H1 Binding Unwinds DNA

EVIDENCE FROM TOPOLOGICAL ASSAYS*

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The preference of the linker histones to bind to superhelical DNA in comparison with linear or relaxed molecules suggests that these proteins might, in turn, change the twist and/or writhe of DNA molecules upon binding. In order to explore such a possibility, we looked for changes in the linking number of plasmid pBR322 caused by H1 binding, using assays that involve nicking and rescaling of DNA strands. Two types of enzymes were used, eukaryotic topoisomerase I and prokaryotic DNA ligase. The results revealed that H1 binding causes unwinding of the DNA, with the unwinding angle being approximately 10°. The globular domain of histone H1 is also capable of unwinding DNA, but to a lesser degree.

It has been known for a number of years that the linker histones (H1, H5, and their variants) exhibit a preference to bind to superhelical DNA over linear or relaxed DNA (1–3). A reasonable corollary to this observation is that these proteins, in binding to superhelical DNA, might change the twist of the double helix and/or the writhe of the helical axis in space. Consistent with this notion, we have recently shown that titration of superhelical plasmid DNA with increasing amounts of histone H1 changes the patterns of cleavage by single strand-specific nuclease, causing disappearance of preexisting nuclease-sensitive sites at low and moderate H1 levels, followed by the appearance of new sites at high protein levels (4). A possible explanation for the disappearance of preexisting sites is that histone H1 binding absorbs some of the negative superhelical stress in the molecule (unwinds the DNA), so that the decreased stress leads to loss of stress-dependent sensitive sites.

Previous work on the binding of histone H1 or H5 to superhelical plasmids has led to contradictory interpretations. Bina-Stein and Singer (5) interpreted their data as indicating stabilization of preexisting superhelical turns by H1 binding, with no new superhelical turns being created. Stein (6) and Morse and Cantor (7), on the other hand, did not observe stabilization of preexisting superhelical turns. More recently, Shellen et al. (8) reported that histone H1 was not able to change the linking number of superhelical DNA when assayed in the topoisomerase I-mediated relaxation assay (see below).

What is required is a very careful analysis using more than one technique, since the effects might well be small. We decided to utilize both the topoisomerase I-mediated relaxation assay and the ligase-mediated supercoiling assay. The results indicate that linker histones binding unwinds DNA.

EXPERIMENTAL PROCEDURES

Preparation of Plasmid DNA and Histone H1—Plasmid pBR322 was prepared by CsCl purification and phenol extraction (9). Chicken erythrocyte histone H1 was obtained under non-denaturing conditions (10) and checked for purity by SDS-containing polyacrylamide gel electrophoresis (11). The globular domain of histone H1 was prepared as outlined in Krylov et al. (3). The concentration of the protein stock solutions was estimated by scanning of Coomassie-stained polyacrylamide gels, using bovine serum albumin as a standard.

Topoisomerase I-mediated Relaxation Assay—Two µg of pBR322 were relaxed by incubation, at 37 °C, with 2–6 units of calf thymus topoisomerase I (Life Technologies, Inc.) in 240 µl of reaction mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and increasing amounts of histone H1 for the time specified in the figures. After incubation, NaCl and SDS were added to final concentrations of 1 M and 1%, respectively, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. The resulting topoisomer distributions were analyzed by two-dimensional gel electrophoresis.

Ligase-mediated Supercoiling Assay—Nicked DNA containing a limited number of nicks per molecule was prepared by digestion of pBR322 with DNase I (0.068 unit/µg of DNA) in the presence of 0.3 µg of EtBr/ml for 1 h at 30 °C (12). The reaction was stopped by adding EDTA to 3 mM and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The nicked preparation contained no supercoiled topoisomers and only trace amounts of linear molecules. For the ligation reaction, 2 µg of nicked DNA were incubated with 6 units of T4 or Escherichia coli ligase (New England Biolabs), in the buffers recommended by the manufacturer; the concentration of MgCl2 was reduced to 2 mM to improve the binding of H1 to DNA. Reactions were allowed to proceed for 1 h at 32 °C for the T4 ligase and at 25 °C for the E. coli ligase. After purification, DNA was analyzed by two-dimensional agarose-gel electrophoresis.

Gel Electrophoresis—Two-dimensional electrophoresis of DNA was performed in 1% agarose gels in either Tris acetate/EDTA (9) or in the same buffer containing 20 mM sodium acetate (13); the concentration of chloroquine in the second dimension was 2.5 µg/ml. Electrophoresis was carried out at 1 V/cm at room temperature. Gels were washed for several hours to remove the chloroquine, stained with 1 µg of EtBr/ml, and photographed using type S5 positive/negative Polaroid film. Scanning was performed on an EPSON ES-120C scanner using the Adobe Photoshop 3.0 program (Adobe Systems, Inc., Mountain View, CA).

