NAP1 Catalyzes the Formation of either Positive or Negative Supercoils on DNA on Basis of the Dimer−Tetramer Equilibrium of Histones H3/H4†

Sharon Peterson, Rachel Danowit, Ann Wunsch, and Vaughn Jackson*

Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

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ABSTRACT: We have studied the tetramer−dimer equilibrium of histones H3/H4 and its effect on DNA supercoiling. Two approaches were found to shift the equilibrium toward dimer. In both instances, when deposited on DNA, the dimers formed positively coiled DNA. The first approach was to modify cysteine 110 of H3 with 5,5′-dithio-bis(2-nitrobenzoic acid (DTNB) and to directly add the histones to DNA at physiological ionic strength. The second approach involved adding an excess of the histone chaperone, nucleosome assembly protein 1 (NAP1) to the H3/H4 prior to deposition on the DNA. It was also observed that when H3/H4 were deposited in the tetrameric state, negatively coiled DNA was formed. The topological state of the DNA prior to deposition was also found to influence the final conformational state of H3/H4. It is proposed that in the tetrameric state, the H3−H3 interface has a left-handed pitch prior to binding DNA. In the dimeric state, the H3−H3 interface is not established until bound to DNA, at which point either the left or right-handed pitch will form on the basis of the initial topology of the DNA. Formaldehyde cross-linking and reversal were applied to identify the histone−histone interactions that facilitate the formation of positive stress. Higher-order interactions between multiple H3/H4 dimers were required to propagate this specific conformation. Changes in the conformational state of H3/H4 were also observed when the histones were bound to DNA prior to treatment with NAP1. It is proposed that these conformational changes in H3/H4 are involved in promoter activation and transcription elongation through nucleosomes.

The DNA of a eukaryotic cell is condensed into a highly organized array of structures referred to as nucleosomes. Each nucleosome consists of two each of histones H2A, H2B, H3, and H4, which form an octameric complex in which 145 bp of DNA are wrapped on the outer surface in a 1.8 left-handed supercoil (*1*−*3*). Upon release of the histones from a nucleosome, the DNA contains the equivalence of one negative coil (*4*). Inter-nucleosomal interaction within the array produces higher levels of compaction that greatly influences the transcriptional activity of a gene (*5*). At physiological ionic strength and in the absence of DNA, H2A and H2B exist as a stable dimer (*6*). Histones H3 and H4 also form a stable dimer, but these dimers tend to prefer a tetrameric state in which a stable H3−H3 interaction is established (*7*−*9*). Because of the stable form of the tetramer, it is generally accepted that this tetramer is first deposited on the DNA to establish the initial left-handed helical pitch of the DNA. The two H2A/H2B dimers then bind the two sides of the tetramer and further extend the left-handed coil (*10*). Histone chaperones such as CAF-1 and Asf1 have been shown to interact with H3/H4 (*11*−*15*) and are considered to be the major means of initiating the deposition. Another chaperone, nucleosome assembly protein 1 (NAP1[1]), has been implicated in the subsequent deposition of the H2A/H2B dimers (*16*). NAP1 has been found to import H2A/H2B into the nucleus (*17*, *18*) and has been shown to facilitate deposition of H2A/H2B (see review in ref *19*). However, it has been observed with *in vitro* studies that NAP1 has a higher preference for binding to H3/H4 than to H2A/H2B (*20*) and as a result will facilitate the complete assembly of a nucleosome through the initial deposition of H3/H4 followed by H2A/H2B (*21*−*24*). NAP1 has been found to be a part of chromatin remodeling complexes at promoters and in the remodeling of nucleosomes during transcription (*25*−*32*). This remodeling is generally considered to be primarily through the displacement of H2A/H2B (*33*−*35*), although it has also been observed that NAP1 facilitates nucleosome sliding as well, which is an indication that NAP1 also interacts with H3/H4, while H3/H4 remain bound to DNA (*36*). The deletion of NAP1 significantly alters gene expression in yeast (*37*).

In 1996, Prunell and his colleagues (*38*) made the important observation that the H3/H4 tetramer could undergo what has been referred to as a chiral transition. When prebound to a minicircle DNA containing a positive topoisomer, subsequent relaxation of the DNA by topoisomerase I showed a significant maintenance of positive stress. This transition was considered to be a change from a left-handed

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* To whom correspondence should be addressed: Phone: (414) 456-8776. Fax: (414) 456-6510. E-mail: jacksonv@mcw.edu.

[1] Abbreviations: NAP1, nucleosome assembly protein 1; 2-ME, 2-mercaptoethanol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); IAA, iodoacetic acid; CsCl−EtBr, cesium chloride−ethidium bromide; SD, superhelical density; ccc, circular, covalently closed; EMSA, electrophoretic mobility shift assay; TEA, triethanolamine.

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to a right-handed pitch at the H3−H3 interface. It was later reported that if the two cysteines at position 110 of the H3−H3 interface were modified with DTNB, the level of positive stress that was maintained after topoisomerase I treatment was substantially increased (*39*−*40*). In this conformation, it was observed that the H2A/H2B dimer was unable to bind this H3/H4. These studies were an indication that the topological state of the DNA could affect the conformational state of the H3/H4. A potential *in vivo* source for such topological changes is the action of RNA polymerase (*41*−*45*). With the formation of positive stress in front of the polymerase and negative stress in its wake, the H3/H4 tetramer could potentially undergo this right-handed conformational change, providing H2A/H2B were displaced during the process. Levchenko et al. (*32*) observed that transcription-induced positive stress could readily displace H2A/H2B and that NAP1 facilitated that process. These experiments were unable to determine the mechanism whereby NAP1 facilitated the release of H2A/H2B from H3/H4.

