High Mobility Group Protein 1 Preferentially Conserves Torsion in Negatively Supercoiled DNA†

Lowell G. Sheflin and Stephen W. Spaulding*
Department of Medicine, Buffalo Veterans Administration Medical Center, State University of New York at Buffalo, Buffalo, New York 14215

Received September 20, 1988; Revised Manuscript Received January 23, 1989

ABSTRACT: HMG 1 is known to bind to a variety of DNAs and to unwind nicked and closed circular DNA. We now report evidence that it has a significantly higher unwinding angle on negatively supercoiled DNA than on the other torsional forms. The degree of unwinding observed on nicked circular DNA depends on the purity of the HMG 1 preparation used. HMG 1 from CM-Sephadex has an unwinding angle of 28.8°, compared to 7.2° for the purer preparation obtained from Mono S, suggesting that contaminating strand-separating activity is removed by the additional purification step. The subsequent studies on closed circular forms of DNA were all performed using the purer HMG 1. After preincubation of highly negatively supercoiled DNA ($\sigma = -0.040$) with HMG 1, the DNA–protein mixture was relaxed with *Escherichia coli* topoisomerase I. At molar ratios of less than 100:1 (HMG 1 to DNA), negatively supercoiled DNA displays a dose-dependent change in the linking number, indicating an unwinding angle of 57.6°. HMG 1 protects 50% of highly negatively supercoiled DNA from *E. coli* topoisomerase I at a molar ratio of 100:1, and protects all supercoils at a molar ratio of 200:1, indicating saturation of the DNA at this concentration. HMG 1 also protects highly negatively supercoiled DNA from calf thymus topoisomerase I, with an apparent unwinding angle of 57.6°. Moderately negatively supercoiled DNA ($\sigma = -0.018$), but not moderately positively supercoiled DNA ($\sigma = +0.011$), competes for the protective effect of HMG 1 on highly negatively supercoiled DNA. Preincubation of negatively supercoiled DNA with HMG 1 also protects it from mung bean nuclease cleavage, but this protection is not complete. The data suggest that HMG 1 preferentially interacts with negatively supercoiled DNA, absorbing its tension, which prevents formation of supercoil-dependent secondary structures. The role HMG 1 plays in the regulation of transcription and DNA replication thus may be related to its ability to preferentially relax negatively supercoiled domains in chromatin. This general mode of action of HMG 1 would permit conservation of higher order DNA structures and could facilitate the binding of sequence-specific regulatory factors.

The eukaryotic chromosomal proteins high mobility group proteins 1 and 2 (HMG 1 and 2)[1] are the most prevalent non-histone chromatin proteins (Goodwin et al., 1978) and are assumed to have a structural role in chromatin related to their ability to unwind DNA (Javaherian et al., 1978, 1979). HMG 1 and 2 have been implicated in a variety of nuclear functions, including DNA replication (Bonne-Andrea et al., 1986; Alexandrova et al., 1984; Alexandrova & Belchev, 1988), cellular differentiation (Seyedin et al., 1981; Karhu et al., 1988), and gene expression (Levy-Wilson et al., 1979; Watt & Molloy, 1988; Stoute & Marzluff, 1982; Tremethick & Molloy, 1986, 1988). The mechanism by which HMG 1 was originally thought to produce negative supercoils in nicked circular DNA was by strand separation, which was suggested by an early thermal denaturation study (Javaherian et al., 1979). More recent thermal denaturation studies in which protein aggregation was monitored, however, do not support the theory that HMG 1 acts to destabilize the DNA duplex (Shastri et al., 1982; Marekov et al., 1984, 1986; Butler et al., 1985). In fact, the helix destabilizing activity in one HMG 1 preparation was shown to be due to the presence of a contaminating unwinding protein (UP 1) (Marekov et al., 1986).

HMG 1 affects several processes that involve the unwinding of DNA, such as the assembly of nucleosomes under physiological conditions (Bonne-Andrea et al., 1984). HMG 1 inhibits formation of Z-DNA in negatively supercoiled plasmid DNA (Waga et al., 1988) and increases the binding of a transcription factor to DNA containing the adenovirus major late promoter region (Watt & Molloy, 1988). HMG 1 also enhances in vitro transcription in both these systems (Waga et al., 1988; Tremethick & Molloy, 1986), and in the latter case, it facilitates the formation of an active initiation complex (Tremethick & Molloy, 1988). We have therefore examined the effects of HMG 1 on the torsional properties of DNA under a variety of torsional states, in order to understand how HMG 1 may affect DNA conformation.

Materials and Methods

Isolation and Purification of Highly Supercoiled DNA. pBR322 DNA was prepared from HB101 cells and was purified in CsCl gradients (Holmes & Quigley, 1981). The superhelical density of the purified plasmid DNA was determined to be −0.040 by chloroquine (CQ) titration and band counting on 0.7% agarose gels (Keller, 1975; Shure et al., 1977).

Production of Nicked Circular DNA. Supercoiled pBR322 DNA was nicked with mung bean nuclease at high enzyme to DNA ratios (30 units/μg) at neutral pH and low ionic strength (Kowalski, 1984) for 20–30 min at 37 °C. The resultant nicked circular DNA was extracted with phenol and ethanol-precipitated (Sheflin & Kowalski, 1984). The extent

† Supported by funds from the Research Service of the Veterans Administration.

[1] Abbreviations: Mono S, a strong cationic exchange resin containing sulfonic acid functional groups (Pharmacia LKB Biotechnology Inc.); CM, carboxylmethyl; HMG 1, high mobility group protein 1; CT, calf thymus; CQ, chloroquine; ds, double stranded; ss, single stranded.

of nicking was determined by electrophoresis on 1% agarose gels.

Production of Moderately Positively and Moderately Negatively Supercoiled DNA. Highly negatively supercoiled pBR322 DNA was partially relaxed with either calf thymus (CT) topoisomerase I or *Escherichia coli* topoisomerase I (1 unit per 0.5 μg of DNA in a 50-μL reaction). The two enzymes produce a spectrum of positive or negative topoisomers, respectively, as determined by 2D gel analysis. The topoisomers formed by CT topoisomerase are resistant to *E. coli* topoisomerase I, since the prokaryotic form of the enzyme can relax only negative supercoils (Wang, 1971), whereas the eukaryotic enzyme can relax both negative and positive supercoils (Champoux & Dulbecco, 1972).

