High Mobility Group Protein 17 Cross-links Primarily to Histone H2A in the Reconstituted HMG 17-Nucleosome Core Particle Complex*

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The "neighbor relationship" of lamb thymus high mobility group (HMG) protein 17 to native HeLa nucleosome core particle histones in the reconstituted complex has been studied. 125I-Labeled HMG 17 was cross-linked to core histones using the protein-protein cross-linking reagent 2-iminothiolane. Specific cross-linked products were separated on a two-dimensional Triton-acid-urea/sodium dodecyl sulfate-gel system, located by autoradiography, excised, and quantified. Disulfide bonds in the cross-links were then cleaved, and the protein constituents were identified by sodium dodecyl sulfate-gel electrophoresis. HMG 17 cross-linked primarily to histone H2A while lower levels of cross-linking occurred between HMG 17 and the other histones. In contrast, cross-linking between 2 HMG 17 molecules bound on the same nucleosome core particle was relatively rare. We have concluded that H2A comprises part of the HMG 17 binding site. Less contact occurs between HMG 17 and the other core histones, and there is little contact possible between the 2 bound HMG 17 molecules. These results are in agreement with the current model for the structure of the nucleosome and the proposed binding sites for HMG 17.

High mobility group (HMG) proteins 14 and 17 are small basic chromosomal proteins (100 and 89 residues, respectively) which appear to play a role in the structure of active and potentially active chromatin. Structurally, and possibly functionally, these two proteins are very similar. They are unusual in having few nonpolar groups, no aromatic groups, an unusual C-terminal (1). The proteins behave as random coils in solution (2-6). They presumably acquire a more defined conformation upon binding to chromatin, and their relatively large number per mammalian cell nucleus (approximately 10^6) suggests a structural role in chromatin (7). Although these proteins have been well characterized, with regard to their primary sequences and various post-translational modifications (e.g., phosphorylation, acetylation, ADP-ribosylation, and glycosylation (8-15)), neither their structure when bound to chromatin nor their function in vivo is presently known.

Active chromatin is characterized by increased sensitivity to nucleases (16-18). HMG 14 and 17 are released with active regions of chromatin by brief digestion with DNase I (19-23), and their presence is required to maintain this sensitivity, i.e., DNase I sensitivity is lost after washing chromatin with 0.35 M NaCl which removes all HMG proteins. Purified HMG 14 and 17 restore this sensitivity and in addition enhance DNase I digestion of oligonucleosomes, mononucleosomes, and nucleosome core particles (21, 24-26). Thus, HMG 14 and 17 appear to confer upon active chromatin greater sensitivity to DNase I. There are, however, some results which conflict with this conclusion (27-30). Regions of chromatin released by digestion with DNase II and specific nucleosome fractions released with micrococcal nuclease are also enriched in HMG 14 and 17 and in DNA sequences from active and potentially active chromatin (19, 20, 31-43), while again there are some conflicting data (44-47).

HMG 14 and 17 bind preferentially to nucleosomes from active chromatin although they bind to all nucleosomes prepared from bulk chromatin (48). The existence of two specific binding sites has been demonstrated in a number of studies. While H6, HMG 14, and HMG 17 have been found to be associated with core particles after micrococcal nuclease digestion of chromatin (33, 49), Albright et al. (50) have shown that the same species can be reconstituted with Dowex-stripped nucleosomes and 1 or 2 molecules of HMG 17. Additional studies indicating two specific nucleosome binding sites near where the DNA enters and exits involve DNase I digestion of reconstituted core particles (51), thermal denaturation of reconstituted chromatin (52), protein-DNA cross-linking (53), and native polyacrylamide gel analysis (54, 55). The latter two studies show cooperative binding at physiological ionic strength. The use of centrifugation has also demonstrated two cooperatively binding sites with a Hill coefficient of nH = 1.3 and dissociation constants of approximately 30 nM for the first HMG molecule bound and approximately 90 nM for the second bound at 0.15 M NaCl, pH 6.8 (55). Only the core histone octamer and approximately 140 base pairs of DNA are required for the binding of 2 molecules of HMG 14 or 17 although nucleosomes containing longer DNA will bind HMG 14 and 17 more strongly (47, 50). HMG 14 and HMG 17 are interchangeable at each binding site (50, 51). Additional nonspecific binding of HMG 14 and 17 has been observed at high concentrations of HMG relative to core particle (50, 51, 56). Separate binding sites on the nucleosome have been demonstrated for HMG proteins 1 and 2 (55, 57) and for histone H1 (50). These basic proteins do not occupy the

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1 The abbreviations used are: HMG, high mobility group; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride.
binding sites for HMG 14/17 which may be bound to the nucleosome simultaneously nor do they bind nonspecifically to core particles (50, 55).

