High Mobility Group Proteins 14 and 17 Can Prevent the Close Packing of Nucleosomes by Increasing the Strength of Protein Contacts in the Linker DNA*

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High mobility group (HMG) proteins 14 and 17 are abundant chromatin-associated proteins found in all higher eukaryotic nuclei. This observation demonstrates that HMGs 14 and 17 must have an important and universal function with regard to the structure and function of chromatin. What this function is, including how they interact with a nucleosomal array in vivo, is not known. Recently, we have demonstrated that HMGs 14 and 17 can organize nucleosomes into a regular array and increase the repeat length from 145 to about 160–165 base pairs in vitro. In addition, they can increase the apparent repeat length of chromatin deficient in histones H2A/H2B from 125 to approximately 145 base pairs. Importantly, this template was transcriptionally active. In this study, we report five new observations that begin to address the mechanism by which HMGs 14 and 17 space nucleosomal particles. First, we demonstrate that both human placenta HMG 14 and HMG 17 can space nucleosomes to produce a chromatin template with a repeat length around 160 base pairs. This result further highlights the similarity between these proteins in terms of protein structure and perhaps function. Second, we show that digestion of HMG containing chromatin with micrococcal nuclease produces DNA fragments that were approximately 10 and 20 base pairs longer than nucleosome core-particle DNA. This suggests that HMG 14 or HMG 17 can protect, directly or indirectly, at least an additional 10 base pairs of linker DNA from micrococcal digestion. However, this HMG-containing particle does not produce a strong kinetic block, and further digestion results in the eventual accumulation of DNA fragments 145 base pairs in length. Third, by comparing the full-length protein with different domains, we demonstrate that the acidic carboxyl-terminal domain is absolutely required for nucleosome spacing; neither the nucleosome binding domain of HMG 14 or HMG 17 nor the amino-terminal domain plus the nucleosome binding domain of HMG 14 could space nucleosomes. Fourth, we demonstrate that extensive micrococcal nuclease digestion of chromatin deficient in histones H2A/H2B led to the accumulation of DNA fragments about 110 base pairs in length, which is presumably the length of DNA associated with a nucleosomal particle deficient in one H2A/H2B dimer. Incorporation of either HMG 14 or HMG 17 into this chromatin results in the disappearance of this band and increase in the accumulation of fragments around 140–150 base pairs in length.

Finally, in contrast to spacing of complete nucleosomes, we find that the nucleosome binding domain of HMG 17 (but not the nucleosome binding of HMG 14) is the only domain required for spacing of H2A/H2B-deficient chromatin.

A key regulatory step in the process that leads to the transcriptional activation of a eukaryotic promoter is the binding of activator proteins to a nucleosomal template. In vivo footprinting experiments of a small number of genes have demonstrated that the structure of an active promoter is highly organized and complex, often involving specific interactions between histones, transcription factors, and DNA (1, 2). The molecular steps involved in generating such active structures from a nucleosomal template are not known. Clearly, the reproduction of an active from an inactive chromatin structure in vitro will be crucial for unravelling these molecular steps.

Much information has been generated concerning the involvement of histones in the establishment of a transcription complex within a chromatin environment. For example, the precise rotational and/or translational positioning of nucleosomes, the direct interaction between the amino-terminal domains of histones and activator proteins, and histone modifications have all been shown to play a role in the transcriptional activation process (1–5). However, considerably less is known about the role of chromatin-associated non-histone proteins in the transcription process. One important class of non-histone proteins includes HMGs1 14 and 17.

HMGs 14 and 17 are abundant proteins found in the nuclei of all higher eukaryotes. Given their ubiquitous distribution, it is assumed that they have a critical basic function in the nucleus (6, 7). Both HMGs 14 and 17 have an unusual amino sequence. They have a high content of charged amino acids that are asymmetrically distributed along their primary structure and, remarkably, lack any hydrophobic aromatic residues. Despite their similarities in primary structure, HMG 14 and HMG 17 are significantly different to suggest that they may be involved in distinguishable nuclear functions. Their precise function remains to be determined.

HMGs 14 and 17 are found in both transcriptionally inactive and active regions of chromatin but appear to be enriched in active regions (6–8). This led to the suggestion that they may modify the structure of chromatin to facilitate transcription. Strong evidence supporting this hypothesis has come from recent transcription studies using in vitro assembled chromatin as templates (9–11). The molecular mechanism by which HMGs 14 and 17 activate transcription is not known but presumably it involves altering or modifying histone-DNA inter-
actions. Therefore, to understand this process, it will be crucial to understand how these proteins interact with chromatin. To date, most studies have only employed single nucleosome-core particles as acceptors.

Recently, we have shown that HMGs 14 and 17 can bind to and increase the nucleosomal repeat length of chromatin assembled in vitro (12, 13). Moreover, this increase in repeat length was correlated with an increase in the transcriptional competence of the template (9). In this report, we begin to investigate the mechanism of the spacing reaction by further characterizing the structure of spaced chromatin templates and by examining the protein domains of HMG 14 and HMG 17 required for spacing nucleosomes and nucleosomal particles deficient in H2A/H2B.