We have studied the tetramer−dimer equilibrium of histones H3/H4 in order to determine the effects on DNA topology when deposited as either a tetramer or dimer. We have found that the DTNB modification of H3 sufficiently disrupts the H3−H3 interface to shift the equilibrium to the dimeric state. In a highly cooperative manner, high levels of positively coiled DNA were formed by the dimer. When high levels of NAP1 were pre-incubated with H3/H4, a similar effect was observed, which we interpret to indicate that NAP1 can alter the tetramer−dimer equilibrium of H3/H4. These collective observations provide insight into the conformational changes of H3/H4 that may be facilitated by NAP1 in order to displace H2A/H2B during transcription.

EXPERIMENTAL PROCEDURES

Preparation of Histones. Histones were purified by a modification of the procedure of Simon and Felsenfeld (*46*). Purified nuclei from chick erythrocytes were prepared by four washes of 1% Triton X-100, 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM Tris (pH 8.0). Chromatin was then prepared by one wash with 10 mM Tris and 10 mM EDTA (pH 8.0), and one wash with distilled water. The chromatin was sheared by sonication, adjusted to 0.7 M NaCl, 50 mM KH_2P0_4, and 5 mM 2-ME (pH 8.0), and applied to a hydroxylapatite column (Bio-Rad). Histones H2A and H2B were eluted in a stepwise gradient of 0.8 to 1.1 M NaCl. Histones H3 and H4 were eluted in 2.0 M NaCl. Further purification of H3 and H4 was done by applying these fractions to a Mono S column (Pharmacia) and eluting with a gradient of 0.4 to 1.1 M NaCl. Pooled fractions were concentrated on Amicon filters and stored at −70 °C. The histone concentrations were determined with an extinction coefficient of 4.2 cm^{-1} mg^{-1} at 230 nm (*47*).

For some experiments, the N-termini of H3 and H4 were removed. This was done by trypsin treatment using a modified protocol of Ausio et al. (*48*). In summary, micrococcal nuclease-solubilized chromatin (from chicken erythrocytes) was exposed to trypsin-coated beads and the digestion terminated by addition of trypsin soybean inhibitor and removal of the beads. The chromatin immobilized on the hydroxylapatite column, and the histones eluted with increasing NaCl concentrations. The remaining small level of contamination of H2A and H2B was removed by application to a Mono S column. The residues protected from trypsin cleavage in the nucleosome (fragments P1−P5, see ref *49*) are residues 27−129 (fragment P1) for H3 and residues 18−102 (fragment P4) and residues 20−102 (fragment P5) for H4. These H3/H4s are referred to as ΔN-H3/H4.

For the preparation of chemically modified histones, H3/H4 were adjusted to a concentration of 3 mg/mL and dialyzed against 2.0 M NaCl and 30 mM $KHP0_4$ (pH 8.0) at 4 °C for 10 h to remove 2-ME. The sample was then adjusted to either 1 mM IAA or 1 mM DTNB from a 20 mM stock solution. After an incubation of 90 min at 23 °C, the samples were dialyzed for 10 h at 4 °C against fresh 2.0 M NaCl solution and then stored at −70 °C.

Preparation of Topoisomerases I and NAP1. Eukaryotic (MSB) topoisomerase I was isolated from MSB cells using a modification (*50*) of the procedure of Liu and Miller (*51*). One unit is defined as that quantity that achieves 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C.

Yeast NAP1 was prepared from *E. coli* strain BL21, which contained plasmid pTN2. The procedure for isolation was a modification (*45*) of the procedure of Fujii-Nakata et al. (*22*). NAP1 concentrations were determined by absorbance using a calculated molar extinction coefficient of 36,100 M^{-1} cm^{-1} at 276 nm (*52*, *53*).

Preparation of DNA. The plasmid 2T7/T3-19 (2255 bp) was prepared and purified on CsCl−EtBr density gradients (*32*). As isolated from the gradients, the ccc plasmid is negatively coiled with an approximately −0.05 SD and directly used for these studies. In order to produce the partially positively coiled DNA, this negatively coiled DNA was treated with topoisomerase I at 0 °C in buffer conditions of 10 mM $MgCl_2$ and 10 mM Tris (pH 8.0). At this reduced temperature, ionic strength, and increased Mg^{2+} concentration, the helical pitch of the DNA substantially decreases (*54*, *55*). Relaxation under this condition results in a DNA that exhibits an average of 2.5 positive coils (avg + 0.01 SD) when subsequently incubated at 35 °C under isotonic conditions. The DNA concentrations were determined using an extinction coefficient of 20 cm^{-1} mg^{-1} at 260 nm.

Reconstitution of Histone−DNA Complexes. Reconstitutions were done with three different methods: (1) direct addition, (2) NaCl stepwise dialysis, and (3) NAP1-facilitated deposition. Reconstitution by direct addition involved diluting the H3/H4 (containing 2.0 M NaCl) to 0.1 M NaCl in 40 mM Tris, 0.1 mM EDTA, and 5 mM 2-ME (pH 8.0) (ISOTONIC buffer) to a concentration of 100 μg/mL. After incubation for 10 min at 23 °C, 50 μL quantities were incubated with increasing amounts of DNA (either the +0.01 SD or −0.05 SD) that was initially added to the side of the tubes prior to rapid mixing by vortexing. The histone to DNA ratio was from 0.2 to 1.0 (wt:wt). Incubation was continued for 10 min to allow association of the H3/H4 to the DNA. Aggregation of the complexes occurred during this time, the extent of which was dependent on the histone/DNA ratio. Topoisomerase I (20 u/μg DNA) was then added and the incubation extended for 4 h at 35 °C. This level of topoisomerase I will relax DNA within 5 min at 35 °C. The extended length of incubation is required to allow the time-dependent conformational changes of H3/H4 to occur in the aggregate. The samples were then centrifuged for 4 min at 10,000*g* to obtain a supernatant and pellet fraction. Recon-

