Two Mutations of Basic Residues within the N-Terminus of HMG-1 B Domain with Different Effects on DNA Supercoiling and Binding to Bent DNA†

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ABSTRACT: High mobility group (HMG) 1 protein and its two homologous DNA-binding domains, A and B ("HMG-boxes"), can bend and supercoil DNA in the presence of topoisomerase I, as well as recognize differently bent and distorted DNA structures, including four-way DNA junctions, supercoiled DNA and DNA modified with anticancer drug cisplatin. Here we show that the lysine-rich part of the linker region between A and B domains of HMG-1, the ^{85}TKKKFKD^{91} sequence that is attached to the N-terminus of the B domain within HMG-1, is a prerequisite for a preferential binding of the B domain to supercoiled DNA. The above sequence is also essential for a high-affinity binding of the B domain to DNA containing a site-specific major 1,2-d(GpG) intrastrand DNA adduct of cisplatin. Mutation of Arg^{97}, but *not* Lys^{90} [Lys^{90} forms a specific cross-link with platinum(II) in *major* groove of cisplatin-modified DNA; Kane, S. A., and Lippard, S. J. (1996) *Biochemistry 35*, 2180−2188], to alanine significantly (>40-fold) reduces affinity of the B domain to cisplatin-modified DNA, inhibits the ability of the B domain to bend (ligase-mediated circularization) or supercoil DNA, and results in a loss of the preferential binding of the B domain to supercoiled DNA without affecting the *structural*-specificity of the HMG-box for four-way DNA junctions. Some of the reported activities of the B domain are enhanced when the B domain is covalently linked to the A domain. We propose that binding of the A/B linker region within the *major* DNA groove helps the two HMG-1 domains to anchor to the *minor* DNA groove to facilitate their DNA binding and other activities.

Chromosomal proteins HMG-1[1] and 2 are members of the large HMG1-box superfamily (*1*). There is mounting evidence implicating the proteins in a number of biological processes including transcription, replication, recombination, and DNA repair, in which they may play architectural roles as DNA *chaperones* by DNA bending/looping or stabilizing underwound DNA, and promoting protein−protein interactions to facilitate assembly of nucleoprotein higher-order structures (*2*, *3*).

HMG-1, like HMG-2, possesses two homologous DNA binding HMG-box domains, A and B, and an acidic C-tail. The HMG-box is a DNA-binding motif of approximately 80 amino acid residues and has a characteristic L-shaped structure formed by the three α-helices and the extended N-terminal strand (*4*, *5*). The A and B domains of HMG-1 have also been shown to bind specifically distorted, bent and underwound, DNA structures such as four-way DNA junctions, cruciforms, DNA modified by anticancer drug cisplatin, and supercoiled DNA (*6*−*10*). In addition, binding of HMG-1 or other HMG-box proteins to bent (cisplatin-modified) DNA further increases the bend angle up to 60° (*11*, *12*). Reported shielding of the major cisplatin adducts on DNA from excision repair in human cell extracts or yeast cells by HMG-1 might suggest a possible involvement of this protein in sensitizing the cells to anticancer drug cisplatin (*13*−*15*).

Photochemical cross-linking detected a specific cross-link of platinum (II) in cisplatin modified DNA to Lys^{90} of the N-terminal strand of the HMG-1 B domain (*16*). Detailed X-ray structure of the HMG-1 A domain bound to a 16-bp DNA duplex modified with cisplatin revealed that the precise positioning of the A domain on cisplatinated DNA is directed by intercalation of Phe^{37} of helix II and by multiple interactions between the DNA and amino acid residues located within the N-terminal strand and helices I and II (*17*).

The HMG-1 B domain is flanked by a lysine-rich N-terminal sequence (corresponding to a part of the region that links the B domain to the A domain in HMG-1, the A/B linker region), and the C-terminal flanking sequence that is attached to the acidic C-tail in HMG-1 (*1*−*3*). DNA binding and bending activities of different HMG-domains were reported to be modulated by their flanking amino acid sequences (*18*−*22*). Whether the flanking sequences of the HMG domains of HMG-1 participate in recognition of specific DNA structures or whether they are required for topoisomerase I-mediated DNA supercoiling has not been clear.

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[1] Abbreviations: cisplatin, *cis*-diaminedichloroplatinum(II); HMG, high mobility group; 4WJs, four-way DNA junctions; WT or wt, wild-type; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; bp, base pairs; DTT, dithiothreitol; D or Asp, aspartic acid; K or Lys, lysine; R or Arg, arginine; F or Phe, phenylalanine; T or Thr, threonine.

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In the present work, we have demonstrated an important role of a lysine-rich part of the A/B linker region of HMG-1, the ^{85}TKKKFKD^{91} sequence, in high-affinity binding of the B domain to DNA modified with anticancer drug cisplatin, as well as in DNA supercoiling and discrimination between supercoiled and linear DNA. We have also shown that Arg^{97} of the N-terminal strand of the B domain is involved in specific binding of the B domain to supercoiled or cisplatin-modified DNA, and in DNA bending and supercoiling. Some of the reported activities of the isolated B domain are enhanced when the B domain is covalently linked to the A domain. We propose that binding of the A/B linker region within the *major* DNA groove helps the two HMG-1 domains to anchor to the *minor* DNA groove to facilitate their DNA binding and other activities.

MATERIALS AND METHODS

Materials. The oligodeoxyribonucleotides were synthesized by the Integrated DNA Technologies (Coralville) and directly used for the PCR. For the preparation of DNA probes, the oligodeoxyribonucleotides were further purified on MonoQ FPLC (Pharmacia) with 0 to 1 M NaCl gradient in 20 mM Tris, pH 7.4. The restriction enzymes, T4 DNA kinase, T4 DNA ligase, and T4 DNA polymerase were from Promega and BioLabs. Isolation of plasmids and DNA fragments from 1% agarose gels was carried out using Qiaprep Spin Miniprep and Qiaquick Gel Purification Kits (Qiagen), respectively. The radioactive labels were from Amersham. Cisplatin was from Lachema, Brno (Czech Republic). All chemicals were of the highest quality and were obtained from Sigma, Merk and Fluka.

Plasmids. The cDNAs encoding the HMG-box A (residues 1−84), HMG-box A1 (residues 1−88), HMG-box B (residues 92−180), HMG-box B2 (residues 90−180), HMG-box B3 (residues 89−180), HMG-box B7 (residues 85−180), and A/B didomain (i.e., HMG-1 lacking the acidic C-tail, residues 1−180) were prepared by means of PCR of the rat HMG1 cDNA (plasmid pT7-RNHMG1, kindly provided by M. E. Bianchi) and cloned either into the expression vector pGEX-4T1 (Amersham Pharmacia Biotech.) or plasmid pAlter-1 (Promega) as previously described (*22*).

Site-Directed Mutagenesis of the B Domain. The cDNAs of the HMG-1 B domain (boxes B or B7) and the A/B didomain were cloned into the plasmid pAlter-1 (Promega). The site-directed mutagenesis was carried out as previously described (*10*, *23*). Corresponding mutations were verified by sequencing using the Sequenase DNA sequencing kit 2.0 (Amersham Pharmacia Biotech.), and the positive clones were re-cloned into the expression vector pGEX-4T1 (Amersham Prarmacia Biotech).

Expression and Purification of the HMG-Box Polypeptides from E. coli. The HMG-box polypeptides were expressed in BL 21 cells (Novagene) as fusion proteins with the glutathionine S-transferase (GST) to allow efficient synthesis and stability in *E. coli*. The expressed HMG-GST fused proteins were selectively retained on glutathionine-Sepharose 4B columns. The GST moiety was removed by cleavage with thrombin as described (*22*). Final purification of HMG-box polypeptides was performed by FPLC purification on a MonoS column (Amersham Pharmacia Biotech) and protein concentrations were determined as previously described (*22*).