EXPERIMENTAL PROCEDURES

Plasmid DNA—The plasmid used in this investigation was pX-bSF201, a 2.9-kilobase plasmid that carries a 240-base pair insert of the Xenopus laevis somatic 5S RNA gene (14).

Chromatin Assembly Reactions—Chromatin assembly reactions employing N1/N2-(H3, H4) (isolated by sucrose gradient centrifugation from a Xenopus laevis oocyte extract), histones H2A and H2B (purified from chicken red blood cells), plasmid DNA and topoisomerase I, in the presence or absence of HMGs 14 and 17 protein (purified from human placenta) or synthesized peptides, were carried out as described previously (9, 14). HMG 14 was separated from HMG 17 on a blue Sepharose column; HMG 14 and HMG 17 eluted from the column at 0.4 mM NaCl and 0.5 mM NaCl, respectively. The amount of histones and HMGs 14 and 17 added to assembly reactions is defined in terms of activity (see Ref. 9 and the legend of Fig. 1 for an explanation, respectively).

Nucleosome Reconstitution and Mobility Gel-shift Assays—Nucleosome core reconstitution, using chicken H1-depleted oligonucleosomes as donor chromatin and a 160- or 180-base pair fragment (42 to 117 and 65 to 117, respectively, of the Fra 2 promoter (15)) as the recipient, was carried out in an identical manner as described in Ref. 16 except that after the final dilution step 2.5 mM MgCl2 was added to precipitate the donor chromatin (17). The aggregated chromatin was then removed by a 30-min spin in a microcentrifuge at 4°C. HMGs 14 and 17 were added to the reconstituted nucleosome after being appropriately diluted with 20 mM Tris, pH 7.8, 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin. The binding reaction was allowed to proceed for 30 min at 4°C. The final conditions in the binding reaction were 50 mM NaCl, 125 mM KCl, 0.1% Triton X-100, 50 mM HEPES, pH 8.0, 0.05 mg/ml bovine serum albumin, 0.05 mM EDTA, 1.25 mM 2-mercaptoethanol, and 2.5 mM dithiothreitol. Following the binding reaction, samples were loaded onto a 6% polyacrylamide gel, 50% TBE (0.044 M Tris, 0.044 M boric acid, 0.001 M EDTA) gel, and run at 4°C.

Micrococcal Nuclease Digestion Analysis and Agarose Gel Electrophoresis—The assembled chromatin was digested with micrococcal nuclease, the DNA fragments purified, and the DNA run either on a 1.5% agarose gel, as described in Ref. 9, or on an 8% polyacrylamide gel. Samples of chromatin constituted from 300 ng of plasmid DNA were removed from the digestion reaction at the time points indicated in the figure legends. Molecular weight markers include a mixture of pUC19 digested with Hpal (fragment sizes are 501, 489, 404, 331, 242, 190, 147, 111, and 67 base pairs) and bacteriophage SPP-1 DNA cut with EcoRI (Brescat, Australia) (fragment sizes are 8510, 7350, 6110, 4840, 3590, 2810, 1950, 1860, 1510, 1390, 1160, 980, 720, 480, and 360 base pairs).

RESULTS AND DISCUSSION

Recently, we partially purified a novel ATP-dependent nucleosome spacing activity from X. laevis oocytes (14). When this activity is added to assembly reactions containing the N1/N2-(H3,H4) complex (both histones are in the acetylated form), histones H2A/H2B, plasmid DNA, and topoisomerase I, regularly spaced nucleosomes are assembled with a repeat length of 160–165 base pairs (12, 13). In the absence of this spacing activity, closely packed nucleosomes are assembled, i.e. 145 base pairs/particle. The spacing activity contained a protein that displayed chromatographic properties and a mobility on different polyacrylamide gels similar to HMGs 14 and 17. For this reason, we tested HMGs 14 and 17 for the ability to space nucleosomes and indeed discovered that HMGs 14 and 17, isolated from human placenta, can mimic the spacing activity and produce a regularly spaced chromatin template with a 160–165-base pair repeat. Strikingly, this repeat length is similar to that observed in lower eukaryotes, such as yeast, which may indicate that similar HMG proteins are involved in spacing in these organisms (18, 19).

HMG 14 and HMG 17 have an unusual amino composition; they are enriched in charged residues and are depleted in hydrophobic residues (7). Human HMG 14 consists of 99 amino acids, 26 are positive and 19 are negative. Similarly, human HMG 17 contains 89 residues of which 26 are positive and 14 are negative. Of the 26 positive amino acids in both proteins, 21 residues are in identical positions when the protein sequences are aligned for maximum similarity. This clearly highlights that their interaction with chromatin is electrostatic in nature and that the position of these positive residues are spatially in very precise positions. It is believed that the polypeptide chain of HMG 14 and HMG 17 only forms an ordered structure upon binding to chromatin (7).

