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Calcium binding to HMG1 protein induces DNA looping by the HMG-box domains

Michal Štros*, Josef Reich, Anna Kolíbalová

Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic

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Abstract

Electron microscopy has shown that non-histone chromosomal HMG1 could induce DNA looping or compaction in the presence (but not in the absence) of Ca^{2+}. The effect of calcium on DNA looping and compaction was interpreted as calcium binding to the acidic C-domain of HMG1. Both individual DNA-binding HMG1-box domains A and B were found to be involved in DNA looping and compaction. Treatment of HMG1 with a thiol-specific reagent, *N*-ethylmaleimide, inhibited the ability of the protein to induce DNA looping and compaction but not the electrostatic interaction with DNA. These results indicated that cysteine-sulfhydryl groups of the HMG1-box domains A and B are specifically involved in DNA looping and compaction, and that in the absence of calcium the acidic C-domain down-regulates these effects by modulation of the DNA-binding properties of the HMG1-box domains.

Key words: DNA looping; DNA compaction; HMG1; HMG box; Calcium; Electron microscopy

1. Introduction

High mobility group (HMG) proteins 1 and 2 are closely related non-histone chromosomal proteins, the cellular function of which remains unknown [1]. In vitro experiments have implicated these proteins in cellular differentiation, chromosomal replication, nucleosomal assembly, and transcription [1]. No strong sequence specificity has been detected with HMG1 or HMG2 for linear double-stranded DNA except for a higher affinity for (A + T)-rich sequences [2]. The high abundance of HMG1 or HMG2 suggests that their functioning in the eukaryotic cell nucleus is very likely structural. This is evident from recent in vitro findings indicating the ability of HMG1 to bind preferentially to DNA four-way junctions [3,4], to induce DNA bending or recognize bent and unwound (cisplatin-modified) DNA [5–7], and to mediate DNA looping and compaction [8]. HMG1 and HMG2 may also function in vivo as dynamic regulators of DNA topology [8–11].

HMG1 and HMG2 have a tripartite domain structure [12]. The basic N-terminal (A) and central (B) domains interact in vitro with DNA [13]. The C-terminal (C) domains of HMG1 and -2 are polyanionic, containing, in the case of rat HMG1, an unbroken run of 30 glutamic and aspartic residues [14], and interact in vitro with histones [15]. The binding of calcium to the acidic C-domain of HGM1 increases the affinity of the protein for naked DNA [16], suggesting that the C-domain of HMG1 modulates the DNA-binding properties of the HMG1 domains A and/or B. DNA-binding regions with a homology to HMG1 and 2 domains A and B (the 'HMG-box' [17]) have been discovered in a number of sequence-specific transcription factors and other DNA-binding proteins [18]. The HMG-box represents a novel DNA-binding motif [19].

Recently we have compared by means of electron microscopy DNA-binding of HMG1 with the product of tryptic removal of its acidic C-domain, termed HMG3. We have found that HMG3, but not native HMG1, induces DNA looping or compaction in a 140 mM NaCl buffer [8]. Here we report that calcium, presumably by binding to the acidic C-domain of HMG1 [16], induces DNA looping or compaction which is manifested by HMG1-box domains A and B and involves their cysteine-sulfhydryl groups.

2. Material and methods

2.1. Isolation of HMG1 and its domains

Calf thymus HMG1 was isolated under non-denaturing conditions as described previously [15]. HMG3 was prepared by limited digestion of calf thymus HMG1 with trypsin (EC 3.4.21.4) [15], and further purified on FPLC MonoQ and MonoS columns as detailed below. Recombinant rat liver HMG1, HMG3, HMG1-box A and HMG1-box B were synthesized in *E. coli* strain BL21 (DE3) under the control of the T7 promoter using plasmids pRNHMG1, pNHMG1/M1-V176,

*Corresponding author. Fax: (42) (5) 4121 1293.