DNA Probes. All oligonucleotides were extensively purified by FPLC chromatography. A 20-bp DNA duplex with the 1,2-intrastrand d(GpG) *cis*-platin adduct was prepared by annealing of the oligonucleotide containing a single intrastrand *cis*-[Pt(NH$_4$)$_2$\{d(GpG)-N7(1),-N7(2)\}] adduct (the upper strand in Figure 1B) with the nonplatinated complementary strand (the bottom strand in Figure 1B) and repurified by FPLC chromatography on a MonoQ column as previously described [ref *9*; this probe is designated in Figure 1B as Pt-DNA (20−mer)]. The presence of one platinum atom at the single d(GpG) site in Pt-DNA was verified by atomic absorption spectrometry (kindly performed by O. Nováková, Institute of Biophysics, Brno) and Maxam−Gilbert sequencing. Control 20-bp DNA duplex was prepared from oligonucleotides of the same sequence but using the upper strand lacking the cisplatin adduct. The 20-bp duplexes were labeled at their 5′-termini with [γ-^{32}P]ATP by T4 polynucleotide kinase and the unincorporated label was removed by passing the samples through Sephadex G-50 (fine) minicolumns. DNA probe of 40-bp was prepared by ligation of two ^{32}P-labeled 20-bp DNA duplexes (with or without a single 1,2-intrastrand cross-link) followed by purification of the ligated products on a 10%-polyacrylamide gel as described (*24*).

Electrophoretic Mobility Shift Assay (EMSA) with Cisplatin-Modified DNA. Different amounts of the HMG-box polypeptides (typically 0.2−5 μM**)** were mixed with ∼4 nM of ^{32}P-labeled DNA probes of 20- or 40-bp (with or without platinum-*cross*-links, see *DNA Probes*) in 0.1 M NaCl, 50 mM KCl, 10 mM Hepes·NaOH, pH 7.2, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 0.05% Nonidet P-40, 4% glycerol, and 200 μg/mL BSA. DNA and proteins were mixed in a final volume of 10 μL and kept on ice for 1 h prior to loading onto pre-run and pre-cooled nondenaturing 10% poly-acrylamide gels [29:1 acrylamide/*N*,*N*′-methylene-bis(acrylamide)] in 0.5 × TBE buffer. The samples were loaded while the gel was running at 50 V/cm and the gel was then run at 250 V/cm for 2−3 h at ∼4 °C, followed by vacuum-drying onto Whatman 3 MM chromatography paper. Quantification of the bands intensity was performed on a PhosphorImager Storm (Molecular Dynamics) using ImageQuant 4.1 software for data processing. For the permanent record, the gel was scanned using a Molecular Dynamics Phorphor-Imager Storm and subsequently adjusted for contrast/brightness using Adobe Photoshop.

EMSA with Synthetic Four-Way DNA Junctions. Synthetic four-way DNA junctions (4WJs) were constructed and labeled at 5′-termini with [γ-^{32}P]ATP as previously described (*10*).

The DNA-binding experiments were typically carried out with ∼1 nM of labeled 4WJs and different amounts of HMG-box polypeptides (typically ∼0.01−1 μM) in the absence or presence of a 5000-fold mass excess of unlabeled competitor DNA (*Kpn*I-linearized plasmid pBluescript II KS+) in a final volume of 10 μL. The binding experiments were carried out in 25 mM NaCl, 5 mM KCl, 10 mM Hepes.KOH, pH 8.0, 1 mM EDTA, 1 mM spermidine, 1 mM dithiothreitol, and 3.5% Ficoll. The samples were incubated on ice for 20 min, loaded onto vertical (0.7 mm thick) pre-run polyacrylamide slab gels (29:1, acrylamide to bis-acrylamide mass ratio) in 0.5 × TBE buffer and subjected to electro-

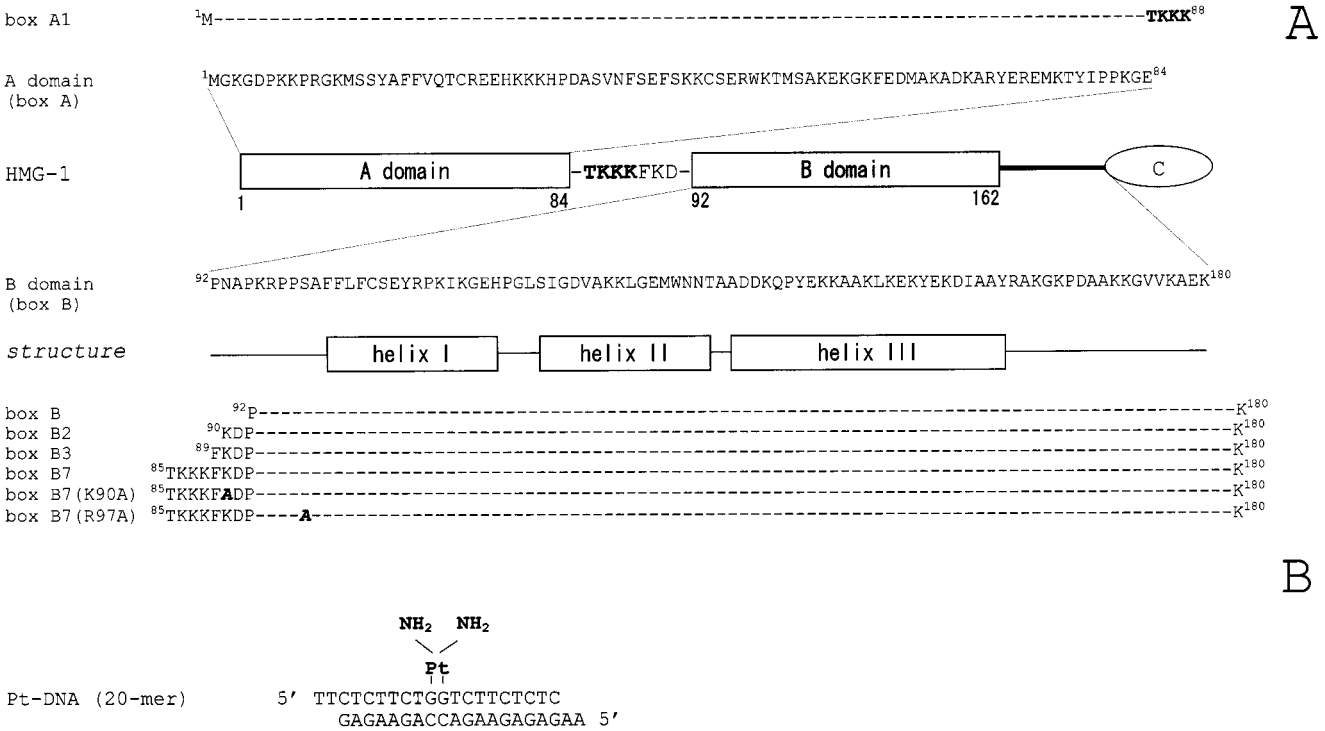

FIGURE 1: (A) HMG-1 and HMG-1 polypeptides. All HMG1-box polypeptides used in this paper were derived from the previously published sequence of the rat HMG-1 protein (GenBank accession no. Y00463). (From top to bottom) Amino acid sequences of the A domain of HMG-1 containing or lacking the ^{85}TKKK^{88} sequence, boxes A1 or A, respectively; domain structure of HMG-1 (residues 1−215) indicating the A and B domains, and the acidic C-domain (depicted as oval, residues 186−215); amino acid sequence of the B domain (box B, residues 92−180); position of the three α-helices within the B domain (34); B domain polypeptides with different N-terminal flanking regions (boxes B−B7). (B) Structure of a 20-bp DNA duplex containing a single site-specific major 1,2-d(GpG) intrastrand DNA adduct of anticancer drug cisplatin.

phoresis at 250 V for 2−3 h at ∼4 °C. The vacuum-dried gels were visualized as indicated for EMSA of Pt-DNA.

