A Role of Basic Residues and the Putative Intercalating Phenylalanine of the HMG-1 Box B in DNA Supercoiling and Binding to Four-way DNA Junctions*

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HMG (high mobility group) 1 is a chromosomal protein with two homologous DNA-binding domains, the HMG boxes A and B. HMG-1, like its individual HMG boxes, can recognize structural distortion of DNA, such as four-way DNA junctions (4WJs), that are very likely to have features common to their natural, yet unknown, cellular binding targets. HMG-1 can also bend/loop DNA and introduce negative supercoils in the presence of topoisomerase I in topologically closed DNAs. Results of our gel shift assays demonstrate that mutation of Arg97 within the extended N-terminal strand of the B domain significantly (>50-fold) decreases affinity of the HMG box for 4WJs and alters the mode of binding without changing the structural specificity for 4WJs. Several basic amino acids of the extended N-terminal strand (Lys96/Arg97) and helix I (Arg110/Lys114) of the B domain participate in DNA binding and supercoiling. The putative intercalating hydrophobic Phe103 of helix I is important for DNA supercoiling but dispensable for binding to supercoiled DNA and 4WJs. We conclude that the B domain of HMG-1 can tolerate substitutions of a number of amino acid residues without abolishing the structure-specific recognition of 4WJs, whereas mutations of most of these residues severely impair the topoisomerase I-mediated DNA supercoiling and change the sign of supercoiling from negative to positive.

HMG (high mobility group) proteins 1 and 2 are relatively abundant and highly conserved chromatin-associated proteins that are present in all vertebrate cell nuclei (1, 2). The proteins contain two homologous repeats of a 70–80-amino acid sequence, HMG box domains (A and B), and an acidic C-terminal tail. Sequences with similarities to the HMG-1 box domains are found in a number of sequence-specific transcription factors and other DNA-binding proteins referred to as HMG-1 box domain proteins (3). This superfamily includes both the non-sequence-specific proteins such as mammalian HMG-1, HMG-D from Drosophila, Xenopus transcription factor UBF, and yeast NHP6A, and the sequence-specific proteins such as sex-determining factor SRY and lymphoid enhancer factor LEP-1 (2, 4).

The tertiary structure of the individual HMG-1 box domains was determined by NMR (4–8). The HMG-1 domain has an unusual untwisted L-shape and is composed of three α-helices and an extended N-terminal strand. The tertiary structure of the DNA-binding domains of all of the HMG-1 box proteins investigated so far is remarkably similar and is more evolutionarily conserved than the amino acid sequences of the HMG-1 box domains (3).

Although HMG-1 and -2 proteins (or the individual HMG-1/2 box domains) bind DNA irrespective of sequence, they are able to recognize and bind preferentially to bent or distorted DNA structures such as four-way DNA junctions (4WJs),1 supercoiled DNA, and kinked and underwound (cisplatin-modified) DNA (9–11). HMG-1, like other proteins of the HMG-1 box family, bends and loops DNA (4, 12–18). In addition to the ability of HMG-1/2 to bind non-B-DNA structures, the proteins can induce (in the presence of topoisomerase I) negative supercoils in topologically closed domains of DNA, stabilize DNA loops in complex nucleoprotein structures, and facilitate binding of certain sequence-specific proteins to their target DNA (11, 16, 19–24). The ability of HMG-1 to bend, loop, and modulate topology of DNA makes the HMG-1 box domain a general “DNA bending/looping/wrapping domain” helping other proteins to facilitate formation of complex nucleoprotein structures required for transcription, recombination, and DNA repair (1, 2).

The NMR structures of the HMG box domains of HMG-1 (5, 6), HMG-D (7, 25), NHP6A (26), LEP-1 (27) and SRY complexed to DNA (28), mutational analyses, and a “domain swap” (12, 29–32; reviewed in Ref. 4) indicated that DNA binding/bending occurs through the concave surface of the L-shaped HMG box containing a cluster of conserved hydrophobic amino acids (hydrophobic core). Partial intercalation of highly conserved hydrophobic residues of helix I of the HMG box (Met48 and Phe48 in NHP6A (26); Met45 and Val136 in HMG-D (25); Met in LEP-1 (27); Ile6 in SRY (28); and Phe137 in HMG-1 box A (33)) between adjacent base pairs causes opening of the DNA minor groove and enables extensive contacts of the N-terminal strand and helices I and II of the HMG box domain with kinks adjacent to the sites of intercalation.

