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Preferential in Vitro Binding of High Mobility Group Proteins 14 and 17 to Nucleosomes Containing Active and DNase I Sensitive Single-Copy Genes†

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ABSTRACT: High mobility group (HMG) proteins 14 and 17 bind to mononucleosomes in vitro, but the exact nature of this binding has not been clearly established. A new method was developed to allow direct membrane transfer of DNA from HMG 14/17 bound and unbound nucleosomes, which have been separated by acrylamide gel electrophoresis. Hybridization analysis of membranes obtained by this method revealed that the HMG 14/17 bound nucleosomes of avian erythrocytes and rat hepatic tumor (HTC) cells were enriched, about 2-fold, in actively transcribed genes and also inactive but DNase I sensitive genes. Nucleosomes containing inactive, DNase I resistant genes were bound by HMG 14/17, but not preferentially. Several factors that have been reported to greatly influence the binding of HMG 14/17 to nucleosomes in vitro were tested and shown to not account for the preferential binding to DNase I sensitive chromatin. These factors include nucleosomal linker DNA length, single-stranded DNA nicks, and DNA bulk hypomethylation. An additional factor, histone acetylation, was preferentially associated with the HMG 14/17 bound chromatin fraction of avian erythrocytes, but it was not associated with the HMG 14/17 bound chromatin fraction of metabolically active HTC cells. The latter finding was true for all kinetic forms of histone acetylation.

The organization of chromatin in the eukaryotic nucleus is thought to play an important part in the regulation of gene activity [for review, see Cartwright et al. (1982)]. At the first level of organization, DNA is assembled with histones into structures called nucleosomes. Although both transcriptionally active and inactive DNA are contained in nucleosomes (Cartwright et al., 1982), distinct structural differences must exist between these states since transcriptionally active chromatin has an enhanced sensitivity to the pancreatic-derived nuclease DNase I compared to the bulk of chromatin (Weintraub & Groudine, 1976). While the ultimate features of chromatin that direct this sensitivity are unknown, the high mobility group (HMG)[1] proteins 14 and 17 have been considered as possible mediators of the enhanced digestion of active chromatin by DNase I. This consideration is based on the observation that 0.35 M salt washing of nuclei or nucleosomes, a treatment that removes a number of non-histone proteins, results in a loss of the nuclease sensitivity of active genes and that this sensitivity can be restored by the readdition of purified HMG 14/17 proteins (Weisbrod & Weintraub, 1979; Gazit et al., 1980). Further attempts to associate HMG 14/17 proteins with transcriptionally active chromatin have given conflicting results and, thus, have been inconclusive (Levy et al., 1977; Levy & Dixon, 1978; Goodwin et al., 1979; Albanese & Weintraub, 1980; Sandeen et al., 1980; Barsoum et al., 1981; Levinger et al., 1981; Weisbrod, 1982; Nicolas et al., 1983; Swerdlow & Varshavsky, 1983). The role of HMG 14/17 proteins in mediating DNase I sensitivity has also been recently questioned (Nicolas et al., 1983; Seale et al., 1983; Goodwin et al., 1985).

The HMG 14/17 proteins are present together in nuclei at about one copy each per ten nucleosomes (Mayes, 1982), and both interact with the nucleosome at the junction between core and linker DNA (Goodwin et al., 1979; Sandeen et al., 1980; Mardian et al., 1980). However, the features of nucleosomal preparations that direct HMG 14/17 binding are not known. Various factors that have been implicated in preferential binding in vitro include nucleosomal DNA length (Goodwin

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[1] Abbreviations: HMG, high mobility group proteins; CRBC, chicken red blood cells; HTC, rat hepatic tumor cell line; TAT, tyrosine aminotransferase; SDS, sodium dodecyl sulfate; bp, base pairs; kbp, kilobase pairs; AZA, 5-azacytidine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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et al., 1979; Swerdlow & Varshavsky, 1983; Stein & Townsend, 1983), single-stranded DNA (Isackson & Reeck, 1981; Weisbrod, 1982), acetylated or hyperacetylated nucleosomal core histones (Weisbrod, 1982; Malik et al., 1984), and hypomethylation of genomic DNA (Weisbrod, 1982).

In this study, we have examined the features of chromatin responsible for the in vitro binding of HMG 14/17 proteins to mononucleosome preparations using acrylamide gel electrophoresis to separate HMG 14/17 bound from unbound nucleosomes and a new method for direct blot hybridization analysis. Our results confirm the 2–3-fold enrichment of adult globin genes in HMG 14/17 bound nucleosomes from adult avian erythrocytes (CRBC) reported previously (Sandeen et al., 1980). The same degree of enrichment was found for the transcriptionally active tyrosine aminotransferase (TAT) gene in HMG 14/17 bound nucleosomes from rat hepatic tumor (HTC) cells. In addition, avian erythrocyte nucleosomes containing the embryonic globin gene ρ were also preferentially bound. The ρ-globin gene is inactive but sensitive to digestion by the nuclease DNase I in adult erythrocytes (Ginder et al., 1984). Although nucleosomes containing inactive and DNase I resistant genes also bound HMG 14/17, there was no enrichment. In our studies, binding did not depend on the length of linker DNA, the extent of single-strand DNA nicks introduced during mononucleosomal preparation, or the bulk hypomethylation of DNA. Interestingly, while histones modified by the fast-kinetic form of acetylation were closely associated with preferential HMG 14/17 binding in nucleosomes prepared from avian erythrocytes, no correlation was found between histone acetylation and HMG 14/17 binding to nucleosomes prepared from HTC cells.

Materials and Methods

Treatment of Cells. CRBC were collected in acid–citrate–dextrose and washed as described previously (Brotherton et al., 1981). HTC cells were grown in suspension culture in Swimms S-77 medium with 5% calf serum. Experimental procedures were performed with cells harvested during their log growth phase [(4–6) $\times$ 10^5 cells/mL].