Isolation, Purification, and Characterization of HMG 1. HMG 1/2 was prepared by extracting calf thymus with 5% perchloric acid (Sanders, 1977) and was further purified by CM-Sephadex C-25 chromatography (Goodwin et al., 1978). "HMG 1" of this degree of purity has been used by several investigators (Javaherian et al., 1978, 1979; Marekov et al., 1984), but it contains variable amounts of HMG 2, degradation products of HMG 1 [particularly "HMG 3" (Cary et al., 1984) and "HGA" (Yoshida, 1987)], and the unwinding protein "UP 1" (Marekov et al., 1986). To obtain a purer preparation of HMG 1, we employed chromatography on a Mono S column, using NaCl gradient elution (Bofinger et al., 1988). The purity of samples was determined by electrophoresis on 18% polyacrylamide–SDS slab gels (Laemmli, 1970) and acid–urea gels and by silver staining (Bofinger et al., 1988). Fractions from Mono S chromatography were pooled and desalted, and concentrated stocks were stored at −85 °C in 0.5 mM DTT to protect the protein from oxidation.

Agarose Gel Electrophoresis. Horizontal 1% GTG agarose gels (Seakem, FMC BioProducts) were run for 16–20 h in TBE buffer (0.089 M Tris–borate, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0) at room temperature at 2.7 V/cm (20–24 °C). For two-dimensional analysis, gels were first run as described above, then soaked in 1.25 μM CQ/TBE for 6 h, rotated 90°, and run in 1.25 μM CQ/TBE for 16 h at 2.7 V/cm.

Enzymes and Other Reagents. CT topoisomerase I was obtained from Bethesda Research Labs, mung bean nuclease from Promega Laboratories, S1 nuclease from Sigma Chemicals, and T4 ligase from New England Biolabs. *E. coli* topoisomerase I was a gift from the laboratory of Dr. J. Wang (Harvard University, Cambridge, MA).

Ligation of Nicked Circular DNA with HMG 1. Nicked circular pBR322 DNA (0.5 μg/50-μL reaction) was preincubated with various concentrations of HMG 1 in 50 mM Tris-HCl (pH 7.8) containing 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, and 50 μg/mL BSA for 15 min at 24 °C. T4 ligase (80 units/mL) was added and the mixture incubated for 60 min at 24 °C. In the absence of HMG 1, greater than 90% of nicked circular formed positive supercoils (+1 to +5) while <5% remained unligated.

Relaxation of Supercoiled DNA. Supercoiled pBR322 (10 μg/mL) was preincubated in the absence or presence of various concentrations of HMG 1 in 1 mM Tris/1 mM Mg^{2+} for 15 min at 37 °C. The protein/DNA complex formed was then relaxed with either *E. coli* or CT topoisomerase I in 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/mL BSA. *E. coli* topoisomerase I reactions contained 8 units/μg of DNA (1 unit relaxes 0.5 μg of form I DNA in 30 min). CT topoisomerase I reactions contained 2–4 units/μg of DNA. Reactions were terminated by incubating with 0.1 volume of 0.5 M EDTA (pH 8.0) and 10% SDS (previously heated to 65 °C for 10 min) for 5 min at 37 °C and then adding 0.1 volume of loading dye (0.25% bromphenol blue in 40% w/v sucrose).

Quantitation of Topoisomers. Gels were stained with 1 μg/mL ethidium bromide and photographed at 300 nm through orange and red filters, using Polaroid positive/negative film (665). The negatives were scanned with a Beckman DU-8B spectrophotometer equipped with a gel scanning attachment.

Determination of the Unwinding Angle for HMG 1. To determine the unwinding angle on nicked circular DNA, we measured the change in the average linking number of closed topoisomers appearing in response to increasing doses of HMG 1 after T4 ligation. This was divided by the molar ratio of HMG 1 to input DNA and then multiplied by 360°. The unwinding angle was determined on negatively supercoiled DNA by the change in average linking number in response to increasing amounts of HMG 1 using *E. coli* topoisomerase I. Determination of the unwinding angle for HMG 1 using CT topoisomerase I was hampered by the fact that at low HMG 1 to DNA molar ratios (<100:1), this enzyme produces positive topoisomers, as determined by 2D gel electrophoresis. At a molar ratio of HMG 1 to DNA equal to 100:1, a peak in the amount of highly negatively supercoiled DNA was observed, indicating total protection of about half the DNA (average linking number −16) from CT topoisomerase I. At higher molar ratios of HMG 1 to DNA (between 100:1 and 200:1), the positive topoisomers are gradually replaced by negative topoisomers, as determined by two-dimensional electrophoresis.

Results

Effect of HMG 1 on the Torsional State of Nicked Circular DNA. The ability of HMG 1 to unwind nicked circular DNA was found to depend on the purity of the preparation. HMG 1 purified only as far as the CM-Sephadex chromatographic step, which contains a number of contaminants (see Materials and Methods), effected a progressive change in the linking number with doses up to 200 mol/mol of nicked DNA, as shown in Figure 1. A dose of 100 mol/mol decreased the linking number produced from (+1 to +5) to (−1 to −8). Both 200 and 400 mol/mol decreased the linking number of the topoisomers produced to −12 to −16 (Figure 1, lanes 4 and 5). On the basis of the decrease in the average linking number with amounts of HMG 1 up to 200:1, the unwinding angle determined for this CM-Sephadex preparation was 28.8°.

HMG 1 that had been further purified by Mono S chromatography had an unwinding angle on nicked circular DNA of only 7.2° (not shown), one-fourth of that determined for the less pure preparation, indicating that contaminants in the CM-Sephadex preparation contribute significantly to the unwinding of nicked circular DNA. The unwinding activity does not appear to affect negatively supercoiled DNA, since the unwinding angle obtained on this form of DNA was much the same for both CM-Sephadex-purified and Mono S purified HMG 1. These findings were confirmed in several preparations from both columns. The subsequent studies on closed circular DNAs all employed Mono S purified HMG 1, in order to eliminate any contaminating strand-separating activity.