Dissociation Constants. The K_d was estimated from the gel mobility shift assays (using a fixed concentration of the labeled Pt-modified 20-bp DNA duplex and varying amounts of the HMG box domains) as the HMG-box concentration at the point in the titration where half of the input DNA had been complexed with protein (i.e., protein concentration at which 50% of the DNA was shifted; refs *8* and *22*). It should be noted that the determination of the K_d as the protein concentration at which half of the DNA is shifted is only valid as long as the DNA concentration is lower than the DNA half-concentration. The accuracy of the K_d values determination was estimated to be ±25%.

Competitions Assays. Cisplatin-modified 20-bp DNA duplex (∼4 nM) was incubated on ice for 1 h with a fixed amount of the HMG box domain (corresponding to a concentration of half-maximal binding estimated from the gel mobility shift assays). After addition of varying amounts of either unlabeled control (unmodified) or cisplatin modified 20-bp DNA with cisplatin, the reaction mixtures were further incubated for an additional 30 min on ice (final volume of 10 μL) and subjected to electrophoresis as described for the EMSA. Quantification of the bands corresponding to free and bound DNA was performed on a Phosphor-Imager Storm (Molecular Dynamics) as described (*25*).

Circularization Assay. Ligase-mediated circularization assay was carried out with ∼1 nM of a ^{32}P-labeled 124-bp DNA duplex and the HMG domains at concentrations of 0.05−1.5 μM as previously described (*22*). The amounts of monomer DNA circles were determined from quantitative analyses of dried gels on a Molecular Dynamics Storm PhosphorImager using the ImageQuant software. For the permanent record, the gels were scanned and adjusted for contrast/brightness using Photoshop (Adobe) as indicated for EMSA.

DNA Supercoiling. Negatively supercoiled plasmid DNA (final concentration ≈ 170 μg/mL) was relaxed in relaxation buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 20% glycerol; Promega) with wheat germ topoisomerase I (4 units/μg DNA; Promega) at 37 °C for 90 min. The relaxed DNA was then diluted in relaxation buffer, followed by addition of a second portion of the enzyme. The DNA mixture was then divided into several tubes, each containing ∼10 nM DNA, and then the HMG peptides were added (as indicated in the legend to figures) 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 mix (5× TBE, 5% SDS, 15% sucrose, 0.1% bromphenol blue, 0.1% xylenecyanol, 1 μg/μL proteinase K) was added and the samples were subjected to further incubation at 37 °C for 1 h. DNA topoisomers were then resolved in the first dimension on 1% agarose gels in 0.5× TBE buffer at ∼3 V/cm for 17 h. The gels were stained with 10000-fold diluted Sybr Green I (Molecular Probes, Eugene), followed by visualization of the DNA topoisomers under UV-illumination (254 nm).

Determination of Sign of DNA Supercoiling. To determine the sign of supercoiling, the gels were soaked after electrophoresis in the first dimension (see *DNA supercoiling*) in chloroquine (2.5 μg/mL) for 1 h, turned horizontally by 90° and resolved in the second dimension in 0.5× TBE contain-

ing chloroquine at ~5 V/cm for 10 h. Gels were stained with Sybr Green I and directly viewed under UV-illumination (254 nm).

Circular Dichroism. Folding of the purified recombinant HMG polypeptides and their mutants was monitored by measuring of their α-helical contents using circular dichroism (CD). The polypeptides were extensively dialyzed against 0.2 M NaCl, 10 mM sodium phosphate, pH 7.4, 0.5 mM EDTA, and 1 mM dithiothreitol. CD spectra of HMG polypeptides (~25−50 μM) were recorded between 300 and 195 nm (resolution step 0.1 nm, 1 mm path length cuvette, 20 °C) using a Jasco J720 spectropolarimeter with nitrogen gas purging. CD spectra are presented as molar ellipticity values [Θ].

RESULTS AND DISCUSSION

Expression and Analysis of Recombinant HMG Polypeptides by Circular Dichroism. Design and nomenclature of the rat HMG-1 A and B domain sequences with different flanking regions as well as the B domain mutants is depicted in Figure 1. HMG-box polypeptides were expressed in *E. coli* as soluble recombinant GST (glutathionine S-transferase) fusion proteins which were subsequently processed by cleavage with thrombin to remove the GST moiety. HMG polypeptides were purified by FPLC chromatography to near homogeneity as judged by Coomassie blue staining of the polypeptides resolved by SDS-polyacrylamide gel electrophoresis (Figure 2A). Correct folding of the polypeptides used in this study was verified by circular dichroic (CD) measurements. The CD spectra of the wild-type B domain (box B7) and its R97A mutant are shown in Figure 2B. The wild-type B domain exhibited a CD spectrum with a distinct minimum at 222 nm and a second minimum at around 208 nm, an indicative for a protein with α-helical structure. Both the R97A mutant (Figure 2B) and the K90A mutant (not shown) exhibited CD spectra nearly identical to that of the wild-type B domain, providing an evidence that the protein folding interactions of the HMG-box polypeptides were not affected by the introduced mutations.

Binding of HMG1-Box Domains to Cisplatin-Modified DNA. It is widely accepted that bent and distorted DNA structures provide a recognition signal for the HMG domain (*3*). It has been shown previously that anticancer drug cisplatin interacts with DNA by forming a major intrastrand adduct at 1,2-d(GpG), and two minor 1,2-d(ApG) and 1,3-d(GpTpG) adducts (reviewed in ref *26*). Modification of N7 atoms of adjacent guanine residues on duplex DNA with cisplatin results in DNA unwinding and bending toward the major groove, with a significant widening and flattening of the minor groove (*27*, *28*). Here we have used an electrophoretic mobility shift assay (EMSA) to investigate the DNA binding potential of the two HMG-1 domains, A and B, with different flanking sequences as well as the effect of two mutations within the N-terminus of the B domain on the specific-binding to a 20-bp DNA duplex containing a site-specific intrastrand 1,2-d(GpG) adduct of cisplatin (Figure 1B).