The putative nucleosome core binding domain covers residues 13 to 43 for HMG 14 and residues 17 to 47 for HMG 17 (20, 21). This region is highly positively charged (11 positively and only 3 negatively charged residues for HMG 14) and displays the greatest similarity between the two proteins (23 out of 30 residues are identical). The carboxyl terminus of both proteins are negatively charged. However, in spite of these similarities, there exists significant differences between the two proteins. For example, in the nucleosome-core binding domain, the two proteins differ at seven positions, and the carboxyl domain of HMG 14 is considerably more negative than the corresponding region of HMG 17. Thus, it is possible that HMG 14 and HMG 17 may have different cellular functions. It was therefore of particular interest to test whether both proteins can space nucleosomes in vitro.

Fig. 1A shows the micrococcal nuclelease pattern of chromatin formed in the presence of human placenta HMG 14 (lanes 2-4) and HMG 17 (lanes 6-8) (the amount of HMG proteins added to assembly reactions is defined in terms of units/100 ng of DNA which is explained in the legend of Fig. 1). The results clearly demonstrate that both HMG 14 and HMG 17 can increase the nucleosomal repeat length. This experiment has been repeated many times with different HMG preparations, and from these results we can conclude that both proteins can produce chromatin with a repeat length of 160–165 base pairs. This observation suggests that HMG 14 and HMG 17 interact with chromatin in a functionally similar way.

To quantitate more accurately the increase in repeat length caused by HMGs 14 and 17, chromatin, assembled in the absence or presence of HMGs 14 and 17, was digested with micrococcal nuclease to yield a greater range of digestion time points. The lengths of DNA in the oligomers were then determined and plotted against digestion time (Fig. 1B). When the length of oligomers is plotted against band number for the 1-min time point, the slope is 145 and 160 base pairs per nucleosome for HMG-deficient and HMG-containing chromatin, respectively (monomer and dimer fragments were not used in this analysis because the sizes of these fragments, in particular the monomer fragment, were significantly greater than the repeat length; a similar observation was also reported when yeast chromatin was digested with micrococcal nuclease (19)). Importantly, Fig. 1B clearly shows that HMGs 14 and 17 space nucleosomes by preventing the close packing of nucleosomes rather than by preventing the sliding of nucleosomes together which may occur during the digestion of chromatin with micrococcal nuclease (19).
Effects of HMGs 14 and 17 on Protein Contacts in Linker DNA

Fig. 1. HMG 14 and HMG 17 can space nucleosomes. A, assembly reactions were carried out using N1/N2-(H3, H4) complex (0.7 unit/100 ng of DNA), histones H2A and H2B (1 unit/100 ng of DNA), HMG 14 or HMG 17 (lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8, respectively), topoisomerase I, and relaxed F201. Each pair of lanes 1-8 represents two time points of digestion for 2 and 4 min. B, molecular weight markers. The amount of HMGs 14 and 17 added to assembly reactions is defined in terms of activity because the spacing activity of different preparations vary and because the spacing activity decreases with time when HMGs 14 and 17 are stored at -70°C. 1.0 unit of HMGs 14 and 17 is defined as the amount of protein needed to convert 100 ng of DNA that had been assembled in nucleosomes with 0.7 unit of N1/N2-(H3, H4) complex and 0.8-1.0 unit of histones H2A and H2B to a nucleosomal template with a repeat length around 160 base pairs. In terms of moles, the activity of different preparations of HMG proteins to produce a spaced template varies between 4 and 10 mol of protein per 100 base pairs of DNA. The preparation of HMG 14 and HMG 17 used in these experiments produced a 160-165-base pair ladder when 10 mol of protein per 160 base pairs of DNA was used. This amount of protein in subsequent experiments is defined as 1.0 unit/100 ng of DNA. An arrow points to the DNA fragment that was associated with three and four nucleosomes, respectively. B, identical assembly reactions were carried in the presence or absence of HMGs 14 and 17 (1.0 unit/100 ng of DNA). The assembled chromatin was digested with micrococcal nuclease (0.03 unit/μl reaction) for the indicated times. After a digestion time of 7 s, the pentamer fragment is completely lost.