In this work, we have introduced mutations into the B domain of HMG-1 to assess the functional importance of a number of highly conserved residues, including the putative intercalating hydrophobic Phe103 of helix I, for the ability of the HMG-1 box domain to introduce supercoils in topologically closed DNA (in the presence of topoisomerase I) and to bind

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**Mutational Analysis of the DNA-binding B Domain of HMG-1**

4WJs. We have shown that the B domain of HMG-1 can tolerate a number of (mainly) nonconservative residue substitutions without losing the ability to bind four-way DNA junctions in a structure-specific manner. On the other hand, mutations of most of these residues can drastically impair the topoisomerase I-mediated DNA supercoiling by the B domain and change the sign of supercoiling from negative to positive. Our data further indicate that hydrophobic Phe103 is required for DNA supercoiling (or protection of supercoiled DNA from relaxation by topoisomerase I), but it is dispensable for binding of the B domain to negatively supercoiled DNA or 4WJs.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes were purchased from Roche Molecular Biochemicals or Promega. Other reagents were of the highest purity either from Sigma or Fluka. [γ-32P]ATP was from Amersham Pharmacia Biotech.

**Plasmids**

The cDNA encoding B domain of HMG-1 (residues 92–180) was amplified by PCR using rat hmg-1 cDNA and specific primers 5′-TCGAGGGATCCATGCCCAATGCCCCAACG-3′ (sense) and 5′-GGAG- GTGCACTCTTTTACCTGGTGG-3′ (antisense) (the initiation and termination codons are underlined). The PCR primers contained restriction sites for BamHI and SalI, as well as the initiation and termination codons (underlined). The PCR protocol was as described previously (34) with the following modification of the PCR cycles: 94 °C for 4 min (denaturation cycle); 94 °C for 40 s, 60 °C for 20 s, 72 °C for 40 s (30 cycles); and 72 °C for 7 min (extension cycle). The amplified HMG box domain was purified on 1% agarose gel, blunted-ended by T4 DNA polymerase and cloned into the Smal site of the plasmid pAlter-1 (Promega). The ligated plasmid was transformed by electroporation and propagated in JM109 cells. Plasmids carrying the HMG box domain were selected on LB plates containing ampicillin and 5-bromo-4-chloro-indolyl-β-D-galactosidase, and the positive (white) clones were sequenced on both strands using the protocol of the Altered Sites II Kit (Promega). Briefly, denaturation was performed at 95 °C for 30 s, followed by incubation of the mixture at 37 °C for 5 min before addition of the termination codons (underlined). The PCR primers contained restriction sites for HI and SalI, as well as the initiation and termination codons (underlined). The PCR protocol was as described previously (34) with the following modification of the PCR cycles: 94 °C for 4 min (denaturation cycle); 94 °C for 40 s, 60 °C for 20 s, 72 °C for 40 s (30 cycles); and 72 °C for 7 min (extension cycle). The amplified HMG box domain was purified on 1% agarose gel, blunted-ended by T4 DNA polymerase and cloned into the Smal site of the plasmid pAlter-1 (Promega). The ligated plasmid was transformed by electroporation and propagated in JM109 cells. Plasmids carrying the HMG box domain were selected on LB plates containing ampicillin and 5-bromo-4-chloro-indolyl-β-D-galactosidase, and the positive (white) clones were sequenced on both strands by the dideoxynucleotide chain termination method using Sequenase kit version 2.0 (Amersham Pharmacia Biotech). The plasmids harboring the rat hmg-1 box B or AB didomain (pARHMG1b or pARHMG1b7) were recloned between the BamHI and SalI sites of the pGEX-4T1 (Amersham Pharmacia Biotech) expression vector.

**Site-directed Mutagenesis of the HMG-1 B Domain**

Site-directed mutagenesis was carried out with pARHMG1b didomain using the protocol of the Altered Sites II Kit (Promega). Briefly, denatured pARHMG1b (containing either the rat hmg-1 box B domain of rat HMG-1 and carrying the tetracycline resistance) was annealed with a tetracycline knockout and an ampicillin repair oligo-primers (from Sigma or Fluka). ([32P]ATP was from Amersham Pharmacia Biotech) fast protein liquid chromatography chromatography on a MonoS column (17). The purity of HMG boxes was routinely checked on SDS/18% polyacrylamide gels (35) that were stained with 0.25% Coomassie Blue R-250. The recombinant HMG box 1 of rUF1 was purified as described previously (36).