For histone labeling, HTC cells were concentrated to (4–6) $\times$ 10^7 cells/mL for 10-min pulses and to 2 $\times$ 10^7 cells/mL for 30-min pulses. CRBC were suspended at 1–2 mL of packed cells in 25 mL of media. Cells were labeled with 1 mCi/mL [^{3}H]acetate (Amersham, 4 Ci/mmol) in Swimms and chased in butyrate as previously described (Brotherton et al., 1981; Covault & Chalkley, 1980). In some experiments, pulse-labeled cells were washed in ice-cold media without butyrate and used immediately for the preparation of nucleosomes. HTC cells were labeled with [*methyl*^{3}H]thymidine (Amersham) at 25 mCi/1500 mL or [*methyl*^{14}C]thymidine (Amersham) at 5 mCi/1500mL by overnight incubation at 37 °C. For experiments using hypomethylated DNA, HTC cells were incubated for 48 h in media containing 3 μM 5-azacytidine (Jones & Taylor, 1980) (see below).

Nuclear Isolation and Mononucleosome Preparation. Nuclei were prepared from CRBC and HTC cells as previously described (Brotherton et al., 1981). Unless otherwise stated, all procedures were carried out in the presence of 5 mM sodium butyrate and 0.1 mM PMSF. Mononucleosomes, stripped of non-histone protein and histones H1 and H5, were prepared by the method of Sandeen et al. (1980) by sucrose gradient centrifugation in 0.35 M NaCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA, and 50 mM sodium butyrate buffer. An alternative method of mononucleosome preparation was by the method described by Perry and Chalkley (1982) in which noncore histone proteins were removed by washing mononucleosomes in 0.45 M NaCl in the presence of Dowex 50W-X2 resin (Bio-Rad) (Bouland & Jones, 1973). Except in the experiments presented in Figure 3, a second digestion with nuclease was always used to remove contaminating polynucleosomes and trim linker DNA. This was done by making the nucleosome suspension in 1 $\times$ TBE (0.089 M Tris-HCl, 0.089 M boric acid, 2.5 mM Na_2EDTA, pH 8.4) 10 mM with $CaCl_2$, warming to 37 °C, and reacting with nuclease (1 unit/25 μg) for 1 min before stopping the reaction by adjusting to 40 mM EDTA. Nucleosome suspensions were used immediately in further reactions or for electrophoresis (see below). Mononucleosomes prepared by either method were free of noncore histones and HMG proteins as assessed by SDS–polyacrylamide gel electrophoresis.

Protein Isolation. HMG 14 and 17 were isolated from CRBC, HTC cells, or adult rat liver nuclei either by the procedure of Weisbrod and Weintraub (1981) using trichloroacetic acid precipitation or by that of Bhullar and Candido (1982) using ammonium sulfate. Both methods yielded preparations containing approximately equal amounts of HMG 14 and 17, free of other proteins as assessed by SDS–acrylamide gel electrophoresis. HMG 14/17 preparations contained between 0.4 and 1 mg/mL protein as assessed by the Lowry colorimetric assay but were used empirically in titration experiments (see below).

Nucleosome Titration with HMG 14/17. Mononucleosome preparations (0.5 mg/mL) in 1$\times$ TBE, 10 mM $CaCl_2$, 40 mM Na_2EDTA were mixed with HMG 14/17 proteins in ratios as indicated in each experiment and allowed to react 5 min at room temperature. HMG 14/17 bound and unbound mononucleosomes were then separated by gel electrophoresis in 5% polyacrylamide gels [acrylamide:bis(acrylamide) ratio 20:1] in 1$\times$ TBE (Sandeen et al., 1981). In the experiments shown in Figure 4, incubation with HMG 14/17 was carried out in 1$\times$ TBE, and excised slab gel lanes were cut in 2-mm steps and digested in 0.6 mL of 30% H_2O_2 at 60 °C overnight for use in scintillation counting. In other experiments, gels were processed for fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). Densitometric scans were performed on 0.1% Coomassie blue stained gels and the corresponding fluorograms with a DU-8B scanning spectrophotometer (Beckman).

DNA Preparation and Hybridization. Direct transfer of DNA from nucleohistone gels was accomplished by incubating the gels in 1% SDS and 1$\times$ TAE (10 mM Tris, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.8) overnight at 28–35 °C. SDS was removed by washing the gel in H_2O with mixed-bed resin (Bio-Rad, 5 g/100 mL of H_2O) for 2 h. After impregnation with 1$\times$ TAE, the gel was sealed in a plastic bag, immersed in boiling water for 15 min, and then cooled immediately in ice water to denature DNA. DNA was then directly transferred to nylon membranes (Zeta-Probe, Bio-Rad) by electroblotting at 1 A for 3 h at 4 °C in 1$\times$ TAE in an electrotransfer chamber (Hoefer). Similar results were obtained if gels were incubated in 2$\times$ TBE, 0.2% sarkosyl, and 20 μg/mL proteinase K overnight at 37 °C instead of in 1% SDS. Transfer was documented by restaining the gels with ethidium bromide. Autoradiograms of the gel and the nylon filter before and after electroblotting ^{32}P-labeled nucleosome samples revealed that all the DNA left the gel and that HMG 14/17 bound and unbound mononucleosomal DNA was bound to the membrane with equal efficiency. To corroborate results from direct transfer studies, and for HTC nucleosome studies, DNA was prepared from HMG 14/17 bound and unbound mononucleosomes electroeluted in 0.5$\times$ TBE from excised acryl-