Most recently, using a crude Drosophila assembly extract, it was shown that simply increasing the final KCl concentration from 70 to 155 mM increased the repeat length in a gradual manner from 60 to 110 base pairs (22). A similar increase was also observed with other cations such as Mg2+. Based on this work, it was suggested that the various cations influence the folding of the nucleosomal fiber, and the extent of folding determines the nucleosomal repeat length. We therefore wanted to examine whether salt has a similar influence on the repeat length using our relatively pure nucleosome assembly system. Fig. 2 clearly shows that increasing the Na+ concentration from 60 to 110 mM (higher salt concentrations disrupt the micrococcal nuclease pattern) does not produce a repeat length similar to that observed with HMGs 14 and 17; the repeat length, in the absence of HMG proteins, is about 140 and 145 base pairs at 60 and 110 mM, respectively. In the Drosophila system, a similar increase in KCl concentration of 50 mM raised the repeat length from 165 to about 180 base pairs. Therefore, we can conclude that salt alone cannot replace the function of HMGs 14 and 17 and space nucleosomes in our system. Similarly, the increase in Na+ concentration does not increase the repeat length in the presence of HMGs 14 and 17. To the contrary, at 110 mM salt, spacing by HMGs 14 and 17 appears to be slightly inhibited. With regard to the spacing results obtained using the Drosophila system, it is worth noting that one important parameter that determines the final nucleosome repeat length of chromatin assembled in vitro is nucleosome density (14, 23, 24). In other words, parameters that determine the extent of nucleosome formation on template DNA will determine the final repeat length, i.e., more nucleosomes loaded would produce a short repeat length, fewer nucleosomes would yield a longer repeat length. The high salt or high Mg2+ conditions used to generate the longer repeats in the Drosophila system inhibit the transfer of histones H3 and H4 from the N1/N2-(H3, H4) complex to DNA (25). One explanation for this is that the neutralization of the negative charges of template DNA would weaken the attraction of the basic histones to DNA. Fractionation of the Drosophila assembly extract into components required for nucleosome formation and nucleosome spacing should resolve whether the high salt or Mg2+ conditions influence nucleosome formation and/or nucleosome spacing.

An important question that needs to be addressed is what is the mechanism by which HMGs 14 and 17 increase the repeat length.

2 D. J. Tremethick and L. Hyman, unpublished data.
Effects of HMGs 14 and 17 on Protein Contacts in Linker DNA

One simple explanation is that the binding of HMG 14 or 17 to a nucleosome generates a larger particle, i.e., the HMG protein either directly or indirectly through histone-DNA contacts increases or stabilizes the protein contact within the linker DNA region. This in turn forces nucleosomes apart and prevents them from packing close together. A similar protection of linker DNA could be achieved if the binding of a HMG protein resulted in a conformational change within the nucleosome. As a result of stronger or more extensive protein contacts within the linker, the DNA peripheral to the nucleosome core would become less accessible to the digestion by micrococcal nuclease. Such an explanation can explain the finding by Drew (13) that histone H1 can increase the repeat length from 145 to 170 base pairs; 170 base pairs is about the size of a chromatosome. Other possible scenarios have been put forward by Busin and co-workers (10) based on the mode of action of micrococcal nuclease. For example, HMGs 14 and 17 may prevent nucleosome sliding induced by micrococcal nuclease or reduce the exonucleolytic DNase activity of the enzyme. However, the results of Fig. 1 rule out these possibilities. Moreover, the experiments carried out by Busin and co-workers (10) utilized crude extracts, prepared from X. laevis eggs, that already contained the factors needed to space nucleosomes. Therefore, it is not clear why HMGs 14 and 17 were expected to change the 180-base pair repeat when endogenous spacing factors were already present in the assembly system.

To test the hypothesis that the binding of HMGs 14 and 17 to chromatin increases the quality of protein-DNA interactions in the linker, chromatin assembled in the absence or presence of HMGs 14 and 17 was extensively digested with micrococcal nuclease to produce mostly single nucleosomes. The size of DNA fragments associated with these particles was then analyzed on an 8% polyacrylamide gel. (Fig. 3) to determine whether additional linker is protected from micrococcal nuclease digestion.

Using this type of analysis, thus far, two types of nucleosomes have been described based on the formation of a micrococcal nuclease-resistant particle, the chromatosome that protects about 170 base pairs and the well characterized nucleosome core that protects about 145 base pairs from digestion (26). Fig. 3 shows that a HMG-containing nucleosome can also form a larger particle protecting about 155 base pairs from digestion (compare lane 3 with lane 2). However, this HMG particle does not present a strong kinetic block to further digestion. At 2.5-min of digestion, the major DNA fragment is 155 base pairs in length (lane 3). However by 5 min of digestion, the 155-base pair fragment is still present but is significantly reduced (lane 5). On the other hand, there is a large increase in the accumulation of DNA fragments 145 base pairs in length. This indicates that the HMG and/or histone contacts in the linker DNA, compared to the histone-DNA contacts in the nucleosome core, do not produce a strong barrier to digestion by micrococcal nuclease.