**DNA Supercoiling**

**DNA Supercoiling Assay—Negatively supercoiled pBR322 DNA (final concentration, −170 μg/ml)** was relaxed in high salt relaxation buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 μg/ml glycerol, 1 mM diithiothreitol; Promega) with wheat germ topoisomerase I (4 units/μg DNA; Promega) at 37 °C for 90 min. The relaxed DNA was then diluted (either in 1× high salt relaxation buffer or in the same buffer containing only 10 mM NaCl, referred to as low salt relaxation buffer), and a second portion of the enzyme was added. The DNA mixture was then divided into several tubes, each containing 150 or 225 ng of DNA and the HMG peptides (as indicated in the legends to figures) and adjusted to a final volume of 10 μl. The reactions were allowed to proceed for 1 h at 37 °C, after which 2.5 μl of the termination buffer, (5× TBE, 5% SDS, 15% sucrose, 0.1% bromphenol blue, 0.1% xylene cyanol, 1 μg/μl proteinase K) were added, and the samples were subjected to further incubation at 37 °C for 1 h.

**DNA Protection Assay—Negatively supercoiled pBR322 DNA (150 or 220 ng/reaction)** was incubated with increasing amounts of the HMG peptides in high salt relaxation buffer for 20 min on ice. This was followed by incubation of the mixture at 37 °C for 5 min before addition of topoisomerase I (4 units/μg DNA). The reaction was allowed to proceed for 1 h before termination as indicated for the supercoiling assay. DNA topoisomerases were then resolved in the first dimension on 1% agarose gels in 0.5× TBE buffer at −3 V/cm for 17 h. To determine the sign of supercoiling, the gels were soaked in chloroquine (2.5 μg/ml) for 1 h, turned horizontally by 90° and resolved in the second dimension in 0.5× TBE containing chloroquine at −5 V/cm for 10 h. The gels were stained with 10,000-fold diluted Sybr® Green I (Molecular Probes, Eugene), followed by visualization of the DNA topoisomerases under UV illumination (254 nm).

**Circular Dichroic Measurements**

The HMG boxes (60–100 μM) were extensively dialyzed against a low salt CD buffer (10 mM sodium phosphate, pH 7.4, 0.5 mM EDTA, 1 mM diithiothreitol) for measurements of CD spectra. CD spectra of the B
domain and its mutants (with the exception of the K53E mutant, the CD spectra of which could not be measured because of insufficient yields after purification from E. coli) were recorded in low salt CD buffer or high salt CD buffer containing 200 mM NaCl (base-line buffers). In experiments studying the effect of competitor (linear) DNA on the α-helical structure of the Y155S mutant, linearized pBluescript plasmid was added to the HMG box polypeptide in high salt CD buffer (the amount of competitor DNA corresponded to 20-fold mass excess over the 4WJs in Fig. 2). All CD spectra were recorded using Jasco J720 spectropolarimeter with a 1-mm path length cuvette at approximately 20 °C over the range of 195–260 nm (resolution step 0.5 nm), each of the spectra representing the average of seven scans. CD spectrum of protein-free linearized plasmid DNA in high salt CD buffer was regarded as a base-line spectrum. After the base-line subtraction, the CD spectra were calculated using the J700 program provided by the manufacturer and finally subjected to noise reduction. All CD spectra are presented as molar ellipticity values (θ).

**RESULTS**

*Strategy of the Mutational Analysis and Preparation of B Domain and Mutants—*The aim of this paper was to carry out mutational analysis of the central domain of the chromosomal protein HMG-1 (the “HMG box B” or “B domain”) to identify amino acid residues involved in structure-specific binding to 4WJs or DNA unwinding (supercoiling) in the presence of topo-isomerase I.

We have selected amino acid residues of the HMG-1 B domain (Fig. 1) as targets for site-directed mutagenesis based on the published three-dimensional structure of the HMG-1 B domain, evolutionary conservation, as well as NMR structures of HMG-DNA complexes and other data (see below). Briefly, residues Lys128 and Arg129 are present within the highly conserved motif PKRP of the N-terminal segment of both the non-sequence-specific HMG box proteins and the sequence-specific HMG box proteins, and the previously published mutagenesis analysis of different HMG boxes, a domain swap as a strategy for tagging other HMG box domains, and evolutionary conservation, as well as NMR structures of HMG-DNA complexes and other data (see below) indicated that HMG-1 binds strongly and selectively to cruciform structures and synthetic four-way DNA junctions. It now appears that 4WJs are universal targets for all proteins of the HMG-1 box superfamily (2). However, a detailed mutational analysis of the B domain of HMG-1 with respect to binding to 4WJs and DNA supercoiling is not available so far.