The structural determinants of the correlation among the active state of chromatin, nucleosome sensitivity, and the binding of HMG 14 and 17 remain to be established.

Protein-protein cross-linking has been used extensively to analyze the spatial arrangement of the histones in the nucleosome (58), and recent attention has turned to the study of HMG 1 binding to H2A/H2B dimers and H3/H4 tetramers (59). HMG 14 and 17 binding to H1 in solution (60), and contacts between HMG 14 and other chromosomal proteins (61). Ring and Cole (62) found dimers that appeared to contain H1 and HMG 14 or 17 after cross-linking whole nuclei. The work presented here was undertaken to characterize, by cross-linking, the interaction between HMG 17 and purified by reverse phase high performance liquid chromatography. were pooled after fractionation.

Some evidence concerning the binding of HMG 14 and 17 remains to be established. The major protein cross-linked to HMG 17 was disulfide link between the e-amino groups of two lysine residues (63). The major protein cross-linked to HMG 17 was histone H2A. Cross-linking to the other histones and between HMG 14 and 17 was histone H2A. Cross-linking to the other histones and between 2 HMG 17 molecules also occurred. The results are discussed in relation to the present model of nucleosome structure and evidence concerning the binding of HMG 14 and 17.

**EXPERIMENTAL PROCEDURES**

**Preparation of Samples for Cross-linking**

Mononucleosome core particles were prepared as previously described (64) with the following exceptions. HeLa chromatin was digested using micrococcal nuclease (Sigma) at a concentration of 70 units/mg of DNA for 15 min. After disrupting the nuclei with 8.0 mM NaHPO4, 1.0 mM EDTA (free acid), 0.2 mM PMSF, pH 7.25, and pelleting the insoluble material, oligonucleosomes and H1-containing mononucleosomes were precipitated by addition of 1.0 mM NaCl, 0.2 mM PMSF to a final concentration of 0.1 mM NaCl according to the method of Levy W. et al. (33). The resulting supernatant was applied to 34-ml 5-20% sucrose gradients and centrifuged in an SW 27 rotor at 29,000 rpm for 24 h at 4 °C. The mononucleosome peak fractions were pooled after fractionation.

HMG 17 was isolated from fresh lamb thymus by the method of Nicolas and Goodwin (65), HMG 17 to be iodinated was further purified by reverse phase high performance liquid chromatography. Approximately 100 µg of HMG 17 was dissolved in 0.5 ml of 0.05% trifluoroacetic acid and loaded onto a µBondapak C18 (Waters Associates, Inc.) high performance liquid chromatography column (3.9 mm × 30 cm). Protein was eluted at 0.6 ml/min using an 8-12% acetonitrile gradient for 45 min. HMG 17 eluted at 8.6% acetonitrile as a single peak. The purified HMG 17 was labeled with [35S] (New England Nuclear) using 0.85 ng of Bolton-Hunter reagent (Pierce Chemical Co.) per µg of protein by the method of Banerjea et al. (66). Radiolabeled HMG 17 was extensively dialyzed against 1 X TBE buffer (8.9 mM Tris base, 8.9 mM boric acid, 2.3 mM Na2EDTA, pH 8.3), 0.1 M NaCl, 0.2 mM PMSF at 4 °C and used immediately. Approximately 1 HMG 17 molecule in 10 was labeled under these conditions.

**Reconstitution and Cross-linking**

Mononucleosome cores were dialyzed against 1 X TBE buffer, 0.2 mM PMSF at 4 °C and reconstituted with sufficient HMG 17 (approximately 3 HMG 17 molecules/core) for 2 molecules of HMG 17 to be bound per core nucleosome as determined by electrophoresis in a 5% nondenaturing polyacrylamide gel run with 1 X TBE buffer (54). Each experiment was carried out with 2.0 mg of core particle at 0.2 mg/ml (1.0 mg/ml core DNA A260 nm = 20.0), 290 µg of unlabeled HMG 17 at 2.0 mg/ml in 1 X TBE buffer, 0.2 mM PMSF, and 10 µg of [35S] labeled HMG 17 at 1.0 mg/ml (1.0 mg/ml HMG 17 A260 nm = 6.29). Reconstituted material was dialyzed against cross-linking buffer (50 mM triethanolamine HCl, 1.0 mM Na2EDTA, 0.2 mM PMSF, pH 8.0) at 4 °C and cross-linked with 2.0 mM 2-iminothiolane (Sigma) at 0 °C for 3 h according to Kenny et al. (63). Samples from before and after cross-linking were analyzed on a 3% nondenaturing polyacrylamide gel (15.5 cm × 12 cm × 1.5 mm) to detect possible nucleosome dimers formed by cross-linking. Cross-linked protein and DNA were recovered by precipitation at 0 °C with 25% trichloroacetic acid, washed once with cold acidified acetone (1.8:100 concentrated HCl:acetone), twice with cold acetone, and dried under vacuum.