Immunoblots—Mixtures of 1.75 µg of pBR322 and corresponding

RESULTS AND DISCUSSION

Relaxation of Superhelical pBR322 with Topoisomerase I in the Presence of Histone H1—Acting on superhelical DNA, topoisomerase I will relax superhelical stress, ultimately giving rise to a narrow distribution of topoisomers centered around the relaxed topoisomer with ΔLk = 0 (15). However, in the...
presence of a ligand that generates changes in twisting or writhe, the enzyme will produce topoisomers centered around a topoisomer with $\Delta L_h \neq 0$. As is indicated in Fig. 1, such ligands can, in the general sense, be considered either “winding” or “unwinding,” characterized by the sense of the superhelicity remaining after the topoisomerase I-mediated relaxation and removal of the ligand. The two ligands used as familiar examples in Fig. 1 act by changing the twist of the DNA, negatively or positively. However, in principle, the same effects could be expected from ligands which create or stabilize either negative or positive writhe. For example, the addition of nucleosome cores to relaxed DNA will promote negative writhes by wrapping the DNA in a left-handed coil around the histone octamer; upon topoisomerase treatment followed by protein removal, negative superhelical turns will be generated, whose number corresponds approximately to the number of nucleosomes initially formed (16).

The topoisomers produced in the above assay were separated in 1% agarose gels in the first dimension and further fractionated in the same gels in a perpendicular direction in the presence of 2.5 mg/ml of chloroquine/ml. Such gels allow good resolution of a wide range of topoisomers, from $-15$ to $-15$ (for a more detailed explanation of such two-dimensional gels, see Bowater et al. (17), and the schematic diagrams in Fig. 4, A and B).

The results from the topoisomerase I-mediated relaxation assay performed with increasing amounts of histone H1 are shown in Fig. 2. Addition of histone H1 led to an apparent shift in the distribution of topoisomers to less and less relaxed forms. In Fig. 2, A and B, two different concentrations of topoisomerase I were used. In both cases, two populations of molecules can be seen in the control (no H1) relaxation patterns, one consisting of completely relaxed molecules, and the other population remaining at the original position of highly supercoiled topoisomers. Such a pattern is typical of the products of action of the eukaryotic topoisomerases, which are known to be processive under the salt conditions used here (18). The patterns in Fig. 2, A and B, differ only in the relative proportions of the two subpopulations, the relaxed and the residual supercoiled. Essentially, the same result was observed in either case; the presence of moderate levels of histone H1 led to retention of extra negative superhelical turns in the “relaxed” subpopulation as compared to the control. Thus, in a formal sense, H1 at these levels of concentration acts as “unwinding” ligand.

At high input ratios of histone H1 to DNA, an interesting shift was observed in the portion of the gel that contained the population of highly negatively supercoiled topoisomers, originally resistant to the action of topoisomerase I. These topoisomers also became susceptible to relaxation, the effect being especially well seen under suboptimal conditions for enzymatic activity (see Fig. 2B). Why this subpopulation of molecules, resistant to relaxation at low H1 levels, should become susceptible to the topoisomerization reaction at high H1 levels is not clear at the moment. It may reflect “stimulation” of the enzyme (19, 20), due to changes in the conformation of the substrate; the condensed, highly superhelical molecules may open up due to an unwinding effect of H1 binding, thereby allowing the enzyme molecule more effective access to its substrate. Since under the salt conditions used here the enzyme acts processively and, once bound, cannot be released and transferred from one DNA molecule to another (see above), this observation would suggest that even at the “suboptimal” conditions there are enough enzyme molecules to bind to most DNA molecules. The lack of relaxation of the most highly superhelically stressed molecules in the absence of histone H1 could be due to their compact structure. An alternative, and may be more plausible explanation could lie in the slower kinetics of relaxation at saturating levels of H1 (see Fig. 3A, bottom panel). The 60-min relaxation time used in this experiment might have not been enough to complete the reaction at these high levels of H1.

The interpretation of the results from the relaxation assay may obviously be complicated by a possible effect of histone H1 on the enzyme activity. That histone H1 may inhibit topoisomerase I has been recently reported (21, 22), although previous reports have indicated either no significant effect of the
histone on the enzyme activity (5) or even stimulation of activity (19, 20) (see also above).