Abbreviations: FPLC, fast protein liquid chromatography; EM, electron microscopy; HMG, high mobility group; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

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pT7-HMG1bA and pT7-HMG1bB, respectively [4]. The plasmids were kindly provided by Prof. M.E. Bianchi. Isolation of HMG1 and its domains was carried out by ammonium sulfate precipitation as described [4]. The supernatants after precipitation with ammonium sulfate were then extensively dialysed against buffer A (20 mM HEPES-HCl, pH 7.9, 0.2 mM DTT) containing 50 mM NaCl. The filtrated dialysate was loaded onto an FPLC MonoQ column (Pharmacia) equilibrated with the same buffer. Unbound fractions were collected and loaded onto an FPLC MonoS column (Pharmacia) equilibrated with the same buffer. HMG1, HMG3 or HMG1-box domains were eluted using a linear gradient of 50–800 mM NaCl in buffer A. Proteins were dialysed against buffer B (40 mM NaCl, 10 mM Tris-HCl, pH 7.8, 0.2 mM EDTA) and kept frozen in small aliquots at −25°C. Purity of the isolated HMG proteins was assessed on SDS/18% polyacrylamide gels prepared as described [20]. Gels were stained with 0.2% Coomassie blue R-250 [20]. The concentration of HMG proteins was determined by spectrophotometry [15] or by the Coomassie blue G-250 assay [21].

2.2. *Modification of HMG proteins with* N-*ethylmaleimide*

HMG proteins were incubated in buffer B with *N*-ethylmaleimide (NEM; Sigma), typically 5 mM, at 37°C for 20 min and then extensively dialysed against buffer B. Modification of cysteine-sulfhydryl groups of rat liver HMG1, HMG3 and individual HMG1-box domains with NEM was verified by the inability of DTNB (5,5′-dithio-bis(2-nitrobenzoic acid); Sigma) to form HMG/DTNB adducts at 420 nm [22].

2.3. *Preparation of HMG–DNA complexes*

Plasmid pBR322 DNA was isolated from *E. coli* JRS 856 cells harbouring the plasmid [8]. CsCl-purified plasmid pBR322 DNA was linearized by digestion with *Bam*H1 restriction endonuclease (EC 3.1.23.6) according to the manufacturer's instructions (Sigma). Linear DNA was deproteinized by phenol extraction and dissolved in 10 mM Tris-HCl, pH 7.8, 0.2 mM EDTA. HMG proteins were mixed with linear pBR322 DNA at different HMG/DNA molar ratios either in buffer C (50 mM Tris-HCl, pH 7.8, 10 mM DTT) in the presence or absence of 1 mM $CaCl_2$ (or 10 mM $MgCl_2$) or in 140 mM NaCl buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.8, 0.2 mM EDTA).

2.4. *Electron microscopy*

HMG–DNA complexes were fixed for electron microscopy (EM) in 0.15% (v/v) glutaraldehyde for 15 min at room temperature and prepared for EM by the benzyldimethyl-alkylammonium chloride (BAC) spreading technique using ethidium bromide-activated carbon-coated grids as described previously [8]. The samples were stained with 50 μM uranyl acetate in 70% ethanol, air-dried and rotary shadowed with Pt/Pd alloy. The grids were viewed with a Zeiss EM109 electron microscope operating at 50 kV and routinely photographed at a magnification of 50,000 ×.

