Immunochromic Analysis of the Exposure of High Mobility Group Protein 14 and 17 Surfaces in Chromatin*  

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Michael Bustin, Massimo P. Crippa, and James M. Pash  
From the Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Antisera were elicited against synthetic peptides corresponding either to regions common to all members of the high mobility group 14 and 17 protein family protein or to distinct domains of the HMG 14 or HMG-17 subgroup. The antisera were used to probe the accessibility of various HMG domains in chromatin.

The central region, which contains the DNA binding domain, has a high positive charge while the C-terminal region has a net negative charge. Furthermore, helical wheel projections of the C-terminal regions reveal that the negative charges are clustered on one surface of the helix (3).

One of the unanswered questions pertinent to the organization of HMG proteins in chromatin, and their possible function, is whether their surfaces are accessible and free to interact with other molecules. Recent studies indicate that cooperative and synergistic protein interactions play an important role in transcriptional regulation (13, 14). If HMG proteins participate in such interactions it would be expected that part of the protein surface would be exposed and capable of interacting with other macromolecules. In the present study we use antibodies elicited by peptides derived from various regions of the molecules to probe the accessibility of HMG domains in chromatin.

**MATERIALS AND METHODS**

**Preparation of Antigens and Antisera**—The preparation and characterization of antisera to calf HMG-14 (15) and HMG-17 (16) has been previously described. The various peptides used in this study (see Fig. 1) were custom-synthesized by Peninsula Laboratories, Inc. (Belmont, CA). The purity of the peptides was assessed by high pressure liquid chromatography and amino acid analysis. Peptides 1, 3, and 6 were conjugated to keyhole limpet hemocyanin, and peptides 2, 4, and 5 to ovalbumin. Conjugations were done by both the glutaraldehyde and the 1-ethyl-3,3′-(3-dimethylaminopropyl)carbodiimide (EDC) techniques essentially as described (17). The peptides were coupled to the protein at a 1:15 molar ratio of protein:peptide. For EDC coupling, the protein was dissolved at 1 mg/ml in N,N-dimethylformamide, 2 mM NaHCO3, 70:30), pH 5.5, and EDC was added in aliquots, with stirring, to a final concentration of 0.1 M. After 10 min the pH was adjusted to 5.5 with 20 mM HCl and the protein added to a peptide solution in N,N-dimethylformamide, 2 mM NaHCO3. After 20 min the pH was readjusted to 5.0 and the reaction continued, with stirring, for 6 h. The reaction was then dialyzed to 50 mM ammonium acetate, pH 7.5, and the sample was vacuum-dried and dissolved in ammonium acetate at 2 mg/ml protein. Insoluble samples were dispersed by sonication. For glutaraldehyde coupling, the protein and peptides were dissolved in 0.1 mM sodium phosphate buffer, pH 7.5, at 2 mg/ml protein. The solution was made 5 mM in glutaraldehyde by dropwise addition of 21 mM glutaraldehyde. After cross-linking for 3 h the mixtures were dialyzed against 50 mM sodium acetate, pH 7.5, and treated as described above. The extent of conjugation was judged by electrophoresis in sodium dodecyl sulfate gels.

The free peptides or the peptide protein conjugates were emulsified in 66% complete Freund’s adjuvant and injected at multiple intramuscular, intradermal, and subcutaneous sites. Peptides 1 were injected into rabbits at either 100 μg of peptide or 500 μg of conjugate per rabbit. Peptides 4–6 were injected into goats at the same dose. Two weeks later, the animal received an identical boost. A final boost was administered 2 weeks later by an intravenous injection of the peptide dissolved in phosphate/saline (14).

**Preparation of Chromatin**—Chromatin was prepared from chicken erythrocytes and from HeLa nuclei as previously described (18, 19). The content of histone and of DNA in the chromatin preparations was analyzed by gel electrophoresis.