A chromatosome appears to produce a single relatively strong barrier to micrococcal nuclease digestion that leads to the accumulation of DNA fragments of the same length, 166 base pairs. The extra 20 base pairs of linker DNA protects has been attributed to the binding of the globular domain of histone H5 or H1 to the linker (27). We do not believe that the 155-base pair particle is a distinct particle like a chromatosome. During a narrow time window of digestion, a third DNA fragment 165 base pairs in length can sometimes be seen (e.g., lane 10). Most interestingly, even longer fragments that appear to be multiples of 10 base pairs can be seen under very mild digestion conditions (data not shown); we are currently analyzing these structures in more detail. Since more than one fragment accumulates upon digestion and because occasionally a hint of a 155-base pair fragment can be seen when chromatin formed in the absence of HMGs 14 and 17 is digested, our favored hypothesis is that the HMG proteins are stabilizing existing histone-DNA contacts in the linker region, thus forming stronger barriers to the digestion by micrococcal nuclease. Supporting this hypothesis, it was shown recently that an octamer can indeed organize at least 160 base pairs of DNA with histones contacting DNA as far as 90 base pairs from the dyad (28). In addition, Weischt et al. (29) demonstrated that, under appropriate digestion conditions, the histone octamer can protect 168 base pairs of DNA from micrococcal nuclease digestion. This also clearly shows that histones can interact with DNA outside the core particle. Another possibility that cannot be ruled out at this time is that the HMG proteins may also directly interact with linker DNA. The amino terminus contains the highly positive sequence PKRK that potentially could interact with the linker. Whatever the precise mechanism, Fig. 3 clearly supports the proposal that HMGs 14 and 17 space nucleosomes by increasing the quality of protein-DNA contacts in the linker, thereby preventing the close packing of nucleosome cores.

Originally, based on a number of observations, we hypothesized that phosphorylation of HMGs 14 and 17 may be important for nucleosome spacing perhaps by increasing the spacing activity of the proteins (12, 13). However, recent studies examining the spacing activity of HMGs 14 and 17 after treatment with a number of different phosphatases produced conflicting results. To clarify the importance of phosphorylation, we tested the ability of recombinant HMGs 14 and 17 to space nucleosomes and found that they could space (data not shown). Therefore, we can conclude that phosphorylation of HMGs 14 and 17 is not absolutely required for nucleosome spacing. However, it is still formally possible that phosphorylation may modify the spacing activity. For example, phosphorylation of the carboxyl domain of HMG 14 by casein kinase II (serine 88) may enhance the spacing activity by strengthening the interaction between this domain and histones (see below). On the other hand, phosphorylation of the basic amino end of HMG 14 (serine 6) may be able to decrease the spacing activity by reducing the positive charge of this region and thus weakening its interaction with DNA. Such a complex interplay between phosphorylation sites could explain our inconsistent results with regard to our phosphatase experiments. In any event, phosphorylation studies...
with recombinant proteins should clarify this issue. It is worth noting that the basic amino end of HMG 14 is rapidly phosphorylated during mitogenic stimulation of mouse fibroblast cells, indicating that modification of HMG 14-chromatin interactions may be necessary for gene activation (30).

It has been postulated that HMG 14 and HMG 17 have a modular structure (20). This was based on the finding that a 30-amino acid long peptide, corresponding to the putative nucleosome binding domain of HMG 17, could interact with a nucleosome core in a way similar to the intact protein. This result suggested that the amino terminus and the carboxyl domain may have a role that is distinct from the binding to a core particle. It was of interest, therefore, to determine whether the nucleosome binding domain can space nucleosomes. Fig. 4A clearly demonstrates that the nucleosome binding domain of HMG 14 cannot space nucleosomes (compare lanes 3-5 with lane 2). Arrows show the positions of trimer and tetramer bands in lanes digested to a similar extent. Lane 5 received 400 molecules of peptide per 160 base pairs of DNA. Lower amounts also had no influence on spacing, whereas higher amounts inhibited nucleosome assembly (Fig. 4B, compare lane 4 with lane 1). Identical results were obtained with the nucleosome binding domain of HMG 17 (data not shown). We can therefore conclude from these experiments that the amino-terminal and/or the carboxyl domain are important for nucleosome spacing.

To determine whether the amino-terminal end plus the nucleosome binding domain can prevent the close packing of nucleosomes, a peptide containing amino acids 1-41 was synthesized and tested for nucleosome spacing (Fig. 4B). Like the nucleosome binding domain alone, this peptide could not increase the nucleosomal repeat length to 160-165 base pairs (compare lanes 5 to 7 with lane 2). This result clearly demonstrates that the carboxyl domain of HMG 14 is crucial for nucleosome spacing. However, it is worth pointing out that this experiment does not rule out the possibility that the amino end also plays a role in nucleosome spacing.

Given that the nucleosome binding domain of HMG 14 (and HMG 17) cannot space nucleosomes, it is important to demonstrate that this peptide can indeed bind to a nucleosome. Mobility gel-shift assays were therefore employed to investigate this question. In these experiments, a single nucleosome was reconstituted onto a 160-base pair DNA fragment (see “Experimental Procedures”). Fig. 5A shows that the nucleosome binding domain of HMG 14 does interact specifically with the nucleosome because two discrete complexes with slower mobilities are observed when this peptide is included in the binding reaction (compare lanes 5-8 with lane 1). Based on similar results obtained by Crippa et al. (20), we interpret this result to indicate that two peptide molecules can bind to a single nucleosome. However, under the conditions employed here, we note that the selective binding of the peptide to the nucleosome is reduced since some binding to the free DNA probe occurs concurrently with the binding of the peptide to the nucleosome (compare lanes 5-8 with lanes 1-3). Similar results were obtained using the nucleosome binding domain of HMG 14 plus the amino-terminal region (data not shown). Taken together, these results suggest that the carboxyl domain of HMG 14 (and HMG 17) may make important contacts with the nucleosome and contribute to the overall affinity of the protein for the nucleosome (see below).