stitution by NaCl stepwise dialysis involved premixing H3/H4 and DNA in 2.0 M NaCl, 40 mM Tris, 0.1 mM EDTA, and 5 mM 2-ME, and dialyzing stepwise at 4 °C against that same buffer except that the NaCl concentration was changed from 1.2 M NaCl to 0.6 M NaCl to 0.1 M NaCl with 3 h intervals for each step (*56*, *57*). This reconstitute was then incubated with NAP1 at molar ratios of 1:1 and 0.5:1 (H3/H4 dimer to NAP1 dimer). For reconstitutions by NAP1, the H3/H4 were placed in ISOTONIC buffer at 100 μg/mL and incubated for 10 min at 23 °C after which NAP1 was added to obtain molar ratios of 4:1, 2:1, 1:1, 0.5:1 (H3/H4 dimer:NAP1 dimer). The samples were then incubated for 30 min. DNA was then added to the 50 μL samples and the incubation extended for 30 min at 35 °C. For most experiments, the DNA is preincubated with topoisomerase I (100 u/μg DNA) for 5 min to relax it before the H3/H4−NAP1 complexes were added. In some experiments, however, either +0.01 SD or −0.05 SD DNA was added to the complexes, and these mixtures were incubated for 15 min at 35 °C before topoisomerase I was added. The incubation was then extended for another 30 min. All reactions were terminated by the addition of an equal volume of 2× STOP buffer (0.4% SDS, 20% glycerol, 50 mM Tris, and 25 mM EDTA (pH 8.0)).

Procedures for the Analysis of Histone−DNA Complexes. For the supercoiling assays, the electrophoretic analysis of DNA was carried out on 1.2% agarose (Calbiochem, type C) in the buffer conditions of 50 mM Tris, 45 mM acetic acid, and 1.25 mM EDTA (pH 8.0) at 80 V for 10 h at 4 °C (*57*). In these conditions, negative and positive coils have different mobilities. For any particular number of negative coils, the overall compaction of DNA is less than that for DNA that has the same number of positive coils. The DNA helix can open up and therefore absorb some of the negative stress. Further verification of coiling is done by a second dimensional analysis in which the second dimension is done by electrophoresis in the presence of 15 μM chloroquin (*32*).

For the EMSA analysis, increasing amounts of the histones were preincubated in 50 μL of ISOTONIC buffer for 10 min at 23 °C after which 2.5 μg of the 172 bp fragment from the 5S gene of *L. variegatus* was added. The histone to DNA ratio was from 0.2 to 1.0 (wt:wt). After a incubation for 30 min at 35 °C, the samples were diluted 2-fold with 20% glycerol and loaded on a 6% acrylamide/0.06% bis-acrylamide gel in 9 mM Tris, 9 mM borate, and 0.2 mM EDTA at pH 8.0 at 120 V for 6 h at 23 °C. For analysis of samples on sucrose gradients, the samples were applied to a 5−20% sucrose gradient containing ISOTONIC buffer and sedimented in a SW60 Ti rotor at 55,000 rpm for 3 h at 4 °C. Fractions were collected, diluted by 5× STOP buffer (without glycerol) and aliquots applied to both a 1.2% agarose gel to determine the DNA distribution and an 18% acrylamide−SDS gel to determine the protein distribution.

Formaldehyde Cross-Linking and Reversal. The H3/H4 or the ΔN-H3/H4 were reconstituted onto the +0.01 SD DNA at a histone to DNA ratio of 0.4:1 (wt:wt). The buffer condition was 0.1 M NaCl, 40 mM TEA, and 0.1 mM EDTA (pH 8.0). After incubation for 4 h in the presence of topoisomerase I, the samples were treated with 1% formaldehyde at 4 °C for 60 min. The reaction was terminated by adjusting to 0.4 N H_2S0_4, sonicated for 30 s to displace uncross-linked histones from the acid-insoluble DNA, and then sedimented at 10,000*g* for 10 min. The acid-insoluble pellet was dissolved in STOP buffer and incubated at 37 °C for 2 days to reverse the protein−DNA cross-links (*58*). The samples were then adjusted to 50 mM 2-ME for 10 min before loading on SDS−PAGE. This treatment removes any oxidized H3 that would mistakenly appear as H3−H3 dimers. Electrophoresis was on 18% acrylamide and 0.09% bis-acrylamide/0.1% SDS gel at 4 °C (*57*). For reversal of the protein−protein cross-links, the gel slice from the first dimension was pre-equilibrated in a REVERSAL buffer (1.0% SDS, 125 mM Tris, and 0.5 M 2-ME (pH 6.8) for 2 h and then heated at 95 °C for 30 min in fresh REVERSAL buffer (*58*). The gel slice was re-equilibrated in REVERSAL buffer (without 2-ME) for 2 h and then sandwiched between glass plates for the second-dimensional analysis. The gel slice was cast within a stacking gel consisting of 2.5% acrylamide, 0.13% bis-acrylamide, 0.125 M Tris, and 0.1% SDS at pH 6.8 and electrophoresed into the 18% acrylamide and 0.9% bis-acrylamide/0.1% SDS gel.