Previously published data indicated that one molecule of the isolated A and B domains of HMG-1 binds first to a high affinity site, such as the junction cross-over (complex I), and then a second HMG box molecule binds to a lower affinity site, such as a four-way junction (complex II). Binding with the B Domain of HMG-1 Protein to Four-way DNA Junctions—The original report of Bianchi and co-workers (10) indicated that HMG-1 binds strongly and selectively to cruciform structures and synthetic four-way DNA junctions. It now appears that 4WJs are universal targets for all proteins of the HMG-1 box superfamily (2). However, a detailed mutational analysis of the B domain of HMG-1 with respect to binding to 4WJs and DNA supercoiling is not available so far.

Previously published data indicated that one molecule of the isolated A and B domains of HMG-1 binds first to a high affinity site, such as the junction cross-over (complex I), and then a second HMG box molecule binds to a lower affinity site, represented by the arms of four-way junctions (complex II in Figs. 2 and 3; see also Refs. 5, 6, 10, 32, 40–42). Higher amounts of the HMG-1 domain result in formation of complexes III and higher (often accompanied by intense smearing; Fig. 3), arising likely from binding of three or more HMG box molecules to 4WJs.

The conformation of 4WJs largely depends on metal ions, which favor the “stacked X” conformation over the open, planar conformation with maximally separated arms (43). Here we have assayed binding of the B domain and its mutants to 4WJs mainly in the stacked X conformation (+ Mg2+); see Figs. 2 and 4. However, some of the binding experiments were also carried out with the open conformation of 4WJs (− Mg2+) with similar
Arg97 within the N Terminal of the B Domain Is Involved in Binding to Four-way DNA Junctions—

Gel retardation assays with the B domain with mutated Lys96 of the extended N-terminal strand mutated to glutamic acid (K96E) or alanine (K96A) revealed significantly reduced (>10- and 5-fold for the K96E and K96A mutants, respectively) intensities of complexes I and II relative to the wild type B domain (Figs. 2 and 3). Specificity of the K96E mutant to 4WJs was challenged in the presence of 1000-fold mass excess of unlabeled competitor DNA. Although binding of K96E mutant to 4WJs was seriously compromised in the presence of competitor DNA (>50-fold decrease in intensity of complex I relative to that of the wild type B domain), the structural selectivity of the mutant for 4WJs (i.e. formation of the high affinity complex I) was retained.

A significant observation in respect to binding to 4WJs was made with the B domain mutant in which Arg97 of the extended N-terminal strand had been mutated to glutamic acid (R97E). Titration of 4WJs with increasing amounts of the R97E mutant (Fig. 2) or the R97A mutant (Fig. 3) revealed that the high affinity complex I was not formed but instead complexes II and III were the most prominent bands detected. Although the gel shift experiments with the R97E mutant to 4WJs was challenged in the presence of ~1000-fold mass excess of unlabeled competitor (linear) DNA. Although binding of K96E mutant to 4WJs was seriously compromised in the presence of competitor DNA (>50-fold decrease in intensity of complex I relative to that of the wild type B domain), the structural selectivity of the mutant for 4WJs (i.e. formation of the high affinity complex I) was retained.

Further addition of competitor DNA (~1000-fold mass excess in total) caused complete disappearance of all of the retarded bands, and only free 4WJs were detected in the presence of the R97E (Fig. 2) or the R97A mutants.3 We conclude that the basic residues of the N-terminal strand, Lys96 and Arg97, are crucial for high affinity and correct binding but not for structure-specific binding of the B domain to 4WJs.

Effect of Mutations within α-Helices of the B Domain on Binding to 4WJs—Replacement of Cys106 (C106S) or putative intercalating Phe103 (F103S) of helix I with serine had very little, if any, effect on specific binding of the B domain to 4WJs (Fig. 2). Formation of complexes I and II by the F103S and C106S mutants was only little affected even at 1000-fold excess of competitor DNA. Mutations of Arg110 and Lys114 (helix I) reduced approximately 5- and 3-fold, respectively, the intensity of complex I upon binding to 4WJs (Fig. 2; R110G and K114E). The presence of 1000-fold mass excess of competitor (linear) DNA further decreased (~2-fold) formation of the complex I, indicating that basic residues Arg110 and Lys114 are very likely in direct contact with 4WJs. Replacement of Pro118 (located at the turn between helices I and II) with alanine (P118A) had very little, if any, effect on binding of the B domain to four-way junctions (Fig. 2).