Concerning these gel shift experiments, Fig. 5B shows a novel result concerning the interaction of HMG 14 and HMG 17 with a nucleosome. Previous gel-shift experiments have mostly employed nucleosome-core particles as acceptor molecules, i.e. an octamer associated with 145 base pairs of DNA (20, 21). In these experiments, HMG 14 and HMG 17 interacted with a core particle in a similar way thereby producing a similar retardation of the core particle. Strikingly, if an octamer that had been assembled onto a 180-base pair fragment is used as an acceptor, the binding of HMG 14 or HMG 17 produces
specific complexes that migrate differently (compare lane 3 with lane 2, and lane 6 with lane 5). This demonstrates that under the conditions employed here, HMG 14 and HMG 17 interact differently with a nucleosome, producing different conformational states of the nucleosome. Although HMG 14 and HMG 17 may interact with a nucleosome differently, this does not translate into any detectable differences in the spacing of nucleosomes (see Fig. 1). Whether this difference in the interaction of HMG 14 and HMG 17 with a nucleosome has any functional significance is currently being investigated.

Although the nucleosome binding domain of HMGs 14 and 17 is the region that interacts most strongly with the nucleosome core, at physiological ionic strength, the entire HMG 17 molecule interacts with the nucleosome core including the acidic carboxyl domain (31). Since the carboxyl domain is not required for binding to DNA (Ref. 31 and references therein), it is likely that this negatively charged domain may make important contacts with histones. With regard to this latter study, and most other structural studies, it is important to note that a single nucleosome-core particle has mostly been used as an acceptor, and, therefore, other important HMG-chromatin interactions may exist. When either native or H1/H5-stripped erythrocyte chromatin was used in reconstitution experiments, thermal denaturation studies showed that the binding of HMG 17 occurs both to the linker DNA and to DNA contiguous to the core region (32).

Recently, we demonstrated that HMGs 14 and 17 cannot only space complete nucleosomes but they can also space a chromatin template deficient in histones H2A and H2B; HMGs 14 and 17 were able to increase the apparent nucleosomal repeat length from 125 to about 140–150 basepairs (9). In addition to increasing the repeat length, HMGs 14 and 17 were able to dramatically increase the quality of the ladder of DNA fragments produced by micrococcal nuclease digestion, i.e. the 125-basepair ladder was poorly defined, whereas the 140–150-base pair ladder was well defined. Importantly, the binding of HMGs 14 and 17 to H2A/H2B-deficient chromatin stimulated initiation of transcription from TATA-containing and TATA-less promoters. To characterize further these different chromatin templates, and to examine whether the HMG nucleosome binding domain can functionally replace the whole protein, micrococcal nuclease digestion conditions were selected so that the assembled chromatin was digested principally to dinucleosomes or mononucleosomes. The DNA purified from these digested products was analyzed on an 8% polyacrylamide gel. m. molecular weight markers.

Fig. 6A (lanes 1 and 4) clearly shows that the lack of a defined ladder of DNA fragments observed when chromatin depleted in H2A/H2B is digested with micrococcal nuclease (see Fig. 1 in Ref. 9) is due to the formation of two types of particles; both the monomer and dimer fragments are split in two bands. Further digestion of these chromatin templates and the subsequent analysis of the purified digestion products on an 8% polyacrylamide gel indeed confirm this observation (Fig. 6B, lanes 1 and 3). Two bands approximately 145 and 110 base pairs in length are observed that correspond to a nucleosome
core and a nucleosome core deficient in a single H2A/H2B dimer, respectively. Even when very low amounts of histones H2A/H2B are added to assembly reactions, both bands are observed, indicating that there may be some cooperativity with regard to the binding of the second histone H2A/H2B dimer (data not shown). Therefore, the apparent repeat of 125 base pairs is due to the formation of both of these particles. When HMG 14 or HMG 17 is added to the assembly reaction, the 110-base pair band disappears with a corresponding increase in the band that is between 140 and 145 base pairs in length (compare lanes 3 and 6 with lanes 1 and 4 in Fig. 6A, and lanes 2 and 5 with lanes 1, 3, and 4 in Fig. 6B). This result suggests that HMGs 14 and 17 may have a higher affinity for a nucleosome deficient in a single H2A/H2B dimer (see below).