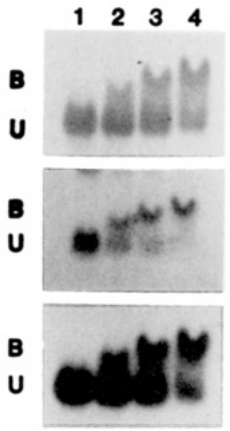

FIGURE 1: Acrylamide gel and blot hybridizations of HMG 14/17 bound and unbound nucleosomes. Mononucleosomes (15 μg) were prepared from CRBC and incubated with increasing amounts of purified HMG 14/17 proteins as described under Materials and Methods. After electrophoresis, the gel was stained in ethidium bromide 1 h and photographed (top panel). DNA was directly transferred to a nylon filter and blot hybridized to nick-translated probes for adult β-globin (middle panel) and a nontranscribed moderately highly repeated sequence (bottom panel). Lane 1 is with no added HMG 14/17 proteins; lane 2 is 1 μL, lane 3 is 2 μL, and lane 4 is 3 μL of added HMG 14/17. (B) HMG 14/17 bound nucleosomes; (U) unbound nucleosomes.

amide gel bands identified by staining with ethidium bromide, 0.4 μg/mL. DNA was then ethanol precipitated after adding 50 μg of high molecular weight calf thymus DNA as carrier. Concentrated nucleosomal DNA was separated from carrier DNA by 1% agarose gel electrophoresis, and after alkali denaturation, DNA was electroblotted in 25 mM phosphate, pH 6.5, to nylon membranes at 1 A for 5 h at 4 °C. Hybridization was carried out as previously described with ^{32}P-labeled nick-translated probes [sp act. (2–4) × 10^8 cpm/μg] (Ginder et al., 1984). Nylon filters were washed at 42 °C for 30 min each in 0.4 N NaOH and 0.1× SSC (1× SSC: 0.15 M NaCl and 15 mM sodium citrate), 0.2 M Tris-HCl, pH 7.5, and 0.5% SDS before rehybridization to additional probes.

Cloned DNA probes for avian β-globin (β), ρ-globin, and ovalbumin genes are described elsewhere (Ginder et al., 1984). The ovalbumin gene probe pOV-1 was provided by Dr. S. McKnight. The TAT gene probe was derived from a 1.05-kbp (*Eco*RI fragment that contains the 3′-exon of TAT (Shinomiya et al., 1984) and was the kind gift of Drs. Wolfgang Schmid and Günther Schutz. A 650-bp probe for the nontranscribed, highly repeated sequence found in the second intervening sequence of embryonic ε-globin was obtained by cutting the 1.8-kbp *Bam–Eco*RI fragment of pCAβG1 with *Hpa*II (Ginder et al., 1979).

Determination of DNA Methylation State. HTC cells that had been incubated in 5-azacytidine were assayed for bulk DNA cytosine methylation by use of an *Hpa*II–methylase enzyme assay (Bestor & Ingram, 1983) with mononucleosomal length DNA as the substrate.

Results

HMG 14/17 Bound Mononucleosomes from CRBC and HTC Cells Are Enriched for Actively Transcribed and Inactive but DNase I Sensitive Genes. Mononucleosomes of uniform electrophoretic mobility were prepared from CRBC and HTC cell nuclei as described under Materials and Methods, allowed to react with purified HMG 14/17, and subjected to acrylamide gel electrophoresis. It should be noted that the nucleosomes were in 1× TBE buffer, 40 mM Na_2EDTA, and 10 mM $CaCl_2$ for reaction with the HMG proteins and subsequent layering on the acrylamide gel. Representative titrations of CRBC nucleosomes with HMG 14/17 are shown in the top panel of Figure 1. With increasing amounts of added HMG 14/17 protein, an increasing proportion of total monosomes migrated as a slower moving band. This slower moving band corresponds to monosomes bound by two molecules of HMG 14/17 as reported previously (Sandeen et al., 1980). Monosomes prepared from HTC cells bound to HMG 14/17 proteins in a similar fashion (not shown).

Table I: Proportion of Total DNA and Active and Inactive Genes in HMG 14/17 Bound Fraction of CRBC and HTC Nucleosomes[a]

CRBC (% bound)				HTC (% bound)	
DNA	β-globin	ρ-globin	ovalbumin	DNA	TAT
27	53	60	21	23	33
33	57	45	24	34	61
48	69	82	45	60	87

[a] DNA was obtained from HMG 14/17 bound or unbound nucleosomes from a single preparation of either CRBC or HTC that was separated by acrylamide gel electrophoresis as described under Materials and Methods. The amount of HMG added was also as described under Materials and Methods. The ratio of total DNA in HMG 14/17 bound and unbound nucleosomes for each quantity of added HMG 14/17 was determined by scanning densitometry of the photographic negative of the ethidium bromide stained gel. The ratio of hybridizing sequences for each probed gene was determined by scanning autoradiograms. Values are corrected for contaminating DNA (or gene sequences) found in the HMG 14/17 bound region of the acrylamide gel when mononucleosomes were electrophoresed without added HMG 14/17 proteins.

DNA in the HMG 14/17 bound and unbound regions of the nucleoprotein gel shown in Figure 1 was transferred to a nylon membrane, and blot hybridization was carried out. As shown in the middle panel of Figure 1, DNA from HMG 14/17 bound CRBC monosomes was enriched about 2-fold for sequences containing the adult β-globin gene. This finding is in agreement with previously published findings (Sandeen et al., 1980). It should be noted that β-globin sequences were equally distributed throughout the mononucleosome DNA band. Thus there is no enrichment for the active gene sequences in monomer DNA of even slightly (3–5 base pairs) greater length. Identical results were obtained with a probe for the embryonic ρ-globin gene (not shown). This gene is sensitive to digestion by the nuclease DNase I in adult erythrocytes, but it does not contain a 5′ DNase I hypersensitive site, nor is it transcribed (Stalder et al., 1980). This finding was not due to cross-hybridization since these probes do not cross-hybridize on Northern blots of primitive and adult CRBC mRNA (Ginder et al., 1984).

