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HMG proteins (1 + 2) form beaded structures when complexed with closed circular DNA

D.J.Mathis, A.Kindelis and C.Spadafora

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France

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ABSTRACT

Structures bearing a resemblance to nucleosomes can be assembled by incubating calf thymus High Mobility Group proteins (1+2) with closed circular DNA. These HMG proteins are capable of forming beads and inducing superhelicity when bound to DNA. However, they do not protect from nuclease digestion the discrete DNA fragments characteristic of nucleosomes. The relationship between HMGs (1+2) and the "primitive" histone-like DNA-packaging proteins from prokaryotes and mitochondria is discussed.

INTRODUCTION

Although it is clear that most eukaryotic DNA is folded around histone cores to form nucleosomes (for reviews, see 1, 2), it is not known to what extent alternative modes of chromatin packaging exist in eukaryotic nuclei. It is conceivable that DNA could be folded by other basic proteins, either alone, or in combination with some of the histones. The resultant structures could bear a superficial resemblance to nucleosomes and thus be difficult to distinguish *in situ* by currently popular methods, e.g. electron microscopic or nuclease digestion analysis.

Likely candidates for any such non-histone DNA packaging proteins include the High Mobility Group proteins, or HMGs, originally defined by Johns (for review, see 3). These proteins can be extracted from chromatin with either 5% perchloric acid (PCA) or 0.35 M NaCl and are characterized by a relatively low molecular weight (<30,000 daltons) and an unusual amino acid composition (approximately 25% basic and 30% acidic amino acids). The calf thymus HMG proteins, the best characterized group to date, consist primarily of four components : HMGs 1, 2, 14 and 17. All four components can bind to DNA, although the modes of

binding differ, as do the effects of binding on DNA structure (4-9). Since the HMGs exhibit little tissue or species specificity and since they are relatively abundant chromatin proteins (10^5-10^6 molecules of each HMG per cell nucleus), John's group has suggested that the HMGs play predominantly a structural role in chromatin organization (see for example, 3). A functional role in either transcription (10,11) or replication (12) has also been hypothesized.

Here we present evidence that calf thymys HMG proteins (1+2) interact with closed circular DNA to form structures superficially resembling nucleosomes. We describe the electron microscopic appearance of these HMG:DNA complexes, measure the topological constraint imposed upon their DNA, and probe their structure by nuclease digestion.

MATERIALS AND METHODS

Isolation of HMGs (1+2)

The following protocol for purifying HMGs (1+2) incorporates procedures previously published by Goodwin et al. (3, 13) and Laskey et al. (14). Unless otherwise stated, all manipulations were carried out at 4°C. Twenty-five grams of frozen calf thymus was homogenized in 30 ml of 5% PCA for 2-3 min at high speed in a Waring blender. The homogenate was centrifuged at 16,000 x g for 10 min. After addition of 30 ml of 5% PCA, the pellet was blended and the homogenate centrifuged as above. The pooled supernatants were filtered through two layers of Whatman 3 MM paper, using a Millipore apparatus. Concentrated HCl was added to the cleared supernatants to make a final concentration of 0 3 M. Histone H1 was eliminated by an overnight precipitation at -20°C with 3.5 volumes of acetone. After the precipitate was pelleted, the HMGs in the supernatant were precipitated overnight at -20°C by the addition of a further 2.5 volumes of acetone. The resulting pellet was washed once with acidified acetone (6 volumes acetone : 1 volume 0.1N HCl) and once with acetone before drying by lyophilization. The partially purified HMGs from 25 g of calf thymus were suspended in 20 ml of 60 mM KCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.5 ml of 6 µg/ml phenylmethylsulphonylfluoride in ethanol was added. After heating

at 80°C for 10-12 min and subsequent cooling to 4°C, the suspension was centrifuged at 10,000 x g for 2 min. The supernatant was further clarified by an additional centrifugation at 10,000 x g for 2 min and then made 10% in trichloroacetic acid. HMGs (1+2) were precipitated during 1 hour at 4°C and pelleted by centrifugation at 1,000 x g for 10 min. The HMGs were washed and dried as above, suspended in 5% PCA - 0.3 M HCl, and subjected to an additional 2.5 volumes of acetone as above. This last step removes any remaining traces of H1. The final pellet was washed and dried as above, suspended at 1 to 5 mg/ml in 200 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA and stored in small aliquots at -20°C. The extinction coefficients for HMGs 1 and 2 were those stated by Goodwin et al. (3). The intactness and purity of the HMG protein preparations were monitored by electrophoresis on low pH urea-acrylamide gels as described in (15).

