Effect of HMG protein 17 on the thermal stability of control and acetylated HeLa oligonucleosomes

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ABSTRACT

Many studies have implicated histone acetylation and HMG proteins 14 and 17 in the structure of active chromatin. Studies of the binding of HMG 14 and 17 to chromatin core particles have shown that there are two binding sites for HMG 14 or 17 located within 20-25 bp of the DNA ends of the core particles [13-15]. Such binding sites may result from the free DNA ends in the core particle being available for the binding of HMG 14 and 17. We have studied the effects of the binding of HMG 17 on the thermal denaturation of DNA in mono, di and trinucleosomes. In each case the binding of 1 HMG 17 molecule per nucleosome reduces the DNA premelt region by 50%, while the binding of 2 HMG 17 molecules per nucleosome abolishes the premelt region. From this it is concluded that there are two HMG 17 binding sites per nucleosome which are located between the entry and exit points to the nucleosome and the strongly complexed central DNA region. Highly acetylated mono, di and trinucleosomes have been isolated from butyrate treated HeLa S3 cells. For this series of acetylated oligonucleosomes, it has been found that there are also two HMG 17 binding sites per acetylated nucleosome.

INTRODUCTION

Evidence for the involvement of histone acetylation and HMG proteins 14 and 17 in the structure of "active" chromatin comes from a range of studies. Allfrey [1] first correlated one of the requirements of transcriptional activity with histone acetylation and this correlation has been found to hold for enhanced levels of transcriptional activity induced by hormones or drugs [2]. Cell cycle studies of histone H4 acetylation show a strong correlation between the behavior of tetraacetylated H4 and ribosomal RNA transcriptional activity [3]. In yeast chromatin which has a very high overall level of transcriptional activity, 63% of the histone H4 molecules were found to be in the di, tri and tetraacetylated forms [4]. Nucleosomes enriched in active DNA sequences from trout testis have been found to contain acetylated histones and a HMG protein, H6, homologous to HMG 14 and 17 [5,6]. Weintraub & Groudine [7] have shown that active genes are preferentially digested by DNase I. This DNase I sensitivity is dependent on the presence of HMG proteins 14 and 17

[8] previously identified and characterized by Johns' group [9-12].

Studies of the binding of HMG proteins 14 and 17 to isolated chromatin core particles have shown that there are two binding sites for HMG 14 or 17 [13-15] and that these sites are available in mononucleosomes in the presence of histones H5 [14] or H1 [15]. From the protection of DNase I cutting sites and preliminary thermal denaturation studies of complexes of core particles with HMG 14 or 17, binding sites have been located within the 20-25 b.p. of the ends of the DNA at the entry and exit points of the core particles. In studies of the interactions of HMG 17 with native and H1, H5 stripped chicken erythrocyte chromatin, less than half of the 1 or 2 molecules of HMG 17 added per nucleosome were found complexed to the chromatin after reconstitution [16]. The loss of the added HMG 17 was attributed to adsorption on the dialysis tube. The results obtained with these much lower stoichiometries supported the findings of the core particle studies concerning the location of the HMG 17 molecules and also indicated that the binding of HMG 17 caused a contraction of the chromatin.

HMG 14 and 17 form a pair of similar proteins with 100 and 89 residues, respectively [9-12]. They contain 21-25% of acidic residues and 28-29% of basic residues to give a total of about 50-55% of charged residues. The charge distributions are asymmetric with the basic residues clustered in the centers of HMG 14 and 17 while the acidic residues are found in the N-terminal and particularly the C-terminal regions. HMG 14 and 17 contain no aromatic residues and very few apolar residues. Their sequence properties show little or no potential for these molecules to form globular structures. Conformational studies of HMG 14 [17] and HMG 17 [18] have shown that over a wide range of pH and ionic strengths these proteins are in random coil conformations. From neutron scatter studies [18] the radius of gyration of HMG 17 has been found to be 3.4 nm, a value close to that expected for a fully disordered chain rather than a compact globular structure which would be expected to have a radius of gyration of 1.1 nm. The main conclusions to be drawn from the conformational studies of HMG 14 and 17 is that they are "designed" not to fold into globular structures and their native conformation must be imposed by their binding sites in chromatin. Their high basic charge suggests that DNA may provide a major binding site for HMG proteins, and nuclear magnetic resonance spectroscopic studies (NMR) of the interactions of HMG 14 [16] and HMG 17 [17] with DNA show that the major HMG binding sites are in their basic N-terminal halves. These regions exhibit extensive homology with a basic segment in the C-terminal half of histone H1. The acidic C-terminal halves of HMG 14 and 17 remain un-