**Immunological Assays**—Enzyme-linked immunosorbent analysis (ELISA) and immunoblotting analysis were performed as previously described (20). The exposure of antigenic determinants in chromatin was assessed by competitive immunoadsorption (20, 21). In this assay, antisera diluted in 5 mM EDTA, 0.1% Trasylol (protease inhibitor, Sigma), 1% bovine serum albumin, and 0.1% normal IgG (either rabbit or goat, depending on the second antibody) are incubated with 100 μg of chromatin in the same buffer. The mixture was shaken for 20077...
FIG. 1. Peptides used as immunogens. Alignment of all known HMG-14/-17 protein sequences. The protein sequences (see reference 3) were aligned with the Beakman Microgen program. Hyphens were inserted to maximize alignments. Dots above the sequences identify positions of invariant amino acids. Boxed regions identify the peptides selected as immunogens. Numbers identify the peptide.

| Antigen | 1 | 2 | 3 | 4 | 5 | 6 | 17 | 14 |
|---------|---|---|---|---|---|---|----|----|
| HMG-14 human PKRKV-SSAEK-KEE PKRRSARLS AKP-PVKAPEPK | 100 | 80 | 0 | 0 | 0 | 0 | 2 | 4 |
| HMG-14 calf PKRKV-SSAEK-KEE PKRRSARLS AKPAPKAVEPK | 100 | 20 | 0 | 0 | 0 | 0 | 100 | 20 |
| HMG-14 mouse PKRKV-S-ADE-KEE PKRRSARLS AKPAPKAVEPK | 300 | 0 | 0 | 0 | 0 | 0 | 100 | 20 |
| HMG-14 chicken PKRKV-S-ADE-KEE PKRRSARLS AKPAPKAVEPK | 300 | 0 | 0 | 0 | 0 | 0 | 100 | 20 |
| HMG-14 mouse PKRKV-S-ADE-KEE PKRRSARLS AKPAPKAVEPK | 300 | 0 | 0 | 0 | 0 | 0 | 100 | 20 |
| HMG-17 human PKRKV-SSAEK-KEE PKRRSARLS AKP-PVKAPEPK | 20 | 5 | 0 | 0 | 0 | 0 | 100 | 50 |
| HMG-17 calf PKRKV-SSAEK-KEE PKRRSARLS AKPAPKAVEPK | 100 | 5 | 0 | 0 | 0 | 0 | 100 | 50 |
| HMG-17 mouse PKRKV-SSAEK-KEE PKRRSARLS AKPAPKAVEPK | 100 | 5 | 0 | 0 | 0 | 0 | 100 | 50 |

30 min at room temperature and for 4 h at 4 °C, then centrifuged in an Airfuge (Beckman) for 90 min at 105,000 × g. Under these conditions over 90% of the chromatin DNA pelleted without affecting the titer of the sera. The resulting supernatant was diluted in phosphate-buffered saline containing 1% powdered milk, 1% bovine serum albumin, 0.1% Tween 20 and added to microtiter plates containing antigens. Studies on the inhibition by peptides were done in the same fashion with peptide substituting for the chromatin. Immune nonadsorbed sera were treated in an identical fashion without the addition of chromatin.

RESULTS AND DISCUSSION

Peptide Immunogens—Over 30% of the sequence of the HMG-14/-17 proteins is invariant from trout to human, an evolutionary span of over 400 million years. Furthermore, the invariant residues are clustered producing evolutionary conserved domains. In addition, the charge distribution along the polypeptide chain is asymmetric. The N-terminal region has a slight positive charge, the central region a high positive charge, and the C-terminal region is negatively charged (3). The peptides used as immunogens were selected either from regions common to all the members of the HMG-14/-17 protein family or from distinct domains of either the HMG-14 or the HMG-17 subgroups. Fig. 1 shows an alignment of all the known members of the HMG-14/-17 protein family and the regions chosen as immunogens. Peptide 2 spans the entire DNA binding domain (22) of human HMG-17. The sequence of the first 14 amino acids in this peptide is virtually invariant among all HMGs, from trout to human. Antibodies elicited by this peptide would be expected to bind to all the proteins of the HMG-14/-17 family. The C-terminal half of peptide 2 contains a proline-rich region which is common to all members of the HMG-17 group and is not present in the HMG-14 group. This 13-amino acid-long peptide, containing 6 proline and 4 lysine residues, is named peptide 1. Antibodies elicited by this region should recognize members of the HMG-17 group. Peptide 3 corresponds to the C-terminal region of HMG-17 which is conserved and has a net charge of (-3). Peptide 4 is derived from the DNA binding domain of HMG-14 (23) and is homologous to peptide 2. The N-terminal half of this peptide is exactly the same as that of peptide 2 while the C-terminal half contains sequences specific for HMG-14. Peptide 5 contains regions which are specific for human and calf HMG-14; however, the last 6 amino acids are invariant among all the HMG proteins. Peptide 6 corresponds to the C-terminal amino acids of the HMG-14 proteins, which is distinct from the C-terminal of the HMG-17 proteins, and has a net charge of -8.