Effect of Mono S Purified HMG 1 on the Torsional State of Highly Negatively Supercoiled pBR322 DNA. (1) Studies with E. coli Topoisomerase I. A preferential interaction of HMG 1 with negatively supercoiled DNA—as compared to relaxed DNA—has been demonstrated previously using competitive binding to ssDNA (Bonne et al., 1980). However, no

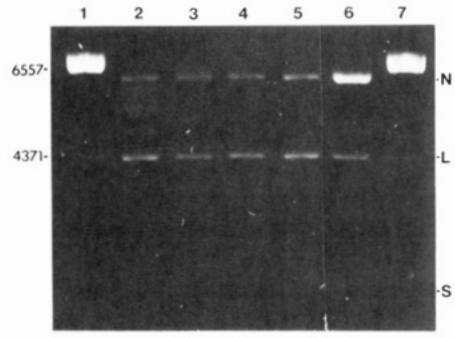

FIGURE 1: Effect of CM-Sephadex-purified HMG 1 on the linking number and sign of nicked circular pBR322 DNA ligated with T4 ligase. Nicked circular pBR322 DNA (0.5 μg/50-μL final reaction volume) was preincubated with increasing concentrations of CM-Sephadex-purified HMG 1 in the Biolabs recommended T4 ligation buffer for 15 min at 24 °C. Four units of T4 ligase were then added, and the mixture was incubated for 60 min at 24 °C. The reactions were stopped by adding 0.1 volume of 0.5 M EDTA plus 10% SDS; then 0.1 volume of loading dye [0.25% bromphenol blue in 40% sucrose (w/v)] was added, and 15–20-μL samples of each reaction were run on a 1% agarose gel in 1 × TBE buffer for 16–20 h at 2.7 V/cm. The gel was then stained with ethidium bromide (1 μg/mL), destained in water, and photographed. The letters along the side of the gel ("N", "L", and "S") denote the electrophoretic position of nicked circular, linear, and supercoiled pBR322 DNA, respectively. Lanes 1 and 7, *Hin*dIII restriction fragments of λ DNA (6557 and 4371 bp); lane 2, control nicked circular DNA treated with T4 ligase (no HMG 1); lanes 3–5, nicked circular DNA preincubated with CM-Sephadex-purified HMG 1 (at protein to DNA molar ratios of 100:1, 200:1, and 400:1, respectively) and then treated with T4 ligase; lane 6, control nicked circular DNA by itself.

analysis of unwinding of this substrate has been reported. In order to assess the unwinding of negatively supercoiled DNA by HMG 1, we have studied the relaxation of the HMG 1/DNA complex with *E. coli* topoisomerase I. Preincubation of highly negatively supercoiled DNA with amounts of HMG 1 up to molar ratios <100:1, followed by *E. coli* topoisomerase I relaxation, decreased the average linking number of the topoisomers produced by this enzyme (Figure 2A, lanes 3–6). This progressive protection of negative supercoils with increasing amounts of HMG 1 is consistent with unwinding of the DNA. On the basis of the average change in the linking number of the resultant topoisomers, the calculated unwinding angle for HMG 1 was determined to be 57.6°. One-dimensional gel analysis showed apparently complete protection of negatively supercoiled DNA at HMG 1 to DNA molar ratios of 100:1 (Figure 2A, lane 7) and greater (not shown). This "complete" protection, however, only reflects the inability of a 1D analysis to resolve topoisomers with linking numbers greater than −16. Two-dimensional analysis (Figure 2B) reveals topoisomers with linking numbers between −16 and −22 at a molar ratio of 135:1 (panel III), while at 235:1 all the DNA is completely negatively supercoiled DNA ($\sigma = -0.04$). This suggests that DNA is saturated by this molar ratio, as confirmed below.

(*2*) *Studies with Mung Bean Nuclease.* HMG 1 protects highly negatively supercoiled DNA from digestion by excess mung bean nuclease, but at doses that lag behind the unwinding of the negative supercoils in the DNA (Figure 2A, lanes 9–13). Progressively more supercoils were protected from mung bean nuclease cleavage over a range of molar ratios of HMG 1 to DNA from 12.5:1 to 100:1 (lanes 10–13). However, only 50% protection from MBN cleavage was observed, even at the highest amount tested (700:1, not shown), even though negatively supercoiled DNA was completely protected from *E. coli* topoisomerase I at a ratio of 200:1. On the other hand, HMG 1 did not protect the negatively supercoiled

