg/ml. HMG concentration 11 μg/ml. Components were mixed in 0.05 M NaCl, 0.0125 M Na phosphate buffer, pH7.15, 0.02 mM EDTA, then dialyzed into 2.5 mM phosphate, pH7.15, 0.02 mM EDTA. The lower ionic strength was used to avoid aggregation at high temperature.

and 21 nucleotides from the 5' end are reduced in intensity, relative to other neighboring bands. Examination of the pattern of cuts near the far end of the core DNA similarly reveals a reduced cutting frequency at the 133 nucleotide position. (The cutting frequency at 122 nucleotides was too weak to use, in both control and experiment).

In comparing the intensities of the bands, it is important to note (Fig. 9a) that the rate of digestion of HMG-nucleosome complexes is considerably slower than that of nucleosome cores alone, as judged by the

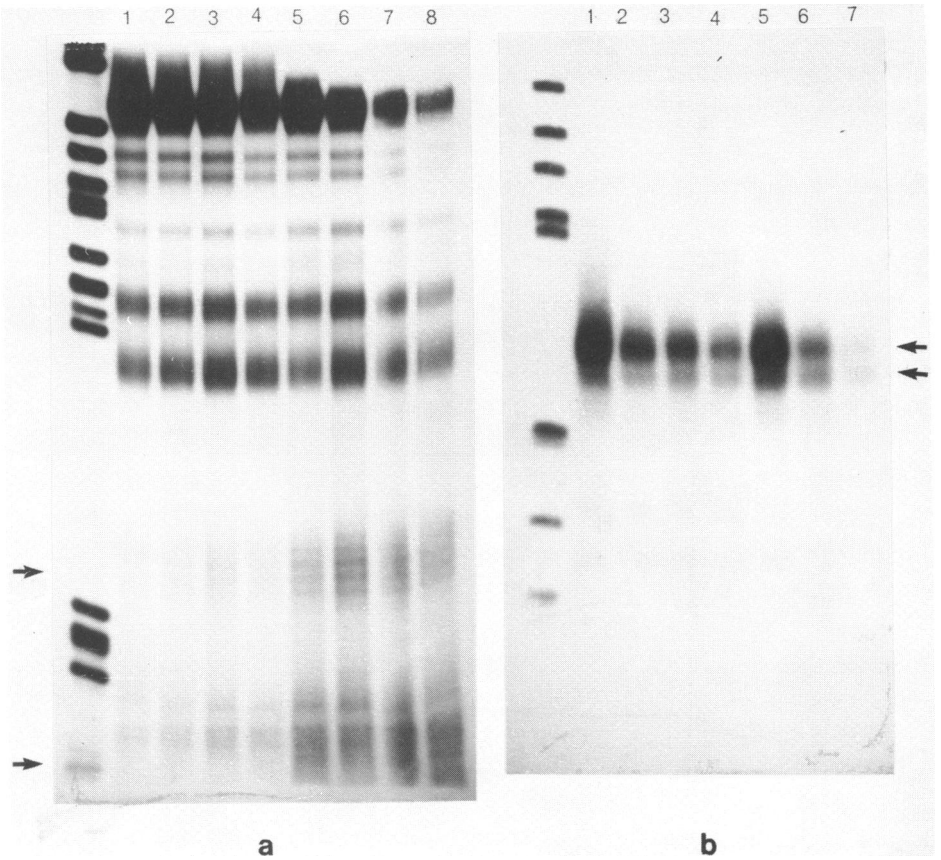

Figure 9. Electrophoretic pattern generated by DNase I digestion of nucleosome core particles ^{32}P labelled at the 5' end. Electrophoresis and digestion conditions are described in Methods. (a) 10% acrylamide gel. Lanes 1-4: Nucleosomes with added HMG14/17 (Nucleosome DNA: HMG mass ratio 2:1), digested 10, 20, 30, or 40 minutes. Lanes 5-8: Nucleosomes only, digested 5, 10, 15 or 20 minutes. Arrows at the left point to the 20 and 10 nucleotide regions of the gel. (b) 6% acrylamide gel. Material in each lane similar to corresponding lane in a). Arrows at the right point to the 140 and 130 nucleotide regions of the gel. Standard at left is 5' terminally labelled HaeIII digest of pBR322.

intensity of the 145 base pair band. For that reason, lanes 3a and 5a, 4a and 6a, or 4b and 6b should be compared. However, at no point in the digestion is the 10-20 nucleotide region as intense in the complex as in the control.

