The Specific Interactions of HMG 1 and 2 with Negatively Supercoiled DNA Are Modulated by Their Acidic C-Terminal Domains and Involve Cysteine Residues in Their HMG 1/2 Boxes†,‡

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ABSTRACT: Sedimentation and gel retardation studies show a stronger interaction of HMG 1 and 2 with negatively supercoiled DNA than with linear, nicked-circular, or positively supercoiled ds-DNA. An apparent unwinding angle of 58° was obtained for HMG 1 and 2 when assayed by protection of negatively supercoiled DNA from topoisomerase I relaxation or when assayed by the supercoiling of nicked-circular DNA with T4 DNA ligase. The protection of negatively supercoiled DNA was linear up to molar ratios of about 250:1. There was little change in binding reactions or in the protection of supercoiled DNA at ratios above 250:1, indicating that both activities saturate and that HMG 1 and 2 have binding site sizes of about 20 bp. P1, the major tryptic fragment of HMG 1 or 2 which retains the two DNA binding HMG 1/2 boxes, displays a 2-fold increase in binding to all types of ds-DNA compared to intact HMG 1 or 2. However P1 protects negatively supercoiled DNA from topoisomerase I relaxation about 5-fold less than intact HMG 1 or 2. Complete protection with P1 occurs at a molar ratio 1040:1, indicating a DNA binding site size of about 4 bp and an apparent unwinding angle of 10°. P1 binding to closed-circular ss-DNA also involves a binding site of about 4 bp. Adding the acidic C-terminal fragment to P1 reversed its binding and allowed topoisomerase I to relax supercoiled DNA. These findings highlight the importance of the acidic C-terminal domains of HMG 1 and 2 in limiting electrostatic interactions of the HMG 1/2 boxes with ds- or ss-DNA. *N*-Ethylmaleimide inhibited the binding of intact HMG 1 or 2 to negatively supercoiled DNA, but did not inhibit the electrostatic binding of HMG 1 or 2 to ss-DNA, or of P1 to any form of DNA (ds or ss). These results suggest that cysteine residues are involved in the specific interaction of HMG 1 or 2 with negatively supercoiled DNA and that the acidic C-terminal domains modulate an intramolecular conformational change involving sulfyhydryls within the HMG 1/2 boxes.

The high mobility group (HMG)[1] proteins 1 and 2 are the most abundant non-histone eukaryotic chromatin proteins and are found in fractions of chromatin that are involved in a variety of DNA metabolic processes. On the basis of their abundance, their ability to supercoil DNA, and their apparent role in both transcription and DNA replication, it has been suggested that they are structural proteins involved in modulating chromatin conformation via changes in DNA topology and/or accessibility (Bustin & Soares, 1985; Singh & Dixon, 1990). The high degree of sequence homology between HMG 1 and 2 suggests that both proteins have similar functions, which are presumably related to their unusual three-domain structure (Reeck et al., 1982; Cary et al., 1984; Carballo et al., 1983): they contain two DNA binding domains (A and B, the HMG 1/2 boxes), which are rich in basic and aromatic amino acids (Davis & Burch, 1992; Jantzen et al., 1990; Bianchi et al., 1992), and a protein binding domain at the C-terminus which is highly enriched in acidic amino acids (25–30 residues in length) (Cary et al., 1984; Bonne-Andrea, 1984).

The level of torsion in double-stranded DNA regulates base-pair stability and DNA conformation (Hsieh & Wang, 1975) and appears to be important in the initiation and regulation of specific DNA metabolic processes (Weintraub, 1985; Tabuchi & Hirose, 1988) as well as in chromatin assembly (Germond, 1975). Since HMG 1 preferentially binds to and protects negatively supercoiled DNA from topoisomerase I relaxation, we have suggested that HMG 1 may transiently regulate or conserve torsion in negatively supercoiled DNA, functions which may be important in a number of different aspects of DNA metabolism related to the regulation of local DNA conformation (Sheflin & Spaulding, 1989). In addition to binding to negatively supercoiled DNA, HMG 1 and 2 have been shown to preferentially bind to AT-rich sequences (Brown & Anderson, 1985), to Z-DNA (Waga et al., 1988), and to a four-stranded ds-DNA structure (Bianchi et al., 1989). A common feature shared by these DNAs is that they are slightly unwound and they can bend. The recent finding that HMG 1 binds specifically to cisplatin-modified DNA, which is also bent and unwound, suggests that DNA bending and/or unwinding are important features required for the preferential interaction of HMG 1 with DNA (Pil & Lippard, 1992).

HMG 1 and 2 share both sequence homology and functional activity with several specific transcription factors (Jantzen et

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[1] Abbreviations: HMG, high mobility group; ds-DNA, double-stranded DNA; ss-DNA, single-stranded DNA; DTT, dithiothreitol; BSA, bovine serum albumin; Topo I buffer, 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM Na_2EDTA, and 30 μg of BSA; TAE, 40 mM Tris–acetate and 2 mM EDTA (pH 8.0); TBE, 89 mM Tris–borate, 89 mM boric acid, and 2 mM EDTA (pH 8.0); SDS, sodium dodecyl sulfate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; GTG, genetic technology grade; EtdBr, ethidium bromide; CT, calf thymus; PBE, polybuffer exchange resin; NEM, *N*-ethylmaleimide; BME, 2-mercaptoethanol.

al., 1990; Singh & Dixon, 1990; Wen et al., 1989). Probably the most important feature is the "HMG 1/2" box which has been found in a variety of DNA binding proteins (Davis & Burch, 1992; Jantzen et al., 1991). This novel DNA binding motif is conserved in other abundant nuclear (hUBF) and mitochondrial (mtTF1 and ABF2) transcription factors which bind specifically to negatively supercoiled DNA and not to ss-DNA (Fisher et al., 1992; Diffley & Stillman, 1992). Another important structural feature of HMG 1 and 2 and HMG T is the highly acidic C-terminal domain. This C-terminal domain apparently modulates the ability of HMG 1 and 2 to interact with core histones to promote nucleosome assembly (Bonne-Andrea et al., 1986; Dimov et al., 1990); with histone H1 to regulate chromatin condensation (Cary et al., 1979; Carballo et al., 1983); with RNA polymerases to regulate transcription (Stoute & Marzluff, 1982); with transcription factors to enhance specific gene expression (Tremethick & Molloy, 1986, 1988); and with DNA polymerase α to enhance DNA replication (Alexandrova & Betchev, 1988; Dimov et al., 1990). The acidic domains of HMG 1 and 2 also appear to regulate the DNA binding characteristics of the intact proteins (Carballo et al., 1983, 1984; Isackson et al., 1983). The combination of the binding of the HMG 1/2 box to negatively supercoiled DNA plus the binding of the acidic domain to other nuclear protein could explain why HMG 1 and 2 are involved in so many different aspects of DNA metabolism.

We have obtained evidence that the acidic C-terminal domain regulates protein conformation of HMG 1 and 2 and is required for their specific binding to negatively supercoiled DNA. We note several features that are conserved in vertebrate HMG 1 and 2 which may be involved in modulating torsional tension in chromatin.

MATERIALS AND METHODS

Materials. Calf thymus (CT) topoisomerase I was obtained from Bethesda Research Labs. GTG-agarose was from FMC (Seakem). Cloned T4 gene 32 protein and *Escherichia Coli* single-strand DNA binding protein (SSB) were from United States Biochemical. Mono-S and PBE 94 columns were from LKB/Pharmacia. M13 closed-circular DNA (7229 bp) was purchased from Boehringer Mannheim Biochemicals. Highly negatively supercoiled pBR322 DNA (4363 bp) was grown and amplified in HB101 cells according to the procedure of Holmes and Quigley (1981). CsCl-purified plasmid DNA was characterized by gel electrophoresis, and its superhelical density (–0.045) was determined by the band-counting technique of Keller (1975) and Shure et al. (1977). DNA concentrations were determined by measuring the optical density of unknown DNA solutions at 260 nm, using calf thymus, λ phage, and *E. coli* DNA as standards.

Purification and Analysis of HMG 1, HMG 2, and Their Respective Tryptic Peptides. HMG 1 and 2 were prepared from fresh calf thymus glands by the nondenaturing salt extraction technique of Marekov et al. (1984) and further purified by anion-exchange chromatography on Mono-S (Pharmacia) (Sheflin & Spaulding, 1989). HMG 1 eluted at concentrations between 0.22 and 0.26 M NaCl, and HMG 2 eluted between 0.27 and 0.32 M NaCl. After desalting, the purified proteins were stored at –80 °C in 0.5 mM DTT to prevent oxidation: all reactions were performed in the presence of 0.5 mM DTT. Under these reducing conditions, purified HMG 1 and HMG 2 ran as single protein bands on both SDS and acid–urea polyacrylamide gels when assessed by silver staining (Bofinger et al., 1988).