Previously we have shown that mutation of Lys128 to glutamic acid (but not Lys127) significantly decreased α-helical content of the B domain and inhibited its binding to supercoiled DNA (21). Here we have demonstrated that both Lys127 and Lys128 were dispensable for specific binding to 4WJs because mutations of either of the residues to glutamic acid had very little, if any, effect on formation of complex I (Fig. 2; K127E and K128E).

Mutation of Lys152 of helix II to glutamic acid (K152E) prevented formation of the complex II and decreased (~2-fold) intensity of complex I, which was further diminished (~5-fold) in the presence of ~1000-fold excess of linear competitor DNA.
were aimed at investigating the possibility of whether the presence of linear DNA (which had suppressed smearing and aggregation shown in Fig. 2; Y155S) could facilitate further refolding of the Y155S mutant. As shown in Fig. 4b, in high salt buffer and in the presence of linear DNA, the Y155S mutant adopted a folded structure comparable with the wild type B domain. These results gave strong evidence that higher ionic strength and the presence of linear DNA could almost fully compensate for improper folding (and binding to 4WJs) of the B domain because of nonconservative substitution of Tyr$^{155}$.

In summary, mutational analysis of the HMG-1 B domain in respect to its binding to 4WJs revealed that Lys$^{96}$ and particularly Arg$^{97}$ of the extended N-terminal strand and also (albeit much less) Arg$^{110}$ and Lys$^{114}$ of helix I are involved in high affinity binding of the B domain to 4WJs. We have also shown that the B domain can tolerate a number of (mainly) nonconservative replacements of highly conserved amino acid residues without abolishing the structural selectivity of the HMG box for 4WJs (see Fig. 8).

**DNA Supercoiling by the B Domain Is Very Sensitive to Amino Acid Replacements**—HMG-1 and its isolated HMG-1 A and B domains can unwind and distort DNA by insertion of negative supercoils (in the presence of topoisomerase I) in topologically closed domains of DNA (11, 16, 19, 20). Here we have analyzed the importance of selected amino acid residues of the B domain for the ability of the HMG box to induce DNA supercoiling in the presence of topoisomerase I. Because DNA supercoiling by the A and B domains of HMG-1 depends on ionic strength (20), topoisomerase-I-mediated DNA supercoiling by the B domain and its mutants was studied either in relaxation buffer containing 50 mM NaCl (high salt relaxation buffer) or in 5-fold diluted buffer (low salt relaxation buffer). We have first assessed the effect of the B domain on DNA unwinding under “DNA Supercoiling Assay.” Briefly, closed circular DNA was incubated with increasing amounts of the HMG polypeptides (all ratios are expressed as molar/molar relationships), and wheat germ topoisomerase I was then added to remove any plec tonic supercoils that formed in protein-free regions of the DNA. DNA was deproteinized, and the extent of supercoiling was analyzed by one-dimensional agarose gel electrophoresis (first dimension). To determine the sign of supercoiling, the gels were upon electrophoresis in the first dimension turned by 90° and further resolved in the second dimension in the presence of intercalating drug chloroquine. Chloroquine intercalates into DNA that results in decreased mobility of negative supercoils and increased mobility of positive supercoils. As shown in Fig. 5, the ability of the B domain to supercoil DNA in high salt buffer was severely compromised by mutations of most of the studied residues (Fig. 5). Mutations of basic residues Lys$^{96}$/Arg$^{97}$ (the extended N terminus), Arg$^{110}$/Lys$^{114}$ (helix I), and hydrophobic residue Phe$^{103}$ (helix I) impaired DNA supercoiling. DNA supercoiling was very low even at relatively high protein/DNA ratios (Fig. 5). In low salt buffer, some DNA supercoiling was apparent with most of the studied mutants (albeit much lower than with wild type B domain), although the B domain peptides with mutated Lys$^{96}$/Lys$^{114}$, or Phe$^{103}$ were still the least efficient in DNA supercoiling (Fig. 6). Interestingly, mutations of most of the studied residues of the B domain changed the sign of topoisomerase-I-mediated DNA supercoiling from negative to positive. In some cases the mutants exhibited negative supercoiling at lower protein/DNA ratios and positive at higher ratios (Fig. 5; P118S K127E, K128E, and Y162S). We have noticed that some positive supercoiling was also detected with the wt B domain, albeit only in low salt buffer (Fig. 6). In addition, we have demonstrated that isolated DNA-binding HMG box 1