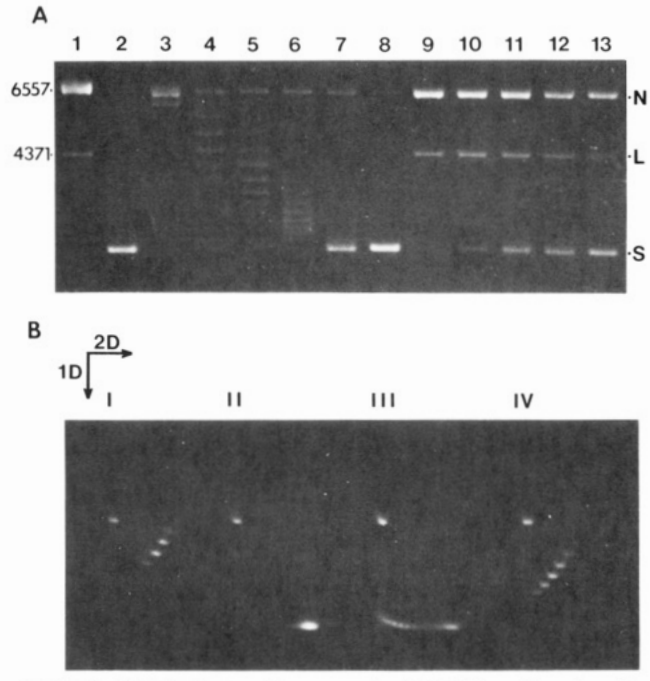

FIGURE 2: Effect of increasing amounts of HMG 1 on the relaxation of highly negatively supercoiled DNA by *E. coli* topoisomerase I and on MBN cleavage. (A) One-dimensional analysis of highly supercoiled DNA relaxed with *E. coli* topoisomerase I or digested by MBN. Highly negatively supercoiled pBR322 DNA ($\sigma = -0.040$, 0.5 μg/50 μL) was preincubated with various concentrations of HMG 1 and then relaxed with 8 units of *E. coli* topoisomerase I for 30 min at 37 °C. Lane 1, *Hin*dIII restriction fragments of λ DNA (6557 and 4371 bp); lanes 2 and 8, control highly negatively supercoiled pBR322 DNA; lanes 3–7, DNA samples relaxed with *E. coli* topoisomerase I; lanes 3 and 9, DNA sample incubated in the absence of HMG 1; lanes 9–13, DNA samples cleaved by MBN (30 units/μg of DNA); lanes 4–7 and 10–13 contained samples of HMG 1 and highly negatively supercoiled DNA preincubated for 15 min at 37 °C in the presence of HMG 1 to DNA molar ratios of 12.5:1, 25:1, 50:1, and 100:1, respectively. (B) Two-dimensional analysis of highly negatively supercoiled DNA relaxed with *E. coli* topoisomerase I. Conditions for relaxing HMG 1/DNA mixtures as above, but with two subsaturating doses of HMG 1 (35 or 135 mol/mol) and one saturating dose of HMG 1 (235 mol/mol). The gel was run in the first dimension as described in the legend to Figure 1, soaked in 1 × TBE buffer containing 1.28 μM chloroquine phosphate for 4–6 h, rotated 90°, and then electrophoresed as above in TBE buffer containing 1.28 μM CQ-PO_4. After soaking in water for 6 h to remove the CQ, the gel was stained and photographed as described in the legend of Figure 1. In each panel, the heavy band at the upper left represents nicked circular DNA. Panels I–IV, supercoiled DNA relaxed with *E. coli* topoisomerase I in the absence (panel I) and presence of HMG 1 at molar ratios to DNA of 235:1, 135:1, and 35:1, respectively.

pBR322 DNA from digestion by rate-limiting amounts of the multiple-cutting restriction endonuclease *Msp*I, even at 200:1 mol/mol, since no alteration in the restriction digest pattern occurred (not shown).

(*3*) *Studies with Calf Thymus Topoisomerase I.* When highly negatively supercoiled DNA is relaxed by CT topoisomerase I in the absence of HMG 1, four to five positive topoisomers are produced (Figure 3A, lane 2). However, preincubation with HMG 1 at molar ratios ≤100:1 changes the relaxation pattern: the intensity of the positive topoisomers decreases (lanes 3–6), while closed circular ("C") and highly negatively supercoiled ("S") DNAs increase (lanes 3–6). The amount of DNA in both C and S peaks at a molar ratio of 100:1 (lane 6). The increase in "S" indicates that negatively supercoiled DNA (linking numbers ≥−16) has been totally relaxed and thus is protected from CT topoisomerase I relaxation. This maximal protection of highly negatively supercoiled DNA at a molar ratio of 100:1 was confirmed by

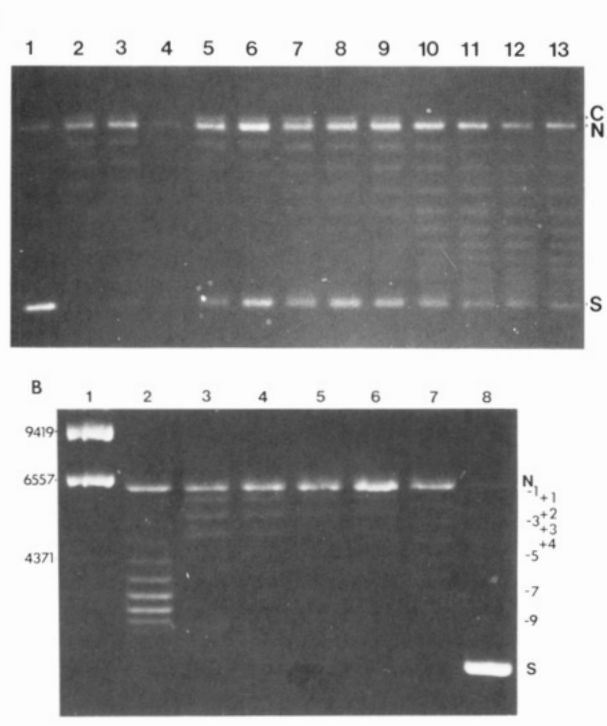

FIGURE 3: Effect of HMG 1 on the relaxation of highly and moderately negatively supercoiled pBR322 DNA by CT topoisomerase I. (A) Highly negatively supercoiled DNA. After preincubation, HMG 1/DNA mixtures were relaxed with CT topoisomerase I (2 units/μg of DNA), and reactions were stopped with 1% SDS. The samples were then phenol-extracted and ethanol-precipitated to remove the HMG 1 and topoisomerase I. The extracted DNA samples were redissolved in 10 mM Tris/1 mM EDTA and, after addition of loading dye, were run on 1% agarose gels as described in the legend to Figure 1. The slowest migrating band is completely relaxed circular DNA ("C"), while the next slowest is nicked circular DNA ("N"); this is followed by a series of topoisomers with linking differences of 1; the fastest moving band is highly supercoiled DNA ("S"). Lane 1, control negatively supercoiled pBR322 DNA; lane 2, same DNA as in lane 1, preincubated without HMG 1 and then relaxed with CT topoisomerase I; lanes 3–13, DNA preincubation with HMG 1 at molar ratios of HMG 1 of 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 275 mol of HMG 1/mol of DNA, respectively, and then relaxed with calf thymus topoisomerase I. (Most of the DNA sample in lane 4 was lost during the loading of the gel.) (B) Moderately negatively supercoiled DNA. Moderately negatively supercoiled pBR322 DNA (σ = –0.018, 0.5 μg/50 μL) was incubated with increasing concentrations of HMG 1 and then relaxed with calf thymus topoisomerase I (2 units/μg of DNA). "N" is nicked circular DNA; "S" is highly negatively supercoiled DNA (σ = –0.04); numbers –1 to –9 refer to the negative topoisomers in lanes 2, 6, and 7; numbers +1 to +4 refer to the positive topoisomers in lanes 3–5. Lane 1, *Hin*dIII restriction fragments of λ DNA (9419, 6557, and 4371 bp); lane 2, control moderately negatively supercoiled DNA (σ = –0.018); lane 3, moderately negatively supercoiled DNA preincubated in the absence of HMG 1 and then relaxed by CT topoisomerase I; lanes 4–7, moderately negatively supercoiled DNA relaxed with CT topoisomerase I after being preincubated with HMG 1 at molar ratios of 31:1, 97:2, 128:2 and 260:1; lane 8, control highly negatively supercoiled pBR322 DNA (σ = –0.040).