Unmodified (control) or cisplatin-modified DNA (Pt-DNA) were titrated with increasing amounts of different HMG-1 polypeptides and analyzed by EMSA. In our preliminary experiments with the B domain lacking the

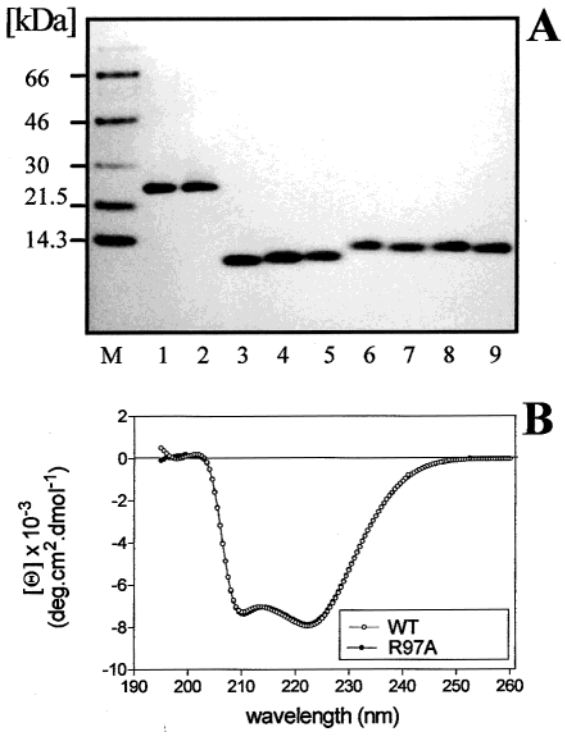

FIGURE 2: Electrophoretic analysis of recombinant HMG-1 polypeptides and CD spectra.(A) HMG-1 polypeptides were expressed in *E. coli* and purified to near homogeneity by FPLC chromatography (Amersham Pharmacia Biotech). Purified polypeptides were analyzed on an SDS/10%-polyacrylamide slab gel in Tris-tricine buffer and stained with Coomassie blue G-250. Lane 1, A/B didomain; lane 2, A/B(R97A) didomain mutant; lane 3, box B; lane 4, box B2; lane 5, box B3; lane 6, box A1; lane 7, box B7; lane 8, B7-(K90A) mutant; lane 9, B7(R97A) mutant. (Left of the panel) Low-range molecular mass protein markers. (B) Circular dichroic (CD) spectra of the wild-type B domain (box B7) and the R97A mutant.

N-terminal ^{85}TKKKFKD^{91} sequence (box B; residues 92−180) we were unable to detect any slow migrating band that would correspond to formation of an HMG/Pt-DNA complex (not shown). However, similar experiments with the B domain that had attached an additional ^{89}FKD^{91} sequence at the N-terminus of the box B revealed a detectable, albeit weak binding ($K_d \approx 1.4 \times 10^{-5}$ M) to cisplatin-modified DNA (Figure 3, box B3, residues 89−180). Further attachment of the ^{85}TKKK^{88} sequence to the N-terminus of the B domain revealed up to ~20-fold increase [$K_d \approx (6-8) \times 10^{-7}$ M] in affinity for cisplatin-modified DNA (Figure 3, box B7, residues 85−180). These results indicated that the presence of the N-terminal ^{85}TKKKFKD^{91} sequence (corresponding to a part of the A/B linker region of HMG-1, see Figure 1A) is a prerequisite for a high affinity binding of the isolated B domain to cisplatin-damaged DNA. Binding experiments with the B domain and Pt-DNA were therefore carried out throughout this paper with the B domain polypeptide containing the ^{85}TKKKFKD^{91} sequence (box B7).

Next EMSA experiments were aimed at investigation whether the ^{85}TKKK^{88} flanking sequence, when attached to the C-terminus of the A domain (box A1 in Figure 1A), could affect binding of the A domain to cisplatin-modified DNA. As shown in Figure 3, binding of isolated A domain to cisplatin-modified DNA was similar irrespective the presence (box A1, residues 1−88) or absence (box A, residues 1−84)

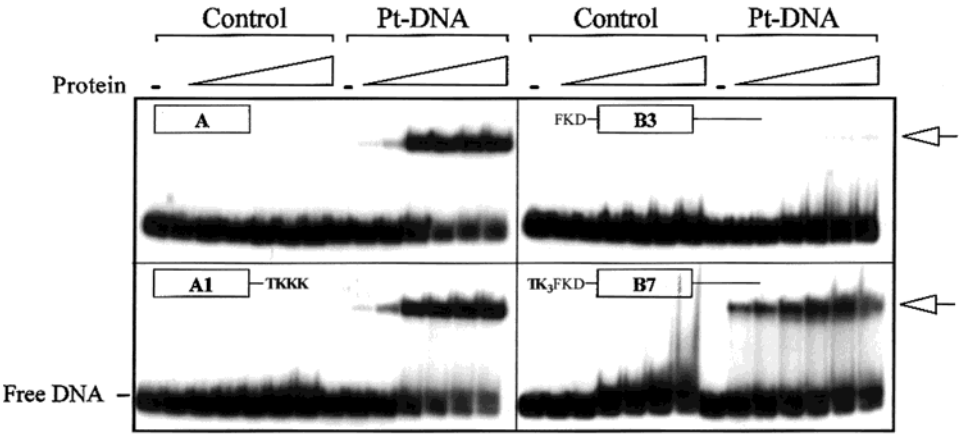

FIGURE 3: Binding of isolated A and B domains of HMG-1 to cisplatin-modified DNA. Cisplatinated DNA (Pt-DNA) was a ^{32}P-labeled 20-bp duplex containing a single 1,2-d(GpG) intrastrand cross-link. Approximately 4 nM of ^{32}P-labeled Pt-DNA or unmodified DNA (Control) was titrated with increasing amounts of individual HMG1-box polypeptides (0.2, 0.8, 1.5, 2.5, 3.5, and 5 μM, left to right). Top (left), A domain (box A); bottom (left), A domain (box A1); top (right), B domain (box B3); bottom (right), B domain (box B7). Dashed lines mean no protein added. Positions of specific complexes of platinated DNA with HMG-1 polypeptides are marked with arrows.

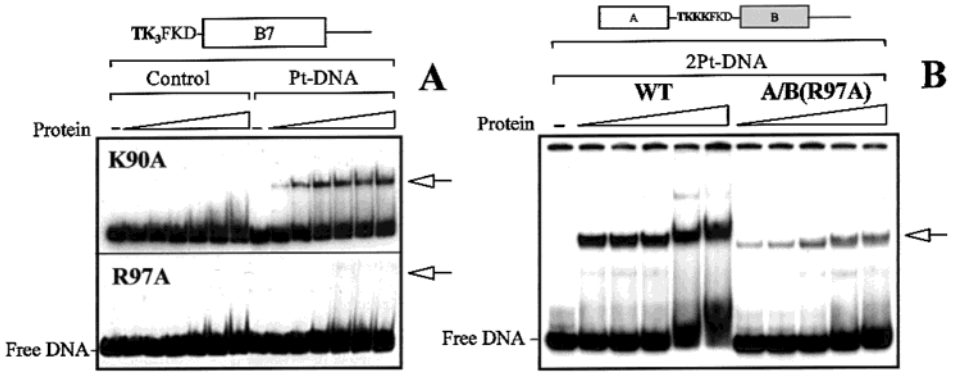

FIGURE 4: (A) Binding of K90A and R97A mutants of the B domain to cisplatin-modified DNA. DNA duplex of 20-bp containing a single Pt adduct (Pt-DNA) was used as in Figure 3. Approximately 4 nM of ^{32}P-labeled Pt-DNA or unmodified DNA (Control) was titrated with increasing amounts of the B domain (box B7) or the mutants (0.2, 0.8, 1.5, 2.5, 3.5, and 5 μM, left to right). (B) Binding of increasing amounts of the A/B didomain or the A/B(R97A) didomain mutant (0.01, 0.02, 0.05, 0.2, and 0.5 μM, left to right) to ~4 nM of ^{32}P-labeled DNA duplex of 40-bp containing two Pt adducts (2Pt-DNA). Positions of specific complexes of platinated DNA with HMG-1 polypeptides are marked with arrows.

of the ^{85}TKKK^{88} sequence ($K_d \approx 8 \times 10^{-8}$ M). Comparison of K_d values for specific binding of the A domain (box A1) and the B domain (box B7) revealed that the isolated A domain exhibited ~10-fold higher affinity for cisplatin-modified DNA than the B domain.