The major tryptic fragment, P1, containing the two DNA binding domains A and B (Reeck et al., 1982; Isackson et al., 1983), was prepared from both HMG 1 and HMG 2 according to the procedure of Isackson et al. (1983). Briefly, the Mono-S-purified proteins were treated with TPCK-treated trypsin (Sigma) at a protein-to-enzyme ratio of 100:1 for 90 min at 4 °C. These digestion conditions routinely gave about 50% digestion of the intact protein, producing the major peptide product "P1" without significant amounts of the smaller products "P2" or "P3" (Isackson et al., 1983), as determined by Coomassie staining of acid–urea polyacrylamide gels. The digest was then diluted 10-fold with 10 mM sodium borate and repurified on the Mono-S column. The fractions containing P1 from HMG 1 or 2 eluted separately from undigested HMG 1 or 2, at concentrations between 0.34 and 0.43 M NaCl. When the fractions containing the P1 fragment were characterized on acid–urea polyacrylamide gels, oxidized and reduced forms could be resolved under nonreducing and reducing conditions, respectively. Peaks containing only P1 were pooled and desalted, and dithiothreitol was added (0.5 mM), before storage in aliquots at –80 °C. All studies were performed with freshly-thawed aliquots of proteins or peptides.

The acidic C-terminal fragments (Reeck et al., 1982; Isackson et al., 1983) from tryptic digestion of HMG 1 and HMG 2 were purified by anion-exchange chromatography on a PBE-94 column (Yoshida, 1987), and their amino acid compositions were determined. Amino acid analysis revealed the C-terminus of calf HMG 1 contained 2 Lys, 21 Glu, and 8 Asp [on the basis of cDNA analysis, it should contain 2 Lys, 21 Glu, and 9 Asp (Kaplan & Duncan, 1988; see Figure 5)]. The C-terminus of calf thymus HMG 2 contained 1 Lys, 1 Pro, 20 Glu, and 5 Asp; on the basis of the cDNA analysis for pig thymus HMG 2 it should contain 1 Lys, 1 Pro, 19 Glu, and 5 Asp (Shirakawa et al., 1990). [cDNA analyses in several species have confirmed that the acidic C-terminal region of HMG 2 is slightly shorter than HMG 1, has an increased Glu/Asp ratio, and contains Pro (Tsuda et al., 1989; Shirakawa et al., 1990; Majundar et al., 1991; Davis & Burch, 1992).]

The concentrations of the purified proteins and peptides were determined spectrophotometrically (Beckman DU-8B) on solutions in distilled water at 214 nm, using BSA as a standard.

Retardation of DNA Mobility by the Binding of HMG 1/2 and P1 Fragments. 1. Interactions with Single-Stranded DNA. Gel retardation of ss-DNA was assessed as described by Lohman et al. (1986) with slight modifications: M13 ss-DNA was preincubated with the various purified proteins in the buffer described in the figure legends, in a final volume of 25 μL for 15 min at 37 °C. Loading dye containing Bromphenol Blue (0.25%), sucrose (40%), and 20 mM EDTA was then added (1/10 volume), and 25-μL samples were immediately loaded onto horizontal 0.4% GTG–agarose gels and subjected to electrophoresis for 5 h at 3.5 V/cm and then for 15 h at 0.25 V/cm in 1 × TAE buffer at room temperature. The gels were stained with 0.5 μg/mL ethidium bromide in 1 M NaCl/1 mM EDTA for 1h, destained in H_2O for 30 min, and photographed using Polaroid 665 film. The relative migration of the M13 ss-DNA in the presence of various amounts of the different proteins was compared to DNA run alone on the same gel. Percent retardation of DNA was determined by subtracting the mean distance migrated by the DNA–protein complex from 1. Thus control DNA in the absence of any exogenous protein has a percent retardation value of 0.

2. Sucrose Gradient Analysis. The effect of HMG 1 or 2 on the sedimentation of nicked-circular and negatively supercoiled pBR322 DNA was assessed on linear 10–20% sucrose gradients (containing 5 mM β-mercaptoethanol) as described by Bonne et al. (1980). The percent retardation of DNA (expressed as the change in the mean peak position of the DNA) was plotted against the molar ratio of HMG 1 or HMG 2 to the input DNA.

3. Interactions with Double-Stranded (ds-) DNA. Gel retardation of different forms of pBR322 ds-DNA (negatively and positively supercoiled, linear, and nicked-circular forms) was assessed by preincubating various concentrations of the proteins or tryptic fragments with DNA in topoisomerase I buffer (BRL) for 15 min at 37 °C in a reaction volume of 50 μL. Half the reaction volume was used to assess the effect of calf thymus topoisomerase I relaxation (see below). The remaining half of the reaction was placed on ice for 30 min and preincubated for 5 min at 37 °C, then loading dye was added, and 20 μL was loaded onto 1% GTG–agarose gels. Samples were subjected to electrophoresis for 18–20 h at 7.5 V/cm min in 1 × TBE buffer at room temperature. The gels were stained with 0.5 μg/mL EtdBr for 1 h, destained, and photographed. The relative migration of supercoiled DNA was determined over the range of protein concentrations indicated in the figure legends. The percent retardation of negatively supercoiled DNA per mole of protein was determined by subtracting the mean migration distance of the DNA–protein complex from 1 as described above for ss-DNA. Note that, at high concentrations of protein, DNA bands were retarded unevenly across the gel slot, producing a characteristic "M" pattern (also observed with high concentrations of H1). We suspect that this reflects a gradient of salt concentrations affecting electrostatic binding to DNA or protein–protein interactions. The fastest migrating DNA in the center of each lane was used to determine the migration of the DNA. This pattern was more pronounced for P1 than for the parent protein at any given concentration. Adding SDS (Figures 1 and 2, "Relaxation") reverses any interaction of the proteins with the DNA.

DNA Supercoiling Assays. 1. Calf Thymus Topoisomerase I Relaxation. The second half of the reaction mixture described in the preceding section was relaxed with calf thymus topoisomerase I (2–4 units/μg of DNA) for 30 min at 37 °C as previously described (Sheflin & Spaulding, 1989). The reaction was then terminated by adding SDS (1% final concentration) for 15 min at 37 °C to dissociate the proteins from the DNA and to inactivate the topoisomerase I enzyme. After the addition of 1/10 volume of loading dye, 20 μL was run on the bottom half of the same gel used for the retardation assay samples described in the preceding section. The relative amounts of the various topoisomers were determined by densitometric scanning of the photographic negatives (using positive/negative Polaroid film type 665) of the gels. The apparent unwinding angle was calculated by determining the relative change in the average linking number produced by a given amount of HMG 1 or 2 or P1 fragment and then multiplying this value by 360°. The change in linking number as measured in this assay and in the T4 DNA ligation assay described below could be due to a change in either twist or writhe (Anderson & Bauer, 1979). We thus express our results as the "apparent" unwinding angle.

2. T4 Ligation of Nicked-Circular DNA. Nicked-circular DNA was prepared by treating negatively supercoiled DNA with mung bean nuclease as previously described (Sheflin & Spaulding, 1989). This DNA was preincubated with increasing concentrations of HMG 1, HMG 2, or their tryptic fragments for 15 min at 37 °C and then incubated with T4 ligase (3 units/μg of DNA) for 1 h at 37 °C. The covalently-closed-circular forms of DNA produced in the absence and presence of the various proteins were assessed by electrophoresis of 15–20 μL of HMG 1, HMG 2, and P1. The apparent unwinding angle for this DNA substrate was calculated as in the preceeding section.

Sulfhydryl Analysis with NEM and DTNB. The effects of the sulfhydryl reagent *N*-ethylmaleimide (NEM) on the HMG 1–DNA interactions were examined: (1) by treating HMG 1 or 2 and their tryptic peptides with NEM at 0, 0.1, 1, and 10 mM NEM, and then adding ds- or ss-DNA; (2) by treating the DNA with the same concentrations of NEM, and then adding the peptides; and (3) by reacting the peptides with DNA, and then adding the NEM. After incubating at 37 °C for 15 min, loading dye was added and the reaction mixtures were subjected to electrophoresis on 1% agarose gels, stained with EtdBr, and photographed. The reactions with negatively supercoiled DNA were split in half: CT topoisomerase I was added to half and relaxation analyzed as described above. We titrated a total of three sulfhydryl groups in calf thymus HMG 1, by absorption of an HMG 1/DTNB adduct at 420 nm as previously described (Ellman, 1959), which is in agreement with that predicted from the cDNA sequence (Kaplan & Duncan, 1985), but differs from the four residues reported for steer HMG 1 (Kohlstedt et al., 1985), which utilized the same technique.