Preparation of DNAs

SV40 DNA Form I was isolated from infected CV1 cells as previously described (16). Relaxed covalently closed DNA (Form I_r) was prepared by treating Form I with an untwisting extract from HeLa cells, followed by repurification of the DNA (16). Plasmid pCR1 DNA Form I was isolated from *E coli* by a method already described in detail (17).

In vitro-assembly of HMG:DNA complexes.

In order to assemble nucleoprotein complexes *in vitro*, HMGs and DNA were incubated at 37°C for 1-2 hours in 100 mM NaCl, 10 mM Tris-HCl pH 7.5. The DNA concentration was always 20 µg/ml and the HMG to DNA ratio was varied as stated in the figure legends.

For the compaction ratio experiments, the supercoiled pCR1 DNA was preincubated for 30 min at 37°C with 10 units of purified untwistase per µg DNA before addition of the HMG proteins. For the experiments analyzing HMG-induced superhelicity, 10 units of purified untwistase per µg DNA were added to the HMGs and SV40 DNA Form I_r at the beginning of the incubation. Purified untwistase was provided by Dr. W. Keller, and we have used his definition for units of untwistase activity (18).

Electron microscopic analysis.

In vitro-assembled HMG:DNA complexes were diluted 5 fold into 15 mM glutaraldehyde buffered with 12 mM triethanolamine (pH 7.5) and were fixed for 5 min at room temperature. The complexes were diluted a further 20 fold in 12 mM triethanolamine (pH 7.5) and spread for electron microscopy as already described (19).

For the compaction ratio experiments, the fixed and diluted complexes were added to an equal volume of a solution of naked DNA, fixed and diluted in an identical fashion. The procedure for computing the compaction ratio has been published (19).

Analysis of HMG-induced superhelicity.

HMG:DNA complexes assembled in the presence of purified untwistase were deproteinized and the DNA analyzed on agaraose-acrylamide gels by previously detailed methods (16).

Nuclease digestion analyses.

A solution of *in-vitro* assembled HMG:DNA complexes was made 3 mM Ca^{++} by the slow addition of 100 mM $CaCl_2$. The complexes were digested at 37°C by 8 units of staphylococcal nuclease (Worthington) per µg DNA for the times indicated in the figure legends. The DNA digestion products were purified as described in (20) and analyzed on 2% agarose gels in 40 mM Tris-HCl ph 7.8, 20 mM sodium acetate, 2 mM EDTA.

Before DNaseI digestion, the solution of HMG:DNA complexes was made 1 mM Mg^{++} by the very slow addition of 10 mM $MgCl_2$. One hundred-twenty units of DNaseI (Sigma) per µg DNA was added, and the digestion carried out at 37°C for varying times. The DNA digestion products were purified as above, and were denatured and analyzed on urea-acrylamide gels by the method of Maniatis et al. (21).

Determination of the percentage DNA acid soluble was by a method already detailed (15).

RESULTS

1. HMGs (1+2) form beaded structures when complexed with DNA.

As illustrated in Fig. 1, our preparation of HMGs (1+2) is

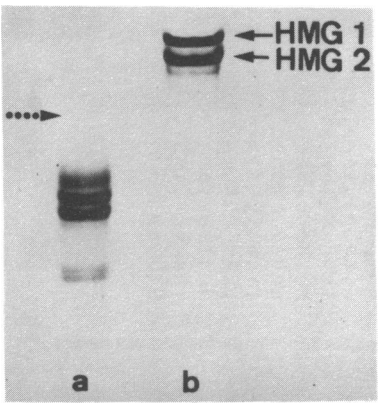

Fig. 1: Electrophoretic analysis of a typical HMG (1+2) preparation. Low pH urea-acrylamide gel electrophoresis of
a) 10 µg of acid extracted core histones from calf thymus and
b) 5 µg HMGs (1+2) from calf thymus.
The dotted arrow marks the position of H1.

not detectably contaminated by other basic proteins; in particular, there is no trace of any of the 5 histones. Also to be noted is that HMG1 and HMG2 are present in approximately equimolar amounts. We have attempted to determine whether these non-histone proteins can package DNA in a nucleosome-like fashion by analyzing *in vitro*-assembled HMG (1+2) : DNA complexes by various techniques commonly employed to characterize nucleosome structures.