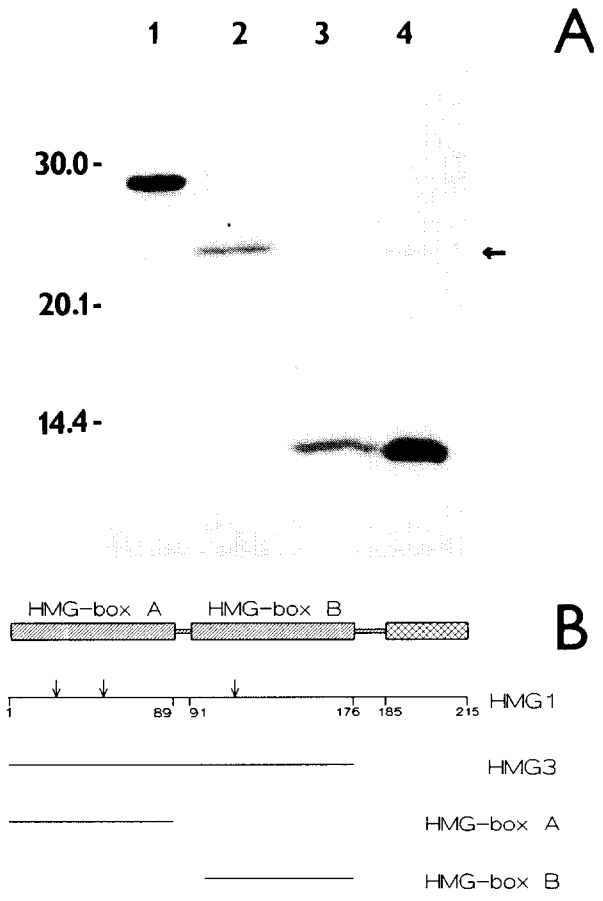

Fig. 1. (A) SDS/18%-polyacrylamide gel electrophoresis. Rat liver HMG1 (lane 1), HMG3 (lane 2), HMG1-box A (lane 3) and HMG1-box B (lane 4). Dimers of the HMG1-boxes A and B are marked with an arrow. The migration of molecular weight marker proteins is indicated on the left (values in kDa). (B) Domain structure of HMG1. Cysteine-sulfhydryl groups at 22 and 44 within the HMG1-box A and at 106 within the HMG1-box domain B are indicated with arrows. The highly acidic C-domain corresponds to residues 185–215.

3. Results

Very recently we have shown [8] by means of electron microscopy (EM) that binding of the product of tryptic removal of the acidic C-terminal domain from calf thymus non-histone chromosomal protein HMG1, which contains two HMG-box DNA-binding domains, A and B, with linear pBR322 DNA (at an HMG3/DNA molar ratio, r, of 140) in 140 mM NaCl buffer (in the absence of divalent cations) results in complexes consisting of one HMG3 bead (presumably an HMG3 oligomer) from which emerges one or several DNA loops of variable size. At a higher HMG3/DNA ratio (r = 420) the beads were larger and sequestered most of the DNA, and instead spherical or somewhat irregular-shaped particles and their aggregates were observed at r = 760. None of the above complexes were visualized by EM with native HMG1 and linear DNA, suggesting that the acidic C-domain of HMG1 down-regulated the ability of basic HMG1-box domains A and/or B to mediate DNA looping and compaction [8].

Experiments in this study were carried out with recombinant rat liver HMG1 (residues 1–215), HMG3 (residues 1–176), HMG1-box A (residues 1–89) and HMG1-box B (residues 91–176), which were purified to homogenity (Fig. 1A). Some of the experiments were performed with complexes of linear pBR322 DNA and calf thymus HMG1 or HMG3 with essentially the same results.

The aim of the present paper was to find a factor relieving the inhibitory effect of the acidic C-domain of HMG1 on the ability of the HMG1-box domains to mediate DNA looping and compaction. We have investigated in this respect the effect of Ca^{2+} since binding of Ca^{2+} to the acidic C-domain of HMG1 in vitro was reported to increase the affinity of the protein for linear