The nontranscribed repeat sequence is found in both HMG 14/17 bound and unbound nucleosomal DNA in the same ratio as in bulk DNA (Figure 1, lower panel). This repeat sequence is mostly DNase I resistant (T. W. Brotherton and G. D. Ginder, unpublished results). Similar results were obtained with a probe for the nontranscribed, DNase I resistant single-copy genes for *c-myb* and ovalbumin (not shown).

To confirm the results obtained by the direct-transfer method, hybridization was also done with DNA electroeluted from gel pieces excised from nucleoprotein gels after the separation of HMG 14/17 bound and unbound nucleosomes. Electroeluted DNA was purified by incubation with proteinase K and sarkosyl followed by extraction with phenol and chloroform–isoamyl alcohol to remove proteins and then subjected to agarose gel electrophoresis. DNA from the gel was transferred to nylon filters and used for hybridization. Results, shown in Table I, confirm those obtained by the direct blot method. This same method was used to analyze DNA isolated

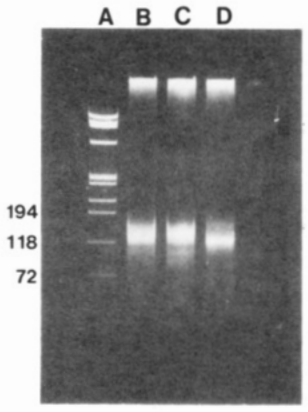

FIGURE 2: Core DNA size in HMG 14/17 bound and unbound mononucleosomes. DNA was electroeluted from mononucleosomes prepared from HTC cells and electrophoresed in an 8% acrylamide nondenaturing gel, along with *Hae*III–ϕX 174 DNA size marker (lane A). (Lane B) DNA from unfractionated HTC mononucleosomes when no HMG 14/17 proteins were added. (Lane C) DNA from unbound HTC mononucleosomes when 34% of total nucleosomes were HMG 14/17 bound. (Lane D) DNA from HMG 14/17 bound HTC mononucleosomes when 34% of total nucleosomes were bound. When 34% of HTC monomer were HMG 14/17 bound, 61% of the active TAT gene were found in the bound fraction (see Table I).

from HTC cell nucleosomes. Blotting with a probe for the TAT gene showed 1.5–2-fold enrichment for this gene in HMG 14/17 bound nucleosomes. Active transcription of the TAT gene in our line of HTC cells was documented by Northern blot analysis (not shown). As shown in Table I, active and inactive but DNase I sensitive genes are preferentially found in HMG 14/17 bound nucleosomes, as illustrated by β-globin and ρ-globin in CRBC, and TAT in HTC, at a level of about 2-fold enrichment. Inactive and DNase I resistant genes are not enriched in the HMG 14/17 bound fraction, as illustrated by ovalbumin in CRBC.

HMG 14/17 Binding Is Not Directed by Nucleosomal Linker DNA Length. The DNA was extracted from the HMG 14/17 bound and unbound bands of HTC nucleosomes separated on a 5% acrylamide gel, when 34% of total nucleosomes were bound. DNA was then electrophoresed in an 8% acrylamide gel. As shown in Figure 2, DNA from monosomes bound by HMG 14/17 (lane d) was not detectably longer than either total monosomal DNA (lane B) or unbound monosomal DNA (lane C). It should be noted that the front-running band of DNA seen in lanes 2 and 3 is not found in lane 4. This band contains DNA of less than 120 bp and corresponds to nucleosomes in which digestion of DNA associated with the nucleosome core has taken place. Since the HMG 14/17 proteins most likely bind at the junction of linker and core DNA (Mardian et al., 1980), it is not unexpected that nucleosomes completely lacking this region of DNA would fail to interact with HMG 14/17. As in the experiments described in the preceding section, a second nuclease digestion was used to prepare the nucleosomes for this experiment. Mononucleosomes of greater than about 150–160 bp have been essentially eliminated by the second nuclease treatment. Therefore, the preferential binding by HMG 14/17 to active and DNase I sensitive nucleosomes shown in Figure 1 and Table I cannot be accounted for by longer linker DNA lengths.

HMG 14/17 Binding Is Not Determined by Single-Strand Nicks. HTC cells were incubated overnight in the presence of either [^{3}H]thymidine or [^{14}C]thymidine. Nucleosomes were then prepared by micrococcal nuclease digestion of isolated nuclei for 10 min ([^{14}C]thymidine) or 1 h ([^{3}H]thymidine). Stripped mononucleosomes were prepared by sucrose gradient centrifugation. A second micrococcal nuclease digestion was