RESULTS

Binding and Protection of Negatively Supercoiled DNA by HMG 1 and HMG 2. HMG1 and 2 retard the mobility of negatively supercoiled ("S") DNA much more than they retard nicked-circular ("N") DNA, when assayed either by gel electrophoresis or by sucrose gradient analysis (Figure 1). Increasing concentrations of HMG 1 and 2 produce a linear response, progressively retarding the mobility of negatively supercoiled DNA up to ratios of 250:1 mol/mol of DNA (4363 bp). Over the linear range, the percent retardation of negatively supercoiled DNA was 0.028%/mol of HMG 1 or 2. The linear retardation responses thus obtained using two independent methods are entirely consistent with earlier sedimentation and unwinding studies which showed that the interaction of HMG 1 with negatively supercoiled DNA is linear, "noncooperative", and saturable (Bonne et al., 1980; Duguet et al., 1981). Ratios greater than 250:1 were much less effective in further retarding the mobility of negatively supercoiled DNA, producing only 0.007% retardation/mol of HMG 1 by gel analysis and no further change in retardation by sucrose gradient analysis.

Previously we have shown that HMG 1 preferentially interacts with negatively supercoiled DNA when compared to positively supercoiled DNA (Sheflin & Spaulding, 1989). These findings are confirmed and extended by the current observations on the effect of HMG 1 on the banding pattern and the percent retardation obtained with different conformational forms of the same DNA (nicked-circular, linear, positively and negatively supercoiled) (Figure 2). In all instances, HMG 1 retards positively supercoiled, linear, and nicked-circular DNAs much less than it does negatively supercoiled DNA (lanes 2, 6, and 10). At concentrations of HMG 1 beyond the linear range of retardation (>250:1), the ladder pattern of the moderately negatively supercoiled topoisomers ($\sigma = -0.02$) and the discrete band of highly negatively supercoiled DNA ($\sigma = -0.045$) were uniformly

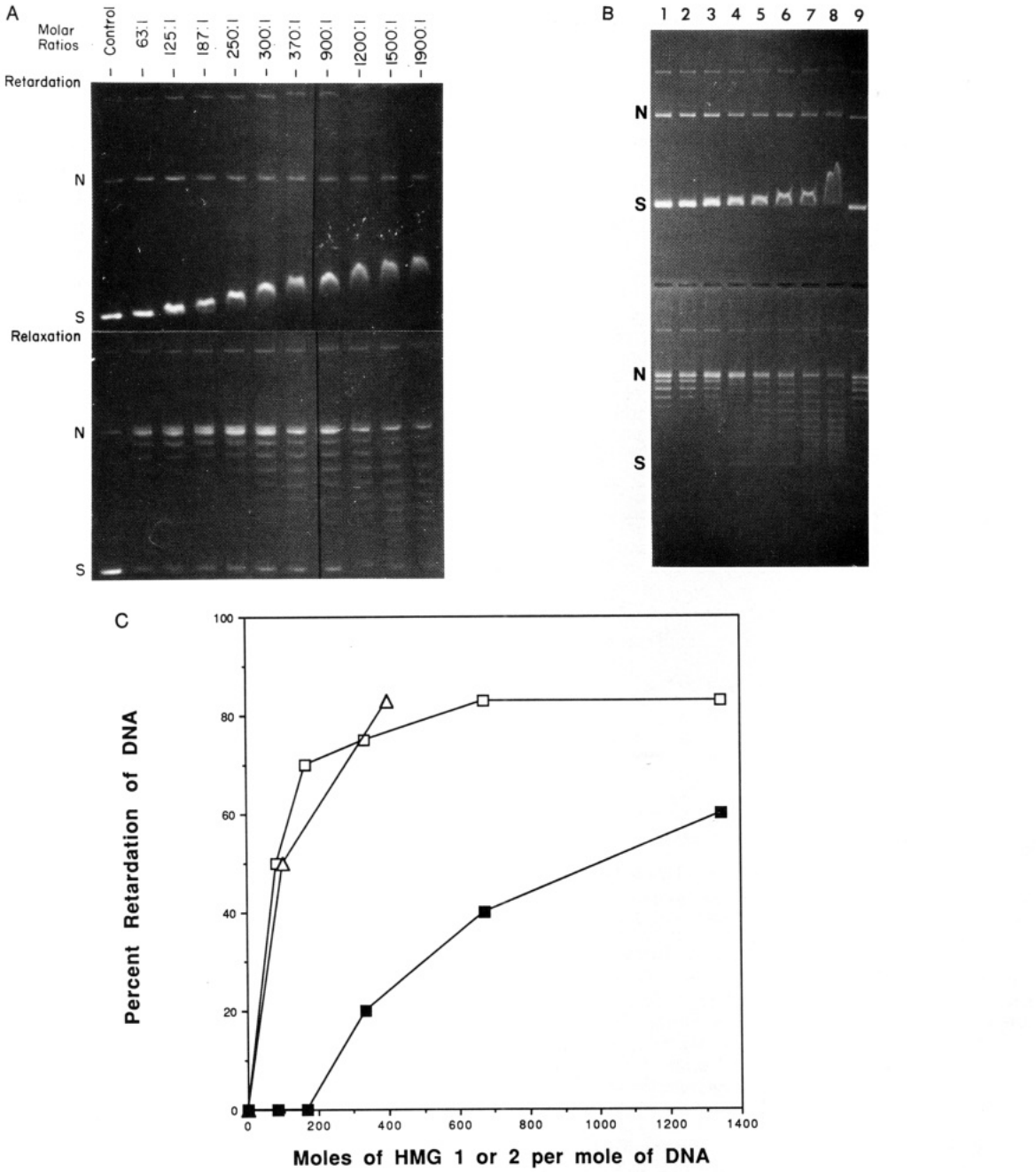

FIGURE 1: (Panels A and B) Preferential retardation and relaxation of closed-circular double-stranded DNA by HMG 1 and HMG 2. Different concentrations of purified calf thymus HMG 1 (A) or HMG 2 (B) were preincubated with highly negatively-supercoiled pBR322 DNA (σ = –0.045) at various molar ratios and mean retardation of the electrophoretic mobility of the DNA was determined (top of each panel). DNA migration distance for lanes containing the "M" pattern produced by high concentrations of protein was estimated at the center of the lane. Aliquots from the same mixtures were also assayed for their ability to protect negatively supercoiled DNA from relaxation by calf thymus topoisomerase I (bottom of each panel). Both assays were run in the same gel. The DNA used contained >90% highly negatively supercoiled DNA ("S") (σ = –0.04) and <10% nicked circular ("N"). (A) Molar ratios of HMG 1 to DNA up to 1900:1. The control lane contained only negatively supercoiled DNA and was not treated with CT topoisomerase I. (B) Various molar ratios of HMG 2 to DNA: lanes 1–8 at 16:1, 32:1, 64:1, 128:1, 192:1, 256:1, and 600:1. Lane 9 contained only negatively supercoiled DNA relaxed by CT topoisomerase I (note: four or five positive topoisomers produced by CT topoisomerase I). (Panel C) Plot of mean percent retardation of negatively supercoiled and nicked-circular DNAs in response to increasing concentrations of HMG 1 and HMG 2 as determined by sucrose gradient analysis. Purified HMG 1 or HMG 2 were preincubated with highly negatively supercoiled DNA (80% negatively supercoiled/20% nicked circular) under the assay conditions of Bonne et al. (1980) for 15 min at 37 °C, then loaded onto a 5–20% sucrose gradient (5 mL), and finally centrifuged at 35000*g* for 3 h in a SW50.1 rotor. The gradient was fractionated from the top, and the fractions were analyzed on 1% agarose gels. The mean percent retardation of DNA is expressed as the change in the mean peak position of the input DNA. Supercoiled DNA retarded by HMG 1 (open squares) or HMG 2 (open triangles); nicked-circular DNA retarded by HMG 1 (closed squares).

retarded (lane 10). On the other hand, the ladder pattern of positive topoisomers (+1 to +7) was maintained and only slightly retarded by these higher levels of HMG 1 (lane 6). Linear DNA was also retarded less than negatively supercoiled DNA (lane 2). Nicked-circular DNA showed even less retardation compared to negatively supercoiled DNA, whether assessed on gels (Figure 2) or by sucrose gradient analysis (Figure 1C).