bound in their complexes with DNA. The sequence properties and conformational behavior of HMG 14 and 17 strongly suggest that these proteins interact in an extended form with chromatin. In a fully extended polypeptide chain adjacent residues are separated by 3.47 Å. HMG 14 with 100 residues could therefore extend over a distance of 347 Å and thus possesses the ability for extensive and widely separated interactions in chromatin. Similar behaviors would be expected for HMG 17 which has 89 residues.

Differential thermal analysis [19-27] has been used to report on the thermal stabilities of different regions of DNA in core particles, oligonucleosomes and chromatin. Core particles exhibit a biphasic profile; a premelt centered at ca 60°C which results from the melting of about 40 b.p. DNA from the two ends of the core particle DNA and a major cooperative melt of about 100 b.p. DNA at ca 74°C attributed to the central DNA of the core particle tightly complexed with core histones [21,22]. The melting profiles of H1 and H5 depleted chicken erythrocyte compact dimers and trimers show that the premelt remains at ca 60°C, while the temperature of the main transition increases from 74-75°C for the core particle to 79°C for dimer and 81°C for the trimer [28]. A new transition is observed for the compact dimer and trimer at ca 73°C. A comparison of the melting profiles of mono, di and trinucleosomes from HeLa S3 cells shows that whereas the premelt is located at 62-64°C for all members of the series, the major transition increases in temperature for the mono, di and trinucleosomes being found at 77°C, 79.2°C and 81.6°C, respectively, while the intermediate transition is found at 74.5°C and 77°C for the di and trinucleosomes [29]. The melting profiles of the di and trinucleosomes are similar to that of native chromatin which show an inflection for the premelt at 57-62°C, an intermediate transition at 72-74°C and a major transition at 82-84°C [19]. The premelt has been attributed to two DNA regions at the DNA ends of each nucleosome, the major transition to the central DNA region strongly complexed with core histones and the intermediate transition to the linker DNA and core DNA flanking the central region [29]. When the core histones are highly acetylated, the temperatures of the intermediate and major transitions are lowered by 1.5 to 2.0°C suggesting that the basic N-terminal regions stabilize both the core DNA and the linker DNA by binding to the DNA at the entry and exit points of the nucleosome [29].

We report on the effects of HMG 17 on the melting behavior of the HeLa S3 control and acetylated mono, di and trinucleosomes. Highly acetylated oligonucleosomes were obtained from butyrate treated HeLa S3 cells. One of the effects of butyrate on cells is to inactivate histone deacetylase and

Figure 1. SDS gel electrophoresis pattern of purified HMG 17 (lane A) and HMG markers (lane B).

this leads to the accumulation of high levels of acetylation of the core histones, particularly histones H3 and H4 [30-33]. The following questions were investigated: i) the binding of HMG proteins to mono, di and trinucleosomes, and ii) how does acetylation of histones in the oligonucleosomes affect the binding of HMG proteins.

MATERIALS AND METHODS

Preparation of Mono, Di and Trinucleosomes

The isolation, fractionation and characterization of mono, di and trinucleosomes were as described previously [29].