We conclude that HMG14/17 bind to nucleosome cores in such a way as to

block access, at least partially, to sites about 10 to 20 base pairs in from the ends of the core DNA.

Selectivity of HMG proteins for nucleosomes containing globin DNA sequences

Studies of the role of HMG14/17 in conferring DNase I sensitivity on chromatin containing transcriptionally active genes suggest (see Discussion) that these proteins bind preferentially to nucleosomes containing such genes (3). We have tested this idea with the HMG-nucleosome complex described here.

Preparative polyacrylamide gels similar to those in Fig. 2 or 4 were run and the bands excised. The β-globin gene content of each of these bands was determined by single-strand probe excess titration. As described in detail in the Methods, we have used a procedure for the nick-translation of cloned DNA and the isolation of labelled single-stranded restriction fragments. These single-strand fragments are then used in probe excess titrations in a manner exactly analogous to cDNA excess titrations. Such titrations have a distinct advantage over hybridization kinetic measurements of gene abundance, as they do not depend on the length of the hybridizing fragments. Isolation of a single-strand probe from a cloned DNA sequence eliminates a major disadvantage of cDNA excess titrations: cDNA excess titrations accentuate any contaminating sequences found in these preparations. The use of a cloned single-strand probe eliminates any contaminating sequences and allows us to obtain probe excess titration curves with great reproducibility, and with smaller quantities of material. The use of a pure probe for the β-globin sequence, free of α-globin sequences, also simplifies interpretation.

Determination of the globin gene content of the HMG-nucleosome complex is shown in Fig. 10. About 550 μg of chicken erythrocyte nucleosome cores were mixed with 30 μg of HMG14/17 in 0.05 M NaCl, 0.015 M Na phosphate buffer, pH7.8, 5 mM Na butyrate. The mixture (along with a control sample of nucleosomes without HMG14/17) was electrophoresed in this solvent on a 4% polyacrylamide gel, the bands excised, and the DNA purified. The β-globin gene content of the control monomer band with no added HMG14/17 was 17.2 cpm/μg chicken DNA, slightly enriched when compared with sonicated chicken DNA (13.7 cpm/μg). When HMG14/17 is added so that about 25% of the DNA is titrated, the band containing the HMG-nucleosome complex is nearly 2-fold enriched in β-globin (30.4 cpm/μg), and the uncomplexed monomer band is correspondingly depleted (14.5 cpm/μg). All the β-globin gene is accounted for in these two bands: the HMG-nucleosome complex has 44% of the input β-globin genes and the depleted monomer 63%.