two-dimensional gel analysis (not shown) and by the resistance of such HMG 1/DNA mixtures to *E. coli* topoisomerase I (shown above in Figure 2, lanes 7). This protection from CT topoisomerase I indicates the unwinding angle for HMG 1 on negatively supercoiled DNA is 57.6°. At ratios between 100:1 and 200:1, both the positive supercoiled topoisomers and closed circular DNA disappear, while moderately negatively supercoiled topoisomers appear in response to CT topoisomerase I (lanes 7–10). A uniform distribution of negative topoisomers, ranging from –1 to –16, was reached at a HMG 1 to DNA molar ratio of 200:1 and above (lanes 10–13). Since there is a decrease in the linking number by 1 for every 6.25 mol of HMG 1 added between 100 and 200 mol of HMG 1 per mole of DNA, this indicates a progressive protection of negative supercoils initially present in the population. Thus, in this range of HMG 1 concentrations, we again estimate that the unwinding angle for HMG 1 on highly negatively supercoiled pBR322 DNA to be 57.6°. Molar ratios of HMG 1 greater than 200:1 did not further alter the topoisomer pattern observed (lanes 11–16), supporting the concept that the DNA becomes totally relaxed by HMG 1 at this ratio, as noted above using *E. coli* topoisomerase I (Figure 2B, panel II).

Several control experiments were performed in order to show that HMG 1 is not capable of directly inhibiting CT topoisomerase I activity. Doubling the amount of topoisomerase I per microgram of DNA does not change the relaxation pattern observed, indicating that the amount of topoisomerase I present was not rate limiting. Even in the presence of high levels of HMG 1, the topoisomerase I is still active, since it was still active when a second dose of DNA was added. Preincubation of CT topoisomerase I by itself at 37 °C reduces enzyme activity by about 50% after 15 min, while preincubation of the enzyme with HMG 1 actually prevents loss of enzyme activity. Thus, HMG 1 does not appear to be a direct inhibitor of topoisomerase activity.

Effect of Mono S Purified HMG 1 on the Torsional State of Relaxed Closed Circular pBR322 DNA. (1) Relaxed pBR322 DNA Preincubated with HMG 1 and Then Relaxed Once More with CT Topoisomerase I. Relaxed DNA was generated by relaxing supercoiled pBR322 DNA with CT topoisomerase I, and then the DNA was purified (see Materials and Methods). Preincubation of this population of relaxed topoisomers (σ = +0.011) with increasing amounts of HMG 1 only slightly affected the pattern produced by subsequent treatment with CT topoisomerase I and generated a maximum of 8–10 negative topoisomers at a molar ratio of 200:1 (not shown). On the basis of the relative change in the total linking number, we estimate the unwinding angle for HMG 1 on this DNA substrate to be 7.2°, a value similar to that determined for the Mono S purified HMG 1 on nicked circular DNA. Molar ratios greater than 200:1 failed to change the linking number further (not shown). This also confirms the saturation observed with *E. coli* and CT topoisomerase I relaxation of negatively supercoiled DNA at a ratio of 200:1 shown above.

(2) Relaxed pBR322 DNA Incubated with HMG 1 in the Continuous Presence of CT Topoisomerase I. Highly negatively supercoiled DNA was first relaxed with CT topoisomerase I. HMG 1 was then added to the tube along with a second dose of CT topoisomerase I, and the reaction was incubated for a further 30 min at 37 °C. Under these conditions, increasing doses of HMG 1 progressively produced up to 8–10 negative topoisomers, which were not produced in control DNA that had only been exposed to the 2 doses of topoisomerase I (not shown). Thus, HMG 1 produces a small change in the linking number of continuously relaxed DNA, with an apparent unwinding angle of about 7.2°, similar to that determined for HMG 1 on nicked circular DNA and CT topoisomerase I relaxed DNA.

(3) Moderately Negatively Supercoiled DNA Preincubated with HMG 1 and Then Relaxed. Samples of moderately negatively supercoiled pBR322 DNA (σ = –0.018) were preincubated with increasing concentrations of HMG 1 and then relaxed with calf thymus topoisomerase I (Figure 3B). Treatment with CT topoisomerase I by itself produced a population of moderately positively supercoiled topoisomer bands (+1 to +4, lane 3), which run with electrophoretic

mobilities between the negatively supercoiled topoisomer bands present in the initial DNA population (−1 to −10, lane 4). Adding increasing amounts of HMG 1 progressively protected the negatively supercoiled topoisomers initially present in the population, since the proportion of DNA in the negative topoisomer bands progressively increased, while the positive topoisomer bands disappeared (lanes 4–7). On the basis of the decrease in linking number observed between lanes 5 and 6, and the relative increase in the ratio of HMG 1 to DNA between these two lanes, the unwinding angle is estimated to be 58°. This is comparable to the value obtained with highly negatively supercoiled DNA. Even at the highest amount of HMG 1, however, the number of negative topoisomers protected (−10) was never greater than those present in the original population (compare lanes 2 and 7). A similar limit to the protection of negative topoisomers was also observed when negatively supercoiled DNA was preincubated with maximally effective amounts of HMG 1 and then relaxed by CT topoisomerase I (molar ratios ≥200:1, Figure 3A). A uniform distribution of negative topoisomers was produced (−1 to −10, lane 7), as was observed with highly negatively supercoiled DNA (−1 to −16).

These results indicate that (1) the protection afforded by HMG 1 does not exceed the torsion initially present and (2) the DNA is apparently saturated by HMG 1 at a molar ratio of 200:1.

Competition between Highly Negatively Supercoiled DNA and Either Moderately Negatively Supercoiled or Moderately Positively Supercoiled DNA for the Protective Effect of HMG 1. Preparations of either moderately negatively supercoiled or moderately positively supercoiled pBR322 DNA were mixed with an equal amount (w/w) of highly negatively supercoiled DNA. The mixtures were incubated with various concentrations of HMG 1 and then relaxed with *E. coli* topoisomerase I (Figure 4). The moderately negatively supercoiled DNA competed for the protection of highly negatively supercoiled DNA by HMG 1, whereas moderately positively supercoiled DNA did not. The protection conferred on highly negatively supercoiled DNA by HMG 1 was reduced by 50% when it was mixed with an equal amount of moderately negatively supercoiled DNA at 200 mol of HMG/mol of DNA (Figure 4B, panel I), but the moderately negatively supercoiled DNA did not reduce the protection of the highly negatively supercoiled DNA at a molar ratio of 400:1 (Figure 4B, panel II). This plateau in protection again suggests that the DNA becomes saturated at a molar ratio of 200:1. Moderately positively supercoiled DNA did not decrease the amount of highly negatively supercoiled DNA protected by a molar ratio of 200:1 (compare panel I with panel II in Figure 4A).