Mutation of Arg^{97} of the B Domain Significantly Decreased Binding of the HMG1-Box to Cisplatin-Modified DNA. From the published X-ray structure of the HMG-1 A domain with cisplatin-modified DNA it is evident that the precise positioning of the HMG-box on platinated DNA is directed by multiple interactions between the DNA and amino acid residues located on the N-terminal strand and helices I and II (*17*).

Two basic residues of the N-terminal strand of the HMG-1 B domain (Lys^{90} and Arg^{97}) were specifically targeted for mutagenesis with the aim to determine the importance of these residues for specific binding to cisplatin-modified or supercoiled DNA and four-way DNA junctions (4WJs), as well as for DNA bending and supercoiling in the presence of topoisomerase I. Lys^{90} corresponds to the amino acid residue of the B domain which forms a specific cross-link to platinum(II) of a single, site-specific 1,2-d(GpG) intrastrand adduct of a 15-bp oligonucleotide (*16*). Arg^{97} is a highly conserved residue in HMG-1/UBF and SRY/SOX families (*1*) and directly contacts the DNA backbone (*29*, *30*).

EMSA assays with the B domain in which Lys^{90} was replaced by alanine (K90A mutant) revealed that specific binding to cisplatin-modified DNA was retained (Figure 4A) and that the affinity of the mutant for platinated DNA was only moderately (>2-fold) decreased ($K_d \approx 1.5 \times 10^{-6}$ M) relative to that of the wild-type B domain [$K_d \approx (6-8) \times 10^{-7}$ M]. These results suggested that Lys^{90} is not critical for binding of the B domain to cisplatin-modified DNA.

Interesting results were obtained with the B domain in which Arg^{97} was replaced by alanine (R97A mutant). As shown in Figure 4A, binding of the B domain to cisplatin-modified DNA was severely compromised since only a very faint band corresponding to the HMG/cisplatin DNA complex was detected (higher amounts of the mutant led to intensive smearing with no apparent production of distinct bands, not shown). We have estimated from competition experiments (by titration of a fixed amount of the wild-type box B7 or B7(R97A) mutant/^{32}P-labeled Pt-DNA complex with increasing amounts of either unlabeled control DNA or Pt-DNA, see Materials and Methods) that the affinity of

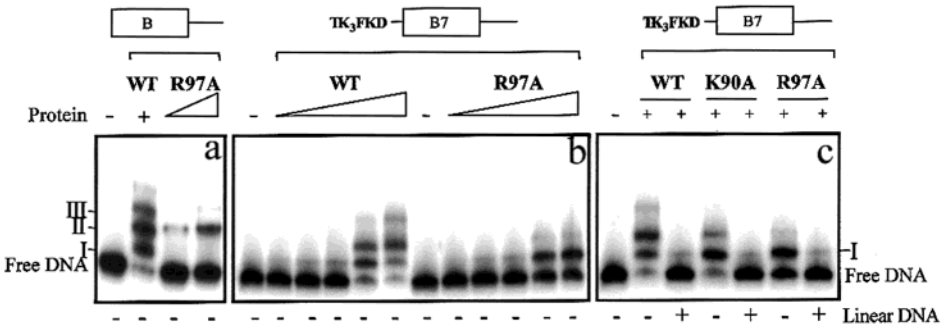

FIGURE 5: Binding of the B domain and mutants to four-way DNA junctions. Gel-retardation assay was carried out with ~1 nM ^{32}P-labeled four-way DNA junctions (4WJs). (A) Binding of the B domain lacking the N-terminal ^{85}TKKKFKD^{91} sequence (box B; 0.5 μM) or the R97A mutant (0.5 and 1 μM, left to right) to 4WJs. (B) Titration of 4WJs with increasing amounts of the B domain containing the additional N-terminal ^{85}TKKKFKD^{91} sequence (box B7; 0.01, 0.025, 0.05, 0.1, and 0.5 and 1 μM, left to right) or the R97A mutant. (C) Binding of the B domain containing the N-terminal ^{85}TKKKFKD^{91} sequence (box B7) and the K90A or R97A mutants to 4WJs in the absence or presence of unlabeled competitor DNA (linearized plasmid pBluescript at ~5000-fold mass excess over labeled 4WJs). The protein−DNA complexes were resolved on 5.8% polyacrylamide gels. Complexes that were formed upon binding of the B domain to 4WJs are indicated as I−III. WT, wild-type B domain.

the R97A mutant for cisplatin-modified DNA was >40-fold lower ($K_d \approx 3 \times 10^{-5}$ M) than that of the wild-type B domain (not shown).

No report is available in the literature whether both HMG domains of HMG-1 protein (and, if so, to what extent) can interact with cisplatinated DNA. However, asymmetric placement of the A domain with respect to the cisplatin adduct on a 16-bp DNA duplex suggests that another HMG domain could be easily accommodated near the cisplatin adduct (*17*). HMG-1 protein contains two covalently linked DNA-binding HMG domains, A and B, and a highly acidic C-tail (Figure 1A). When tested as a single domain, the A domain can bind Pt-DNA with ~10-fold higher affinity than isolated B domain (Figure 3). We have therefore asked whether Arg^{97} is still important for binding to Pt-DNA when the B domain is attached to the A domain. To address this question, recombinant A/B didomain (i.e., covalently linked A and B domains corresponding to the HMG-1 protein lacking the acidic C-tail, residues 1−180, see Figure 1A) and the A/B(R97A) didomain mutant were used. As high-affinity binding of HMG-1 to cisplatin-modified DNA requires multiple cisplatin adducts on DNA (*24*, *31*−*33*), we have used for EMSA with the A/B didomain not the 20-bp DNA duplex containing a single Pt adduct (used throughout this paper for binding studies with isolated HMG-1 domains) but a 40-bp DNA duplex containing two identical 1,2-intrastrand d(GpG) cisplatin adducts spaced by 20 nucleotides (2Pt-DNA). As shown in Figure 4B, binding of the A/B didomain to 2Pt-DNA resulted in appearance of a strong retarded band (corresponding to the HMG/Pt-DNA complex), the intensity of which increased depending on the amounts of the A/B didomain (Figure 4B). Similar EMSA experiments with the A/B(R97A) didomain mutant revealed that mutation of Arg^{97} to alanine significantly decreased (~3−5-fold) binding of the A/B didomain to 2Pt-DNA. This result was an indication that either the affinity of the A domain for cisplatinated DNA was much lower within the A/B didomain than when tested as a single domain or that the A domain of the A/B didomain enhanced binding of the mutated B domain to cisplatin-modified DNA (and also other activities as shown below in this paper). It remains to be elucidated whether the specific recognition of cisplatinated DNA by the two HMG-1 domains is further modulated by the acidic C-tail of HMG-1, as previously shown for some DNA binding and bending activities of HMG-1 (*22*, *34*−*36*).