When HMGs (1+2) are incubated with SV40 DNA Form I_r and the resultant nucleoprotein complexes fixed with glutaraldehyde, beaded structures can be visualized by electron microscopy (Fig. 2a). These beads bear a superficial resemblance to nucleosomes, but are more irregular in size and shape. At least some of the irregularity derives from the tendancy of beads to stick together to form large aggregates, a phenomenon more apparent at higher HMG to DNA ratios (data not shown). The diameter of the HMG (1+2) containing beads ranged from 113 to 233 Å at an HMG to DNA ratio of 4. If only the smaller, clearly individual beads were measured, we obtained values of 137.4 ± 12.2 Å and 144.5 ± 13.9 Å at HMG to DNA ratios of 2 and 4, respectively, under fixation and spreading conditions that gave a value of 135.1 ± 13.3 Å for nucleosomes from rat liver nuclei lysed by treatment with EDTA.

Because of the tendancy for beads to aggregate, we have chosen an HMG to DNA ratio of 4 for the majority of the subsequent analyses. Nevertheless, the average number of HMG-containing beads per DNA molecule increases at least until an HMG to DNA

Fig. 2: Electron microscopic visualisation of HMG (1+2): DNA complexes.
a) HMGs (1+2) were incubated (Materials and Methods) with SV40 DNA Form I for 2 hours at a protein to DNA ratio of 4:1
b) HMGs (1+2) were incubated with pCR1 DNA (Form I preincubated 30 min at 37°C with 10 units of untwistase/µg DNA) for 90 minutes at a protein to DNA ratio of 3:1. The HMG:DNA complex was fixed with glutaraldehyde (see Materials and Methods) and an equal amount of identically fixed naked pCR1 DNA was added just before spreading. The bar indicates 0.3 µm.

ratio of 8 : 4.4 ± 2.1, 7.7 ± 2.5, 16.1 ± 3.4, 19.3 ± 2.3, 22.8 ± 3.1 at ratios of 1, 2, 4, 6, 8, respectively.

Beads could also be observed after incubating HMGs (1+2) with supercoiled SV40 DNA (data not shown). Bead formation was slightly more efficient on supercoiled DNA : an average of 19.1 ± 2.1 beads per supercoiled DNA molecule versus 16.1 ± 3.4 per relaxed molecule at an HMG to DNA ratio of 4. We did not succeed in visualizing HMG-containing beads on linear adenovirus-2 DNA because of problems with aggregation.

It should be emphasized that fixation of the HMG:DNA complexes was required in order to see beads in the electron microscope.This is not the case for nucleosomes, nor for core histone:DNA complexes assembled *in vitro* by the salt reassociation method (see 19, for example). A further illustration of the instability of HMG-containing beads was their extreme sensitivity to divalent cations. At Mg^{++} or Ca^{++} concentrations as low as 10 mM, loss of some beads occurred after incubating the HMG:DNA complexes for short periods (15 min) at room temperature. Again, such an instability is not characteristic of either nucleosomes or core histone : DNA complexes assembled via the salt method.

Fig. 2b depicts an HMG:pCR1 DNA complex adjacent to a naked pCRI DNA molecule, both fixed with glutaraldehyde. It is evident that the former exhibits a reduced contour length. We have attempted to quantitate the degree of DNA compaction induced by HMGs (1+2) employing the same method used previously to calculate the DNA compaction within *in vitro*-assembled core histone: DNA complexes (19). However, we observed a spurious value for the compaction ratio of control glutaraldehyde-fixed core histone : DNA complexes-a value 1.4 times that of the identical material unfixed. Thus we are unable to accurately quantitate the degree of DNA compaction induced by HMGs (1+2), although we can say that the amount of compaction associated with each HMG-containing bead is about 1/3 that associated with core histone-containing beads when both types of complexes are fixed with glutaraldehyde. A further problem was that the plot of HMG:DNA complex contour length versus bead number exhibited a large amount of scatter. This pronounced scatter was observed in several experiments with

several preparations of HMGs, but not with control, glutaraldehyde fixed core-histone DNA complexes. The scatter could possibly be due to a difficulty in scoring beads since they do have a tendancy to cluster; alternatively individual HMG-containing beads could really compact varying amounts of DNA.