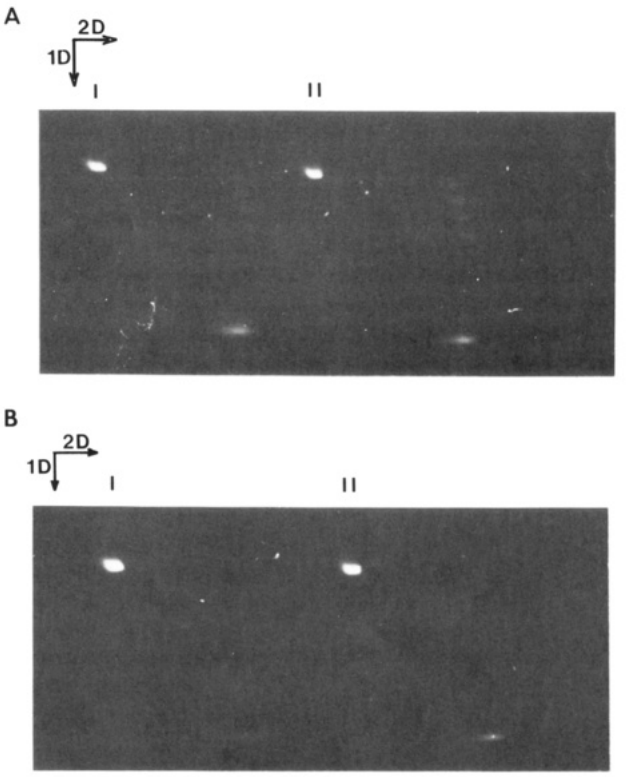

FIGURE 4: Competition for the protective effect of HMG 1 on *E. coli* topoisomerase I mediated relaxation of highly negatively supercoiled DNA by moderately negatively supercoiled DNA but not by moderately positively supercoiled DNA. The two-dimensional gels were run as described in the legend to Figure 2B. (A) Competition with moderately positively supercoiled DNA. Panel I, a mixture of moderately positively supercoiled pBR322 DNA (0.5 μg/50 μL) and highly negatively supercoiled DNA (0.5 μg/50 μL) was preincubated with 200 mol of HMG 1/mol of DNA and then relaxed with *E. coli* topoisomerase I. Panel II, the same DNA mixture as in panel 1 preincubated with 400 mol of HMG 1/mol of DNA before relaxation with *E. coli* topoisomerase I. (Note that the positive topoisomers are unaffected by the *E. coli* topoisomerase I while all the highly negatively supercoiled DNA is protected by preincubation with either dose of HMG 1.) (B) Competition with moderately negatively supercoiled DNA. Panel I, a mixture of moderately negatively supercoiled DNA (0.5 μg/50 μL) and highly negatively supercoiled DNA (0.5 μg/50 mL) was precincubated with HMG 1 (200 mol of HMG 1/mol of DNA) and then relaxed with *E. coli* topoisomerase I. (Note that only half of the highly negatively supercoiled DNA was protected from relaxation, as compared with panel II below and panels I and II above.) Panel II, the same DNA mixture as in panel I, preincubated with 400 mol of HMG 1/mol of DNA. (Note that this higher dose was required to completely protect the highly negatively supercoiled DNA from relaxation.)

Discussion

The major conclusion of our study is that HMG 1 unwinds negatively supercoiled DNA much more effectively than positively supercoiled or nicked circular DNA. At molar ratios of ≥200:1, HMG 1 totally protects negatively supercoiled DNA from relaxation by *E. coli* topoisomerase I; the DNA may actually be slightly positively supercoiled, since it remains partially susceptible to relaxation by the CT topoisomerase I. Javaherian et al. proposed that HMG 1 induces negative supercoils into nicked circular DNA by local separation of DNA strands, based on thermal denaturation data (Javaherian et al., 1979). However, the response to thermal denaturation has now been attributed to protein aggregation and/or to contaminating unwinding protein (Shastri, 1982; Marekov et al., 1986). Helix destabilization therefore does not appear to be a valid explanation for the action of HMG 1. Our observations emphasize the importance of using sufficiently purified HMG 1 and suggest that the higher unwinding angle observed in CM-Sephadex-purified HMG 1 when assayed on nicked circular DNA (Javaherian et al., 1978, 1979; this paper) may be due to contaminating unwinding protein (Marekov et al., 1986). The unwinding angle determined for Mono S purified HMG 1 on either nicked or relaxed closed circular DNA, 7.2°, is similar to values determined for the interaction of various simple electrolytes and core histones with closed circular DNA (Anderson & Bauer, 1978; Baase & Johnson, 1979). We conclude that pure HMG 1 does not substantially unwind either nicked circular DNA or relaxed closed circular DNA by strand separation, confirming and extending recent thermal denaturation studies using linear DNA (Shastri et al., 1982; Marekov et al., 1984, 1986; Butler et al., 1985). The unwinding angle for pure HMG 1 on negatively supercoiled DNA is 57.6°. Thus, HMG 1 is 8 times more efficient at unwinding negatively supercoiled DNA than torsionally re-

laxed DNAs. This preferential unwinding of supercoiled DNA by HMG 1 is fully reversible and occurs with negatively but not with positively supercoiled DNA. In confirmation of the selective effect on negative supercoils, moderately negative supercoiled DNA competes for HMG 1 with highly negative supercoiled DNA, while positively supercoiled DNA does not.