**FIG. 3.** Lys$^{96}$ and Arg$^{97}$ are required for high affinity binding of the B domain to 4WJs. Gel retardation assay was carried out with $\sim$1 nm $^{32}$P-labeled 4WJs and the wt B domain and mutants. a, titration of 4WJs with increasing concentrations of the wt B domain and the K96A mutant (left to right, 0.25, 0.5, 1, 2.5, 5, and 10 μM) in DNA binding buffer containing Mg$^{2+}$. b, titration of 4WJs with increasing concentrations of the wt B domain or the R97A mutant (left to right, 0.01, 0.05, 0.15, 0.5, and 1 μM) in DNA binding buffer without Mg$^{2+}$. The protein-DNA complexes were resolved on 5.5% polyacrylamide gels. Complexes that were formed upon binding of the B domain to 4WJs are indicated as I–III. First lane in each panel are free 4WJs. Unlabeled competitor DNA was linearized plasmid pBluescript in $\sim$200-fold mass excess over the 4WJs. WT, wild type HMG-1 B domain.

The changes observed upon binding of the Y155S mutant to 4WJs in the absence or presence of a small amount of linear DNA over 4WJs (Fig. 2) prompted us to investigate (by means of CD) whether the introduced mutation did not cause any alternation in the protein structure, and if so, whether the excess of linear DNA could help the Y155S mutant to refold prior to its binding to 4WJs. In a low salt CD buffer, the Y155S mutant displayed $\sim$50% loss of α-helical content relative to the wild type B domain as indicated by the decrease of a distinct minimum at 222 nm in the CD spectrum (Fig. 4b). We have observed that in high salt CD buffer (which corresponds approximately to the ionic strength of the buffer used for the gel retardation assays), the Y155S mutant could refold to $\sim$80% of the α-helicity of the wild type B domain (no change in the α-helicity of the wild type B domain was detected in the high salt buffer, not shown; see also Ref. 32). Next, CD experiments

![Image](59x458 to 287x729)
of RNA polymerase transcription factor xUBF could induce positive supercoils ( unlike the full-length xUBF, which was previously reported to form only negative supercoils; Refs. 18 and 52) in closed circular relaxed DNA in the presence of topoisomerase I (Fig. 7a).

We then investigated the effect of the introduced mutations on the ability of the B domain to protect supercoiled DNA from relaxation by topoisomerase I (under “DNA Protection Assay”). A very good correlation between the results of DNA supercoiling and protection was observed. The less efficient the B domain mutant in DNA supercoiling was, the less efficient it was also in protection of supercoiled DNA from relaxation by topoisomerase I (Fig. 7b for the wt B domain, R97A and F103S as an example). We have also found that protection of negatively supercoiled DNA from relaxation by topoisomerase I was more efficient and occurred at lower protein/DNA ratios when the B domain was covalently linked to the A domain (AB didomain of HMG-1).2 Separation of the AB-DNA complexes (and also of the A or B domain-DNA complexes) by agarose gel electrophoresis generally results in gel retardation that is dependent on protein-DNA input ratios (11, 21). However, under certain conditions (electrophoresis in low ionic strength), the resolution of the latter complexes (that were not treated with topoisomerase I) on agarose gels revealed a formation of a series of closely

FIG. 5. DNA supercoiling by the B domain and mutants in high salt buffer. Circular relaxed plasmid pBR322 DNA was incubated with the wt B domain or its mutants (protein/DNA molar input ratios 500 and 2000) in the presence of topoisomerase I. The deproteinized DNA was subjected to one-dimensional agarose gel electrophoresis. To determine the sign of supercoiling, the gels were then soaked in chloroquine and turned by 90°, and the DNA topoisomers were electrophoresed in the second dimension. Upon completion of the electrophoresis, the gels were stained with Sybr Green I. The presence of chloroquine during electrophoresis in the second dimension increased the mobility of positive topoisomers, whereas the mobility of negative topoisomers was decreased. WT, wild type B domain.
with the AB didomain (protein/DNA molar ratios were 50, 100, 200, and negatively supercoiled and linearized plasmid DNA (1:1) was incubated DNA topoisomers were resolved on 1% agarose gels.

ence or absence of the B domain or mutants at a protein/DNA molar ratio of 2000 and then treated with topoisomerase I. Deproteinized DNA topoisomers were resolved on 1% agarose gels, c, a mixture of negatively supercoiled and linearized plasmid DNA (1:1) was incubated with the AB didomain (protein/DNA molar ratios were 50, 100, 200, and 350, left to right) and the nucleoprotein complexes were then resolved on 1% agarose gel. S, supercoiled plasmid DNA. O, covalently closed relaxed plasmid DNA. L, linearized plasmid DNA. N, nicked closed circular plasmid DNA. The agarose gels were stained with Sybr Green I.