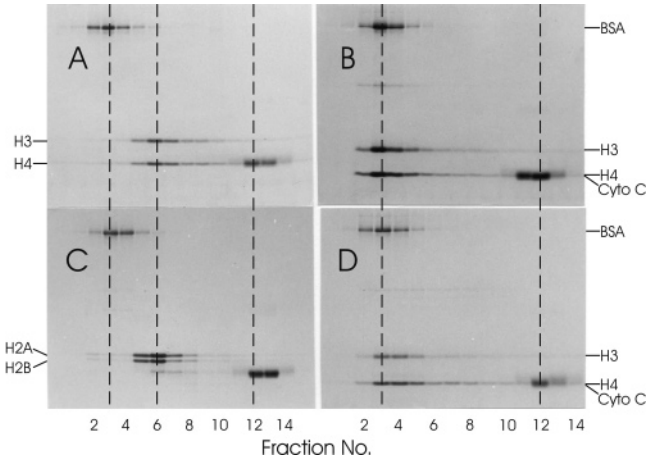

FIGURE 1: Molecular size analysis of histone complexes, as determined by SDS−PAGE of fractions from a Superdex G-75 column. (A) DTNB-treated H3/H4; (B) untreated H3/H4; (C) H2A/H2B; (D) IAA-treated H3/H4. The histones were applied to the column in the ionic condition of 1.0 M NaCl and 40 mM Tris at pH 8.0 and in the presence of the protein size standards, BSA (66 Kd) and cytochrome C (12.4 Kd).

RESULTS

Treatment with DTNB Causes the H3/H4 Tetramer to Form Two H3/H4 Dimers. In order to determine whether NAP1 could affect the tetramer−dimer equilibrium of H3/H4 and have any consequences on the structural state of DNA, we needed to first establish whether the tetrameric or dimeric state would cause any differences in DNA topology. The interface between the two H3s that stabilizes the tetramer consists of a 4-helix bundle, which involves an interaction between the α2 and α3 helices of both H3s (*1*−*3*). The single cysteine in H3 is located on α2 at position 110. When both H3s are in the tetrameric structure, the two cysteines are 7 Å apart. Chemical modification of these cysteines could potentially destabilize the H3−H3 interface and shift the equilibrium toward the dimeric state. Histones H3/H4 were purified and subsequently modified with the cysteine 110-modifying reagents, DTNB and IAA. The samples were then applied on a Superdex G-75 column in the presence of 1.0 M NaCl, and as shown in Figure 1A, the DTNB-treated H3/H4 have a molecular size similar to that of H2A/H2B (Figure

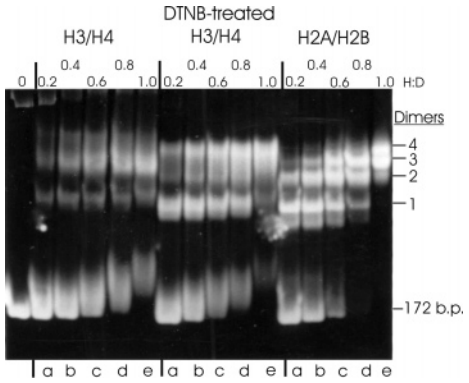

FIGURE 2: EMSA analysis of histone−DNA interactions. Increasing amounts of untreated H3/H4, DTNB-treated H3/H4, or H2A/H2B were added to the 172 bp DNA sequence of *L. variegatus* in isotonic conditions, and after incubation for 30 min at 35 °C, the samples were diluted 2-fold with 20% glycerol and applied to a PAGE gel.

1C). Because it is well established that H2A/H2B exists as a dimer in these conditions (*6*, *9*), this result is an indication that this modification shifts the equilibrium from tetramer to dimer. Figure 1B shows that without DTNB treatment, H3/H4 have a molecular size that is characteristic of a tetramer. Figure 1D shows that when the cysteine is modified with IAA, the equilibrium is not shifted toward the dimeric state. These results are an indication that the more bulky nature of the DTNB adduct tends to be more disruptive of the H3−H3 interaction.

Dimeric Form of DTNB-Treated H3/H4 Is Observable on DNA. The dimeric state of the DTNB-treated H3/H4 that was observed in Figure 1 was in a condition with 1.0 M NaCl. The higher salt condition is required because of the tendency of these histones to adhere to the Superdex matrix. To determine whether this form of H3/H4 persisted when bound to DNA at physiological ionic strength, we did an EMSA analysis with untreated H3/H4 and DTNB-treated H3/H4 or H2A/H2B. We used the direct addition approach, which was first described by Ruiz-Carrillo et al. (*59*) and has since then been used by others (*60*−*63*). These earlier studies showed that substantial nucleosome formation can occur without the involvement of a histone chaperone, particularly when H3/H4 are allowed to bind DNA before H2A/H2B. We incubated the histones at physiological ionic strength with a 172 bp linear DNA from the 5S gene of *L. variegatus.* This particular sequence strongly positions nucleosomes (*56*). As shown in Figure 2, when this incubation was done with increasing levels of H2A/H2B, four different complexes were formed (indicated as containing 1−4 dimers in the Figure). Histones H2A/H2B form a stable dimer (*6*), and with higher histone to DNA ratios, complexes that contain additional dimers will form. This pattern has been described previously (*62*). We then used these H2A/H2B complexes as size markers in order to determine the number of H3/H4 dimers that are present on the DNA. As shown in Figure 2, the untreated H3/H4 formed complexes that were both dimeric and tetrameric (two dimers). The major complex is tetrameric even at the low histone to DNA ratio of 0.4:1. In contrast, the major complex for the DTNB-treated H3/H4 at this ratio was dimeric. The DTNB modification is altering the H3−H3 interface for H3/H4 when bound to DNA. In this experiment, all of the histone−DNA complexes were soluble (data not shown). This result will be different in the following experiment in which a larger ccc DNA was used.