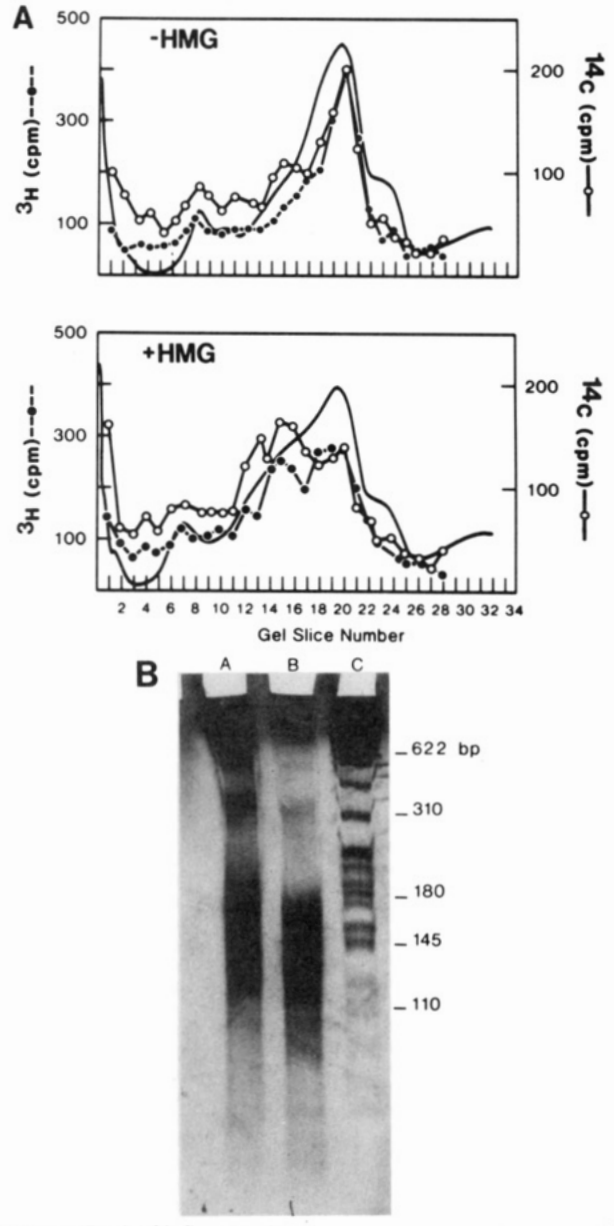

FIGURE 3: Lack of influence of nucleosomal DNA nicking on HMG 14/17 binding. HTC cells were incubated overnight with either [^{14}C]thymidine or [^{3}H]thymidine. Nucleosomes from ^{14}C-labeled cells were prepared with a single 10-min digestion with micrococcal nuclease, while nucleosomes from ^{3}H-labeled cells were prepared with a single 60-min nuclease digestion. Labeled nucleosomes were mixed with ^{3}H-labeled nucleosomes in 10-fold molar excess, with no added HMG 14/17 (top of panel A) or with these proteins added (bottom of panel B). Ethidium bromide stained gels were scanned (solid line) and then sliced in 2-mm steps and counted as described under Materials and Methods for ^{3}H (●) or ^{14}C (○). Gel slices 10–16 are from bound mononucleosomes, while slices 17–22 are from unbound mononucleosomes. (B) DNA from the labeled nucleosome preparations was extracted and denatured with glyoxal. DNA was then run on a 5% acrylamide gel and stained with the silver stain. (Lane A) DNA from ^{14}C-labeled nucleosomes; (lane B) DNA from ^{3}H-labeled nucleosomes; (lane C) *Hpa*II digest of pBR322 plasmid DNA as size marker.

not done in these experiments. Labeled monosomes were then mixed with ^{3}H-labeled nucleosomes in 10-fold molar excess and subjected to acrylamide gel electrophoresis. With no added HMG 14/17 (Figure 3A), most ^{3}H-labeled nucleosomes migrated as a single size of monomer, while ^{14}C-labeled nucleosomes contained both core-sized monosomes and larger sized monomers (so-called chromatosomes), as well as increased amounts of dimeric and higher order nucleosomes.

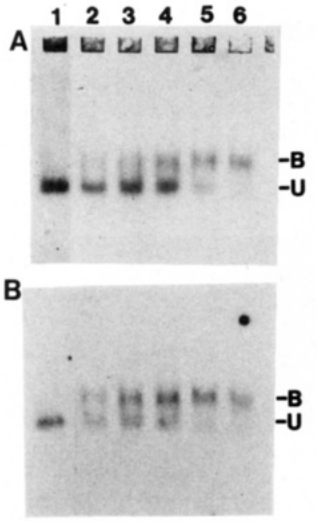

FIGURE 4: Enrichment of histone acetylation in HMG 14/17 bound mononucleosomes from CRBC. Mononucleosomes were isolated from CRBC that had been pulse labeled for 10 min with [^{3}H]acetate and chased 1 h in the presence of 50 mM sodium butyrate. After incubation with increasing amounts of HMG 14/17 proteins, (from left to right) nucleosomes were separated into bound (B) and unbound (U) forms on a 5% polyacrylamide gel, which was then stained with Coomassie blue and photographed as shown in the upper panel (A). The same gel was then subjected to fluorography with the resulting fluorogram shown in the lower panel (B).

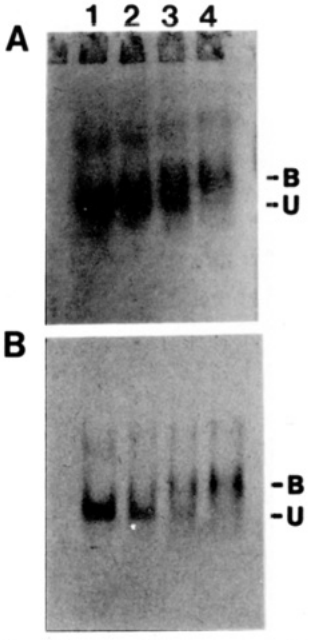

FIGURE 5: Lack of enrichment of histone acetylation in HMG 14/17 mononucleosomes from HTC. Mononucleosomes were isolated from HTC that were pulse-labeled with [^{3}H]acetate as described in Figure 4 and under Materials and Methods. After incubation with increasing amounts of HMG 14/17 (from left to right), nucleosomes were fractionated into bound (B) and unbound (U) forms by electrophoresis on a 5% polyacrylamide gel, which was stained with Coomassie blue and photographed (panel A). The lower panel (B) shows the fluorogram of the same gel.

However, it should be noted that the peak in counts for both ^{3}H- and ^{14}C-labeled nucleosomes was found in the same gel slice, and hence, these nucleosomes contain double-strand DNA of the same size range. Despite more extensive nicking of ^{3}H-labeled nucleosome DNA, the addition of HMG 14/17 (Figure 3B) did not result in preferential movement of ^{3}H-labeled DNA into the slower moving HMG 14/17 bound nucleosomal band. The extent of DNA nicking in ^{14}C- and ^{3}H-labeled nucleosomes as a result of nuclease digestion was measured by extracting DNA from the respective nucleosome preparations, denaturing the DNA with glyoxal (Ginder et al., 1984), and electrophoresing the DNA in a 5% polyacrylamide gel. As shown in lane C of Figure 3B, the ^{14}C-labeled DNA (lane A) migrated more slowly than ^{3}H-labeled DNA (lane B). Thus, the ^{14}C-labeled DNA did in fact contain fewer single-strand nicks than ^{3}H-labeled DNA.