2. The association of HMGs (1+2) with closed circular DNA induces the formation of supercoils.

It is possible to determine whether the binding of a protein induces superhelicity in a closed circular DNA molecule by treating the nucleoprotein complex with untwistase, deproteinizing the DNA and electrophoresing it under conditions which separate DNA molecules by degree of superhelicity (16). Such an analysis (Fig. 3) indicates that the binding of HMGs (1+2) to SV40 DNA Form I_r induces superhelicity. Slots c-g illustrate a progressive increase and then a slight decrease in the degree of superhelicity detected after incubations at HMG to DNA ratios ranging from 1 to 8. At the optimum ratio, 4, the distribution

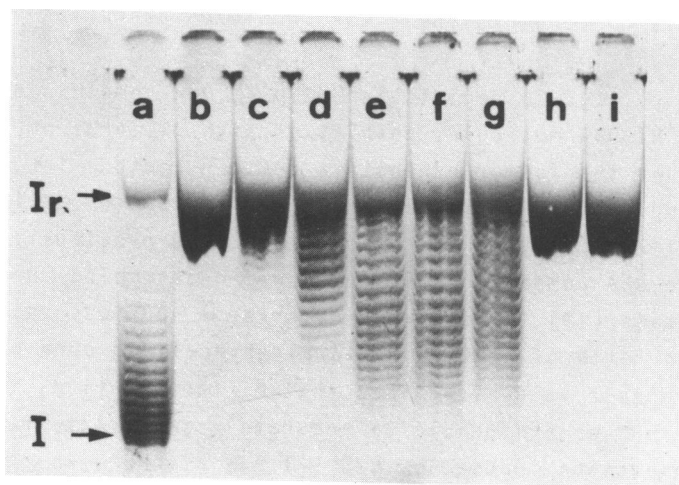

Fig. 3 : Supercoiling of closed circular DNA within HMG (1+2):DNA complexes. Prior to electrophoretic analysis (Materials and Methods) HMGs (1+2) were incubated with SV40 DNA Form I_r for 2 hours at 37° in the presence of 10 units untwistase/µg DNA (b-g) or in the absence of untwistase (h-i). The HMG to DNA ratio was 0 (b), 1 (c), 2 (d), 4 (e,h), 6 (f,i), or 8 (g). Slot (a) shows a sample of SV40 DNA Form I.

of supercoils centers at 9-11. It should be recalled that at this ratio, about 16 beads per DNA molecule could be visualized by electron microscopy, indicating that each bead induces 0.5 to 0.7 turns. At higher HMG to DNA ratios, fewer supercoils are observed, probably due to precipitation of the complexes and subsequent inaccessability to untwistase. This interpretation is supported by electron microscopic observations (data not shown). Slot b indicates that the detection of supercoils is dependent on the presence of HMGs (1+2), while slots h and i demonstrate that untwistase is required, thus showing that the HMG preparation, itself, is devoid of untwistase activity under these assay conditions.

3. HMGs (1+2) and histones do not protect the same discrete DNA fragments from nuclease digestion.

When complexed with DNA, HMGs (1+2) are capable of bead formation and supercoil induction; these properties are also characteristic of the core histones as they interact with DNA to form nucleosomes. Thus, it was of interest to determine whether, like histones, HMGs (1+2) can protect discrete DNA fragments from nuclease digestion. Since the HMG (1+2)-containing beads were sensitive to divalent cations (see Results 1), care was taken to add $CaCl_2$ or $MgCl_2$ to the complexes very slowly and from solutions that were not highly concentrated. Also, the presence of beads after divalent cation addition was always confirmed by electron microscopy before starting the digestion.