**Polyacrylamide Gel Analysis of Cross-linked Proteins**

**Separation and Detection of HMG 17-Histone Cross-linked Dimers** — The cross-linked material was dissolved in 10 µl of 8.0 M urea, 0.9 M acetic acid, 2.0% salmon protamine sulfate (Sigma) (67). Samples were loaded onto a 15% Triton-urea gel (15.5 cm × 24 cm × 1.5 mm) (68) and run at 10 mA until the dye front reached 20 cm from the origin, stained with Brilliant Blue R (Sigma) in 45% MeOH, 10% acetic acid, and destained in 5% MeOH, 10% acetic acid. The protein lanes were cut out separately (3 cm wide) and equilibrated against SDS stacking gel solution (0.1% SDS, 125 mM Tris-HCl, pH 6.8) for 1 h with one change. The strip was laid directly onto the stacking gel of a 17.5% Laemmli/Staehelin SDS gel (30 cm × 60 cm × 2.25 mm) (69) which was electrophoresed (4 °C) at 500 V for 1.5 × time required for the dye (Brilliant Blue R) to run off. The gel was stained and destained as above, photographed, equilibrated against 45% MeOH, 10% acetic acid to facilitate drying, and then dried between two layers of dialysis membrane. Autoradiography was done for 20 h at 25 °C using Kodak X-5 x-ray film.

**Quantitation of Cross-linked Dimers** — The dried two-dimensional gel was placed over the autoradiogram, and the labeled monomer and dimers were cut from the gel and counted in a Searle model 1185 γ-counter.

**Identification of Histones in Cross-linked Complexes** — A two-dimensional gel was run as above, but using unlabeled HMG 17, stained, and destained. Instead of drying the gel, the spots identified previously as HMG 17-histone dimer complexes were cut out, dialyzed against SDS-gel stacking solution containing 5% β-mercaptoethanol, minced, and placed into the individual wells of a 17.5% Laemmli/Staehelin SDS slab gel (15.5 cm × 9.5 cm × 1.5 mm). These pieces were overlaid with sample buffer solution and the gel was run at 150 V for 4 h, Brilliant Blue R stained as above, and then silver stained by the method of Wray et al. (70).

**Statistical Analysis**

Statistical significance was determined by one-way analysis of variance with specific differences between groups located using Duncan's new multiple-range test (71).

**RESULTS**

Fig. 1 shows the histone and HMG proteins used in cross-linking. Fig. 2 shows the 5% polyacrylamide nondenaturing

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**Fig. 1. Proteins before reconstitution and cross-linking; 17.5% Laemmli/Staehelin SDS gel. Lane a shows the core histones of the mononucleosome core particles prepared by sucrose density gradient centrifugation, approximately 2 µg/band. The two lightly staining bands above the core histones are from the top ubiquitinated H2A and HMG 17. Lane b is HMG 17 after high performance liquid chromatography purification, approximately 4 µg. Staining is with Brilliant Blue R.**
FIG. 2. Gel analysis of reconstitution and cross-linking. 5% acrylamide nondenaturing polyacrylamide gel electrophoresis of core nucleosomes before reconstitution with HMG 17 (lane a), after reconstitution with 2 HMG 17 molecules bound per core particle (lane b), and after reconstitution and cross-linking (lane c); 1 µg of DNA/lane. Staining is with ethidium bromide.

The gel used to determine the extent of reconstitution. The HMG 17-nucleosome core complex migrates more slowly than the unbound nucleosome cores due to a reduced net negative charge (72). Lane a shows free nucleosome core particles (1 µg of DNA) after purification on sucrose gradient and before reconstitution; lane b shows core particles reconstituted with 2 HMG 17 molecules bound per core particle, before cross-linking, and lane c shows HMG 17-reconstituted and cross-linked core particles. As can be seen, cross-linking does not change the mobility of the HMG 17-core particle complex. The cross-linking has occurred primarily within the reconstituted complex since no dimers are apparent after cross-linking. An autoradiogram of this gel likewise showed no difference in particle mobility before and after cross-linking (not shown). Fig. 3 shows the two-dimensional gel separation of the cross-linked protein for identification of dimers. Only the monomer and dimer regions of the SDS gel are shown here. The numbers next to the dimers label those which contain HMG 17 as identified by autoradiography and shown in Fig. 4. Dimers 4 and 5 (indicated by circles) stained lightly. Brilliant Blue R stained.