HMG 17 Reconstitution with Oligonucleosomes

The isolation and purification of HMG 17 was as described by Goodwin *et al.* [8]. The purified HMG 17 compared to HMG markers is given in Figure 1. Nucleosomes at a concentration of 1-2 A_{260} units/ml in TBE buffer (0.089 M Tris-borate, pH 8.3, 2.5 mM EDTA and 1 mM NaButyrate) were incubated on ice in a polypropylene test-tube for 15 minutes with amounts of HMG 17 corresponding to 1, 2 or 4 molecules of HMG 17 per nucleosome. A stock solution of HMG protein 17 in TBE was prepared and the concentration determined to be 1.7 mg/ml using the extinction coefficient of $\epsilon_{220}^{1\%} = 62$. This solution was used

Table 1
Stoichiometry of HMG 17/Nucleosomes

Input ratio mol HMG 17 per nucleosome	Control		Butyrate	
	monomers 166 b.p.	dimers 355 b.p.	monomers 170 b.p.	dimers 355 b.p.
1	1.3	0.95	1.3	1.1
2	2.1	2.0	2.2	2.0
4	3.8	3.8	4.1	4.0

Final ratio of molecules of HMG 17/nucleosome determined as described in Materials and Method.

throughout all the reconstitution experiments. Following incubation, the reconstituted samples were passed through a Sephadex G-25 column equilibrated with 0.25 mM EDTA, pH 8.0 and 1 mM NaButyrate. 1 A₂₆₀ unit of reconstituted nucleosomes from the peak fractions were then subjected to thermal denaturation as described by Yau *et al.* [29].

Stoichiometry of HMG 17/nucleosome

¹²⁵I-labelled Boulton Hunter reagent was prepared as described by Banerjee *et al.* [34]. HMG 17 (1 mg) was labelled by reacting with ¹²⁵I-labelled Boulton Hunter reagent. Analysis of ¹²⁵I-labelled HMG 17 was performed by gel electrophoresis and autoradiography. Label was found only in the Coomassie stained HMG 17 band. ¹²⁵I-labelled HMG 17 was reconstituted with nucleosomes as described above. Absorbance at 260 nm was used to determine the amount of DNA; radioactivity in ¹²⁵I-labelled HMG 17 was determined by counting in a Searle 1197 solid state gamma counter and gave the amounts of HMG 17 complexed with the mono and dinucleosomes. The stoichiometries of HMG 17 binding in the control and acetylated mono and dinucleosomes for an input ratio of 1, 2 and 4 molecules of HMG 17 per nucleosome are given in Table I. Thus the reconstitution procedures used did not result in a loss of added HMG 17.

RESULTS

Effect of HMG 17 on the Melting of Control and Acetylated Oligonucleosomes Control and Mononucleosomes

The effects of adding increasing amounts of HMG 17 to control mononucleosomes are shown in Figure 2A. The mononucleosome melting profile con-

Figure 2. Thermal denaturation derivative plots of control (A) acetylated (B) monomers and HMG 17 complexes: alone —; with one molecule HMG 17/nucleosome -----; with two HMG 17/nucleosome - · - · - · - · - ·; with four HMG 17/nucleosome · · · · ·.

tains a broad premelt centered at 61–62°C and a major cooperative transition at 76.0°C. Addition of the equivalent of 1 HMG 17 molecule per nucleosome results in i) a reduction of the premelt which is found at a higher temperature becoming a shoulder at ca 68°C, ii) a shift of the major cooperative transition to 77.8°C, and iii) the appearance of a new higher temperature transition at ca 80.2°C. A further addition of one molecule of HMG 17 to two molecules per nucleosome results in a considerable loss of the premelt region and a more cooperative transition at a T_m of 81°C. At the level of four molecules of HMG 17 per nucleosome the premelt has almost completely disappeared and the single cooperative transition moves to 82.2°C.

Very similar behaviors are observed for the highly acetylated mononucleosomes, Figure 2B. Except for a small decrease in the T_m of the major transition, the melting profile of acetylated nucleosomes is almost identical to that of the control; a broad premelt is centered at 61.3°C and the major cooperative transition occurs at 75.1°C. When one molecule of HMG 17 per nucleosome is added the premelt region is reduced becoming a shoulder at ca 68.0°C and the major transition at 78.8°C shows additional complexity as evidenced by the increased width of the transition. At levels of two and four HMG 17 molecules per nucleosome the premelt decreases and largely disappears while the major transition becomes more cooperative moving to higher melting temperatures, 80.8 and 81.9°C for two and four molecules, respectively, of

Figure 3. Half width of main transitions and hyperchromicity in monomers (A), dimers (B) and trimers (C). Half width of control $\circ$ --- $\circ$ and acetylated $\triangle$ --- $\triangle$ nucleosomes. Percent hyperchromicity of control $\bullet$ --- $\bullet$ and acetylated $\blacktriangle$ --- $\blacktriangle$ nucleosomes in premelt (P), intermediate (I) and main (M) transitions.