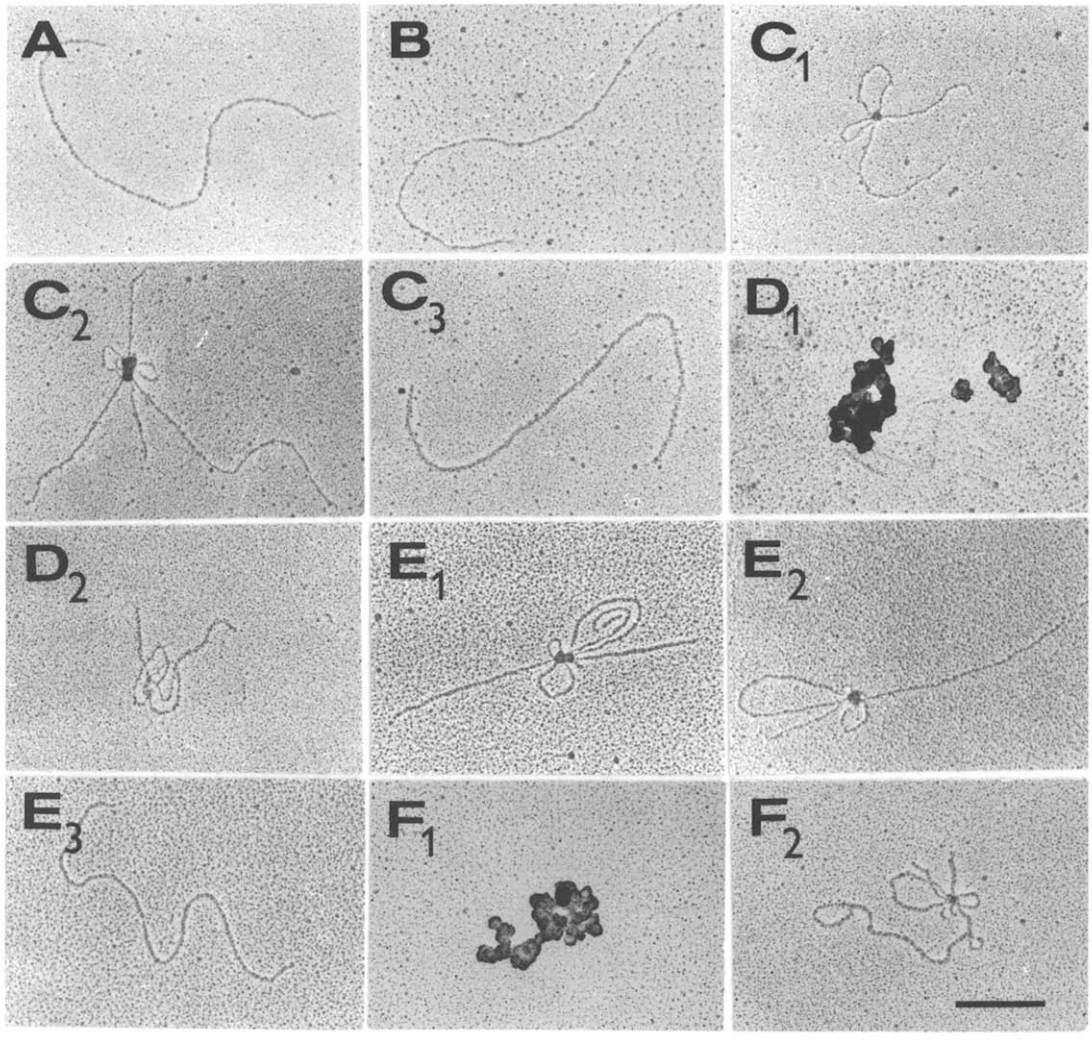

Fig. 2. Electron microscopy of HMG1 (3)–DNA complexes. All experiments were performed with linearized pBR322 DNA. (A) Free linear pBR322 DNA. (B) DNA incubated with HMG1 at r = 720 in buffer C. (C_1–C_3) DNA incubated with HMG1 at r = 240 in buffer C containing 1 mM $CaCl_2$. (D_1,D_2) DNA incubated with HMG1 at r = 720 in buffer C containing 1 mM $CaCl_2$. DNA incubated with HMG3 at r = 240 in buffer C in the presence (E_2,E_3) or absence (E_1) of 1 mM $CaCl_2$. (F_1,F_2) DNA incubated with HMG3 at r = 720 in buffer C containing 1 mM $CaCl_2$. Experiments with HMG1 (C_3,D_2) and HMG3 (E_3,F_2) modified with 5 mM NEM before mixing with DNA. Bar = 250 nm.