Recognition of Cisplatin-Modified DNA and DNA Bending by the B Domain Is More Sensitive to Mutation of Arg^{97} Than Binding to Four-Way DNA Junctions. It was shown earlier that both individual A and B domains of HMG-1 selectively recognize synthetic four-way DNA junctions (4WJs) that are very likely to have features common to their natural, yet unknown, cellular binding targets (reviewed in refs *1* and *3*). Gel-retardation assays revealed that the affinity of the isolated B domain for 4WJs was higher than that of the A domain (*21*). The HMG domain binds 4WJs by formation of a high-affinity complex I (arising likely from binding to the DNA junction crossover), followed by formation of complex II (binding of a second HMG-box molecule to low-affinity 4WJ arms) or higher (refs *6* and *40* and also Figure 5A). Recently, we have demonstrated that mutation of Arg^{97} to alanine significantly (>50-fold) diminished the affinity of the B domain (box B) for 4WJs and altered the mode of binding by producing only complexes II or higher (but *not* complex I) without changing the structural selectivity for 4WJs (ref *10*, see also Figure 5A). Although the attachment of the additional ^{85}TKKKFKD^{91} sequence to the N-terminus of the wt B domain (box B) resulted in only moderate increase (~2−3-fold) in binding affinity for 4WJs (not shown), the presence of the seven-residue N-terminal sequence within the R97A mutant could fully restore the normal binding mode of the B domain to 4WJs (i.e., formation of complexes I and II, Figure 5B). The above N-terminal sequence within the B domain also significantly increased (>15-fold) the affinity of the B7-(R97A) mutant for 4WJs relative to the B(R97A) mutant. Nevertheless, the affinity of the B7(R97A) mutant for 4WJs was still >3-fold lower than that of the wild-type B domain (box B7), suggesting that Arg^{97} is required for binding of the B domain to 4WJs even if the N-terminal flanking ^{85}TKKKFKD^{91} sequence is attached to the B domain. On the other hand, gel-retardation assays with the K90A mutant of the B domain (box B7) revealed that the affinity (as well as mode of binding) was similar to the wt B domain (Figure 5C, lanes 2 and 4, left to right). In addition, binding specificity (i.e., formation of complex I) for 4WJs was

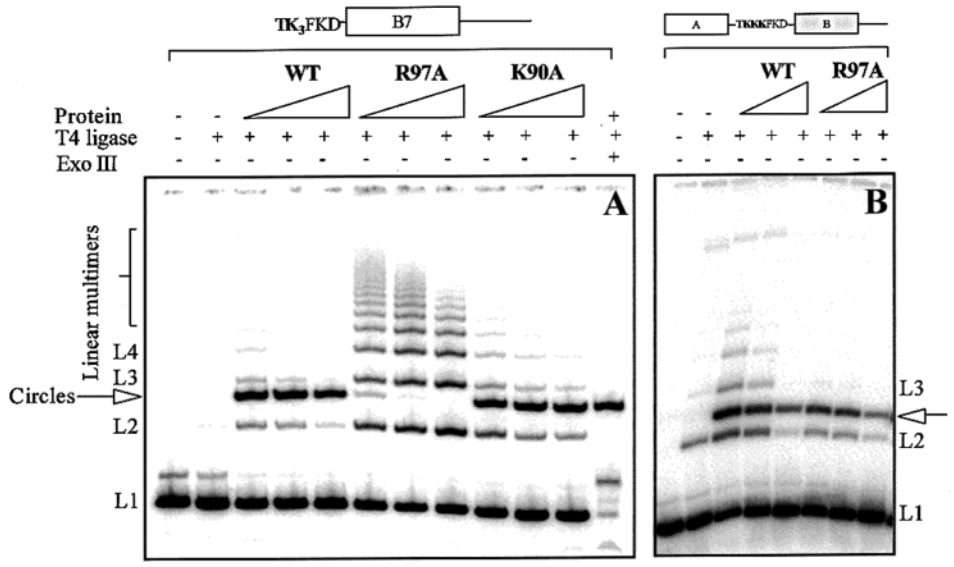

FIGURE 6: DNA bending by the B domain, A/B didomain and mutants. The 5′-end ^{32}P-labeled 123-bp DNA duplex (~1 nM) was preincubated with different concentrations of the HMG-1 polypeptides. (A) The B domain (box B7) and the K90A or R97A mutants (0.05, 0.25, and 0.5 μM; left to right). (B) The A/B didomain and the R97A mutant (0.5, 0.75 and 1.5 μM; left to right). The preincubation was followed by ligation with T4 DNA ligase at 30 °C for 20 min. The deproteinised ligation products were then subjected to electrophoresis on a 5% nondenaturing gel in 0.5× TBE buffer. Exo III digestion was used to verify the production of DNA circles (marked by arrows). L1, L2, L3, and L4 stand for linear monomers, dimers, trimers, and tetramers, respectively (position of longer linear multimers is also indicated).

retained for both the K90A and R97A mutants (box B7) as suggested from competition experiments in the presence of a large excess of linear DNA (Figure 5C, lanes 3, 5, and 7, left to right).

HMG-1 protein or its B domain could not only recognize prebent and distorted DNA structures, it could also effectively bend/loop DNA (*11*, *12*, *21*−*23*, *41*−*44*). In the present paper, we have tested the importance of Lys^{90} and Arg^{97} for the ability of the B domain (box B7) to bend DNA using a ligase-mediated circularization assay. This assay measures the efficiency with which T4 DNA ligase forms circles from fragments of DNA that are shorter than the persistence length (<150 bp). In the absence of internal curvature, the stiffness of these DNA fragments prevents intramolecular alignment of their ends so that circles (resistant to exonuclease III digestion) are detected only in the presence of proteins that bend DNA.

Previously, we have demonstrated using the circularization assay that the ability of the HMG-1 B domain to bend DNA was dependent on the presence of the N-terminal ^{89}FKD^{91} sequence (box B3) and that Lys^{90} within the latter sequence was crucial for an efficient flexure by the B domain (*22*). In this paper, we have extended the latter study by showing that the B domain with the N-terminal ^{85}TKKKF*A*D^{91} sequence [Lys^{90} of the box B7 mutated to alanine, the B7-(K90A) mutant] was able to promote circularization of an 124-bp DNA fragment with a comparable efficiency to the wild-type B domain (box B7; Figure 6A). The latter result suggests that Lys^{90} is dispensable for DNA bending if the B domain is flanked at the N-terminus with the ^{85}TKKK^{88} sequence (Figure 6A). Circularization assay with another studied B domain mutant, B7(R97A), revealed a significantly reduced (>10-fold) or even completely suppressed (at higher amounts of the mutant added) formation of DNA minicircles relative to the effect of the wt B domain (Figure 6A). This finding suggests that arginine at position 97 is involved in DNA bending by the isolated B domain (enhanced formation of linear DNA multimers in the course of DNA ligation in the presence of the HMG domain, as well as decreased production of DNA minicircles at higher amounts of the A/B didomain, are consistent with previous reports, refs *22*, *43*, and *50*).

The single A domain was previously shown to be very inefficient in formation of DNA minicircles (*22*). Although the DNA bending potential of HMG-1 appears to be due predominantly by the B domain (box B7), covalent attachment of the A domain to the B domain (A/B didomain of HMG-1) was shown to be necessary for efficient DNA flexure (*22*). In this paper we have asked whether attachment of the A domain to the B domain having Arg^{97} mutated to alanine [A/B(R97A) didomain mutant] could influence the ability of the polypeptide to bend DNA. As shown in Figure 6B, the A/B(R97A) didomain mutant could produce DNA minicircles with the efficiency only less than 2-fold reduced relative to the wild-type A/B didomain (Figure 6B). From these results it might follow that the A domain, when linked to the B domain (A/B didomain of HMG-1), either directly promotes efficient DNA flexure (unlike the single A domain; ref *22*) or enhances the ability of the B domain to bind and bend DNA and thus compensates for the effect of replacement of Arg^{97} by alanine.