HMG 17.

The effect of HMG 17 on the melting profiles of acetylated and control mononucleosomes is summarized in Figure 3A for the changes in hyperchromicity of the premelt region and the width at half height of the major transition. Addition of one and two molecules of HMG 17 per nucleosome results in a large linear decrease in hyperchromicity of the premelt from 34% to 13% for the acetylated mononucleosome and from 32% to 15% for the control mononucleosomes. Addition of a further two molecules of HMG 17 causes a further smaller decrease in hyperchromicity for the acetylated and control mononucleosomes. The major change in hyperchromicity is caused by the first two molecules of HMG 17. For the control mononucleosomes the widths of the main melting transition change on the addition of HMG 17 are as follows: 6.2°C (0 HMG 17), 8.5°C (1 HMG 17), 7.0°C (2 HMG 17) and 6.3°C (4 HMG 17). A similar pattern of behavior is observed for the acetylated mononucleosomes with the following transition widths: 7.1°C (0 HMG 17), 9.1°C (1 HMG 17), 7.0°C (2 HMG 17) and 6.3°C (4 HMG 17). In Figure 2A, it can be seen that the increased width of the major transition on addition of one HMG 17 to the control mononucleosome is due to the appearance of a third higher temperature transition and a similar behavior probably applies to the acetylated mononucleosome although there no distinct inflection is observed as for the control mononucleosome. The appearance of this third higher temperature melting transition is accompanied by a reduction in the hyperchromicity of the premelt, Figure 3A, and of the

Figure 4. Thermal denaturation derivative plots of control (A), acetylated (B) dimers and HMG 17 complexes. Symbols used were the same as in Figure 2.

major cooperative transition of the uncomplexed mononucleosomes. This change is largely completed by the addition of two HMG 17 molecules. The addition of a further two molecules of HMG 17 results in an increased but smaller stabilization probably due in large part to non-specific interactions between the mononucleosomes and HMG 17. The above described behaviors support the proposals that HMG 17 binds at least to the two DNA ends of both the control and acetylated mononucleosomes. These DNA ends which contribute to the premelt are thus stabilized by the binding of HMG 17; this stabilizes further the melting of the central DNA region complexed with core histones to give a single higher temperature transition. Because of the conformational properties of HMG 17, the possibility that it also interacts with the central DNA region on the core histones cannot be excluded. The data are fully consistent with two HMG 17 binding sites for both the control and acetylated mononucleosomes.

Control and Acetylated Dinucleosomes

The effects of binding of 1, 2 and 4 molecules of HMG 17 to the control dinucleosomes are shown in Figure 4A. The melting profile of the dinucleosome shows a broad, complex premelt at ca 62°C, an intermediate transition at 74.3°C and a major cooperative transition at 79.7°C. It has been suggested that the premelt region results from the DNA ends of the dimer, the intermediate transition results from the linker region and the major cooperative transition re-

sults from the central DNA region of the nucleosomes [32]. This interpretation is most probably an oversimplification because the relative size of the premelt region is larger than would be expected just for the two DNA ends of the dimer.

Addition of the equivalent of one molecule of HMG 17 per nucleosome results in the reduction of the size of premelt and a change of T_m to ca 67 to 68°C. The intermediate and major transitions become broader and have slightly higher T_m 's, at 75°C and 80.5°C, respectively. The width of the major cooperative transition increases from 5.8°C for the control dinucleosome to 7.4°C for the complex with HMG 17. At the level of two HMG 17 molecules/nucleosome, the premelt has largely disappeared and the intermediate and major transitions are HMG 17. Addition of a further two molecules of HMG 17 results in a sharpening of the major transition and an increase in the melting temperatures of both the intermediate transition to 78°C and the major transition to 83.5°C. The data support a model in which two molecules of HMG 17 bind to specific sites on the acetylated nucleosome. Acetylation at the levels achieved by butyrate treatment of HeLa cells does not therefore result in an increase in the number of specific binding sites. Additional HMG 17 molecules can bind non-specifically and increase the thermal stability of the complexed nucleosome.