DNA as revealed by a nitrocellulose filter binding assay [16]. In agreement with our previous report [8], no DNA looping was observed by EM with HMG1 and linear DNA in 140 mM NaCl buffer (not shown) or buffer C up to the highest HMG1/DNA molar ratio studied (Fig. 2B; at r = 720). However, binding of HMG1 to linear pBR322 DNA at r = 240 in buffer C containing 1 mM $CaCl_2$ (Fig. $2C_1$ and C_2) resulted in DNA looping (ca. 81% of the 100 complexes scored with ca. 15% of compact aggregates and ca. 4% of free unlooped DNA). Most of the complexes contained single DNA molecules with an HMG1 bead (presumably an HMG1 oligomer) at the site of DNA looping (ca. 90% of 150 complexes scored; Fig. $2C_1$), while others contained two or more linear DNA molecules joined at a bead (Fig. $2C_2$). DNA looping observed with HMG1 and linear DNA was not specific to calcium since similar results were obtained in buffer C containing 10 mM $MgCl_2$ (not shown; no looping was observed in buffer C containing 1 mM $MgCl_2$). Looping of linear DNA induced by HMG3 was visualized irrespective of the presence (Fig. $2E_2$; 1 mM $CaCl_2$/buffer C) or absence of calcium (Fig. $2E_1$; buffer C; similar results were obtained in a 140 mM NaCl buffer [8]). In agreement with our previous report with HMG3 and linear forms of pBR322 or M13 DNA [8] the positions of DNA loops on linear pBR322 DNA with HMG1 were also variable.

Mixing of HMG1 with linear DNA at r = 720 in the presence of 1 mM $CaCl_2$ (Fig. $2D_1$) or 10 mM $MgCl_2$ (not shown) resulted in the appearance of compact irregular-shaped particles or their aggregates (> 95% of the complexes scored with very little free DNA). With HMG3 at r = 720, compact irregular-shaped particles or their aggregates (> 99% of the complexes scored) were also visu-

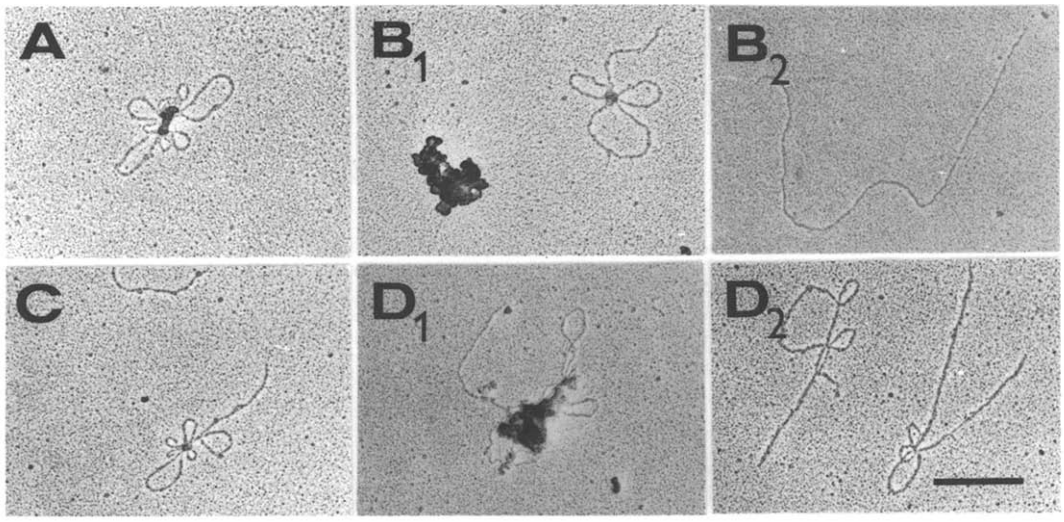

Fig. 3. Electron microscopy of HMG1-box–DNA complexes. All experiments were performed in buffer C/1 mM $CaCl_2$ with linearized pBR322 DNA. (A) DNA incubated with HMG1-box domain A at r = 720. (B_1,B_2) DNA incubated with HMG1-box domain A at r = 1440. (C) DNA incubated with HMG1-box domain B at r = 720. (D_1,D_2) DNA incubated with HMG1-box domain B at r = 1440. Experiments with HMG1-box domain A (B_2) and HMG1-box domain B (D_2) modified with 5 mM NEM before mixing with DNA. Bar = 250 nm.

alized, irrespective of the presence (Fig. $2F_1$; similar results were obtained with Mg^{2+}, not shown) or absence of Ca^{2+} in buffer C (not shown; see also [8] for complexes in 140 mM NaCl buffer). These results suggested that the effect of calcium (and very likely magnesium as well) on DNA looping and compaction was very probably manifested via calcium binding to the acidic C-domain of HMG1 (see section 4).