DTNB-Treated H3/H4 Forms Positive Coils and IAA-Treated H3/H4 Forms Negative Coils. In order to determine the potential topological effects on DNA caused by the binding of H3/H4, increasing amounts of the modified and unmodified histones were adjusted to isotonic conditions and incubated for 10 min with ccc DNA (2255 bp) that was either partially positively coiled (+0.01 SD) or negatively coiled (−0.05 SD). During this incubation, the histones bound the DNA, and substantial aggregation was observed. Topoisomerase I was then added, and incubation continued for 4 h at 35 °C. At this time, the samples were centrifuged for 4 min at 10,000*g* to collect the aggregated complexes. As shown in Figure 3A, when DTNB-treated H3/H4 were added to the +0.01 SD, even at a low histone to DNA ratio of 0.2:1 (wt:wt), very high levels of positively coiled DNA were observed in this aggregated material. Approximately 20% of the DNA was in this aggregate. A much lower level of supercoiling was observed for the DNA in the supernatant. As the histone to DNA ratio was increased toward 0.8:1 (H:D), even more of the DNA aggregated and formed this high level of positive supercoils. These results indicate that a highly cooperative process has occurred by which we mean that the H3/H4 dimers preferentially bind select plasmid molecules to the point that aggregation occurs. Because of the larger size of the DNA, we are now able to observe these histone−histone interactions. The smaller 172 bp DNA in Figure 2 does not provide sufficient size to form these higher-order interactions, and therefore, those complexes remain soluble. It is remarkable that topoisomerase I is able to access this aggregated DNA and that during the 4 h incubation period, the histones form such highly positively coiled DNA. We have previously shown a time course of this process, which demonstrates that 4 h are required to complete supercoiling (see Figure 1 of ref *32*). We next repeated this experiment with the −0.05 SD DNA. As shown in Figure 3A, the histone to DNA ratio that was required to observe an equivalent level of supercoiled DNA needed to be greater than 0.4:1. That this DNA is also positively coiled was verified by applying a second dimensional gel analysis (Figure 3A). These results are an indication that the topological state of the DNA prior to the addition of the H3/H4 dimers can significantly determine the extent to which positive coils are formed by them.

We next determined the conformational state of untreated H3/H4 and the IAA-treated H3/H4. Both of these forms promote the tetrameric state of H3/H4 (Figure 1). As shown in Figure 3B, when deposited on the +0.01 SD DNA, supercoils were observed, and the formation of these coils also exhibited cooperativity, although the extent of cooperativity in the IAA-treated H3/H4 was much reduced. In this instance, the supercoils were in the supernatant, and as shown in the second dimensional gel analysis (Figure 3B), these coils were negative. Increasing the histone to DNA ratio did not result in an increase in these negative coils, but rather there was an increase in aggregation. Topoisomerase I was unable to access the DNA in this aggregate, as indicated by the presence of the same topological state of the initial DNA (+0.01 SD) in the pellet fraction. This is in contrast to the DTNB-treated H3/H4, although equally inclined to cause aggregation (Figure 3A), it still remained sufficiently dy-