Control and Acetylated Trinucleosomes

The melting profile of the control trinucleosomes shows more complexity in the intermediate melting region than found for the dinucleosomes. This is probably due to the melting of regions of DNA associated with the central nucleosome. The effect of the binding of HMG 17 on the melting profiles of the control trinucleosomes is given in Figure 5A and summarized for the premelt and main transitions in Figure 3C. The hyperchromicity of the premelt centered at 62.5°C is reduced by about one half and moves to 68-69°C on the addition of one molecule of HMG 17 per nucleosome. Addition of two molecules of HMG 17 per nucleosome largely abolishes the premelt region. This loss of hyperchromicity of the premelt region is accompanied by an increase in the hyperchromicity of the main transition. At the level of one HMG 17 per nucleosome the melting profile of the control trinucleosomes shows additional complexity compared to the uncomplexed trinucleosome profile and the profiles with two and four molecules of HMG 17. This is shown also in the increase in the width of the main transition for the addition of one molecule of HMG 17. On addition of two molecules of HMG 17 this complexity disappears and only the intermediate and main transitions remain. These transitions show increases in melting temperature with increasing numbers of HMG 17; for the uncomplexed form and for the complexes

Figure 5. Thermal denaturation derivative plots of control (A), acetylated (B) trimers and HMG 17 complexes. Symbols used were the same as in Figure 2.

with 1, 2 and 4 molecules of HMG 17 the intermediate transitions are at 74, 76, 76.5 and 77.3°C and the main transition at 80.4, 82.3, 82.5 and 83.5°C, respectively. The intermediate transition is reduced for the complex of the control trinucleosome with four HMG 17 molecules per nucleosomes and the hyperchromicity of the main transition is increased.

For the acetylated trinucleosomes the melting profile is very similar to that of the control trinucleosomes except that the melting temperatures of the intermediate and main transitions are lower. Additions of 1, 2 and 4 molecules of HMG 17 give a similar pattern of behavior as found for the control as shown in Figure 5B. These behaviors are summarized in Figure 3C. The same description could be given for these behaviors as has been given for the control trinucleosomes except for a small reduction in the melting temperatures of the transitions. The premelt is abolished by the addition of two molecules of HMG 17 per nucleosome. Addition of a further two molecules of HMG 17 results in a reduction of the hyperchromicity of the intermediate transition and an increase in that of the main transition.

DISCUSSION

It has been shown that there are two binding sites for HMG 17 at the DNA ends of the core particles [13-15]. This finding, however, may be a consequence of the free DNA ends of the core particle being available for the binding of HMG 17 rather than being specific binding sites. If this were the

case then there should also be a maximum of two binding sites for HMG 17 in each of the mono, di and trinucleosomes. This is not found to be the case and the binding and melting data of the HMG 17:oligonucleosome complexes are all fully consistent with two HMG 17 binding sites per nucleosome: 2, 4 and 6 HMG 17 molecules for the mono, di and trinucleosomes, respectively. The effect of the binding of 1 and 2 HMG 17 molecules on the premelt hyperchromicity, shows that the HMG 17 molecules bind initially to those DNA regions which contribute to the premelt transition at 62-64°C for the series of oligonucleosomes. For all members of the series, one HMG 17 per nucleosome reduces the premelt by 50% while two HMG 17 per nucleosome almost completely abolishes the premelt transition. This shows that the premelt transition can be attributed to two DNA regions per nucleosome and not just to the free DNA ends. This would explain why the hyperchromicity of the premelt region is about 30% of the total hyperchromicity for the mono, di and trinucleosomes and doesn't decrease along the series of oligonucleosomes as would be expected if only the free ends contributed to the premelt. Because the premelt regions of the di and trinucleosomes are identical to that of the mononucleosome it is probable that the premelts of the oligonucleosomes all have the same origin which for the chromatin core particle [25] has been attributed to the DNA regions flanking the central DNA regions; the central DNA region of about 100 b.p. of DNA is stabilized by interactions of the core histones while the flanking regions of about 40-50 b.p. DNA are more weakly stabilized, probably by interaction of the N-terminal regions of histones H3 and H4. It is known [35] that the basic N-terminal regions of H2A and H2B are not bound within the core particle and must have binding sites on DNA outside of the core particle 146 b.p. DNA, probably the linker DNA. The intermediate transition is attributed to the DNA outside of the core particle, i.e. largely the linker DNA. Although the major effects of binding of HMG 17 on the melting profiles of the di and trinucleosomes are caused by two molecules of HMG 17 per nucleosome, additional effects are observed on the binding of another two molecules of HMG 17; these are an increase in the hyperchromicity of the main transition paralleled by a decrease in the hyperchromicity of the intermediate transition and both transitions move to a higher temperature. These additional HMG 17 molecules appear to bind to the linker DNA stabilizing these regions against melting and further stabilizing the whole nucleosome.