The relaxation studies using CT topoisomerase I also revealed saturation of negatively supercoiled DNA at molar ratios of HMG 1 of about 250:1 (bottom of Figures 1 and 2, "Relaxation"). Previously we reported that HMG 1 unwinds

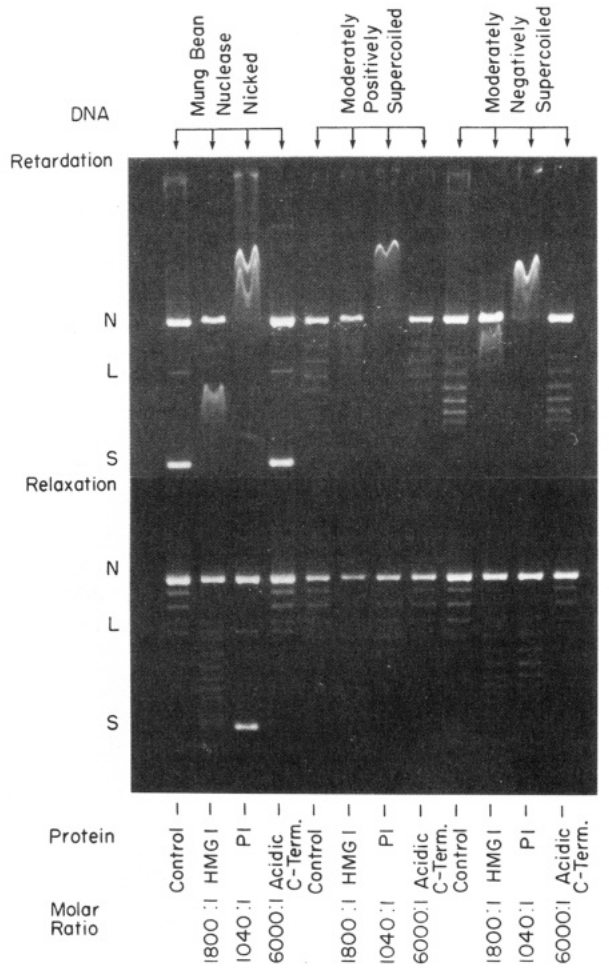

FIGURE 2: Retardation and relaxation of closed-circular double-stranded DNA by HMG 1 and its major tryptic fragment. Mung bean nuclease-nicked DNA (left four lanes) contained 45% negatively supercoiled ("S"), 45% nicked-circular ("N"), and 10% linear DNA ("L"). Moderately positively supercoiled pBR322 DNA (middle four lanes; $\sigma = +0.02$) contained topoisomers +1 to +7. Moderately negatively supercoiled pBR322 DNA (right four lanes; $\sigma = -0.02$) contained topoisomers –1 to –7. These various DNAs were preincubated either with HMG 1, with the major tryptic fragment from HMG 1 ("P1"), or with the purified acidic C-terminal region of HMG 1. Upper and lower panels represent the same assays as in Figure 1.

negatively supercoiled DNA ($\sigma = -0.04$) with an unwinding angle of 58°, using either calf thymus or *E. coli* topoisomerase I (Sheflin & Spaulding, 1989) up to a ratio of 200:1. We have now extended these earlier findings, using molar ratios of HMG 1 to DNA up to 1900:1. HMG 1 caused progressive change in the average linking number up to molar ratios of 250:1 mol of HMG 1/mol of DNA (Sheflin & Spaulding, 1989). Ratios above 250:1 caused no further changes in the relaxation pattern produced by calf thymus topoisomerase I (Figure 1), again indicating a saturation of the ability of HMG 1 to effect a change in the average linking number. Since the relaxation pattern was unchanged with doses of HMG 1 up to 1900:1, such "supersaturating" levels of HMG 1 do not appear to nonspecifically inhibit the action of the topoisomerase. In fact, when a second negatively supercoiled DNA, PM2, was added at the end of the first relaxation assay, it was relaxed by the remaining topoisomerase I activity to the same degree as when no HMG 1 was present in the reaction. HMG 1 totally protects highly negatively supercoiled DNA from *E. coli* topoisomerase I relaxation at molar ratios above 250:1 [data not shown; see Sheflin and Spaulding (1989)], confirming that HMG 1 apparently unwinds negatively supercoiled DNA to produced positive supercoils which are resistant to *E. coli* topoisomerase I (Wang, 1971) but not to CT topoisomerase I.

HMG 2 unwound and partially protected negative supercoils from CT topoisomerase I relaxation in a manner very similar to that of HMG 1: with increasing molar ratios of HMG 2 up to 250:1, positive topoisomers (produced by CT topoisomerase I) were progressively replaced with negative topoisomers, indicating protection of the negatively supercoiled DNA by HMG 2 (Figure 1B). Note that at a molar ratio of 128:1 (lane 4) the number of positive topoisomers equals the number of negative topoisomers and that the fraction of DNA that is highly negatively supercoiled DNA is approximately equal to the fraction that is present as closed-circular DNA. At a molar ratio of 256:1 only negative topoisomers are present. Again, molar ratios above 250:1 did not further alter the relaxation pattern, suggesting that HMG 2 has totally relaxed the torsion and that the change in average linking number was saturable. Up to a ratio of 250:1, the apparent unwinding angle for HMG 2 is 58°, a value comparable to that for HMG 1 [above and Sheflin and Spaulding (1989)].

We also utilized a different approach to assess supercoiling activity of HMG 1 and 2, namely, analysis of the change in average linking number of topoisomers formed by T4 ligase on nicked circular DNA ($\sigma = 0$) in the presence of increasing concentrations of HMG 1 and 2. These studies confirmed that HMG 1 and 2 supercoil this relaxed DNA with an apparent unwinding angle of 58° up to a molar ratio of 125:1 (higher molar ratios did not further result in a change in the linking number, suggesting saturation; data not shown). Thus the change in the average linking number produced by HMG 1 and 2 is not a function of the DNA substrate or of the enzyme used to measure the change in linking number, and is not due to some nonspecific inhibitory effect of the proteins on calf thymus topoisomerase I.

Other DNA Binding Proteins Do Not Protect Negatively Supercoiled DNA in the Same Fashion as HMG 1 and 2. Histone H1, T4 gp32, and *E. coli* SSB all preferentially retarded negatively supercoiled DNA, which is similar to the effect of HMG 1 or 2, but we found none of these other proteins were able to change the linking number of this torsionally stressed DNA when assayed with either CT or *E. coil* topoisomerase I (data not shown). Furthermore, none of these proteins displayed a supercoiling activity using nicked-circular DNA with T4 ligase (data not shown). Thus the mode by which HMG 1 and 2 bind to and protect negatively supercoiled DNA is different from that proposed for H1 (Bina-Stein & Singer, 1977; De Bernardin et al., 1986) and for prokaryotic SSBs (Williams et al., 1983; Srivenngopal et al., 1987).

Binding and Protection of Negatively Supercoiled and Supercoiling of Nicked-Circular DNAs by the P1 Fragments, But Not the C-Terminal Fragments, Assayed Individually and Combined. During preliminary studies we observed by PAGE analysis that, upon repeated freezing/thawing of some preparations of HMG 1 and 2, these proteins spontaneously degrade to generate increasing amounts of "HMG 3", a peptide analogous to the major tryptic fragment of HMG 1 or HMG 2, P1 (Isackson et al., 1983). Concomitantly, we found that these preparations produced a greater retardation of all forms of DNA and conferred total protection on negatively supercoiled DNA from relaxation by CT topoisomerase I. An example of this change in activity for an HMG 2 preparation which contains equal amounts of HMG 2 and "HMG 3" is shown in Figure 3A. The retardation of both negatively supercoiled and nicked-circular DNA by this HMG 2/HMG