We estimate the "HMG 1 binding site" to be approximately 20 bp, since pBR322 DNA is apparently saturated by HMG 1 at a molar ratio of 200:1 regardless of its torsional tension. A similar level of saturation was observed for moderately and highly negatively supercoiled DNA as well as for nicked circular and positively supercoiled DNA. This "binding site" value is in agreement with several earlier estimates using ss and ds linear DNA (Shooter et al., 1974; Shepelev et al., 1982; Brown & Anderson, 1986; Watt & Molloy, 1988). Since all forms of DNA display saturation at approximately the same molar ratio, HMG 1 binding does not appear to require negative supercoiling to induce a ss site 20 nucleotides in length. The amount of HMG 1 that interacts with negatively supercoiled DNA at saturation appears to be proportional to the length of the DNA, and not to its superhelical density per se [this paper and unpublished data with negatively supercoiled PM2 DNA, $\sigma = -0.080$, which has eight early denaturation regions and an A + T content of 57%, as compared to pBR322 which has only two early denaturation regions and an A + T content of 46% (Sheflin & Kowalski, 1984)].

The features recognized by HMG 1 in supercoiled DNA are not clear. Supercoiling can selectively alter the conformation of DNA in certain sequences, increasing their susceptibility to single-strand-specific endonucleases (Lilley, 1980; Panayotatos & Wells, 1981; Sheflin & Kowalski, 1984, 1985). Adding HMG 1 to supercoiled DNA *decreases* the ability of single-strand-specific endonucleases to cleave potential ss sequences (Cockerill & Goodwin, 1983; Hamada & Bustin, 1984; Bustin & Soares, 1985; Butler, 1986; this paper). If HMG 1 were simply stabilizing the ss regions that form in negatively supercoiled DNA, one would expect it to *increase* ss nuclease digestion, as observed with *E. coli* SSB protein (Glikin et al., 1983). In fact, SSB has been shown to recognize and denature sites that are different from the S1 nuclease sensitive sites, but which are similar to sites recognized in chromatin by micrococcal nuclease and DNase I (Glikin et al., 1983). Since we have found that HMG 1 dramatically *lowers* the superhelical density of negatively supercoiled DNA, it should actually prevent the formation of secondary structures. This provides a more logical explanation for the observed protection of specific DNA sequences (Cockerill & Goodwin, 1983; Bustin & Sources, 1985; Hamada & Bustin, 1985; Butler, 1986) and the overall resistance of negatively supercoiled DNA to ss-specific endonucleases conferred by HMG 1 (Hamada & Bustin, 1985; this paper). It thus appears that HMG 1 recognizes some feature(s) of negatively supercoiled DNA other than single-stranded regions.

The binding of HMG 1 to DNA involves ionic interactions between the basic regions of HMG 1 and the negative charges in the phosphate backbone in DNA (Shooter et al., 1974). Negatively supercoiled DNA has fewer bases per helical turn than torsionally relaxed or positively supercoiled DNA (Richardson et al., 1988). Since helical twist directly affects the angular position of phosphate groups (Trifonov, 1982), the preferential binding of HMG 1 to negatively supercoiled DNA may be related to altered accessibility of some feature in the sugar–phosphate backbone. Presumably, relaxing the negative torsion in the DNA produces a conformational change in HMG 1 as well. Such a conformational change has been observed when HMG 1 binds to ss- or dsDNA (Butler et al., 1985). It is interesting that HMG 1 also preferentially binds to bent regions in linear DNA, specifically to AT-rich sequences (Brown & Anderson, 1986) and origins of DNA replication (Bottger et al., 1988). This suggests that such sequences could contain features similar to those which HMG 1 recognizes in negatively supercoiled DNA.

HMG 1 prevents the formation of certain altered secondary structures in negatively supercoiled prokaryotic DNA and stimulates in vitro transcription (Waga et al., 1988). HMG 1 also stimulates binding of a specific transcription factor to the promoter region of adenovirus major late gene product, enhancing transcription and altering DNase I sensitivity of this region (Watt & Molloy, 1988). Additionally, HMG 1 enhances promotion of transcription with RNA polymerase II in vitro (Tremethick & Molloy, 1986; Stoute & Marzaluff, 1982) and removes a transcriptional block in prokaryotic DNA caused by Z-DNA formation (Waga et al., 1988). These observations indicate that HMG 1 may act in a general manner to promote transcription. Taken with the recent observation of Waga et al. (1988), our findings further suggest that the relaxing effect of HMG 1 on underwound DNA may facilitate the formation of a stable initiation complex by preventing formation of supercoil-dependent secondary structures.

Dynamic changes in DNA conformation are believed to play a role in regulating gene expression and DNA replication. When HMG 1 binds to underwound DNA, its torsion is protected from topoisomerase activity, which could alter local DNA conformation in a reversible fashion. Transcription in eukaryotic cells can be accompanied by the underwinding of upstream DNA and the generation of positively supercoiled domains downsteam from the transcriptional complex (Wu et al., 1988). Similar supercoiled domains may be generated by other processes involving "macromolecular translocations along DNA" (Liu & Wang, 1987). If HMG 1 were available to selectively bind and neutralize negative domains, only the positive domains would be relaxed by eukaryotic topoisomerase activites. Conservation of the negative torsion generated during eukaryotic transcription by HMG 1 could prevent the formation of altered secondary structures, as well promote the binding of specific regulatory proteins (Watt & Molloy, 1988; Tremethick & Molloy, 1988). Underwinding is also required for nucleosome formation. When nucleosomes are removed from a stretch of DNA, they leave it underwound: HMG 1 could bind to the DNA to conserve its torsion, protecting it from topoisomerase activity and serving as a "bookmark" to promote the localized re-formation of the nucleosomes.

Acknowledgments

We thank Nancy Fucile for expert technical assistance in the purification and characterization of HMG 1, Drs. Richard Lynn and James Wang for the kind gift of *E. coli* topoisomerase I, and Drs. Edward Niles, Robert Noble, and Robert Umek for constructive comments on the manuscript.

References

Alexandrova, E. A., & Beltchev, B. G. (1987) *Biochim. Biophys. Acta 915*, 399–405.

Alexandrova, E. A., & Beltchev, B. G. (1988) *Biochem. Biophys. Res. Commun. 154*, 918–927.

Alexandrova, E. A., Marekov, L. N., & Beltchev, B. G. (1984) *FEBS Lett. 178*, 153–156.

Anderson, P., & Bauer, W. (1978) *Biochemistry 17*, 594–601.

Baase, W. A., & Johnson, W. C., Jr. (1979) *Nucleic Acids Res. 6*, 797–814.