As has been shown, the binding of HMG 17 to the acetylated oligonucleosomes produced almost identical effects to those observed for the control oligonucleosomes. Two molecules of HMG 17 per nucleosome cause the loss of the

Figure 6. A proposed model of HMG 17 binding sites to the DNA coils in a trinucleosome. HMG 17 binding sites-----.

premelt region for mono, di and trinucleosomes; addition of a further two molecules of HMG 17 to the di and trinucleosomes result in a reduction in the hyperchromicity of the intermediate transition and an increase in that of the main transition. Although the levels of acetylation are high [H_4 (10%), Ac_1H_4 (15%), Ac_2H_4 (19%), Ac_3H_4 (25%) and Ac_4H_4 (31%)] this does not result in the binding of additional HMG 17 molecules. The effects of the binding of HMG 17 on the melting profiles of both the control and acetylated oligonucleosomes are consistent with two specific binding sites per nucleosome involving the two DNA regions flanking the central 90-100 b.p. DNA. Similar binding sites have been proposed for the binding of HMG 17 to core particles [13-15]. For the di and trinucleosomes additional HMG 17 molecules can bind to the linker DNA. This model is shown for the trinucleosome in Figure 6. Because of the conformational properties of HMG 17 [17], which is a flexible random coil in solution under a range of conditions of pH and ionic strength, the possibility that there are additional weaker binding sites for HMG 17 on the nucleosome DNA or protein cannot be excluded.

Active regions of chromatin have been associated with core histone acetylation and the binding of HMG proteins 14 and 17 together with other changes, e.g., in DNA methylation. The effects of histone acetylation and the binding of HMG proteins on chromatin structure and function are not understood. It has been suggested that histone acetylation may destabilize the 34 nm chromatin supercoil [3,31,36] and this, together, with a loss of H1 may provide binding sites for HMG proteins 14 and 17. Transitions between supercoiled and extended chromatin will be determined in part by the continuity of the DNA and by the path of the DNA at the entry and exit points of the nucleosome and in linker DNA. It is significant that the major HMG 17 binding sites are the exit and entry regions of the DNA and that additional binding may take place at the linker DNA. The effects of HMG proteins on chromatin structure, however, ap-

pear not to be consistent with a role whereby HMG proteins destabilize the 34 nm supercoil of nucleosomes. The binding of HMG 17 to chicken erythrocyte chromatin "produces an overall stabilization and condensation of structure" [16]. It has also been shown that the binding of HMG 14/17 to chicken erythrocyte polynucleosomes "do not significantly interfere with nucleosome packing in the 30 nm fiber nor do they prevent important stabilizing interactions" [37]. In these studies, however, HMG proteins are complexed with bulk chromatin which contains histones H1 and H5 and the core histones are largely in the nonacetylated forms. A more significant finding is that HMG 14/17 did not induce "an extended configuration in the chromatin of the chicken adult β -globin gene, in cells where this gene is known to be DNase I sensitive" [37]. However, there is a strong possibility that changes in DNA supercoiling may be involved in gene activation and these effects probably induced by protein interaction would be lost in studies of pieces of chromatin with free ends.

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