B Domain Is the Primary Determinant of a Preferential Binding of HMG-1 to Supercoiled DNA. HMG-1 protein, like its truncated form that lacks the highly acidic C-terminal region (A/B didomain), preferentially binds to supercoiled DNA over linear or relaxed closed circular DNAs, suggesting that the two HMG1-box domains (and *not* the acidic C-tail) determine the preferential binding of the protein to supercoiled DNA (*7*, *23*). Previously, we have found using gel retardation assay that the isolated B domain (box B) exhibited no preference for supercoiled DNA over other DNA forms (*23*). In this paper, we have shown that attachment of the ^{89}FKD^{91} sequence to the N-terminus of the box B did not confer any preference of the B domain to supercoiled DNA

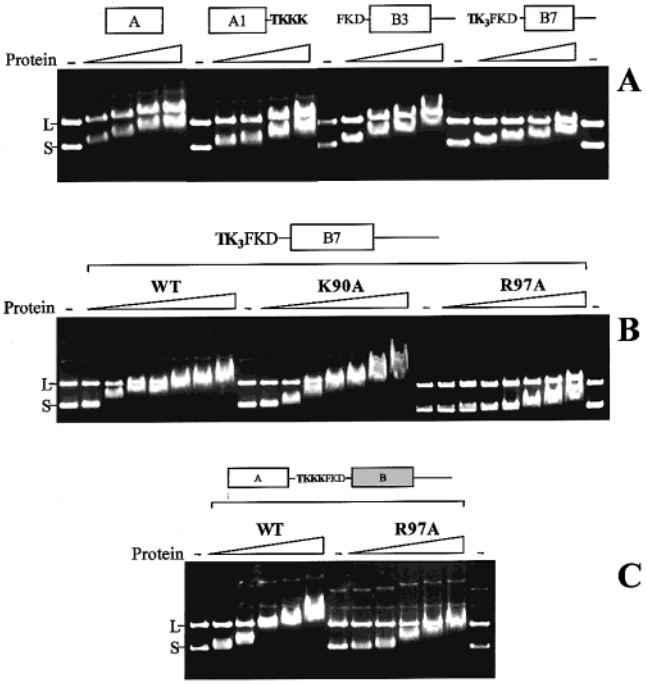

FIGURE 7: (A) B domain is the primary determinant of a preferential binding of HMG-1 to supercoiled DNA. HMG-1 polypeptides at concentrations of ~0.7, 0.8, 1.1, and 1.4 μM (boxes A1 and B3), and ~0.4, 0.5, 0.6, and 0.7 μM (boxes A and B7) were preincubated on ice with equimolar mixtures of supercoiled and linearized plasmid DNA (~10 nM), followed by resolution on 1% agarose gels. (B) Preferential binding of B domain to supercoiled DNA is abolished by mutation of Arg^{97} to alanine. Gel retardation experiments were carried out as in panel A but at ~0.3, 0.6, 0.9, 1.1, 1.25, 1.55, and 1.85 μM of the HMG-1 polypeptides (left to right). (C) A/B didomain preferentially binds supercoiled DNA regardless the mutation of Arg^{97} within the B domain. Gel retardation experiments were carried out as in panel A but at ~0.17, 0.33, 0.67, 1, and 1.3 μM of the wt A/B didomain or the A/B(R97A) didomain mutant. Agarose gels were stained with SybrGreen. S and L stands for supercoiled and linear plasmid DNA, respectively. WT, wild-type.

[Figure 7A (box B3)]. However, when the additional ^{85}TKKK^{88} sequence was linked to the N-terminus of the box B3, the resulting B domain polypeptide (box B7) exhibited up to 3-fold higher affinity for supercoiled DNA and it could bind supercoiled DNA with a clear preference over linear DNA (i.e., retardation of linear DNA was not detected until the retarded supercoiled DNA reached the position of linear DNA; Figure 7A). Although the presence of the ^{85}TKKK^{88} sequence at the C-terminus of the A domain visibly increased retardation of supercoiled DNA relative to linear DNA, the single A domain did not exhibit any exclusive binding to supercoiled DNA, Figure 7A (boxes A or A1). These results strongly suggest that the primary determinant for the preferential binding of HMG-1 to supercoiled DNA is the B domain containing the N-terminal ^{85}TKKKFKD^{91} sequence.

We have then asked whether residues Lys^{90} and Arg^{97} are important for the preferential binding of the B domain (box B7) to supercoiled DNA. As shown in Figure 7B (K90A), replacement of Lys^{90} to alanine had only very little, if any, effect on a preferential binding (and affinity) of the B domain to supercoiled DNA. On the other hand, affinity of the R97A mutant for supercoiled DNA was significantly lower than that of the wild-type B domain and the preferential binding of the mutant to supercoiled DNA was visibly compromised [i.e., retardation of linear DNA was detected before the retarded supercoiled DNA reached the position of the originally unretarded linear DNA; Figure 7B (R97A)]. Interestingly, the effect of mutation of Arg^{97} to alanine on the ability of the B domain to preferentially bind to supercoiled DNA was fully eliminated if the B domain was covalently linked to the A domain [Figure 7C, A/B (R97A) didomain mutant]. Thus, the A domain can promote (directly or indirectly) the ability of HMG-1 protein to preferentially recognize and bind supercoiled DNA, a function which is manifest primarily through the B domain with the lysine-rich N-terminal flanking sequence promoting the high-affinity binding of the HMG domain to DNA.

Arg^{97}, but Not Lys^{90}, Is Involved in DNA Supercoiling by the B Domain. HMG-1 and its isolated 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 (*21*, *34*, *35*, *45*). Previously, we have shown that a number of amino acid residues within the N-terminus and helix I of the B domain (box B) are required for topoisomerase I-mediated DNA supercoiling (*10*). In this paper, we have investigated the importance of Lys^{90} and Arg^{97} on topoisomerase I-mediated DNA supercoiling by the B domain containing the additional N-terminal ^{85}TKKKFKD^{91} sequence (box B7).

Two assays were used to assess the ability of the B domain and mutants to supercoil DNA. First, relaxed closed circular DNA was incubated with increasing amounts of the B domain in the presence of topoisomerase I to remove any plectonemic supercoils that formed in protein-free regions of the DNA ("DNA supercoiling assay"). Second, the B domain was preincubated with supercoiled DNA and topoisomerase I was then added to study the extent of protection of supercoiled DNA from relaxation ("DNA protection assay"). Distribution of DNA topoisomers was analyzed on 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 intercalative drug chloroquine. Chloroquine intercalates into DNA which results in decreased mobility of negative supercoils and increased mobility of positive supercoils.

Here we have demonstrated that the wild-type B domain containing the N-terminal ^{85}TKKKFKD^{91} sequence (box B7) was only slightly more efficient in DNA supercoiling (or protection of supercoiled DNA from relaxation by topoisomerase I) than the B domain lacking the latter sequence (box B), Figure 8A. Substitution of Lys^{90} to alanine had very little, if any, effect on the ability of the B domain (box B7) to supercoil DNA or protect supercoiled DNA from relaxation by topoisomerase I (Figure 8A). Two-dimensional agarose gel electrophoresis in the presence of chroroquine revealed that the sign of DNA supercoiling generated by the K90A mutant was negative, similarly to the wild-type B domain (Figure 8B).