If histone H1 merely inhibits topoisomerase I, then the reactions done at higher H1 concentrations may simply have not gone to completion during the 1-h incubation with topoisomerase I used in the above experiment (Fig. 2). In order to resolve this question, the kinetics of the topoisomerization reaction were followed either in the absence of H1 or in its presence at increasing amounts of the histone. As shown in Fig. 3A, three top panels, there was a fast initial isomerization (5 min), either in the absence of H1 or in its presence at levels up to 1 molecule of H1 per 40 bp.1 This latter concentration of H1 will approximately saturate the DNA lattice; only at H1 levels above saturation, when the histone tightly covers the entire DNA template (Fig. 3A, bottom panel), was there a significant lag time before the enzyme started relaxing.

At an H1/DNA ratio of 1 molecule of H1 for 40 bp, the initial fast phase of relaxation was followed by a phase or relatively slow further relaxation which continued for at least another hour (Fig. 3A, third panel from the top). We attribute this slower change to a possible displacement of some of the linker histone molecules from the DNA by the acting topoisomerase I. Also, in view of the known preference of H1 to superhelical DNA (see Introduction) (23), we would expect the histone to redistribute from the more relaxed to the less relaxed topoisomers during these more prolonged incubations.

The results from the kinetics experiments showed that an end point in the topoisomer distribution could be closely approached, under the conditions of our assay, by performing the topoisomerase reaction anywhere between 5 and 15 min. The major changes in the topology of the starting population of superhelical molecules took place during this initial period; the additional changes that occurred at longer times with higher H1/DNA ratios were very small and can be neglected. Comparison of the topoisomer patterns obtained early during relaxation shows that the apparent superhelicity end point was different for the different amounts of histone. Quantitation of these patterns by scanning (Fig. 3B) indicates a shift of the centers of the distributions to increasingly negative values with increasing amounts of histone H1 present.

**Ligase-mediated Supercoiling Assay**—Because of possible ambiguities in interpretation of the data from the topoisomerase assay we turned to an alternative assay, ligation of single strand nicks in circular DNA molecules in the absence and presence of histone H1. In the absence of H1, the action of ligase should produce a Boltzmann distribution of topoisomers, centered around the relaxed state2 (15, 24). On the other hand, if addition of H1 to the nicked plasmid either produces a change in twist or induces looping or bending of the DNA, this should be reflected in a change in the distribution of topoisomers generated after sealing of the nick and removal of the proteins.

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1 The abbreviations used are: bp, base pair(s); GH1, globular domain of histone H1.

2 We have recently shown that the commonly used T4 and E. coli DNA ligases are themselves DNA unwinding proteins (Vanchenko, M., van Holde, K., and Zlatonova, J. (1996) Biochem. Biophys. Res. Commun. 226, 498–505. However, for their effect to be seen as a shift in the topoisomer distributions, very high concentrations of the enzymes have to be present, at least 50–100-fold more than the ones used in this work.  

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FIG. 3. A, kinetics of topoisomerase I-mediated relaxation at different levels of histone H1 present (the H1/DNA ratios, expressed as molecules of histone H1 per bp are denoted to the right of each panel). C denotes the starting supercoiled population; M in the top two panels denotes the marker ladder of consecutive topoisomers spreading from 0 to ~25–30 negative superhelical turns (prepared according to Singleton and Wells (29)). B, quantitation of the topoisomer distributions obtained during the initial 5 min of incubation with topoisomerase I: the histone/DNA ratios are 0, 1/50, and 1/40 molecules of histone H1 per bp in a, b, and c, respectively. Since topoisomers ~1, 0, and +1 are not well resolved in these gels, their sum is denoted by the bar at zero. The centers of the distributions are denoted by arrows.
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FIG. 4. Ligase-mediated supercoiling assay. DNA containing single-strand nicks was prepared by mild DNase I treatment of supercoiled pBR322 and was ligated with T4 ligase in the absence or presence of increasing amounts of histone H1. The products of ligation were analyzed by two-dimensional agarose gels. N, nicked circle; L, linear molecule. A, titration with intact histone H1. B, titration with GH1. The protein/DNA ratios were 0, 1/70, 1/50, and 1/30 molecules of H1 per bp in A, and 0, 1/50, 1/33, and 1/20 molecules of GH1 per bp in B. Higher protein/DNA ratios could be tested in the case of GH1, since it inhibited T4 ligase less than did the intact protein (25). Note also that the starting DNA preparation in A contained more nicked material than that in B, since the DNase I treatment was carried out in the absence of EtBr (see “Experimental Procedures”). To facilitate evaluation of the topoisomer patterns, schemes of the patterns are provided for the highest protein/DNA ratios tested: numbered lines represent topoisomers with the respective number of superhelical turns during the electrophoretic analysis. C, quantitation of the topoisomer distributions obtained in the GH1 titrations at histone/DNA ratios of 0, 1/50, 1/33, and 1/20 (molecules of H1 per bp) in a, b, c, and d, respectively. The centers of the distributions are denoted by arrows. Because we could not resolve bands corresponding to topoisomers −1, 0, and +1 well enough to quantitate them separately, the sum of these three is denoted by the bar at zero. This accounts for the seemingly anomalous height of this one bar.