There are at least three possible explanations for this result. First, HMGs 14 and 17 may facilitate the binding of more H2A/H2B to template DNA. We previously ruled out this possibility since the relative amount of histones H2A/H2B versus histones H3/H4 bound to plasmid DNA does not change (9). Second, HMGs 14 and 17 may facilitate the proper incorporation of histones H2A/H2B, already associated with the DNA, into a nucleosome. Finally, HMGs 14 and 17 may be able to functionally replace the histone H2A/H2B dimer to form a larger particle. We favor the last possibility because if HMGs 14 and 17 were facilitating the proper incorporation of histones H2A/H2B, combined with its ability to prevent the close packing of complete nucleosomes, a repeat length of 160 and not 145 base pairs would be expected. Furthermore, it has been reported that HMGs 14 and 17 can bind to a nucleosomal particle deficient in one H2A/H2B dimer (33). In addition, since we have never observed a 155-base pair or longer fragment under these H2A/H2B limiting conditions (this experiment has been repeated many times), we believe that the principal particle formed is an HMG-containing, H2A/H2B-depleted nucleosomal particle. Clearly though, a fine detailed structural analysis of this chromatin template is required to prove this proposal.

Next, we determined whether the nucleosome binding domain of HMG 14 and HMG 17 can space a chromatin template depleted in histones H2A/H2B (Fig. 7). In contrast to the inability of the nucleosome binding domain of HMG 17 to space nucleosomes, the addition of this peptide to assembly reactions can mimic the activity of the whole protein with regard to the generation of 140–145 base pair particles (compare lane 5 and lane 2 with lane 1). However, in terms of the number of moles of peptide or full-length protein added to assembly reactions, 15 times more peptide was required to observe the spacing. Surprisingly, the nucleosome binding domain of HMG 14 could not space an H2A/H2B-depleted chromatin template. Therefore, at least in this particular structural assay, the nucleosome binding domains of HMG 14 and HMG 17 do not behave in an identical manner. In addition, this result also argues against the possibility that the action of the nucleosome binding domain of HMG 17, with regard to generating a 145-base pair ladder, is nonspecific because the nucleosome binding domain of HMG 14 does not function in this assay. Given this result, it was of particular interest to investigate whether the peptide containing the amino end plus the nucleosome binding domain of HMG 14 can space H2A/H2B-deficient chromatin. Under identical assembly conditions, in contrast to the nucleosome binding domain, this peptide indeed could generate chromatin with a 140–145-base pair repeat (Fig. 7B). It can therefore be concluded that the nucleosome binding domain of HMG 14 and HMG 17 are not identical and that the acid carboxyl domain present in both proteins is not required for spacing of an H2A/H2B-deficient chromatin template.

As discussed above, the binding of HMGs 14 and 17 to a chromatin template depleted in histones H2A/H2B stimulates initiation of transcription from different RNA polymerase II promoters (9). We have also observed stimulation of transcription from chromatin templates with normal levels of H2A/H2B, i.e. chromatin with a repeat length of 160–165 base pairs. However, the histone/HMG composition of the active templates (which only represents a small percentage of the total number of templates) under these conditions remains to be determined. In addition, we are currently testing the ability of the above different peptides, and the role of phosphorylation, in activating RNA polymerase II transcription. Recently, using an extract prepared from Drosophila embryos, Kadonaga and co-workers obtained results similar to ours in which it was clearly demonstrated that HMG 17 could increase the repeat length and activate initiation of transcription from a polymerase II promoter (11). Most interestingly, this induction was dependent on the presence of an upstream activator therefore indicat-

![Figure 7](image-url)
Effects of HMGs 14 and 17 on Protein Contacts in Linker DNA

hing that HMGs 14 and 17 may be viewed as a transcriptional co-activator.

An important question that requires answering is what is the molecular mechanism by which HMGs 14 and 17 stimulate transcription from a chromatin template. One possibility is that these proteins function as true transcription co-activators, i.e. they directly interact with the transcription machinery. In this scenario, the nucleosome binding domain would target HMGs 14 and 17 to nucleosomes, and another region of the HMG protein would interact with the transcription apparatus. Alternatively, HMGs 14 and 17 may modify the structure of the nucleosome to facilitate the binding of transcription factors. However, this possibility creates a paradox because it has been reported that these HMG proteins stabilize the structure of a core particle (20, 21). Therefore, HMGs 14 and 17 may not function at the level of a nucleosome but at the level of the chromatin fiber. For example, in the absence of histone H1, HMGs 14 and 17 may activate transcription by preventing the close packing of nucleosomes or nucleosomal particles. This would ensure individual particles are separated by an appropriate distance thus preventing strong core-core interactions. The appropriate spacing of nucleosomes would ensure greater accessibility of linker DNA and consequently greater transcription factor access. Also at the level of the chromatin fiber, Trieschmann et al. (10) have postulated that HMGs 14 and 17 may facilitate the transcription process by inducing an extended conformation in the chromatin fiber. Even though HMGs 14 and 17 may stabilize the structure of a core particle overall, they may weaken histone-DNA interactions at critical sites in the core particle thereby facilitating transcription factor binding. For example, the strongest interaction between histones and DNA occurs at the dyad. A cross-linking study has shown that HMGs 14 and 17 are located near the dyad and interact with histone H3 (34). It is possible that the carboxyl domain of these proteins may be involved in this interaction.