Antiser specificity—Immunoblotting with intact proteins (not shown) reveals that, as expected, antipeptides 1 and 3 were specific for HMG-17, antipeptides 5 and 6 for HMG-14, and that antipeptides 2 and 4 reacted with both HMG-14 and HMG 17. For a more quantitative analysis, the specificity was tested by ELISA, at several sera dilutions, with various concentrations of each antigen. Table I summarizes the antisera specificity as detected by ELISA. The 100% reaction was taken as the value obtained by reacting the antisera with the immunogen (i.e. antipeptide 1 with peptide 1) at a 1:500 dilution of both the first and second antibody (see "Materials and Methods"). The concentration of the antigen added to the well was 5 μg/ml peptide or 1 μg/ml protein, and the reaction was stopped when the A540 reached 0.8–1.0. Antisera to peptide 1 reacted specifically with peptide 1 and, as expected, also recognized peptide 2. The weak reactivity with HMG-14 is due to the similar concentration of the protein which is about 50 times smaller than the peptide concentration. At higher HMG-17 concentrations the reaction between antipeptide 2 and HMG-17 was similar to that obtained with the peptide. Antipeptide 2 reacts with both HMG-14 and HMG 17 as well as with peptide 2. Peptide 3 did not bind to the ELISA plate; therefore, the reaction with HMG-17 was
taken as 100%. The specificity with the peptides derived from HMG-14 was similar to those derived from HMG-17 in that antipeptide 4 reacted with both HMG-14 and HMG-17 as well as with peptide 2 but not with peptide 1. Antipeptide 6 reacted better with HMG-14 than HMG-17.

Accessibility of HMG Antigenic Determinants in Chromatin—The availability of antigenic determinants was assessed by competitive ELISA assays (20, 21). Antisera to HMG proteins were preincubated with chromatin for 1 h, and the incubation mixture was centrifuged to remove the chromatin and the antibodies bound to it. The unadsorbed antibodies, remaining in the supernatant, were then tested by ELISA. The ability of chromatin to inhibit the reaction of anti-HMG-17 with HMG-17 is shown in Fig. 2. The antiserum, diluted 1:20,000, was incubated with 100 ng of chromatin containing approximately 1 μg of HMG proteins. Higher amounts of chromatin resulted in nonspecific interactions. As shown in Fig. 2, the reaction was inhibited by approximately 60% suggesting that not all the antibodies elicited by the free protein reacted with the chromatin-bound protein. In contrast, chromatin treated with 0.4 M NaCl, a concentration which extracts HMGs from chromatin (3), did not adsorb antibodies to HMG proteins. Control antibodies did not interact with chromatin (21). We conclude therefore that the interaction of anti-HMG-17 with chromatin is specific and that only a fraction of the antibodies, generated by HMG-17, binds to chromatin. The lack of complete binding could be due either to steric hindrance or conformational change in the protein upon binding to chromatin.

Binding of Antipeptide Sera to Chromatin—Preincubation of antibodies to peptide 1 with chromatin inhibited the reaction with HMG-17 by 85% suggesting that this region of the protein is exposed and available to interact with antibodies (Fig. 3A). Preincubation of antisera elicited by peptide 2 with chromatin inhibited its reaction with HMG-17 to only about 25% (Fig. 3B). Peptide 2 encompasses the entire sequence of peptide 1, and therefore a fraction of the antibodies elicited by peptide 2 cross-reacts with peptide 1. Since antibodies to peptide 1 bind to chromatin, we assume that at least part of the antipeptide 2 antibodies that bind to chromatin were elicited by the portion corresponding to peptide 1. Since the overall binding of antipeptide 2 is significantly lower than that of antipeptide 1, we conclude that antibodies elicited against the region specific to peptide 2 (i.e. absent from peptide 1, see Fig. 1) bind poorly to chromatin. These findings suggest that access to that region is sterically hindered or that the conformation of that region in chromatin is significantly different from that of the protein free in solution. Antibodies to peptide 3 encompassing the C-terminal of the molecule were efficiently adsorbed by chromatin so that the reaction with HMG-17 was inhibited by about 80% (Fig. 3C), suggesting that this region of the protein is exposed and able to interact with other molecules.