Bofinger, D. P., Fucile, N. W., & Spaulding, S. W. (1988) *Anal. Biochem. 170*, 9–18.
Bonne, C., Duguet, M., & DeRecondo, A.-M. (1980) *Nucleic Acids Res. 8*, 4955–4966.
Bonne, C., Sautiere, P., Duguet, M., & DeRecondo, A.-M. (1982) *J. Biol. Chem. 257*, 2722–2725.
Bonne-Andrea, C., Harper, F., Sobczak, J., & DeRecondo, A.-M. (1984) *EMBO J. 3*, 1193–1199.
Bonne-Andrea, C., Harper, F., Puvion, E., Delpech, M., & DeRecondo, A.-M. (1986) *Biol. Cell 58*, 185–194.
Bottger, M., Vogel, F., Platzer, M., Kiessling, U., Grade, K., & Straus, M. (1988) *Biochim. Biophys. Acta 950*, 221–228.
Brown, J. W., & Anderson, J. A. (1986) *J. Biol. Chem. 261*, 1349–1354.
Bustin, M., & Soares, N. (1985) *Biochem. Biophys. Res. Commun. 133*, 633–640.
Butler, A. P. (1986) *Biochem. Biophys. Res. Commun. 138*, 910–916.
Butler, A. P., Mardian, J. K. W., & Olins, D. E. (1985) *J. Biol. Chem. 260*, 10613–10620.
Carballo, M., Puigdomenech, P., Tancredi, T., & Palau, J. (1984) *EMBO J. 3*, 1255–1261.
Cary, P. D., Turner, C. H., Leung, I., Mayes, E., & Crane-Robinson, C. (1984) *Eur. J. Biochem. 143*, 323–330.
Champoux, J. J., & Dulbecco, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 143–146.
Cockerill, P. N., & Goodwin, G. H. (1983) *Biochem. Biophys. Res. Commun. 112*, 547–554.
Duguet, M., Bonne, C., & DeRecondo, A.-M. (1981) *Biochemistry 20*, 3598–3603.
Earnshaw, W. C. (1987) *J. Cell Biol. 105*, 1479–1482.
Germond, J.-E., Rouviere-Yaniv, J., Yaniv, M., & Brutlag, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3779–3783.
Giaever, G. N., & Wang, J. C. (1988) *Cell 55*, 849–856.
Glikin, G. C., Gargiulo, G., Rena-Descalzi, L., & Worcel, A. (1983) *Nature 303*, 770–774.
Goodwin, G. H., Walker, J. M., & Johns, E. W. (1978) *Biochim. Biophys. Acta 519*, 233–242.
Hamada, H., & Bustin, M. (1985) *Biochemistry 24*, 1428–1433.
Holmes, D. S., & Quigley, M. (1981) *Anal. Biochem. 114*, 193–197.
Isackson, P. J., Fishback, J. L., Bidney, D. L., & Reeck, G. R. (1979) *J. Biol. Chem. 254*, 5569–5572.
Isackson, P. J., Beaudoin, J., Hermodson, M. A., & Reeck, G. R. (1983) *Biochim. Biophys. Acta 748*, 436–443.
Javaherian, K. J., Liu, L. F., & Wang, J. C. (1978) *Science 199*, 1345–1346.
Javaherian, K., Sadeghi, M., & Liu, L. F. (1979) *Nucleic Acids Res. 6*, 3569–3580.
Karhu, I., Mahonen, A., & Palvimo, J. (1988) *J. Chromatogr. 426*, 65–73.
Keller, W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4876–4880.
Kowalski, D. (1984) *Nucleic Acids Res. 12*, 7071–7086.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Levitt, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 640–644.
Levy-Wilson, B., Connor, W., & Dixon, G. H. (1979) *J. Biol. Chem. 254*, 609–620.
Lilley, D. M. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6468–6472.
Liu, L. F., & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7024–7027.
Marekov, L. N., Beltchev, B. G., & Pivec, L. (1984) *Biochem. Biophys. Res. Commun. 120*, 782–788.
Marekov, L. N., Demirov, D. G., & Beltchev, B. G. (1986) *Biosci. Rep. 6*, 137–142.
Panayotatos, N., & Wells, R. D. (1981) *Nature (London) 289*, 466–470.
Richardson, S. M. H., Boles, T. C., & Cozzarelli, N. R. (1988) *Nucleic Acids Res. 16*, 6607–6616.
Sanders, C. (1977) *Biochem. Biophys. Res. Commun. 78*, 1034–1042.
Seyedin, S. M., Pehrson, J. R., & Cole, R. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5988–5992.
Shastri, K., Isackson, P. J., Fishback, J. L., Land, M. D., & Reeck, G. R. (1982) *Nucleic Acids Res. 10*, 5059–5072.
Sheflin, L. G., & Kowalski, D. (1984) *Nucleic Acids Res. 12*, 7087–7104.
Sheflin, L. G., & Kowalski, D. (1985) *Nucleic Acids Res. 13*, 6137–6154.
Shepelev, V. A., Kosaganov, Y. N., & Lazurkin, Y. S. (1984) *FEBS Lett. 172*, 172–176.
Shooter, K. V., Goodwin, G. H., & Johns, E. W. (1974) *Eur. J. Biochem. 47*, 263–270.
Shure, M., Pulleyblank, D. E., & Vinograd, J. (1977) *Nucleic Acids Res. 4*, 1183–1205.
Stoute, J. A., & Marzluff, W. F. (1982) *Biochem. Biophys. Res. Commun. 107*, 1279–1284.
Tremethick, D. J., & Molloy, P. L. (1986) *J. Biol. Chem. 261*, 6986–6992.
Tremethick, D. J., & Molloy, P. L. (1988) *Nucleic Acids Res. 16*, 11107–11123.
Trifonov, E. N. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 271–278.
Waga, S., Mizuno, S., & Yoshida, M. (1988) *Biochem. Biophys. Res. Commun. 153*, 334–339.
Wang, J. C. (1971) *J. Mol. Biol. 55*, 523–533.
Wang, J. C., & Giaever, G. N. (1988) *Science 240*, 300–304.
Watt, F., & Molloy, P. L. (1988) *Nucleic Acids Res. 16*, 1471–1486.
Wu, H.-Y., Shyy, S., Wang, J. C., & Liu, L. F. (1988) *Cell 53*, 433–440.
Yoshida, M. (1987) *J. Biochem. 101*, 175–180.