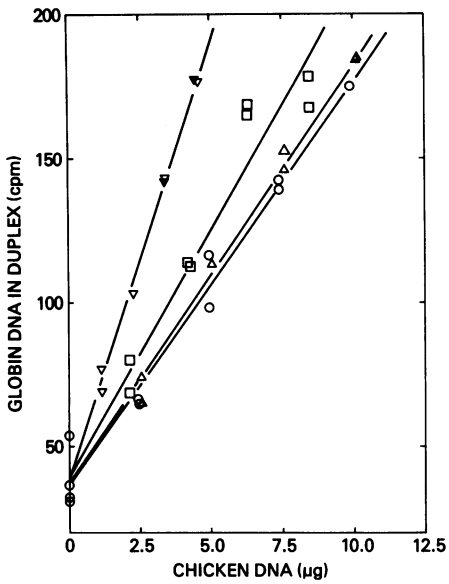

Figure 10. Titration of HMG-nucleosome complexes and other samples with β-globin single-strand probe. Nucleosomes from chicken erythrocytes were partly titrated with HMG14/17, electrophoresed on a 4% acrylamide gel, and stained with ethidium bromide. The bands were excised, DNA isolated, and titrated with globin probe (see Methods). Each titration contains 7700 cpm of ^{32}P in single-strand probe, about 110 cpm/pg. (○) Chicken DNA control. (□) Monomer DNA, with no HMG, carried through the electrophoresis and isolation procedures. (△) Lower band (nucleosomes uncomplexed with HMG) from a titration experiment. (▽) Upper band (nucleosome-HMG complex) from the same experiment.

In a control experiment (data not shown) HMG proteins were used to titrate DNA purified from erythrocyte nucleosomes (for example Fig. 5b, lane 9). In this experiment no β-globin gene enrichment in the HMG-DNA complex was found.

DISCUSSION

The titrations shown in Figs. 1-4 demonstrate that there are two strong binding sites on nucleosome cores for HMG14 or 17. In sufficiently dilute Tris-borate buffer, core particles with one or two molecules of bound HMG protein can be distinguished from each other. In gel electrophoresis at higher ionic strength (Fig. 2), only a single band is observed; it corresponds to the complex containing two HMG protein molecules per

nucleosome core (Fig. 1).

It seems likely that the difference between the results in Figs. 1 and 2 reflects a difference in the reaction mechanisms at the two different ionic strengths, and not merely a loss of resolution on the gel. The fact that no singly-reacted intermediate is observed at higher ionic strength (see Fig. 2 and 3) means that the reaction is cooperative under such conditions. The appearance of the singly-reacted intermediate at low ionic strength can be explained if the reaction is reversible but non-cooperative. It is also possible that at low salt concentration, binding is irreversible, so that the intermediate structure is trapped. We have ruled out the latter possibility by showing that mixture of HMG and core particles in high salt, followed by dilution to low salt, gives the same pattern obtained by direct mixture in low salt. Thus, the reaction at low ionic strength is non-cooperative and reversible.

We have been particularly concerned with the questions of reversibility and strength of the HMG-nucleosome interaction, and we have therefore carried out tests of reversibility and relative binding affinities under a variety of conditions. The most informative of these experiments involve competition for HMG between DNA of monomer size and nucleosome cores. The results obtained by gel electrophoresis and by sucrose gradient sedimentation are in good agreement. They show that DNA itself can bind HMG14/17, that nucleosome cores compete effectively for HMG14/17, and that neither altering the order of addition of the components, nor diluting into low salt after mixing in high salt solvents, has any effect on the apparent binding. Under all conditions we have employed, nucleosome cores bind HMG14/17 more tightly than does DNA of nucleosome core size. This may simply be accounted for by the higher negative charge density of the DNA in the nucleosome core, where the turns of duplex are in close proximity.

Similar conclusions about the stoichiometry of binding of HMG14 and 17 have been drawn from electrophoresis experiments by Garrard (21), who also showed that HMG14/17 could exchange freely among nucleosomes under moderate ionic conditions, confirming the observations of Rill (22). Our results are entirely consistent with these observations.

The binding of the HMG proteins does not appear to affect the overall conformation of the nucleosomes. The sedimentation coefficient of the complex is very slightly smaller than that of the core particle itself; this is the result of a slight increase in frictional coefficient balancing the small effective mass increment.

Clearly, these HMG proteins do not unfold nucleosomes. The thermal denaturation results (Fig. 8) show that nucleosome core DNA is stabilized against melting by the presence of HMG. Most of the stabilizing effect occurs in the early-melting region; the denaturation curve in the late-melting region is hardly perturbed. It has been demonstrated that the early denaturation step observed in nucleosome cores at low ionic strength is associated with liberation and melting of about 20-25 base pairs at the DNA termini (23-25). The HMG proteins affect the denaturation of these termini.