FIG. 3. Two-dimensional gel separation of cross-linked proteins. First dimension is a 15% Triton-acid-urea gel, and the second dimension is a 17.5% acrylamide Laemmli/Staehelin SDS gel. Only the monomer and dimer regions of the SDS gel are shown here. The proteins in a given spot must be 1:1 since they run as dimers in the SDS-gel dimension. The extent of cross-linking to HMG 17 was greatest for histone H2A (Fig. 5, bar 1), but there was also cross-linking to the other core histones. By contrast, there was little cross-linking between two HMG 17 molecules bound on the same nucleosome core particle.

DISCUSSION

The HMG proteins 14 and 17 bind to nucleosomes, and their presence is correlated with DNase I sensitivity and the transcriptional activity of the chromatin. The occurrence of major conformational changes in the nucleosome, such as...
HMG 17 Cross-linking to Core Histones

The relatively greater yield of cross-links to histone H2A compared to the other histones suggests that HMG 17 binds to the nucleosome core particle nearest to H2A, while it also makes contact with the other core histones. The preferential cross-linking to H2A is significant at p < 0.01. The location of HMG 17 near H2A is consistent with a variety of other experimental data summarized in the 7 Å structure of the nucleosome which places H2A at sites where the ends of the DNA enter and leave the nucleosome core particle (78).

DNA-histone cross-linking data of Bavykin et al. (79) demonstrate that the two major DNA-H2A cross-links are immediately flanking the regions between 120 and 140 bases from the 5' ends of the nucleosome core DNA. These are the same regions of DNA shown by Mardian et al. (51) to be protected by HMG 14 from DNase I digestion. Furthermore, Shick et al. (53) have recently found HMG 14 and 17 to cross-link to the DNA of core particles at the 3' and 5' termini of both strands and at sites 25 and 125 base pairs from the 5' ends. It is interesting to note that histone H1, which shows sequence homology between its random coil terminus and HMG 14 and 17 and is thought to bind to the nucleosome where the DNA enters and exits, also specifically cross-links to H2A (at H2A C-terminal half) (80, 81) and to the DNA near this location (82). While H1 and HMG 14 or 17 bind simultaneously to the same nucleosome, HMG 14 and 17 may modulate the binding of the H1 C-terminal by competing for a site on the same nucleosome or between nucleosomes (7).

The data presented here suggest that HMG 17 binds near the globular domain of H2A. If HMG 17 were cross-linking to the free N-terminal domain of histone H2A one would also expect a comparable amount of cross-linking to the free N-terminal of histone H2B. This is particularly true since the N-terminal of H2B has three times the number of lysine residues (i.e. 12 in calf thymus H2B) available for cross-linking as has the N-terminal of H2A (i.e. 4 in calf thymus H2A) (83, 84). Instead, the extent of cross-linking of HMG 17 to H2B is similar to that seen between HMG 17 and H3 or H4, the latter of which have nucleosome-bound N termini (84). There is no statistically significant difference in the extent of cross-linking of HMG 17 with the three core histones in this group suggesting a possible nonspecific "background" against which the cross-linking to H2A may be compared.

In sharp contrast to the interaction between HMG 17 and the core histones, HMG 17 cross-links very little to a second HMG 17 molecule bound on the same nucleosome core particle. This indicates that the two binding sites for this protein are situated at some distance from each other, effectively restricting contact between the bound HMGs.

Our model, based on the above data and that in the literature, places the central lysine-rich DNA binding region of HMG 17 near the globular region of H2A where it is in association with approximately 25 base pairs at each end of the DNA.
the core particle DNA (Fig. 7). The acidic C terminus of HMG 17 may interact with the histones to release partially some core DNA from histone interaction. Furthermore, each HMG 17 is localized on an opposite side of the nucleosome core particle, providing little opportunity for direct interaction between the two HMG 17 proteins bound to the same nucleosome.

There are limitations to the cross-linking technique as it is used in the present experiment. Cross-linking is statistically biased toward the lysine-rich proteins and regions of proteins studied. This does not appear to be a deciding factor in determining the difference seen in cross-linking between HMG 17 and the core histones as mentioned above. Second, we studied only the dimer fraction of the cross-linked material, and polymer formation greater than dimers might affect the relative ratios of the dimers. Consequently, a low concentration of 2-iminothiolane was used to decrease the amount of polymer formation.

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