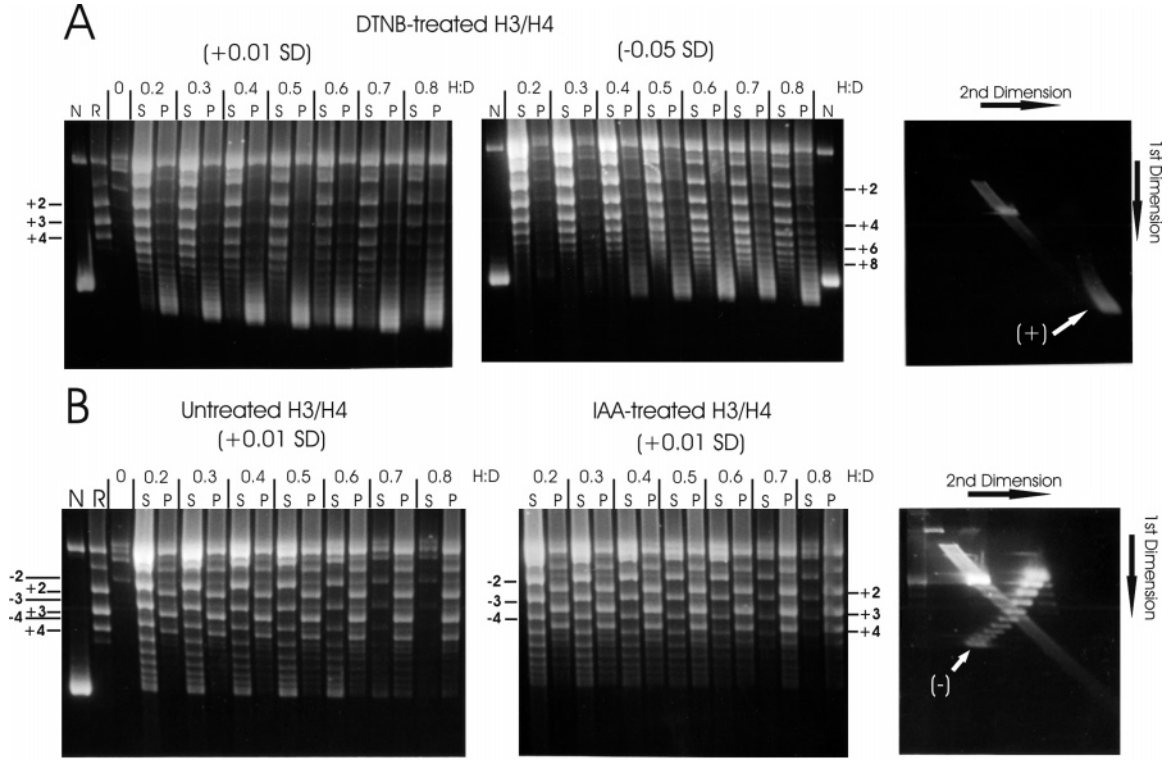

FIGURE 3: Analysis of the coils that were formed by H3/H4 when directly deposited on either +0.01 SD or −0.05 SD DNA. (A) DTNB-treated H3/H4 deposited on +0.01 SD DNA and −0.05 SD DNA. (B) Untreated H3/H4 and IAA-treated H3/H4 deposited on +0.01 SD DNA. The second dimensional gel of panel A is of the pellet fraction from the deposition on −0.05 SD DNA at a ratio of 0.8:1 histone to DNA (H:D, wt:wt). The second dimension gel of panel B is of the supernatant fraction from the deposition of untreated H3/H4 at 0.2:1 (H:D). The second dimension electrophoresis was done in the presence of 15 μM chloroquin. After the 4 h incubation of the histones with DNA, the samples were centrifuged to obtain the supernatant (S) and pellet (P). R refers to the +0.01 SD DNA, and N refers to the −0.05 SD DNA. The numbers on the side of the panel point to the DNA bands, either negative or positive coils. This gel electrophoretic system is able to distinguish between positive and negative coils.

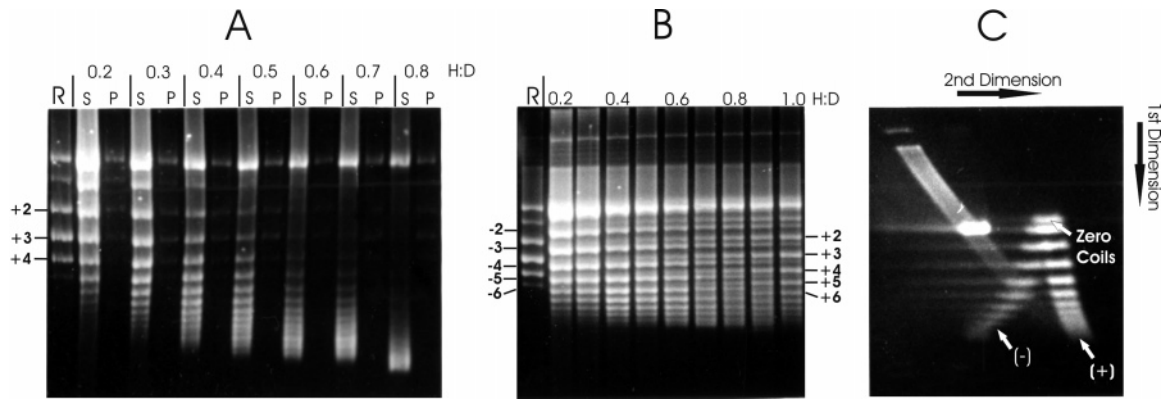

FIGURE 4: Analysis of the coils that were formed by ΔN-H3/H4 when on +0.01 SD DNA. (A) DTNB-treated ΔN-H3/H4 or (B) untreated ΔN-H3/H4 were added to the DNA at the indicated H:D ratios, and after 10 min, topoisomerase I was added, and the incubation continued for 4 h. Samples were centrifuged to obtain the supernatant (S) and pellet (P). Because no pellets were observed for the untreated H3/H4, only the supernatant fractions are shown. (C) The second dimensional gel analysis is of the untreated H3/H4 at the 0.8:1 H:D ratio. R is the +0.01 SD DNA that was used as the substrate for this experiment.

namic so that topoisomerase I could access the DNA. The aggregation brought about by the dimeric state of H3/H4 is significantly different from the tetrameric state.

N-Termini of the H3/H4 Are Responsible for the Cooperativity That Is Observed with Both Untreated and DTNB-Treated H3/H4. From the crystallographic structure of the nucleosome, it is known that the four N-termini of the H3/H4 tetramer extend from the inner core of histones to interact with the DNA on the outside surface of the nucleosome (*1*−*3*). The N-termini are important in establishing internucleosomal interactions that are characteristic of high order nucleosome−nucleosome compaction (*5*, *64*−*66*). The cooperativity that was observed with both untreated and DTNB-treated H3/H4 may be due to interactions between adjacent H3/H4 complexes through these N-terminal regions. To determine whether the N-termini are involved in this cooperativity, we tryptically removed them from both H3 and H4 (ΔN-H3/H4) and incubated them with the +0.01 SD DNA. As shown in Figure 4A, the DTNB-treated ΔN-H3/H4 continued to produce positive coils, but these coils were entirely in the supernatant. There was a gradual increase in a Gaussian distribution of positive coils as the histone to