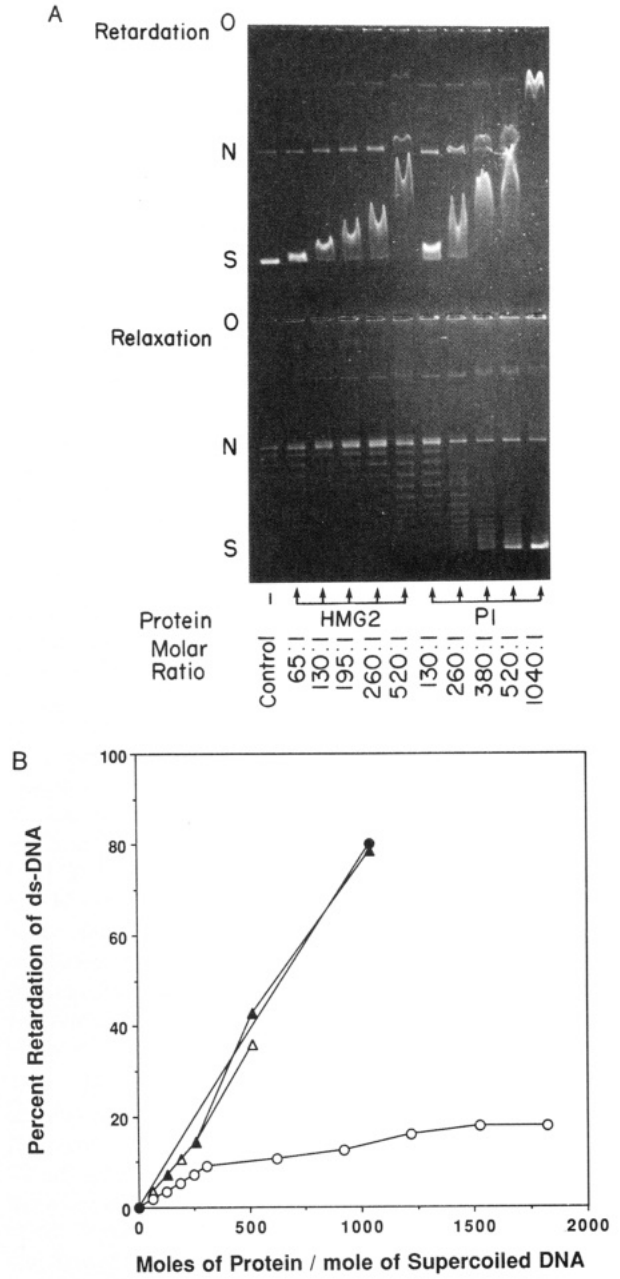

FIGURE 3: (A) Retardation and relaxation of highly negatively supercoiled closed-circular ds-DNA by HMG 2 (freeze/thawed) and its major tryptic fragment. A freeze/thawed preparation of HMG 2 (containing about 50% spontaneously generated "HMG 3") and the major tryptic fragment ("P1") from HMG 2 were preincubated with highly negatively supercoiled pBR322 DNA at molar ratios of up to 520:1 and 1040:1, respectively, and then assayed for gel retardation and for protection from CT topoisomerase I relaxation. (B) Plot of mean percent retardation of negatively supercoiled DNA by increasing concentrations of HMG 1 and 2 and P1 tryptic fragments determined by gel electrophoresis. Data from Figures 1 and 2 and panel A, and data (not shown) for purified HMG 2, were used to plot the mean percent retardation produced by various molar ratios of the protein/peptide to negatively supercoiled pBR322 DNA. Open circles: HMG 1 or HMG 2; closed circles: the major HMG 1 fragment; open triangles: freeze/thawed HMG 2; closed triangles: the major HMG 2 fragment.

3 preparation is greater than that of pure HMG 2 (Figure 1B) and is similar to that of pure P1 from HMG 2 (Figure 3A, compare 260:1 and 520:1 for both HMG 2 and P1). The relaxation pattern of the contaminated preparation shows less change in the average linking number per mole of protein and conferred total protection on negatively supercoiled DNA at high molar ratios >1040:1 (not shown), similar to the total protection observed with the pure P1 from HMG 1 (Figure 2) or pure P1 from HMG 2 (Figure 3A) at molar ratios of 1040:1.

To confirm that the increase in the retardation and change in the relaxation pattern of DNA was due to the "HMG 3" generated, we prepared its equivalent tryptic fragment, P1 (Isackson et al., 1983; Reeck et al., 1982). We found that P1 fragment from either HMG 1 or HMG 2 was substantially more potent than the native protein in retarding negatively supercoiled DNA (Figures 2 and 3A, "Retardation"). For example, HMG 1 retarded negatively supercoiled DNA by 0.024%/mol over the linear range of retardation, up to 250:1, whereas the percent retardation produced by P1 from HMG 1 was twice as great (0.055%) and was linear up to a molar ratio of 1040:1 (Figure 3B). The P1 fragment from HMG 2 also enhanced retardation of negatively supercoiled DNA compared to intact HMG 2 (Figure 3A, "P1 Retardation"; Figure 3B). Thus P1 from HMG 1 or 2, which contain the same two DNA binding regions as the native proteins, retarded DNA much more than did intact HMG 1 or 2. These results suggest that the acidic "protein-binding" C-terminus, which is removed by tryptic digestion, modulates the binding of HMG 1 or 2 to negatively supercoiled DNA and hence may be involved with unwinding of DNA.

Although the P1 fragments are more potent in retarding negatively supercoiled DNA, they are substantially less potent in protecting negatively supercoiled DNA than the parent HMGs, the apparent unwinding angle determined for P1 being only about 10° ("Relaxation" gels in Figures 2 and 3A). P1 fragments changed the average linking number over a much broader range of concentrations than the intact native proteins using CT topoisomerase I relaxation (linear range up to 1040:1). Molar ratios above 1040:1 were ineffective in further changing the average linking number. Taken in conjunction with the retardation results reported above, these results indicate an apparent plateau in P1 binding at molar ratios of 1040:1, a value about 4 times that obtained above for intact HMG 1 or 2, suggesting an apparent binding site size of 4 bp [binding site sizes for P1 may be larger than this, because there may be protein–protein interactions at high P1 concentrations; some oligomer formation can be detected by gel filtration and is disrupted by SDS, as confirmed by SDS–glycerol–PAGE analysis (data not shown)]. Data on the P1 fragment from HMG 2 are shown in Figure 3A; identical titration results were obtained using P1 from HMG 1 (data not shown). In no case did either P1 fragment induce more supercoils than were initially present in the positively or negatively supercoiled DNA preparations, indicating that the apparent unwinding activity of P1 is limited to the torsion initially present in the DNA (Figure 2, "Relaxation"). At molar ratios ≥1040:1, however, the P1 fragments totally protected all the negatively supercoiled DNA from CT topoisomerase I relaxation, whereas similar molar ratios of the intact proteins did not (Figures 2 and 3A; "Relaxation"). A similar apparent unwinding angle of about 10° was obtained from the P1 from HMG 1 and 2 when assayed on nicked-circular DNA using T4 ligase (see Figure 4A). When P1 was added by itself, a maximum of 16 topoisomers could be formed on nicked-circular DNA, producing a plateau at a molar ratio of 500:1, a value which is 4 times that obtained with the intact HMG 1 or 2. Again these results confirm an apparent unwinding angle of about 10° and P1 saturation of the nicked-circular DNA substrate at a molar ratio 4 times that of the intact proteins (Figure 4A).

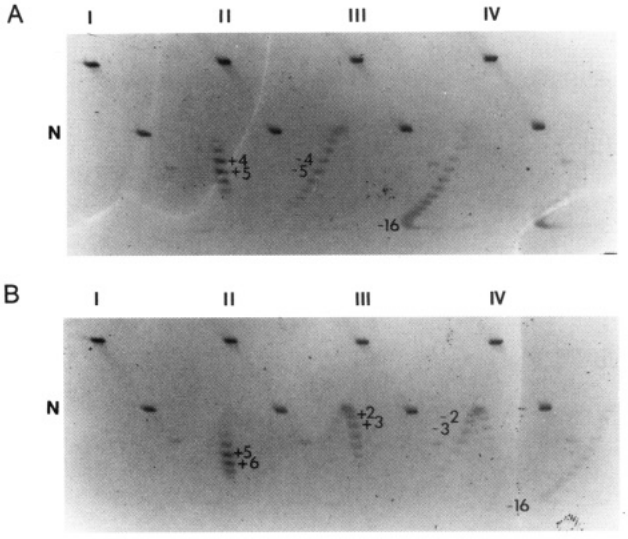

FIGURE 4: Two-dimensional analysis of "P1" unwinding of nicked-circular DNA. Increasing concentrations of purified "P1" from HMG 1 were preincubated with nicked-circular DNA (see Materials and Methods) in T4 ligation buffer and then ligated with T4 ligase. The resultant closed-circular DNA was analyzed by 2-D gel analysis by the procedure of Sheflin and Spaulding (1989). Panel I: No "P1". Panels II–IV: Molar ratio of "P1" to DNA, 125:1, 250:1, and 500:1, respectively. "N" indicates the migration position of nicked-circular DNA. Relaxed closed-circular DNA migrates at the top of the arc of topoisomers. "+" indicates positive topoisomers present in panel I, and "–" indicates negative topoisomers present in panels II and III. The assigned numbers represent linking number differences of 1 produced by T4 ligase in the absence and presence of P1. (A) Ligation in the absence of the acidic C-terminal region. (B) Ligation in the presence of the acidic C-terminal region at an input molar ratio to DNA of 6000:1.