Figure 4 indicates that the digestion of HMG (1+2):DNA complexes by micrococcal nuclease does not result in an oligomeric series of DNA digestion products, as would be expected from nucleosomal complexes. Under identical conditions, an oligomeric series was detected after the digestion of histone:DNA complexes assembled *in vitro* by the salt reassociation method (22). With the HMG:DNA complexes there is an accumulation of material at approximately 105 base pairs at advanced stages of digestion; at even later stages, all of the DNA on the gel appears in this band, the size of which is gradually reduced to about 70 base pairs (85% DNA digestion-data not shown). The same digestion pattern has been obtained with several HMG (1+2) preparations, at ratios of

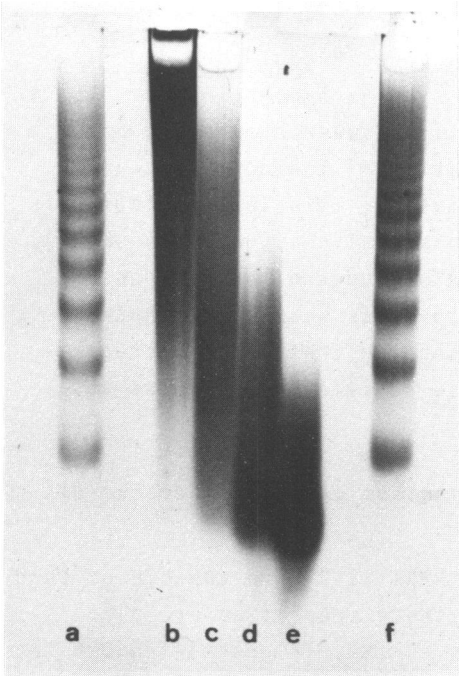

Fig. 4 : Electrophoretic pattern of the DNA fragments produced by micrococcal nuclease digestion of HMG (1+2):DNA complexes. The complexes were assembled as in Fig. 2a. After addition of $CaCl_2$ and nuclease (Materials and Methods), the complexes were incubated for 1 (b), 2 (c), 5 (d), or 10 (e) min at 37°C, resulting in percent DNA acid solubilities of 2.1, 4.3, 15.2, and 50.8%, respectively. The DNA digestion products were purified and analyzed on 2% agarose gels. Slots a and f show micrococcal nuclease digests of rat liver nuclei used as markers for band size determination. The band sizes from monomer to hexamer are : 177, 365, 569, 781, 970 and 1190 base pairs.

HMGs:DNA as high as 6, and with HMG:DNA complexes fixed as for electron microscopy and then dialyzed 4 hours against 100 mM NaCl - 10 mM Tris (pH 7.5).

Similarly, the digestion of HMG (1+2):DNA complexes by DNaseI does not result in a discrete pattern of single-stranded DNA digestion products (Fig. 5). No evidence of a ten-base ladder was observed with several HMG preparations, and at HMG to DNA ratios ranging from 2-6.

DISCUSSION

Similarities and differences between nucleosomes and the beaded structures of HMG (1+2) : DNA complexes.

This study has attempted to determine whether HMGs (1+2) can package DNA in a nucleosome-like fashion. We have demonstrated that *in vitro*-assembled HMG (1+2): DNA complexes resemble core histone:DNA complexes in two important characteristics : when bound to DNA, these HMGs are capable of forming beads and of

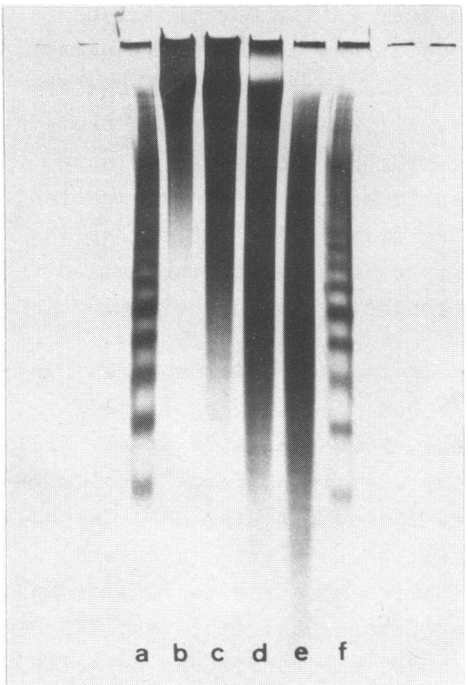

Fig. 5 : Electrophoretic pattern of the single-stranded DNA fragments produced by digestion of HMG (1+2): DNA complexes with DNaseI. The complexes were assembled as in Fig. 2a. After addition of $MgCl_2$ and DNaseI (Materials and Methods), the complexes were incubated for 0.5 (b), 1 (c), 3.5 (d), and 7 (e) min at 37°C resulting in percent DNA acid-solubilities of 5.5, 15.1, 63.5 and 82.1, respectively. The DNA digestion products were purified, denatured and analyzed on urea-acrylamide gels. Slots a and f show DNaseI digests of rat liver nuclei; the typical 10-base ladder is evident.