DNA ratio was increased. This increase in solubility and Gaussian distribution indicates that cooperativity has been substantially suppressed. We next examined whether this loss of cooperativity would be observed with the untreated ΔN-H3/H4. As shown in Figure 4B for the supernatant fractions, a Gaussian distribution of coils was also formed. These coils were negative and gradually increased from 0.2 to 0.6 (H:D), an indication of a largely non-cooperative process. A curious effect, however, was observed when the histone to DNA ratios was increased greater than 0.6:1. Positive supercoils began to appear. To verify that these coils were indeed positive, the sample at the 0.8 (H:D) ratio was analyzed on a second dimensional gel (Figure 4C). There was a substantial increase in positive coils beyond the number of coils that were present in the initial DNA (+0.01 SD). By increasing the histone content, we have increased the interaction between the ΔN-H3/H4 molecules and simulated the effect of a DTNB modification of the cysteine 110. This similarity might be interpreted as indicating that the tetramer−dimer equilibrium has been altered and that some of the ΔN-H3/H4 is being deposited as a dimer. However, it has been reported (*9*, *67*), and we have independently verified, using the procedures that were applied for Figures 1 and 2, that removal of the N-termini does not alter this equilibrium (data not shown). Because the positive coils are only observed at high histone to DNA ratios, we interpret this observation as indicating that an increase in histone−histone contacts promotes a transition in the ΔN-H3/H4 tetramer when bound to DNA. This transition simulates the interactions that are formed when H3/H4 dimers are deposited. These interactions would have to occur between the core regions of the ΔN-H3/H4 complexes. There is an additional point of interest regarding these protein−protein interactions. As observed in the second dimensional gel, both positive and negative coils were observed equally in the same sample (Figure 4). If the H3/H4 were to randomly form a structural state that can form either positive or negative coils, then the DNA upon which it is associated should show a Gaussian distribution that averages at zero coils. The distribution is clearly non-Gaussian. Therefore, even though there is a lack of the high levels of cooperativity observed when the N-termini were present, the core regions of the ΔN-H3/H4 do interact and promote a particular conformation that is conducive for forming either positive or negative coils on any particular DNA molecule.

H3−H3, H3−H4, and H4−H4 Interactions within the Core Regions of the Histones Facilitate the Formation of Positive Coils. As protein−protein interactions are promoting the formation of these positive coils, we applied formaldehyde cross-linking to identify the interactions that facilitated this process. DTNB-treated H3/H4 were added to the +0.01SD DNA at a ratio of 0.5:1 (H:D) and incubated in the presence of topoisomerase I for 4 h. These conditions will form the positively coiled DNA of Figure 3A. The samples were then cooled to 4 °C and treated with formaldehyde for 60 min. Cross-linking was terminated (see Experimental Procedures) and after reversal of the cross-link between the histones and DNA, the proteins were electrophoresed on SDS−PAGE in the first dimension. As seen in Figure 5, cross-linked dimers were observed, but their composition remained undefined. We determined their composition by reversing the cross-links between the proteins and electrophoresing the proteins in the second dimension. All three possible dimers were observed, an H3−H3 dimer, an H3−H4 dimer, and an H4−H4 dimer. The highest quantity of dimer was the H3−H4 dimer, which would be expected considering the intimate interactions that occur between the histone folds of H3 and H4 (*1*−*3*). The cross-link between two H3s was also observed and is an indication that when the H3/H4 dimer is deposited, H3−H3 interactions are re-established. These interactions do not form the characteristic left-handed pitch of the normal tetramer, which would form negatively coiled DNA. Rather, a right-handed pitch must be established by the H3−H3 interaction to form the positive coils. The presence of the H4−H4 dimer is an indication that there is substantial interaction between two different sets of H3/H4 tetramers, which may explain the high level of cooperativity seen in Figure 3A. This analysis does not indicate whether the cross-link occurs between the N-termini or core regions of the histones. In order to determine whether the core regions can establish an intimate interaction, we repeated the cross-linking with the DTNB-treated ΔN-H3/H4. Using a 0.8:1 H:D ratio, which will form high levels of positive coils (Figure 4), the reconstitute was treated with formaldehyde, and the cross-linked dimers were analyzed. As shown in Figure 5, the relative levels of the cross-linked products remained the same. In particular, the persistent presence of the H4−H4 dimer is an indication that there is a tendency for the core regions of the ΔN-H3/H4 tetramers to stack upon each other by way of that interaction.

High Levels of NAP1 Facilitate the Formation of Positive Coils, Providing the Histone to DNA Ratio Is Greater than 1:1. Now having established the consequences of deposition in the tetrameric versus the dimeric forms of H3/H4, we determined whether NAP1 could affect the tetramer−dimer equilibrium. It is known based on hydrodynamic (*68*) and structural (*53*, *69*) studies that the histone chaperone NAP1 forms a very stable homodimer. It has been shown to bind H3/H4 with a variable stoichiometry from 2:1 to 1:1 (H3/H4 dimers to NAP1 dimer) (see Figure 2C of ref *20*). As previously indicated, the preferred state of H3/H4 in physiological ionic strength is a tetramer (*7*−*9*). At low levels of NAP1, the tetramer would tend to be stabilized by NAP1. At high levels of NAP1, the competition for binding to the H3/H4 tetramers would tend to shift the equilibrium toward the dimer. Each dimer will bind an NAP1 dimer (*20*). The deposition of H3/H4 in this latter condition might be expected to form positive coils on DNA, similar to those in Figure 3A. In contrast, the stabilized tetramer might be expected to form negative coils similar to those in Figure 3B. It is also known that a major site of interaction between NAP1 and H3/H4 is their N-termini (*20*). Thus, a part of the mechanism in which NAP1 facilitates deposition is through negating the highly basic charge density of those regions. The second observation that might be expected is that when NAP1 is used to deposit H3/H4, the cooperative process that was observed in Figure 3 should be reduced. The results may look similar to those when ΔN-H3/H4 were deposited on DNA (Figure 4). However, for positive coils to be observed with ΔN-H3/H4, high histone to DNA ratios were required. Similar high ratios may be required during histone deposition by NAP1. To test these predictions, the following experiments were done. Increasing amounts of NAP1 were incubated with H3/H4 for 30 min and then added to DNA