Adding purified acidic C-terminal fragments from HMG 1 or 2 (at protein-to-DNA ratios ≤6000:1) reduced the linking number produced by a given amount of P1 fragment by about 50% using the T4 ligase reaction (Figure 4B). Similarly, mixing a 5-fold molar excess of C-terminal fragment with its complementary P1 fragment (at 1040:1 mol/mol of DNA) also decreased both the gel retardation and the relaxation pattern produced by CT topoisomerase I on negatively supercoiled DNA, compared to the effect of the major peptide alone (data not shown). Since the acidic C-terminal fragment by itself did not change the linking number produced by T4 ligase or by CT topoisomerase I (Figure 2), these results suggest that the acidic C-termini act by modulating the interaction of the DNA binding domains in P1 with ds-DNA, and not by altering the enzyme or the DNA substrate. Thus both on relaxed and on negatively supercoiled DNA, the isolated acidic C-terminal fragment reverses the interactions of the P1 fragment with DNA, suggesting P1 fragments bind to DNA and unwind DNA only via weak electrostatic interactions that are partly neutralized by the presence of the highly acidic C-terminal fragment.

When the acidic C-terminal fragments (from either HMG 1 or HMG 2) were assayed by themselves for DNA binding/unwinding effects, they did not retard any torsional form of ds-DNA (or ss-DNA, see below), nor did they protect the negatively supercoiled DNA from topoisomerase I relaxation, even at very high molar ratios (e.g., 6000:1, Figure 2). Thus, as expected, the acidic C-terminal regions do not appear to bind directly to negatively supercoiled DNA to alter its electrophoretic mobility or its linking number, and they do not adversely affect topoisomerase I activity. When assayed for unwinding activity on nicked circular DNA, the C-terminal fragments actually caused a slight increase in the number of positive topoisomers produced by T4 ligase under the ionic conditions present in T4 ligation buffer (Figure 4B), opposite to the effect which would have been expected if the acidic C-terminal fragment were interacting with DNA directly to unwind it [as had been suggested by Yoshida et al. (1987)]. This suggests that high concentrations of these highly negatively charged peptides actually further overwind the nicked-circular DNA, possibly by changing the ionic environment around the DNA (Anderson & Bauer, 1979).

NEM Specifically Inhibits the Binding of HMG 1 and 2 to Negatively Supercoiled DNA. NEM is a sulfhydryl reagent that has been shown to inhibit the DNA binding activity of the herpes simplex virus major DNA binding protein (Ruyechan, 1988). We found that increasing concentrations of NEM progressively inhibited the binding of HMG 1 to negatively supercoiled DNA (at a molar ratio of 250:1), with about 30% inhibition at 0.1 mM, 55% inhibition at 1 mM, and 90% at 10 mM (Figure 5). The same degree of inhibition was observed whether the NEM was added to the protein first, to the DNA first, or after the protein had been incubated with the DNA. These results indicate that sulfhydryl groups are involved in the binding of HMG 1 to negatively supercoiled DNA and are accessible to NEM even when HMG 1 is bound to DNA. The binding of HMG 1 to the other conformational forms of ds-DNA displayed less sensitivity to NEM. While 1 mM NEM inhibited the binding to negatively supercoiled DNA by about 55%, the binding to linear DNA was only inhibited by about 35%, and binding to nicked-circular ds-DNA was not inhibited at all. Thus the major effect of low concentrations of NEM is to inhibit the binding of HMG 1 to negatively supercoiled DNA. In fact, 0.1 mM NEM also appeared to reverse the unwinding of negatively supercoiled DNA produced by HMG 1 (Figure 5, bottom), as would be expected if binding were being inhibited; however, since 10 mM NEM inhibited topoisomerase I activity by itself, we have reservations about drawing a definite conclusion about the action of NEM on HMG 1 supercoiling activity.

NEM Does Not Inhibit the Binding of P1 Fragments to DNA. On the basis of the similar binding/supercoiling of P1 fragments from either HMG 1 or 2, we tested only the P1 fragment from HMG 2 for NEM sensitivity. The binding of P1 fragment from HMG 2 to negatively supercoiled (or to nicked-circular) DNA at a molar ratio of 340:1 showed only a slight inhibition at the highest concentration of NEM (10 mM; data not shown). Thus over the same range of concentrations of NEM (0.1–1 mM) which progressively inhibited the binding of intact HMG 1 to negatively supercoiled DNA, the binding of the P1 fragment from HMG 2 to negatively supercoiled DNA was not affected. These results indicate that sulfhydryl groups are not a dominant factor in the electrostatic binding of P1 to DNA. NEM treatment at 0.1 or 1 mM also did not appear to alter the effect of P1 on the relaxation of supercoiled DNA by CT topoisomerase I, but we reiterate the reservations mentioned above concerning the effects of NEM on topoisomerase I activity itself.

Binding of Closed-Circular Single-Stranded DNA by HMG 1 and HMG 2 and Their P1 Fragments Compared with Prokaryotic Single-Strand DNA Binding Proteins. To study HMG 1 and 2 interactions with cc-ss-DNA directly, we employed the gel retardation assay method of Lohman et al. (1986). HMG 1, HMG 2, and their P1 fragments behave very differently from the well-characterized single-stranded DNA binding proteins, T4 gp32 and *E. coli* SSB (Figure 6), which display a concentration-dependent increase in their binding to closed circular ssDNA, consistent with cooperative

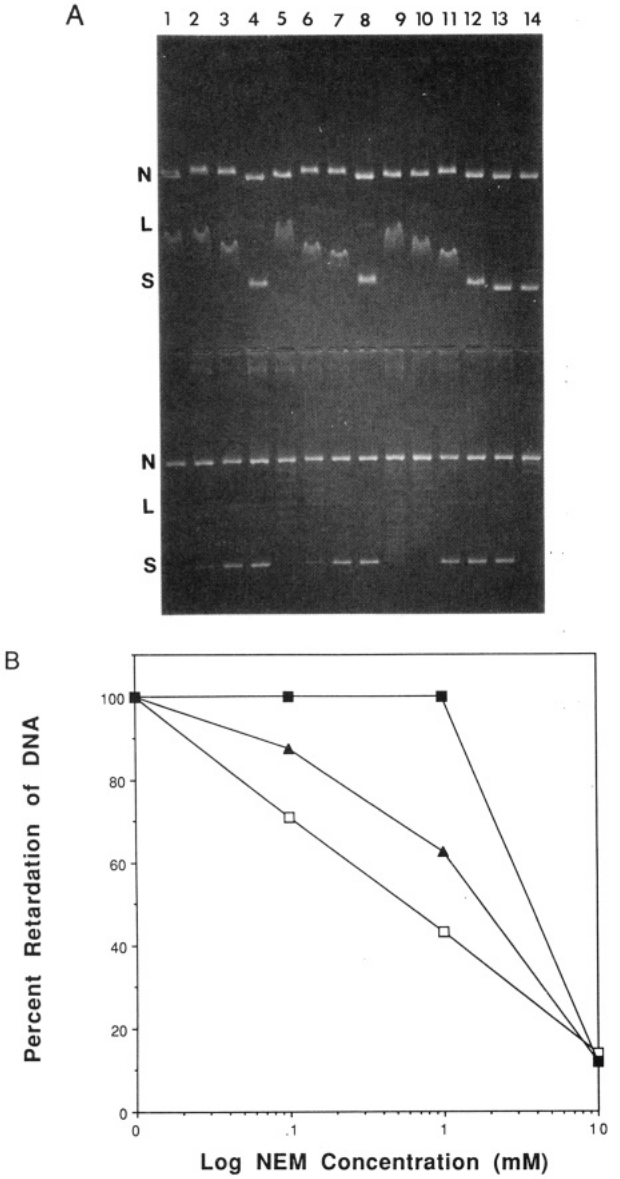

FIGURE 5: (A) NEM inhibition of HMG 1 binding/unwinding of negatively supercoiled DNA. Top: Retardation. Bottom: Relaxation. Lanes 2–5: Highly negatively supercoiled DNA [55% "S" (negatively supercoiled), 40% "N" (nicked-circular), and 5% "L" (linear)] was incubated with HMG 1 at a molar ratio of 250:1 and then treated with increasing concentrations of NEM (0.1–10 mM). Lanes 6–9: The same DNA was first incubated with increasing amounts of NEM, and then HMG 1 at a molar ratio of 250:1 was added. Lanes 10–13: HMG 1 was first incubated with increasing concentrations of NEM, and then the DNA was added. The top of the gel shows the retardation of the DNA and the bottom of the gel shows the unwinding of the DNA as assayed using CT topoisomerase I. Lanes 1, 5, 9, and 14 not treated with NEM. lanes 2, 6, and 10 treated with 0.1 mM NEM. Lanes 3, 7, and 11 treated with 1.0 mM NEM. Lanes 4, 8, 12, and 13 treated with 10 mM NEM. (B) Plot of the effect of NEM concentration on HMG 1 retardation of negatively supercoiled, linear, and nicked-circular ds-DNAs. The mean percent retardation of each ds-DNA was determined in the absence and presence of increasing concentrations of NEM based on the data shown in panel A. A maximum of 100% retardation of DNA by HMG 1, at a molar ratio of 250:1 in the absence of NEM, was established as the reference. Open squares represent negatively supercoiled DNA, closed triangles represent linear DNA, and closed squares represent nicked-circular DNA.