As an additional test for the specificity of the sera, we examined the ability of heterologous peptides to inhibit the reaction between a peptide and its antibody under the same
Table II
Inhibition of antibody binding by chromatin

The ability of 100 μg of chromatin to inhibit the reaction between a particular sera and either HMG-17 (for anti-HMG-17 and antipeptides 1–3) or HMG-14 (for antipeptides 4 and 6) is given as percentage of inhibition. Values are presented for two points of ELISA assays (see Figs. 2 and 3).

| Antisera to Peptide | Point at which examined | 50% | 100% |
|---------------------|-------------------------|-----|------|
| HMG-17              |                         | 64  | 83   |
| Peptide 1           |                         | 70  | 83   |
| Peptide 2           |                         | 4   | 14   |
| Peptide 3           |                         | 84  | 80   |
| Peptide 4           |                         | 22  | 11   |
| Peptide 6           |                         | 78  | 83   |

![Diagram of the binding of HMG-14/17 proteins to nucleosomes.](image)

**Fig. 4. Model of the binding of HMG-14/17 proteins to nucleosomes.** The model is a modification of that suggested by Cook et al. (24). The proteins bind near the entry/exit points of the core DNA (5, 6); part of the DNA binding domain is between histone H2A and the DNA (6, 24). The structure of the proteins is diagrammed in the upper right corner. The charge density and the ability of the various regions to bind antibodies are indicated below the structure. DNA binding domain (22, 23) open box has a high net positive charge while the C-terminal region has a net negative charge.

conditions as the inhibition studies described above. As shown in Fig. 2D, the interaction of antipeptide 1 with HMG-17 was fully inhibited with peptide 1, only 40% inhibited by preincubation with peptide 2, and not inhibited by peptide 4. Likewise, the binding of antipeptide 4 to HMG-14 was fully inhibited by peptide 4, partially inhibited by peptide 2, and not inhibited by peptide 1 (not shown). These results verified that the antibodies elicited by the peptides recognize different regions of the proteins.

### Differential Exposure of HMG Protein Surfaces in Chromatin

The ability of chromatin to inhibit the reaction between an antibody and its antigen depends on the exposure of the antigenic site in chromatin. The data shown in Table II indicate that the ability of chromatin to inhibit the interaction between an antibody and its antigen varied among the various antibodies. The table documents the ability of chromatin to inhibit the reaction between various antisera and HMG-14 or HMG-17. The percent inhibition is reported for two points in the ELISA assay, the midpoint of the maximal reaction and 100% reaction obtained between the antisera and the protein. The 100% reaction is an arbitrary point taken 30–60 min after the addition of substrate when the A0 observed reached 0.8–1.0. For example, at 50% of the maximal reaction chromatin inhibited only 4% of the reaction between antipeptide 2 and HMG-17 while at the end point (100% reaction) chromatin inhibited the ELISA by 14%. The data indicate that antisera to peptides 1, 3, and 6 bind to chromatin to a significantly larger degree than antisera to peptides 2 and 4. Peptide 1 has partial homology with a region of histone H1, and although the reaction with histone H1 is only 5% of that obtained with HMG-17 (not shown), it is feasible that part of the reaction is due to binding to this histone. The weak binding of the antibody to peptides 2 and 4 suggests that this region is not accessible to antibody binding, either because of steric hindrance or conformational change. This region constitutes the DNA binding domain of the proteins (22, 23); therefore we assume that it is closely associated with DNA.

This association may cause steric hindrance or conformational changes, both of which would decrease the binding of antibodies to this protein domain. The negatively charged C-terminal region of the proteins is recognized by the antisera, suggesting that this region is free to interact with other components. The accessibility of the various regions of the molecule to antibody binding parallels the interaction of the molecule with nucleosomes. Cook et al. (10) reported that the basic central region of the molecule binds more strongly to the core DNA than the acidic C-terminal region. The model presented in Fig. 4 incorporates the information obtained here with that available from the literature. The proteins are located near the entry/exit points of the core DNA, the central region of the molecule is near histone H2A, and the C-terminal region is relatively exposed.

The transcription activating domain of certain regulatory proteins resides in a negatively charged region of the molecule (13, 14). By analogy, the C terminus of the HMG-14/17 proteins may be involved in a similar function. A minimum requirement for this function is that this region should be exposed and available for interactions with other molecules. The results presented here indicate that the C terminus of the proteins is exposed and available to participate in reactions which require contact with other proteins.

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