Histone Acetylation Is Closely Associated with HMG 14/17 Binding to CRBC Nucleosomes. Mononucleosomes stripped of all but core histone proteins were obtained from CRBC incubated for 10 min in the presence of [^{3}H]acetate and chased 1 h in 50 mM butyrate. Such treatment results in isotopic label being present on histones capable of undergoing the fast kinetic form ($t_{1/2}$ = 3 min) of acetylation (Brotherton et al., 1981). It is this form of acetylation that has been closely associated with transcriptional activity (Nelson et al., 1978). These labeled monosomes were titrated with increasing amounts of HMG 14/17 proteins and subjected to polyacrylamide gel electrophoresis. Figure 4A shows a representative gel stained for protein with Coomassie blue. The proportions of HMG 14/17 bound and unbound monosomes in each lane, as determined by densitometry, were the same as those found after ethidium bromide staining for DNA (not shown). Histone acetate content was assayed by fluorography (Laskey & Mills, 1975). As shown in Figure 4B, with increasing amounts of HMG 14/17, an increasing proportion of acetate label was contained in the HMG 14/17 bound nucleosome fraction. By comparison of lane 3 in panels A and B of Figure 4, when about 25% of total monosomes were bound by HMG 14/17, over 50% of isotopic acetate was in the bound fraction of nucleosomes. Since acetate was exclusively associated with core histones, these results demonstrate an apparent 2-fold enrichment for histones modified by the fast kinetic form of acetylation in mononucleosomes bound by HMG 14/17.

Histone Acetylation Is Not Associated with HMG 14/17 Binding to HTC Nucleosomes. Histone acetylation occurs on only a minority of the total histones in avian erythrocytes (Brotherton et al., 1981). Bulk hyperacetylation does not occur even after prolonged incubation of these cells in butyrate (Brotherton et al., 1981). By contrast, in HTC cells and other metabolically active cells, 80% of total histone protein undergoes a slow form of acetylation ($t_{1/2}$ = 30 min), and a distinct population representing 10–20% of total histone undergoes a rapid form of acetylation (Covault & Chalkley, 1980). We thus turned to HTC cells to study further the association between histone acetylation and nucleosomal binding of HMG 14/17 in a metabolically active cell. Stripped HTC monosomes from cells pulse labeled with [^{3}H]acetate for 10 min and chased in 50 mM butyrate for 1 h were titrated with HMG 14/17 proteins (Figure 5A). Acetate label incorporated into the fast kinetic pool of histones was detected by fluorography (Figure 5B). In contrast to the results obtained with CRBC nucleosomes, no enrichment for acetylated histone was found in HMG 14/17 bound nucleosomes. HMG 14/17 proteins derived from HTC or CRBC gave similar results. In other experiments, acetate label was introduced into histones modified by the slow kinetic form of acetylation by pulse labeling for 30 min, followed by a 15-min chase in the absence of butyrate, and then a 1-h chase in 50 mM butyrate (Covault & Chalkley, 1980). Again, no preferential association of HMG 14/17 with nucleosomes containing acetate-labeling histones was found (data not shown).

Butyrate has many effects on cellular metabolism in addition to the inhibition of histone deacetylases (Covault et al., 1982). To rule out the possibility that butyrate was affecting the

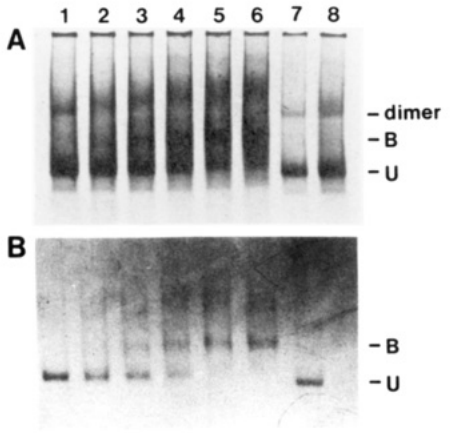

FIGURE 6: Competition between nucleosomes from sodium butyrate treated vs. untreated HTC. Mononucleosomes from unlabeled HTC cells treated with 50 mM sodium butyrate for 1 h were mixed in slight molar excess (1.5×) with nucleosomes from HTC cells pulse labeled with [^{3}H]acetate in the absence of butyrate. Subsequent titration with HMG 14/17 and acrylamide gel electrophoresis (panel A) and fluorography (panel B) were done as in Figure 4 and described in the text. Lanes 1–6 have increasing amounts of HMG 14/17 added to a mixture of butyrate-treated and acetate-labeled nucleosomes. (Lane 7) Acetate-labeled nucleosome; (lane 8) butyrate-treated nucleosomes. (A) Unbound mononucleosome; (B) HMG 14/17 bound mononucleosome; (dimer) dimeric nucleosome. Note that by lane 4 all of the dimer band has slower mobility than the control dimer (lanes 1, 7, and 8), whereas only about 30% of monomeric nucleosome is HMG 14/17 bound (see Discussion).