spaced bands (Fig. 7c) resembling the topoisomerase ladder produced by partial relaxation of supercoiled plasmid by topoisomerase I. Deproteinization of the latter nucleoprotein complexes followed by electrophoresis on agarose gel showed that the mobility of protein-free supercoiled plasmid DNA was fully restored, indicating that the AB didomain did not have topoisomerase strand passage activity that would change the linking number. Although transitional relieving the torsional stress in the supercoiled plasmid (without changing the linking number) by the AB didomain is not excluded (see Ref. 11), the extent of contribution of gel retardation to the “topoisomerase-like” ladder by preferential binding of the HMG-1 peptide to supercoiled DNA (Fig. 7c and also Ref. 21) awaits further investigation (see “Discussion”).

We have previously verified binding of the B domain and the mutants used in this study to negatively supercoiled DNA by retardation of the nucleoprotein complexes in agarose gels (21). The binding of the K96A, R97A, R110G, and K114E mutants (and also the K128E mutant) to negatively supercoiled DNA was the most reduced from all of the studied B domain mutants (Fig. 8). All other mutants exhibit either similar or only modestly decreased (up to <2-fold; P118A, Y155S, and Y162S) affinities for the supercoiled DNA. 
Phe103 of the B domain was the only residue that when mutated significantly impaired DNA supercoiling (Fig. 5) with very little, if any, effect on retardation of the nucleoprotein complexes followed by electrophoresis on agarose gel. The supercoiled nucleoprotein complexes followed by electrophoresis on agarose gel. The supercoiled nucleoprotein complexes followed by electrophoresis on agarose gel showed that the mobility of protein-free supercoiled plasmid DNA was fully restored, indicating binding activity by the B domain or mutants.

In conclusion, our results indicated that putative intercalating hydrophobic Phe103 of helix I is important for topoisomerase I-mediated DNA supercoiling by the HMG-1 B domain, with a number of basic residues of the extended N terminus (Lys106/Arg107) and helix I (Arg110/Lys114) of the B domain contributing likely to DNA supercoiling via direct DNA binding (Fig. 9).

**DISCUSSION**

HMG-1 is a member of a subclass of the HMG-1 box superfamily that binds DNA non-sequence-specifically but can recognize prebent (distorted and untwisted) DNA regions (such as cisplatin adducts, 4WJs, and supercoiled DNA) and unwind DNA (reviewed in Refs. 2 and 46). In this report we have analyzed the effect of a number of (mainly) nonconservative amino acid replacements on the ability of the B domain of HMG-1 to unwind DNA (in the presence of topoisomerase I) and to bind 4WJs. 4WJs have been proposed to be the central intermediates of gene recombination (43), a process recently shown to require HMG-1/2 proteins (22). Although 4WJs are unlikely to be the binding targets for HMG-1 in the cell, distorted (bent and unwound) DNA structures in regulatory and/or damaged regions of genome with certain similarities to 4WJs might be good candidates for HMG-1 binding. Recent data indicated that the two linked HMG-1 box domains (AB didomain or HMG-1 lacking the acidic C-tail) are not functionally equivalent in 4WJs binding (45). The A domain of the AB didomain binds to the center of the junction, whereas the linked B domain makes contacts along one of the arms. However, the isolated B domain was shown to mediate structure-specific recognition by binding to the center of the junction (41, 42, 45). Here we have demonstrated that Lys96 (which corre-
sponds to Arg10 of the A domain of HMG-1, which when mutated to glycine reduced ~10-fold the affinity of the mutant for 4WJs; Ref. 29) and particularly Arg87 of the extended N-terminal strand of the B domain are required for high affinity and proper binding to 4WJs. The observed mode of binding of the R97E(A) mutants to 4WJs (formation of complex II or higher but not complex I) indicates that the binding kinetics of the B domain was altered and became highly cooperative. Thus, formation of complex II upon binding of the R97E(A) mutants to 4WJs is likely a result of simultaneous binding of one HMG-1 box molecule to a high affinity site (junction center) and a second HMG-1 box molecule to low affinity sites (the double-stranded junction arms). We have also noticed that residues of the B domain that are important for binding to 4WJs (Arg87 > Lys96 → Arg110 > Lys114), are also important for topoisomerase I-mediated DNA supercoiling and binding to negatively supercoiled DNA.