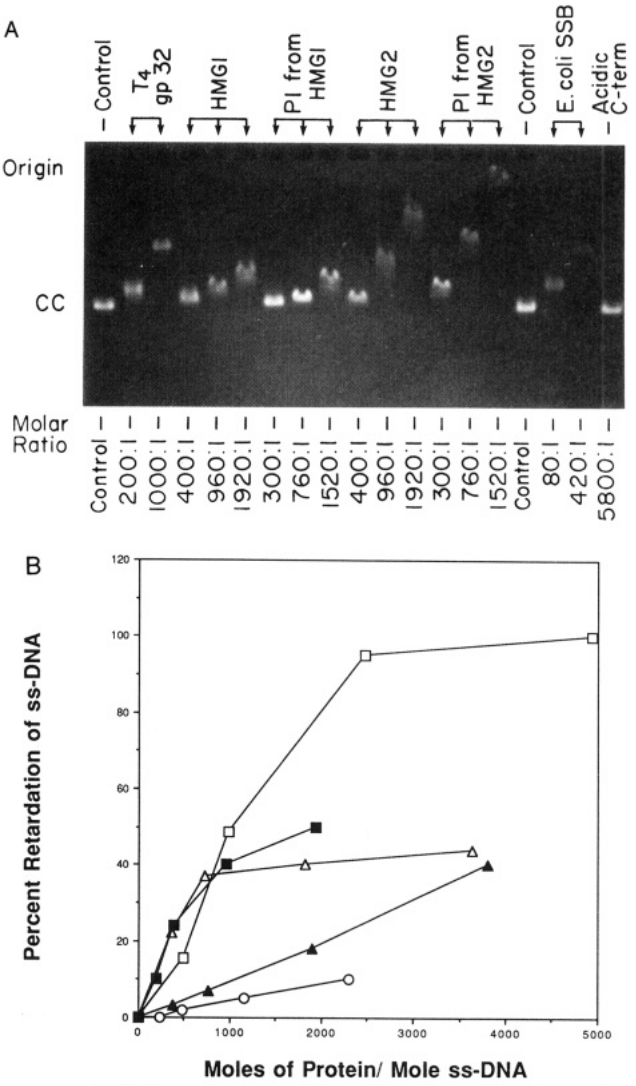

FIGURE 6: (A) Gel retardation of closed-circular ss-DNA by HMG 1, HMG 2, and their major tryptic fragments, compared to the effects of T4 gp32 and *E. coli* SSB. Different concentrations of HMG 1, HMG 2, the major tryptic peptide fragment from each ("P1" from HMG 1, "P1" from HMG 2), and the acidic C-terminus from HMG 1, as well as T4 gp32 and *E. coli* SSB, were preincubated with closed-circular M13 DNA and then assayed for gel retardation according to the method of Lohman et al. (1986). (B) Plot of mean percent retardation of cc-ss-DNA by SSB binding proteins, HMG 1 and 2, and their major tryptic fragments. Data from panel A and other experiments (not shown) were used to plot the mean percent retardation of closed-circular M13 ss-DNA. Open triangles: *E. coli* ssb; closed squares: T4 gp32; open circles: HMG 1 and the major HMG 1 tryptic fragment; closed triangles: HMG 2; open squares: the major HMG 2 tryptic fragment.

binding (Lohman et al., 1986; Toulme et al., 1984).

HMG 1 is about half as effective as HMG 2 in retarding the mobility of ss-DNA (Figure 6). The apparent binding site size for HMG 1 on ss-DNA is about 4 bases/mol over the linear range, twice that of HMG 2. The apparent binding site size and the percent retardation values determined for HMG 1 on cc-ss-DNA are both about one-fourth the values for HMG 1 determined on negatively supercoiled DNA (above; Sheflin & Spaulding, 1989), and as independently determined for ds-DNA (Shooter et al., 1974; Butler et al., 1985) and ss-DNA (Butler et al., 1985). P1 from HMG 1 retards closed-circular ss-DNA with a percent retardation of 0.010, similar to that of intact HMG 1 (Figure 6B).

HMG 2 retards cc-ss-DNA in a linear, noncooperative manner up to a molar ratio of 3800:1 (Figure 6B). The percent retardation of ss-DNA per mole of HMG 2 over this linear range is 0.024. On the basis of the slope of this linear response, the ss-DNA binding site size appears to be about 2 nucleotides/mol of HMG 2. HMG 2 retarded both ds- and ss-DNA to the same extent, whereas the binding site size determined for HMG 2 is about one-eighth the respective value obtained on

negatively supercoiled ds-DNA (see above). The P1 fragment from HMG 2 retards M13 ss-DNA 0.055%/mol, twice as much per mole as native HMG 2 up to 500;1 (Figure 6B), indicating that the interaction with closed-circular ss-DNA has been changed by removal of the acidic C-terminal. The P1 fragment from HMG 2 retards ss-DNA in a nonlinear fashion, also being twice as potent as its parent in retarding negatively supercoiled DNA (see above). A plateau in the percent retardation of the ss-DNA was reached at a molar ratio of 2250:1 (Figure 6B), which suggests saturation of this DNA and indicates an apparent ss-DNA binding site size of 4 nucleotides, similar to that obtained above with intact HMG 1.

Thus, despite the fact that the P1 fragments from either HMG 1 or 2 bind to negatively supercoiled DNA in a similar fashion, the P1 fragments as well as the parent proteins show a differential interaction with ss-DNA. As expected, the acidic C-termini of HMG 1 (Figure 6A) and HMG 2 (not shown) were ineffective in retarding the electrophoretic mobility of ss-DNA, as we also observed with ds-DNA (Figure 2).

NEM Does Not Substantially Inhibit the Binding of HMG 1 and 2 or Their P1 Fragments to ss-DNA. NEM was much less effective in inhibiting the binding of HMG 1 and 2 to ss-DNA than in inhibiting binding to negatively supercoiled DNA (about 10% inhibition of binding to ssDNA at 10 mM NEM; data not shown). NEM (at 10 mM) did not affect the binding of P1 fragments to ss-DNA at all [over the molar ratios (100–1000):1]. This latter result is similar that obtained with negatively supercoiled DNA, thus indicating that NEM does not alter the electrostatic binding of P1 fragments to any type of DNA.

DISCUSSION

We have found that HMG 2, like HMG 1, preferentially interacts with negatively supercoiled DNA. This is perhaps not surprising in view of the high degree of homology between their DNA binding domains (85%; Walker, 1982) and their similar tertiary structures (Reeck et al., 1982; Isackson et al., 1983; Cary et al., 1982). Bustin and Soares (1985) previously reported that HMG 2 displayed a greater interaction with negatively supercoiled DNA than did HMG 1, using a different assay (nitrocellulose filter binding; pH 4.5, 4 °C). However, spontaneous contamination of HMG 1 or HMG 2 with "HMG 3" could result in greater interactions with ds-DNA, as confirmed in our studies with P1 fragments equivalent to "HMG 3". Additionally, Bianchi et al. (1992) recently reported that the recombinant DNA binding A/B domain, which contains the same HMG 1/2 boxes as our P1 from HMG 1, has a greater DNA binding activity than does the intact protein. Despite the greater binding of P1 to negatively supercoiled DNA, as well as to other types of ds-DNA, the ability of P1 to protect supercoiled DNA is about 5-fold less than intact HMG 1 or 2, indicating the importance of the acidic C-terminal domains in modulating DNA topology. The 2-fold increase in DNA binding by P1 coincides with a doubling of the net positive charge of the peptide when compared to intact protein. Our findings extend those of Carballo et al. (1984) and of Isackson et al. (1983) and indicate that the negatively-charged C-terminal region interacts electrostatically with the positively-charged DNA binding domains and modulates their DNA binding activities. At high concentrations, the P1 fragments can totally protect negatively supercoiled DNA from relaxation by topoisomerase I, whereas the intact proteins are never more than partially effective. The apparent unwinding angle for intact HMG 1 and 2 is 58°, while that for purified P1's which contain the same DNA binding domains is about 10°, regardless of the circular ds-DNA substrate. This low value is comparable to the unwinding angle reported for core histones and for simple polyelectrolytes (Anderson & Bauer, 1978; Baase & Johnson, 1979) and thus is consistent with P1 DNA binding being primarily electrostatic (Shooter et al., 1974; Goodwin et al., 1975; Isackson et al., 1983).