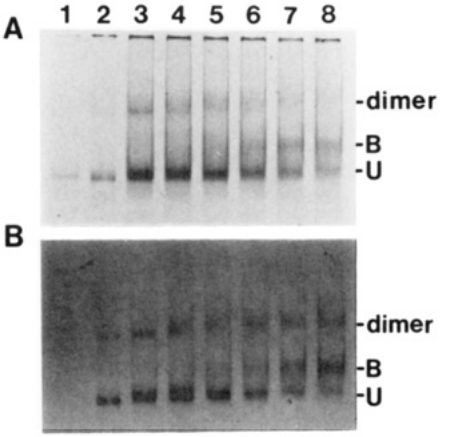

FIGURE 7: Competition between nucleosomes from 5-azacytidine-treated vs. untreated HTC cells for HMG 14/17 binding. Mononucleosomes from HTC cells treated with 3 μM 5-azacytidine (AZA) for 48 h were mixed in equal portions with nucleosomes from HTC cells pulse labeled with [^{3}H]acetate in the absence of butyrate. Subsequent titration with HMG 14/17, acrylamide gel electrophoresis (panel A), and fluorography (panel B) were done as in Figure 4 and described in the text. (Lane 1) 2 μg of AZA-treated nucleosome; (lane 2) 5 μg of [^{3}H]acetate-labeled nucleosomes; (lanes 3–8) 10 μg each of AZA and [^{3}H]acetate-labeled nucleosomes with 0, 3, 5, 7, 9, and 12 μL of HMG 14/17.

interaction of HMG 14/17 with HTC-derived nucleosomes by a mechanism other than histone acetylation, the following two control experiments were performed. First, nucleosomes were prepared from HTC cells pulse labeled for 10 min with [^{3}H]acetate and then immediately processed on ice in the presence of 5 mM butyrate. Titration of these acetate-labeled nucleosomes with HMG 14/17 revealed no enrichment of ^{3}H label in the HMG 14/17 bound fraction (not shown). In the second experiment, nucleosomes from those cells pulse labeled with [^{3}H]acetate but not butyrate chased were mixed with unlabeled nucleosomes prepared from HTC cells that had been incubated for 1 h in 50 mM butyrate. Significant levels of bulk histone hyperacetylation are produced by incubating HTC cells in 50 mM butyrate for 1 h (Covault & Chalkley, 1980). Unlabeled hyperacetylated nucleosomes were in slight (1.5×) molar excess. The mixture of labeled and unlabeled nucleosomes was then titrated with HMG 14/17 proteins. If butyrate, by some unknown mechanism, altered the interaction of HMG 14/17 with nucleosomes derived from HTC cells so that the close association between histone acetylation and HMG 14/17 binding seen in CRBC-derived nucleosomes and described by others for chromatin from HeLa cells (Malik et al., 1984) were lost, then it would be predicted that the [^{3}H]acetate-labeled nucleosomes would be enriched in the HMG 14/17 bound fraction relative to the unlabeled nucleosomes from butyrate-incubated cells. As shown in Figure 6, at low levels of added HMG 14/17, no preferential association of the HMG 14/17 with nucleosomes containing [^{3}H]acetate was seen (lanes 1–3). At higher levels of HMG 14/17, some enrichment of acetate label in the bound nucleosomes was seen (lanes 4–6). The cause for this is unknown but suggests that butyrate treatment of cells may result in a moderate decrease in HMG 14/17 binding to isolated nucleosomes. However, comparison of the results presented in Figure 1 and Table I with the results of the above histone acetylation studies indicates that acetylation was neither closely associated with the transcriptionally active TAT gene in HTC nor a necessary determinant of preferential HMG 14/17 binding in vitro.

Treatment of HTC Cells with 5-Azacytidine Demethylates DNA But Does Not Affect HMG 14/17 Binding. It has been shown that DNA recovered from the active chromatin fraction isolated by HMG 14/17 affinity chromatography is undermethylated compared to bulk DNA (Weisbrod, 1982). To test whether this observation is due to a direct effect of DNA methylation state on HMG 14/17 binding, we have performed HMG 14/17 binding competition assays between [^{3}H]-acetate-labeled HTC nucleosomes and unlabeled HTC nucleosomes derived from 5-azacytidine- (AZA-) treated cells. AZA treatment was carried out by incubating HTC cells in log-phase growth in the presence of 3 μM AZA for two to three cell generations (Jones & Taylor, 1980). DNA hypomethylation was documented by a modification of the methylase assay of Bestor and Ingram (1983) (not shown). [^{3}H]Acetate-labeled nucleosomes were mixed with nucleosomes from AZA-treated cells, with the latter in slight molar excess (Figure 7A). If DNA hypomethylation alone were an important determinant of HMG 14/17 binding, then nucleosomes from AZA-treated cells should preferentially bind available HMG proteins and thereby exclude ^{3}H-labeled nucleosomes from the HMG 14/17 bound fraction. However, as shown in Figure 7B, there was no depletion of ^{3}H label in the HMG 14/17 bound fraction. Thus, DNA hypomethylation does not appear to be a significant direct determinant of HMG 14/17 binding. A similar conclusion was reached by Felsenfeld and co-workers (Felsenfeld et al., 1983) in studies of reconstituted nucleosomes.

Discussion

We have investigated some characteristics of mononucleosomes reported to influence the binding of HMG 14/17 proteins in vitro. Our results confirm previously published work indicating that HMG 14/17 bind preferentially to globin gene containing nucleosomes derived from avian erythrocytes. When 25–50% of the total nucleosomes were bound, enrichment for β-globin was 2–3-fold in the bound fraction. This same enrichment was found for the inactive but DNase I sensitive ρ-globin gene in HMG 14/17 bound nucleosomes

from CRBC and for the TAT gene in bound nucleosomes from HTC cells. The TAT gene is actively transcribed in HTC cells and, in the absence of glucocorticoid stimulation, is transcribed in low copy number (Plesko et al., 1983). In addition, we have demonstrated that nontranscribed single-copy genes in the mature erythroid cell, such as ovalbumin and *c-myb*, were not preferentially associated with HMG 14/17 proteins. These findings indicate that HMG 14/17 proteins have an enhanced affinity in vitro for monosomes derived from actively transcribed and DNase I sensitive chromatin, as compared either to bulk chromatin or to nucleosomes containing nontranscribed and DNase I resistant genes. Furthermore, this selective affinity was not limited to the special cell product globin but was also observed with the constituitive-type gene TAT. Thus, the observation that HMG 14/17 proteins help mediate selective DNase I digestion of genes in nuclei (Weisbrod & Weintraub, 1979; Gazit et al., 1980) appears to be correlated to the in vitro binding of mononucleosomes by these proteins. However, our results do not necessarily indicate a causative role for HMG 14/17 proteins in DNase I sensitivity.