In order to find out which of the HMG-box domains is involved in DNA looping and compaction, binding of individual HMG-box domains A or B was studied. Electron microscopy revealed that interaction of linear pBR322 DNA with the isolated HMG-box domain A at r = 720 resulted in DNA looping (ca. 40–50% of the complexes scored; Fig. 3A). In addition to DNA structures with one or more loops (ca. 50% of the complexes scored), compact particles and their aggregates were apparent at r = 1,440 (Fig. $3B_1$). Similar structures were visualized with linear DNA and the isolated HMG-box domain B (Fig. 3C), although the percentage of the looped DNA molecules at r = 720 was higher (ca. 81%) and, consequently, more compact particles or their aggregates were visualized at r = 1,440 (Fig. $3D_1$). The above results indicated that both individual HMG-box domains A and B could mediate DNA looping and compaction, although HMG1 and HMG3 seemed stronger in these effects.

In order to study the possible mechanism by which HMG1 could bring about DNA looping and compaction, the involvement of cysteine-sulfhydryl groups of HMG1 in the latter functioning was investigated using a highly specific thiol-modifying reagent, *N*-ethylmaleimide (NEM). Mixing of NEM-modified HMG1 with DNA at r = 240 (1 mM $CaCl_2$/buffer C) resulted in ca. 80% inhibition of DNA looping (Fig. $2C_3$) relative to HMG1–DNA complexes with unmodified HMG1 at the same ratio (Fig. $2C_1$ and C_2). With unmodified HMG1 at r = 720, more than 95% of the complexes scored were visualized as compact particles or their agregates (Fig. $2D_1$). However, the same experiment at r = 720 but with NEM-modified HMG1 indicated an inhibition of DNA compaction by 95% and instead DNA molecules with mainly several loops were apparent (ca. 82% of the complexes scored with about half of the looped DNA molecules without visible beads at the base of the cross-overs; Fig. $2D_2$). Similar results were obtained with HMG3 (compare Fig. $2E_2$ with E_3 and Fig. $2F_1$ with F_2).

Rat liver HMG1 contains three cysteine-sulfhydryl groups, two cysteine residues at 22 and 44 within the HMG1-box domain A and a single cysteine at 106 within the HMG1-box domain B [14] (Fig. 1B). Modification of the HMG1-box domains A (Fig. $3B_2$) or B (Fig. $3D_2$) with NEM at r = 1440 inhibited by ca. 80% the ability of the HMG1 peptides to induce DNA looping (unlike experiments with the NEM-modified HMG1-box domain A, we have observed, in experiments with NEM-modified HMG1-box domain B, a considerable amount of free DNA with one or several loops but without apparent beads at the base of the cross-overs), and also markedly suppressed DNA compaction, suggesting that cysteine-sulfhydryl groups of both DNA-binding HMG1-box domains are involved in the latter effects.

Modification of HMG1 or HMG3 with NEM (0.1–5 mM) had very little, if any, effect on the overall binding of the proteins to linear pBR322 DNA (Ca^{2+}-containing buffer), as suggested from an agarose gel retardation assay (not shown), in agreement with previously published results [11]. These results indicated that free

sulfhydryl groups of HMG1 did not affect the electrostatic interaction of HMG1 with linear DNA but, on the other hand, facilitated specific interactions such as DNA looping and compaction (NEM-modified HMG1 very likely binds to DNA predominantly as a monomer which can not be visualized by EM due to its small size).

4. Discussion

The goal of this study was to find the conditions under which native HMG1 would mediate DNA looping and compaction, and to identify the domain (or amino acid residues) of HMG1 involved in these processes. Using electron microscopy we demonstrated that native HMG1 induced looping or compaction of linear DNA in the presence of Ca^{2+}. Similar results were obtained with Mg^{2+}, suggesting that the latter effect was not specific to calcium. This is also supported by the fact that $^{45}Ca^{2+}$-binding to HMG1 reported earlier [16] was completely abolished above a 10-molar excess of Mg^{2+} over $^{45}Ca^{2+}$ (Štros, unpublished results).