This observation is consistent with the pattern of cuts obtained when the complex is digested with DNase I (Fig. 9). The cutting sites near both ends of the DNA (10, 20 and 130 nucleotides from the 5' end) are partly protected against nuclease attack. The absence of special protection elsewhere along the DNA backbone is of course not evidence for the absence of binding elsewhere, but these results, together with the thermal denaturation data, do suggest that the binding of HMG14/17 involves the terminal regions of the core DNA. At least part of the site of contact between these proteins and the nucleosome core must be in these regions.

If the contact sites are inset roughly 10 base pairs from the termini of the nucleosome core's duplex, they are separated by about 1 1/2 turns of supercoil, assuming that there are about 83 base pairs per turn. Thus the sites are on opposite sides of the nucleosome from one another. Garrard (21) has reported that nucleosomes carrying H1 histone are still able to bind HMG14/17. We confirm this observation, and find that chicken erythrocyte nucleosomes carrying H5 still bind HMG14/17, as judged by gel electrophoretic experiments (data not shown). It is thought that the major binding site of H1 or H5 is in the region of the 166 base pair chromatosome where the ends of two full turns of DNA emerge (26). This is of course outside the nucleosome core itself, so that H1 need not be expected to interfere with the binding of HMG14/17, if the latter are bound as suggested above.

Recent studies have shown that the presence of HMG14/17 is correlated with the sensitivity of transcriptionally active chromatin to digestion by DNase I (3). Removal of these HMG proteins abolishes the sensitivity, but adding them back restores sensitivity. The phenomenon has also been observed with individual nucleosomes: Weisbrod et al. (4) have carried out a titration of chicken erythrocyte nucleosomes (depleted of HMG) with HMG14/17. DNase I sensitivity of the globin gene is restored to about 2/3 its original value when one HMG molecule has been added for each twenty nucleosomes.

Such a result could be explained if HMG14/17 were bound only by a small

subclass of nucleosomes which included those containing globin genes. Our results show that this is not the case: under a wide variety of solvent conditions where the reaction is reversible, all nucleosome cores and nucleosomes bind HMG14/17 more tightly than does naked DNA. The results of DNase I sensitivity titration therefore imply that nucleosomes containing globin genes have an even greater affinity for HMG14/17 than do normal nucleosomes. The experiment shown in Fig. 10 directly demonstrates the correctness of this conclusion. When a nucleosome core preparation from chicken erythrocytes is partially titrated with HMG14/17, the electrophoretic band containing the complex is enriched in β-globin gene content, and the uncomplexed monomer is correspondingly depleted. The measured enrichment is only half the maximum possible, but this may be an underestimate, since under our annealing conditions the adult β globin probe will also react with embryonic β globin sequences. It has been reported (27) that chromatin containing the embryonic genes is relatively insensitive to DNase I in adult erythrocyte nuclei, and monomers containing these genes may fail to show selective binding of HMG14/17.

The extent to which binding is selective also depends upon the ratio of the affinity constants for binding to globin gene-containing particles compared to normal nucleosomes, under our experimental conditions. Preliminary experiments at higher salt concentrations, just sufficient to suppress binding to bulk nucleosomes, show that particles containing globin genes are still capable of binding HMG proteins.

Although we have discussed the nucleoprotein particles containing globin genes as though they were nucleosomes, it is in principle possible that they are quite different. On the other hand, those particles which complex preferentially with HMG proteins behave, in terms of mobility on gels, exactly like normal nucleosomes. Major differences in protein composition between globin-sequence containing particles and other nucleosomes therefore seem unlikely.

It remains to be determined what features of nucleosomes containing transcriptionally active gene sequences confer the ability to bind HMG14/17 so tightly, and to render the entire complex specially sensitive to DNase I. (We note that HMG14/17 has the effect of reducing the DNase I sensitivity of bulk nucleosomes.) Histone acetylation or other modifications are at least two possible ways in which these nucleosomes might be "marked". Such hypotheses can be tested by the methods described here.

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