The helix I of the HMG-1 B domain is exposed to solvent as revealed by NMR studies (5, 6). Corresponding residues by sequence alignment of the sequence-specific HMG proteins LEP-1 (Met) and SRY (Ile) and sequence-nonspecific HMG proteins HMG-D (Met) and yeast NHP6A (Met) were previously demonstrated to protrude into the DNA from the minor groove and to produce base pair unstacking (25–28). We have demonstrated that binding of the F103S mutant of the B domain to 4WJs or supercoiled DNA (like the ability to stabilize DNA loops of supercoiled DNA by protein–protein associations, Ref. 21) is unaffected by the introduced mutation and that the mutant is inefficient in inducing DNA supercoiling or protection of supercoiled DNA from relaxation by topoisomerase I. Molecular dynamics simulation and modeling studies revealed (47) that hydrophobic residues Phe103 of the B domain of HMG-1 has the potential to intercalate into DNA base pairs (corresponding residue in the A domain of HMG-1 is Ala17 which cannot intercalate). We propose that Phe103 of the concave surface of the HMG-1 B domain, by virtue of its ability to intercalate in the DNA minor groove, is involved in topoisomerase I-mediated DNA supercoiling. A number of basic residues of the extended N terminus (Lys96/Arg97 and helix I (Arg110/Lys114) of the B domain are likely to contribute to DNA supercoiling by directing proper DNA binding, which enables intercalation of Phe103. It remains to be established whether, in addition to Phe103, another putative putative intercalating residue Ile122 of the B domain is also involved in DNA supercoiling (this residue corresponds to Phe38 of the A domain of HMG-1 that was shown to intercalate into a hydrophobic notch created at the platinum-DNA crosslink (33), as well as to intercalating Phe48 and Val32, which are located at the analogous positions in yeast NHP6A and HMG-D, respectively (26).

We have shown that resolution of complexes of AB didomain with negatively supercoiled DNA on agarose gels results in the absence of topoisomerase I in formation of a series of closely spaced bands (this paper) resembling to the ladder produced by partial relaxation of supercoiled plasmid by topoisomerase I. The latter electrophoretic pattern bears resemblance to that previously reported upon binding of tumor suppressor protein p53 to negatively supercoiled DNA (49). Although the electrophoretic pattern of the resolved AB-DNA (and p53-DNA; Ref. 49) complex is likely affected by the gel retardation, the formation of the topoisomerase-like ladder may also be due to transitional relieving the torsional stress in the supercoiled plasmid (without changing the linking number). The latter idea is in agreement with the previously reported HMG1-mediated DNA unwinding (11, 50). Thus, the protection of supercoiled DNA from relaxation by topoisomerase I by the AB didomain (and very likely also by HMG-1 or its individual HMG box domains) may be a consequence of relaxation of the underwound DNA that had occurred upon binding (and before addition of topoisomerase I) of the HMG polypeptide to negatively supercoiled DNA. The relatively high angle of DNA unwinding by HMG-1 (~60°; Refs. 11 and 50) and calculated additional increment in free energy in the binding reaction (51) explain why the protein preferentially binds to negatively supercoiled DNA even at low values of superhelix density.

The isolated B domain (like HMG-1 or AB didomain, Ref. 11 and unpublished) introduces negative supercoils into topologically closed DNA domains in the presence of topoisomerase I. A change of the superhelix density from negatively supercoiled DNA, via the relaxed, to the positively supercoiled state observed with most of the studied B domain mutants suggests that supercoiling by the HMG-1 box domain is very sensitive to amino acid replacements. This is also likely to be the case of another HMG box containing protein, RNA polymerase I transcription factor xUBF, which in the presence of topoisomerase I induces negative DNA supercoiling (52), whereas isolated HMG box 1 of xUBF induces only positive supercoiling (this paper). Although it is not known whether the topoisomerase I-mediated transition of topologically closed DNA from underwound (negative) to overwound (positive and more compact) by HMG-1 occurs in vivo, a change in the DNA binding properties of HMG-1 (that is likely required for such a transition) could be mediated by interactions with other parts of the same protein or with other cellular factors (11, 16, 50, 53). HMG-1 has often been implicated in transcription and facilitation of binding of certain sequence-specific proteins to their target DNA by both protein–protein interaction and/or manipulation of DNA structure by bending/looping or conservation of torsional stress (2, 22, 23, 24). Generation of more flexible positively supercoiled DNA relative to negatively supercoiled DNA (54) could then favor binding of regulatory proteins at multiple sites.

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