The fact that DNA looping and compaction induced by HMG3 was not affected by the presence of Ca^{2+} (or Mg^{2+}), together with our previous results on calcium binding to HMG1 [16], suggested that the effect of calcium (and very likely magnesium as well) is to be explained as a result of calcium binding to the highly acidic C-domain of HMG1. Thus, HMG1-induced DNA looping and compaction in the presence of calcium (or magnesium) might be a consequence of sequestering or shielding the negative charge of the acidic C-domain by Ca^{2+} (or Mg^{2+}), which would subsequently relieve the inhibitory effect of the acidic C-domain on the interaction of the basic HMG1-box domains with DNA. Alternatively, binding of divalent cations to the acidic C-domain of HMG1 might suppress the electrostatic repulsion between the acidic C-domain and the negatively charged DNA phosphates.

The variable positions of DNA loops seem to argue against the sequence-specific binding of HMG1 or HMG3. This conclusion is in agreement with so far available reports indicating no strong sequence preference of HMG1 and HMG2 for DNA [25–27], but, on the other hand, a preference for different DNA structures such as kinked (cisplatin-modified) DNA [7], bent regions in linear double-stranded DNA, in particular AT-rich regions [2], and DNA four-way junctions [3,4]. In addition to the latter findings recent reports demosntrated the ability of HMG1 (in a low-salt buffer containing 10 mM $MgCl_2$) to mediate curvature (bending) in double-stranded DNA, as revealed by T4 DNA ligase-dependent cyclization of short DNA fragments [5,6]. Thus, DNA looping observed here with HMG1 and previously with HMG3 [8] could be a result of either the recognition of sequence(type)-dependent structural distortions (e.g. AT-rich regions) or HMG1-induced bending accompanied by protein–protein interactions between distant binding sites. This explanation seems to be supported by recent fidings (using a gel electrophoretic assay) that HMG1 could mediate Hin invertasome assembly which requires interaction of the Hin and Fis proteins bound at distant sites with looping of the intervening DNA sequences [5], and is facilitated by HMG1-induced DNA bending.

We have shown that both HMG1-box domains A or B are able to induce DNA looping and compaction. The fact that the individual domains A or B were slightly less effective in DNA looping and compaction (particularly the HMG1-box A) than the AB bi-domain (i.e. HMG3) or native HMG1 suggested that, for efficient DNA looping and compaction, both covalently attached HMG1-box domains A and B are required, and that in the absence of divalent cations the highly acidic C-domain of HMG1 further modulates the DNA-binding properties of the HMG-box domains. In the cell nucleus, in addition to divalent cations, other factors such as basic histones may interact with the acidic C-domain of HMG1 and thus relieve the basic AB bi-domains of HMG1 and 2 for their interaction with DNA.

Using a thiol-specific chemical modifying reagent, *N*-ethylmaleimide, we have demonstrated that Cys residues (at 106 within the HMG-box B and at 22 and/or 44 within the HMG-box A) are involved in the effect of HMG1 on DNA looping and compaction. The importance of sulfhydryl groups of HMG1 for its interaction with DNA has recently been demonstrated by several groups. Modification of sulfhydryl groups of HMG1 with *N*-ethylmaleimide was shown to reduce binding of HMG1 to negatively supercoiled DNA but not to linear DNA by a gel retardation assay [11]. Recognition of kinked and unwound (cisplatin-modified) DNA by HMG1 was also markedly inhibited by oxidation or covalent blockade of sulfhydryl groups of the protein [23]. Thus it seems firmly established that free cysteine-sulfhydryl groups of HMG1 are involved in the recognition of bent DNA [11,23] and induction of DNA looping and compaction (this paper).

The cellular role of HMG1 and HMG2 proteins remains undefined. In vitro experiments performed to date suggest that HMG1 or HMG2 can bind bent DNA [3,4,7] and manipulate DNA structure by bending [5,6], looping (to permit protein–protein interactions between proteins boud at distant DNA sites [5], which can explain the previously published stimulatory effects of HMG1 on DNA replication and transcription [1]), compaction ([8] and this paper), as well as by changes in DNA topology [8–11].

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