Recently, we have shown that the R97A mutant of the B domain (box B) was very little efficient in DNA supercoiling or protection of supercoiled DNA from relaxation by topoisomerase I (ref *10*; see also Figure 8A). The sign of the inserted DNA supercoils by the latter mutant was shown

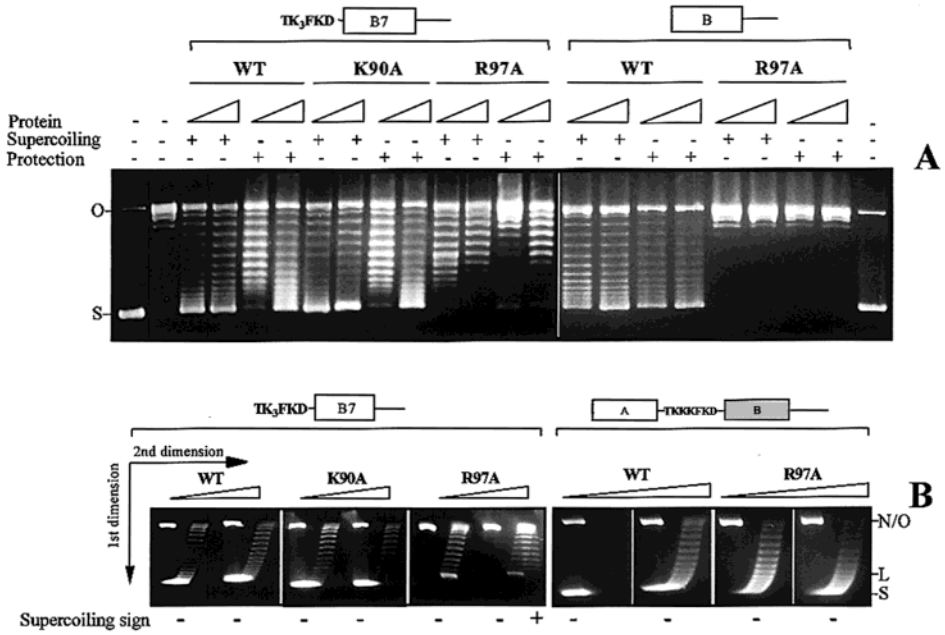

FIGURE 8: DNA supercoiling and protection from relaxation by topoisomerase I. (A) Supercoiling assay: circular relaxed plasmid pBR322 DNA was incubated with the B domain (boxes B or B7) or mutants at protein/DNA molar ratios of 500 and 2000 (left to right) in the presence of topoisomerase I. Protection assay: supercoiled plasmid DNA was preincubated with HMG polypeptides at molar ratios indicated above and then treated with topoisomerase I. Deproteinised DNA samples were subjected to electrophoresis on 1% agarose gels (first-dimension). (B) Electrophoresis in the second-dimension. Deproteinised DNA topoisomers (generated by incubation of relaxed closed circular plasmid DNA with box B7, A/B didomain, and mutants at molar ratios 500 and 2000 in the presence of topoisomerase I) were resolved on agarose gels in the first-dimension, and the gels were then soaked in chloroquine, turned by 90° and subjected to electrophoresis in the second-dimension. The gels were stained with Sybr Green. L, linearized DNA; S, supercoiled plasmid DNA. O, relaxed closed circular plasmid DNA. N/O, nicked and intact relaxed closed circular plasmid DNA. WT, wild-type.

Table 1: Summary of Relative Activities of Different HMG-1 Domains

HMG-1 polypeptides	Binding to Pt-DNA	Binding to 4WJs	Binding to scDNA	DNA supercoiling	Supercoiling sign	Preference for scDNA	DNA bending
A	++++	+++[a]	++++[c]	++++[c]	-[c]	no	+[b]
A1-TKKK	++++	n.d.	++++[c]	++++[c]	-[c]	no	+[b]
B	-	++[d]	+++	++	-[d]	no[e]	-[d]
B(R97A)	-[c]	-	+[d]	-	+[d]	no[c]	-[c]
TKKKFKD-B7	+++	++++	+++	+++	-	yes	+++
TKKKFAD-B7(K90A)	++	++	+++	+++	-	yes	+++
TKKKFKD-B7(R97A)	-	++	++	+	+/-	no	+
A-TKKKFKD-B	++++	++++[f]	++++	++++	-	yes	+++[b,f]
A-TKKKFKD-B(R97A)	+	n.d.	+++	+++	-	yes	+++

[a] Ref *21*. [b] Ref *22*. [c] (Unpublished results). [d] Ref *23*. [e] Ref *10*. [f] Ref *43* and this paper. Relative activities of the individual HMG-box polypeptides: (++++) >100%; (+++) 50−100%; (++) 30−50%; (+) 10−30%; (−) <5%. n.d., not determined. scDNA, supercoiled DNA; 4WJs, four-way DNA junctions; Pt-DNA, cisplatin modified 20- or 40-bp DNA duplex modified with cisplatin, Supercoiling sign: (−) negative; (+) positive; (±) mixture of positive and negative topoisomers.

to be exclusively positive (*10*). Here we show that the attachment of the additional N-terminal ^{85}TKKKFKD^{91} sequence to the R97A mutant of the B domain (box B) can partially restore the ability of the B domain to supercoil DNA or to protect supercoiled DNA from relaxation [Figure 8A (box B7)]. Resolution of the DNA topoisomers in the second dimension in the presence of chloroquine revealed negative supercoils at low protein/DNA molar ratios but both negative and positive DNA supercoils at higher ratios [Figure 8B, B7 (R97A)]. The latter results indicated that the presence of the lysine-rich N-terminal sequence within the B domain can partially compensate for the effect of mutation of Arg^{97} on DNA supercoiling. In addition, as in the case of binding to supercoiled DNA (Figure 7C), mutation of Arg^{97} to alanine had very little, if any, effect on the distribution of introduced DNA topoisomers (and did not bring about a change in the

sign of supercoiling) when the B domain was covalently linked to the A domain [Figure 8C, A/B (R97A) didomain mutant].

In summary, we have demonstrated an important role of a basic part of the A/B linker region of HMG-1 (the ^{85}TKKKFKD^{91} sequence) and Arg^{97}, in DNA binding, bending, supercoiling, and discrimination between supercoiled and linear DNA by the B domain (Table 1). Some of the above-reported activities of the B domain were enhanced when the B domain was covalently linked to the A domain.

CONCLUDING REMARKS

Cisplatin-modified DNA, has a single kinked site for which the HMG-boxes of HMG-1 have increased affinity and specificity (*17*, *46*). We propose that the observed differences in binding of the two B domain mutants (R97A and K90A) to cisplatinated DNA are likely due to distinct contacts of Lys^{90} and Arg^{97} with DNA. Whereas Lys^{90} (and very likely also the lysine-rich part of the A/B linker region that flanks the HMG-box, the ^{85}TKKKF*K*D^{91} sequence) is expected to interact in the DNA *major* groove where the cisplatin adduct is localized (as suggested from photoaffinity cross-linking using a photoaffinity label attached to platinum; ref *16*), Arg^{97} contacts the widened *minor* groove of DNA where the HMG-box binds (*17*, *29*, *30*, *37*−*39*).

We have shown that evolutionarily highly conserved lysine-rich ^{85}TKKKFKD^{91} sequence (*1*), that is a part of the A/B linker region of HMG-1, is absolutely essential for a high-affinity binding of the HMG-1 B domain to cisplatin-modified DNA and for preferential binding to supercoiled DNA. The latter sequence is also a prerequisite for efficient DNA flexure by the B domain (*22*). Increased DNA-binding specificity by the longer B-domain peptide (box B7) may be due to the generation of favorable contacts of the N-terminal and C-terminal flanking sequences helping the HMG domain to anchor to DNA (this paper and refs *22*, *43*, and *47*). The observed enhancement of DNA binding activities of the B domain within the A/B didomain (this paper; see also refs *22* and *43*) does not likely originate from mutual interactions of the two HMG domains since no interactions between A and B domains were detected by NMR in free solution (*43*). We conclude that the A/B linker region is likely required for stable binding and proper positioning of the A and B domains of the HMG-1 protein in the widened *minor* groove of the differently bent and distorted DNA molecules (*30*, *37*, *48*. *49*). It remains to be established whether the interactions of the two HMG domains of HMG-1 with cisplatinated DNA may be selectively "tuned up" by the acidic C-terminal tail as previously shown for other DNA structures (*34*−*36*, *51*).

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