The enrichment of actively transcribed genes in HMG 14/17 bound nucleosomes could not be accounted for by a reciprocal depletion in the nontranscribed genes that we have examined. It is interesting in this regard to note that rDNA genes have been found to be depleted in HMG 14/17 bound mononucleosomes (Levinger et al., 1981). It may be that chromatin that is not transcribed by RNA polymerase II, as for example the rDNA genes (Lewin, 1980), does not readily bind these proteins regardless of the state of transcriptional activity.

The association between nucleosomal histone acetylation and HMG 14/17 protein binding has been previously studied (Weisbrod, 1980; Malik et al., 1984). With HMG 14/17 specific antiserum, a strong correlation between antibody binding and acetylated histones in a partially purified polynucleosome preparation was found (Malik et al., 1984). Weisbrod (1980) demonstrated a weak association between bulk hyperacetylation and nucleosome binding to an HMG 14/17 protein affinity column. We have shown that CRBC nucleosomes that bind HMG 14/17 were enriched in histones modified by the fast kinetic form of acetylation. However, nucleosomes prepared from metabolically active HTC cells were not enriched in histones modified by either the fast or the slow kinetic forms of acetylation. Our studies go beyond previous attempts to relate HMG 14/17 binding to histone acetylation since we have rigorously employed a protocol (Covault & Chalkley, 1980) to selectively label the two distinct pools of histones that undergo either fast or slow kinetic forms of acetylation. In addition, we present evidence that indicates that although the treatment of HTC cells with butyrate can alter the subsequent interaction of isolated nucleosomes with HMG 14/17 proteins, overall binding is actually decreased in association with bulk hyperacetylation. Finally, because we were able to show no consistent correlation between acetylated histones and HMG 14/17 binding, we conclude that core nucleosome modification by either the fast or slow kinetic forms of histone acetylation is not a general feature of transcriptionally active chromatin. However, these studies do not exclude the possibility that specific acetylation of a small number of the possible sites contained in the four core histones is necessary for transcriptional activity or for HMG 14/17 binding. A similar conclusion was reached by Perry and Chalkley (1982) using different methods.

It has been suggested that regions of single-stranded DNA, possibly created during the preparation of mononucleosomes, may be responsible for selective in vitro binding by HMG 14/17 (Swerdlow & Varshavsky, 1983). Isackson and Reeck (1981) have shown preferential binding by HMG 14/17 for naked single-stranded DNA over double-stranded DNA, and Weisbrod (1982) has shown that single-stranded DNA elutes from matrix-bound HMG 14 and 17 at higher salt concentrations then do so-called active nucleosome fractions. However, these reports do not entirely assess whether single-strand regions, produced in the course of preparing mononucleosomes, are responsible for the apparent preferential binding by HMG 14/17 to active chromatin fractions. The micrococcal nuclease nicking experiments reported here, which were designed to directly test this question, show that single-strand regions introduced by nuclease digestion do not account for HMG 14/17 binding to active nucleosomes. This is in accordance with the increased thermal stability of double-stranded DNA in HMG 14/17 bound nucleosomes as described by Sandeen et al. (1980).

Varshavsky and co-workers (Barsoum et al., 1981; Levinger et al., 1981) and others (Nicolas et al., 1983; Goodwin et al., 1985) have found poor correlation between HMG 14/17 binding and gene activity. Instead, they have proposed that HMG 14/17 bind preferentially in vitro to mononucleosomes with longer DNA lengths, and evidence for this has been presented (Swerdlow & Varshavsky, 1983; Stein & Townsend, 1983). Our results presented here show that while preferential binding of HMG 14/17 to nucleosomes containing longer DNA can occur, this effect is lost in nucleosome preparations subjected to a second micrococcal nuclease digestion after removal of all proteins except core histones. Thus, DNA linker length does not appear to account for the preferential HMG 14/17 binding to active nucleosomes observed in our studies. It has been previously noted by Felsenfeld and co-workers (Sandeen et al., 1980) that, by increasing the strength of the buffer in which the reaction between HMG proteins and nucleosomes was carried out, a smaller portion of total nucleosomes became bound for any given amount of re-added HMG 14/17. Weisbrod and Weintraub (1981) report that nonspecific binding of inactive nucleosomes to an HMG 14/17 affinity column can be reversed by increasing salt. Thus, it may be that we have detected preferential binding to nucleosomes containing DNase I sensitive genes because our HMG 14/17–nucleosome reactions were carried out in higher ionic strength buffers (1× TBE with added 40 mM Na_2EDTA), as well as with mononucleosome preparations free of 165–180 bp sized particles. While the degree of in vitro preferential binding seems relatively low, it may well be possible to greatly increase this preference by further optimizing reaction conditions. We are currently testing these possibilities.

The mechanism(s) that determine(s) the binding of HMG 14/17 proteins to chromatin remain(s) unknown. Our studies indicate that under appropriate conditions these proteins can bind preferentially to mononucleosomes containing DNase I sensitive genes in vitro. This preferential binding is not determined by transcriptional activity, linker DNA length, DNA hypomethylation, single-strand DNA nicks, or histone acetylation. It would appear then that some as yet undescribed feature(s) intrinsic to the nucleosome core is (are) responsible for preferential binding, and one likely mechanism would be core histone modifications that would affect ionic charge interactions. Clearly, further effort is needed to delineate the function of the HMG 14/17 proteins in the cell. The results presented in this paper suggest that the interactions of these proteins with isolated chromatin in vitro may be a reasonable

model system for the study of the function of HMG proteins in intact chromatin.

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