On the basis solely of the structural similarity noted between the HMG 1-like proteins (Wen et al., 1989) and nuclear "acid blob" proteins (Sigler, 1988), it has been suggested that HMG 1 and 2 may function as "generic transcriptional regulatory proteins" (Ptashne, 1988). The acidic C-terminal "protein binding" domains of HMG 1 and 2 can regulate access of other proteins to the DNA to which the HMG 1 or 2 is bound (Tremethick & Molloy, 1988; Carballo et al., 1983). We have found that purified C-terminal fragments do not interact with DNA and do not affect topoisomerase I activity directly. However, the addition of a molar excess of the C-terminal fragment can reverse the DNA binding and unwinding activities of P1. Thus electrostatic interactions occurring between the acidic C-terminal domains and the positively charged DNA binding domains may allow DNA topology modifying enzymes access to the DNA that otherwise is completely shielded by the A/B domain.

Our data suggest a DNA binding site size for HMG 1 and 2 of about 20 bp, a value similar to what has been reported for the binding of HMG 1 to linear ss- and ds-DNA (Butler et al., 1985; Shooter et al., 1974), and for the binding of HMG 2 to linear ds-DNA (Goodwin et al., 1975). Removal of the acidic C-terminal domain from HMG 1 and 2 reduced the apparent size of the DNA binding site to about 4 bp and eliminated the ability of NEM to selectively inhibit binding to negatively supercoiled DNA. These data indicate that the specific interaction of intact HMG 1 or 2 with negatively supercoiled DNA is dependent upon intramolecular protein conformational changes which involve the acidic C-terminal domain and sulfhydryl groups present in the HMG 1 boxes.

The binding specificity of intact HMG 1 and 2 for negatively supercoiled DNA is due to some feature inherent in negatively supercoiled DNA other than the torsion-induced formation of single-stranded regions, since HMG 1 and 2 interact less specifically with closed-circular ss-DNA than with negatively supercoiled DNA. In conformation of this, Bianchi has recently demonstrated that recombinant HMG 1 does not bind to ssDNA (Bianchi et al., 1992). Furthermore, we find that the binding HMG 1 and 2 to ss-DNA is insensitive to NEM and is significantly different from the binding of T4-gp32 and *E. coli* SSB, which have ss-DNA binding specificity.

HMG 1 and 2 have also been shown to bind preferentially to DNA with altered structures, such as AT-rich sequences (Brown & Anderson, 1985), Z-DNA (Waga et al., 1988), and a novel four-stranded structure lacking ss-loops (Bianchi et al., 1989, 1992). A feature shared by these structures may be the bending of the DNA backbone (Bianchi et al., 1992; Sheflin & Spaulding, 1989). An increased distance between phosphate groups in bent DNA (Trifonov, 1982) might facilitate more specific secondary interactions, possibly involving the intercalation of regularly-spaced aromatic amino acids present in both HMG 1/2 boxes in the intact HMG 1 or 2 (see below and manuscript in preparation). Since the binding specificity of HMG 1 to cisplatin-modified DNA has been correlated with the degree of unwinding and not just the bending of the DNA by this anticancer agent (Pil & Lippard, 1992), the combination of unwinding and bending of the DNA

may be an even more important DNA binding requisite. Recently, mitochondrial transcription factors from man (mtTF1) and yeast (ABF2) have been shown to contain two HMG 1/2 boxes (Diffley & Stillman, 1992; Fisher et al., 1992). Both of these proteins, which lack an acidic C-terminal domain and cysteine residues, also display supercoiling activity on relaxed DNA and display preferential binding to negatively supercoiled DNA as compared to ss-DNA (Fisher et al., 1992; Diffley & Stillman, 1992).

The conservation of the acidic C-terminal domain, cysteine residues, and sequentially-spaced aromatic residues in the two HMG 1/2 boxes suggests that the abundant vertebrate nonhistone chromatin proteins HMG 1 and 2 and HMG T have maintained structurally equivalent domains which are involved in the modulation of both DNA torsional tension and the binding of other DNA binding proteins in nuclear chromatin. All known mammalian HMG 1 and 2 proteins, as well as those from chicken and fish (HMG T), contain two HMG 1/2 boxes (Davis & Burch, 1992; Jantzen et al., 1991) and also an acidic C-terminal domain (Isackson & Reeck, 1982; Bianchi et al., 1992). A striking feature of the "HMG 1/2 box" (Davis & Burch, 1992; Bianchi et al., 1992) is the conservation of four regularly-spaced aromatic amino acids, separated by 10, 10, and 6 residues. The HMG 1-like protein from Tetrahymena contains only one HMG 1/2 box with four regularly-spaced aromatic amino acids, but lacks cysteine residues (Hayashi et al., 1989). Thus the precursor for HMG 1, 2, and T, as well as mtTF1 and ABF2, probably evolved two HMG 1/2 boxes via gene duplication, and the cysteine residues present in HMG 1, 2, and T evolved after this (Tsuda et al., 1990). Wen et al. (1990) have suggested that cysteine residues in each HMG 1/2 box, in conjunction with the linker region between the two HMG 1/2 boxes acting as a swivel, may permit formation of an intramolecular disulfide bridge between the two DNA binding domains. This could explain the specific binding to negatively supercoiled DNA that we have found to be selectively sensitive to NEM. Intramolecular protein conformational changes mediated by disulfide bridge formation or metal binding are suggested by physical changes we have observed in preparations of HMG 1 and 2 by both HPLC and acid–urea gel electrophoresis (unpublished data), and which have been reported by others to be highly dependent on storage conditions and the presence or absence of reducing or metal chelating agents (Kolstedt et al., 1988). We propose that the torsional tension of the negatively supercoiled DNA provides the driving energy needed to promote this conformational change in HMG 1 and 2, even under the reducing conditions in our experiments, which would also be the case in vivo in the nucleus (Kolesteadt et al., 1985). Since the mitochondrial transcription factors mtTF1 and ABF2 lack the cysteine residues, oxidation/reduction-dependent conformational change should not play a role in their interaction with DNA in mitochondrial chromatin. Furthermore, the lack of the acidic C-terminal domain in these mitochondrial proteins suggests that this highly negatively-charged region is not necessary for the action of other proteins in mitochondrial chromatin.

It has been suggested that a general means of regulating chromatin condensation/decondensation involves either the binding of HMG 1, or alternatively of H1, to the linker region of eukaryotic chromatin (Kohlstaedt et al., 1989; Karhu et al., 1989; Mosevitsky et al., 1989; Levy et al., 1979). H1 has been shown to preferentially interact with negatively supercoiled DNA (De Bernardin et al., 1986), but it does not change the linking number (Bina-Stein & Singer, 1977). We confirmed both of these findings for H1, whereas we found that HMG 1 and 2 which also preferentially bind to negatively supercoiled DNA do change the linking number. HMG 1 and 2 also enhance the accessibility of the DNA to DNA topology-modifying enzymes, apparently through the acidic C-terminal domains. The binding of HMG 1 and 2 to negatively supercoiled DNA domains generated during transcription (Giaever & Wang, 1988) would provide a means to locally modulate negative torsional tension (White et al., 1988; Sheflin & Spaulding, 1989). With the torsion removed, other chromatin structural proteins like the nucleosome histone octamer may be more easily assembled onto the DNA, perhaps by binding via the acidic C-terminal region of HMG 1 and 2 (Bonne-Andrea et al., 1984; Stein et al., 1979; Stros & Kolibalova, 1987). The binding of HMG 1 and 2 to the linker region could cause displacement or reconfiguration of H1 or nucleosomes to make the DNA in chromatin more accessible to gene-specific regulatory proteins with affinities for specific nucleotide sequences (Laybourn & Kadonaga, 1991), while conserving the negative torsion required for nucleosome reassembly (Germond et al., 1975; Bode et al., 1991). In support of this model, the binding of histone H5 to HMG 1 requires the presence of its acidic C-terminal domain (Stros & Vorlickova, 1990). A specific protease in the nucleus has been reported to remove the C-terminal domain of HMG 1 and 2 (Dyson & Walker, 1984) and, given the data provided in this report, would provide a means to dramatically modulate the tertiary conformation and the ability of HMG 1 and 2 to modulate DNA torsional tension and/or to bind other nuclear proteins (Cary et al., 1984; Isackson et al., 1983; Sterner et al., 1979; Carballo et al., 1983).

ADDED IN PROOF

While the manuscript was being reviewed, Billings et al. (1992) demonstrated that the binding of HMG 1 or 2 to cisplatin-modified DNA was sensitive to NEM. This provides added support to the concept that cysteine residues modulate intramolecular protein conformational changes necessary for specific DNA binding by these proteins.

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