inducing superhelicity. (This latter property has also been detected by Javaharian et al. (8) using a different method). However, the HMG-containing beads differ from core histone-containing beads in several respects. The former are more irregular in size and shape, and compact less DNA. In addition, while the core histones can induce about one superhelical turn per bead (16), the HMGs induce only 0.5-0.7 turns per bead (at a ratio of 4:1 HMGs to DNA). Finally, HMGs (1+2) and the core histones do not protect the same discrete DNA fragments from nuclease digestion.

An important implication of these findings is that structural characteristics shared by HMG:DNA and nucleosomal complexes (e.g. the presence of beads or the detection of superhelicity) can no longer be used to define nucleosomes. More rigorous criteria, particularly the nuclease digestion patterns, need to be satisfied.

It is relevant to consider at this point that our *in vitro*-assembled HMG (1+2): DNA complexes might not be identical to any

in vitro HMG:DNA complexes, should they exist. Our isolation procedure, which involves the use of strong acids, might damage HMGs (1+2). We have found such extensive purification necessary to avoid problems of precipitation. Alternatively, other proteins might be required to stabilize the HMG:DNA complexes. We have excluded HMGs 14 and 17 from our analyses, because, as expected, they promote DNA precipitation at relatively low HMG:DNA ratios (5-7). The effect of including histones during the incubation of HMGs (1+2) and DNA will be the subject of a future communication.

HMG (1+2) : DNA complexes are similar to HU : DNA complexes; are HMGs (1+2) "primitive" histone-like DNA packaging proteins ?

While this work was in progress, Rouvière-Yaniv et al. (23) reported that the *E.coli* DNA-binding protein HU forms "nucleosome-like" structures when complexed with closed circular DNA. The HU:DNA complexes resemble the HMG (1+2):DNA complexes in several ways : they both contain beads which are more varied in size and shape than those observed for core histone:DNA complexes; HMG:DNA and HU:DNA complexes are unstable under the conditions normally used to visualize nucleosomes by electron microscopy; and both HMGs (1+2) and HU induce supercoils in SV40 Form I_r, but never as many per DNA molecule as the core histones are capable of. In addition, Rouvière-Yaniv et al. were not able to detect discrete protected DNA fragments after nuclease digestion of the HU:DNA complexes.

HMG:DNA and HU:DNA complexes do, however, exhibit some differences in bead specifications. HU containing beads have a diameter of 180 ± 23 Å, larger than that measured for HMG-containing beads at any HMG:DNA ratio studied. Each HMG-containing bead induced only about 0.5-0.7 supercoils, while there was approximately a one to one ratio between beads and supercoils for the HU:DNA complexes. HU:DNA and core histone:DNA complexes appeared to have a similar compaction ratio, defined as the contour length of naked DNA divided by the contour length of the DNA within highly beaded complexes. However, since neither the naked DNA nor the histone:DNA complexes were fixed with glutaraldehyde, as were the HU:DNA complexes, we must question the significance of this result in light of our observation that glutaraldehyde

fixation may cause spurious results.

Rouvière-Yaniv et al. have hypothesized the existence of "primitive" histone-like proteins which package DNA in a manner analogous to that by histones in nucleosomes. The *E.coli* HU protein and a similar protein from *Thermoplasma acidophilum* are included in this group, and a similarity in amino acid composition between these proteins and the HMGs has been noted (24). Yeast mitochondria seem also to contain a histone-like packaging protein, HM, which induces supercoils when complexed with SV40 DNA but does not protect discrete DNA fragments from nuclease digestion (25). HM also has an amino acid composition similar to that of HMGs (1+2). The similarities in amino acid composition and DNA binding properties between HMGs (1+2), HU, and HM lead one to question whether HMGs 1 and 2 should be included in the group of "primitive" histone-like DNA packaging proteins.

In conclusion, HMGs (1+2) can complex with DNA to form structures which superficially resemble nucleosomes. It remains to find evidence of such structures *in vivo*, but the existence of compositionally similar "primitive" histone-like DNA-packaging proteins in prokaryotes and yeast mitochondria may provide some precedent for their existence.

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