Based on our results (9), we favor the hypothesis that replacement of a histone H2A/H2B dimer in a nucleosome with HMGs 14 and 17 creates and/or maintains a nucleosomal particle in an active conformation. Recently, Walter et al. (35) demonstrated that removal of histones H2A/H2B from a nucleosome by nucleaseplasm enhances transcription factor binding. If such a mechanism operates in vivo, a possible function of HMGs 14 and 17 is to replace a missing H2A/H2B dimer thereby maintaining the nucleosomal particle in a potentially active conformation. The association of HMGs 14 and 17 may also further weaken certain histone-DNA contacts. The overall stabilization of the particle by HMGs 14 and 17 may ensure that the overall integrity of the particle is maintained, thus preventing the complete disruption of the nucleosome which could be detrimental to the accurate regulation of transcription of an RNA polymerase II gene. This proposal can be tested experimentally.

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REFERENCES

1. Grunstein, M. (1990) Trends Genet. 6, 395–400
2. Lu, O., Wallrath, L. L., and Elgin, S. C. R. (1994) J. Cell. Biochem. 55, 83–92
3. Wolffe, A. P. (1990) Trends Genet. 6, 395–400
4. Workman, J. L., and Buchman, A. R. (1993) Trends Biochem. Sci. 18, 90–95
5. Owen-Hughes, T., and Workman, J. L. (1994) Crit. Rev. Eukaryotic Gene Expression 4, 403–441
6. Elinck, L., and Bustin, M. (1985) Exp. Cell Res. 156, 295–310
7. Bustin, M., Lehn, D. A., and Landsman, D. (1987) Biochim. Biophys. Acta 1049, 231–243
8. Seale, R. L., Annunziato, A. T., and Smith, R. D. (1983) Biochemistry 22, 5008–5015
9. Trieschmann, D. J. (1994) J. Biol. Chem. 269, 28436–28442
10. Trieschmann, L., Alfonso, P. J., Crippa, M. P., Wolfe, A. P., and Bustin, M. (1993) EMBO J. 12, 1478–1489
11. Paranjape, S. M., Krumm, A., and Kadonaga, J. T. (1995) Gene & Dev. 9, 1978–1991
12. Trieschmann, D. J., and Drew, H. R. (1993) J. Biol. Chem. 268, 11389–11393
13. Drew, H. R. (1993) J. Mol. Biol. 230, 824–836
14. Trieschmann, D., and Frommer, M. (1992) J. Biol. Chem. 267, 15041–15048
15. Foletta, V. C., Sonobe, M. H., Suzuki, T., Endo, T., Iba, H., and Cohen, D. R. (1994) Oncogene 9, 3305–3311
16. Adams, C. C., and Workman, J. L. (1995) Mol. Cell. Biol. 15, 1405–1421
17. Schwarz, P. M., and Hansen, J. C. (1994) J. Biol. Chem. 269, 16284–16289
18. Thomas, J. O., and Furber, V. (1976) FEBS Lett. 66, 274–280
19. Godde, J. S., and Widom, J. (1992) J. Mol. Biol. 230, 1029–1025
20. Crippa, M. P., Alfonso, P. J., and Bustin, M. (1992) J. Biol. Chem. 267, 442–449
21. Alfonso, P. J., Crippa, M. P., Hayes, J. J., and Bustin, M. (1994) J. Mol. Biol. 236, 189–198
22. Blank, T. A., and Becker, P. B. (1995) J. Biol. Chem. 270, 305–313
23. Stein, A., and Mitchell, M. (1988) J. Mol. Biol. 203, 1029–1043
24. Shimamura, A., Trieschmann, D., and Worell, A. (1988) Mol. Cell. Biol. 8, 4257–4269
25. Zucker, K., and Worcel, A. (1990) J. Biol. Chem. 265, 14487–14496
26. van Holde, K. E. (1989) Chromatin, Springer Publishing Co., New York
27. Hayes, J. J., Pruss, D., and Wolfe, A. P. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7817–7821
28. Pruss, D., and Wolfe, A. P. (1993) Biochemistry 32, 6810–6814
29. Weischet, W. O., Allen, J. R., Riedel, G., and Van Holde, K. E. (1979) Nucleic Acids Res. 6, 1843–1862
30. Barratt, M. J., Hazzalin, C. A., Zhelev, N., and Mahadevan, L. C. (1994) EMBO J. 13, 4524–4535
31. Cooke, G. R., Mindh, M., Schröth, G. P., and Bradbury, E. M. (1989) J. Biol. Chem. 264, 1799–1803
32. Sasi, R., Huvos, P. E., and Fasman, G. D. (1982) J. Biol. Chem. 257, 11448–11454
33. Gonzalez, P. J., and Palacian, E. (1990) J. Biol. Chem. 265, 8225–8229
34. Brawley, J. V., and Martinson, H. G. (1992) Biochemistry 31, 364–370
35. Walter, P. L., Owen-Hughes, T. A., Cote, J., and Workman, J. L. (1995) Mol. Cell. Biol. 15, 6178–6187