With this assay, inhibition of the enzyme will not lead to ambiguity: more DNA will remain at the position of the nicked circle, but a shift in the distribution of the ligated products over the control (no H1) distribution will still reflect only the H1-dependent changes in topology.

To perform the ligation assay, single strand nicks were first introduced in the supercoiled plasmid population by mild DNase I treatment. The nicked DNA was then incubated with T4 ligase, increasing amounts of histone H1 were added, and the ligation reaction was allowed to proceed by addition of ATP. Alternatively, H1 was added to the DNA first, followed by the enzyme and the cofactor. The ligase reaction was gradually inhibited by increasing amounts of histone H1, as evidenced by more DNA remaining at the position of the nicked circle (Fig. 4A) (see also Ray et al. (25)). The inhibition was independent of the order of addition of components. A similar degree of inhibition was also observed when E. coli ligase was used (not shown). Notwithstanding the inhibition, a small but reproducible effect of H1 binding could be observed (Fig. 4A). Under the combination of ligation and electrophoresis conditions used here, all ligated topoisomers in the control (no H1) reaction were positively supercoiled on the gel. Increasing the H1/DNA input ratio led to a redistribution of the ligated molecules among the individual topoisomers. The peak of the distribution moved to less positive values, with topoisomers +6, +5, and +4 being significantly reduced in amount. More obvious was the change in the upper portion of the topoisomer ladder where at least three negative topoisomers appeared (for interpretation of the pattern at the highest H1 level used, see the scheme in Fig. 4A). The shift in the topoisomer distribution toward more negative values means that histone H1 is unwinding the DNA, in accordance with the results from the topoisomerase I-mediated relaxation assay. Qualitatively the same shift was obtained when H5 (the major member of the linker histone family in chicken erythrocytes) was used instead of H1.

The above effect was much more clearly seen when isolated globular domains of either H1 or H5 were used instead of the intact protein. The linker histones are known to consist of three domains, a structured central globular domain flanked by N- and C-terminal portions which are very basic and probably lack regular structure. The globular domain is implicated in a number of DNA-binding properties of the intact molecule, including its preference for four-way junctions (26, 27), its preferential binding to superhelical DNA (3), and so forth.

The results of the ligation assay performed in the presence of increasing amounts of the globular domain of histone H1 (GH1) are presented in Fig. 4B and the quantitation of the respective patterns is shown in Fig. 4C. From both the visual inspection of the topoisomer distributions on the gel and their histograms it becomes clear that the center of the distribution shifts from around +4 in the absence of the protein to around 3.1, 2.7, and 1.0 in the presence of GH1 at one molecule per 50, 33, and 20 base pairs, respectively.

Estimation of the Unwinding per H1 Molecule Bound—We have presented evidence that the binding of histone H1 to DNA affects superhelicity of closed circular DNA molecules. The observed changes in the topoisomer distributions in both the topoisomerase and the ligase assays suggest an apparent un-
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The effect of linker histone binding on DNA superhelicity is relatively small, and partially hidden by the inhibitory effects of H1 on the enzymes used. Perhaps this explains why it has not been reported previously. Nevertheless, the reliability of the results is supported by the fact that two independent assay methods have been used. The unwinding effect of histone H1 binding should be considered in any analysis of the structure of the chromatin fiber. Finally, we must emphasize that our experiments cannot reveal the mechanism of the unwinding detected, in particular whether twist, writhe, or both are affected.

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FIG. 5. A, agarose gel (left panel) and nitrocellulose replica stained with anti-H1 antiserum (right panel). S denotes the position of supercoiled pBR322; N denotes nicked pBR322. B, the same amounts of H1 as in A, but applied to a nitrocellulose membrane by slot-blotting and stained with the antiserum. C, graph comparing the intensity of H1 spots in A with the intensity of H1 spots in B.

FIG. 6. Graphs of the centers of the topoisomer distributions shown in Figs. 3B and 4C plotted as a function of the histone H1/DNA ratio. The lower line corresponds to the topoisomerase assay of H1-produced unwinding (Fig. 3B), whereas the upper line corresponds to the ligase assay data on GH1 (Fig. 4C).
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