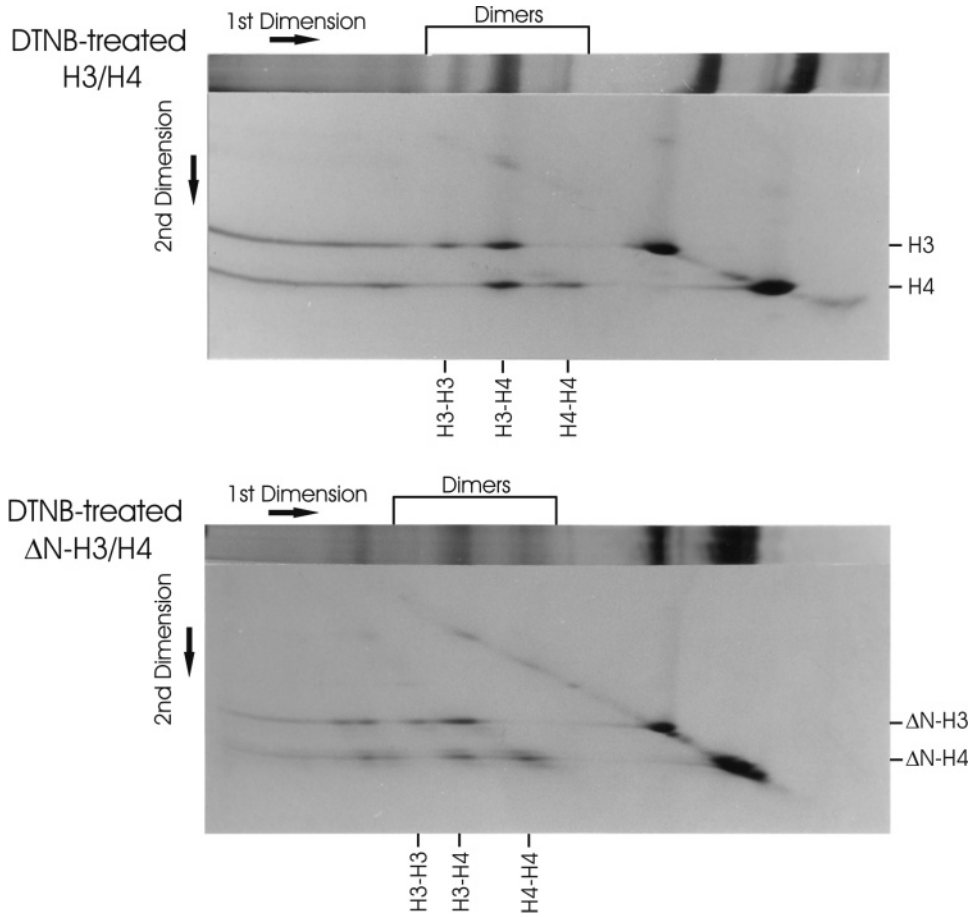

FIGURE 5: Analysis of the formaldehyde cross-linked products that were formed on reconstitutes that contain either DTNB-treated H3/H4 or DTNB-treated ΔN-H3/H4. Reconstitutions were on +0.01 SD DNA at a 0.5:1 (H:D) ratio. After cross-linking, the samples were processed as described in Experimental Procedures, and samples were electrophoresed in the first dimension to separate the cross-linked products and in the second dimension to identify the proteins in the products.

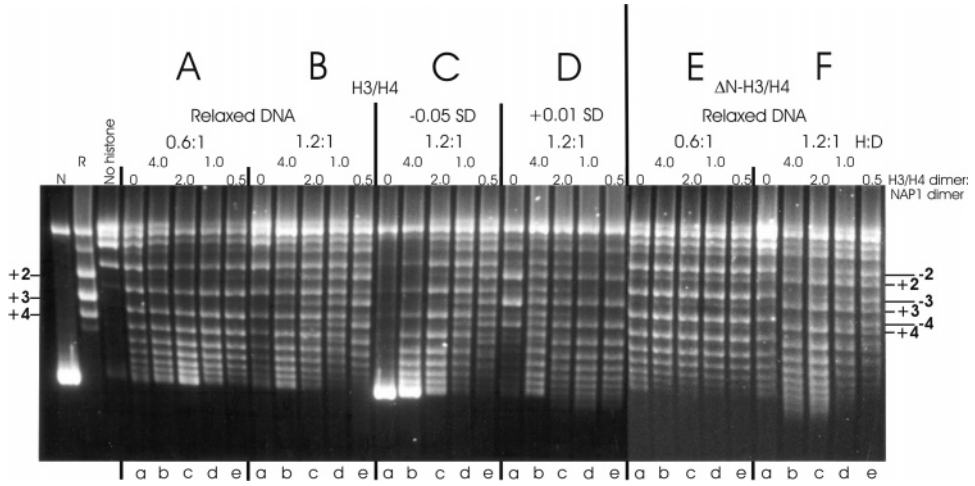

FIGURE 6: Analysis of the supercoils that are formed when NAP1 is used to deposit H3/H4 on DNA. (A) Deposition on relaxed DNA with a H:D ratio of 0.6:1. (B) Deposition on relaxed DNA with a H:D ratio of 1.2:1. (C) Deposition on −0.05 SD DNA with a H:D ratio of 1.2:1. (D) Deposition on +0.01 SD DNA with a H:D ratio of 1.2:1. (E) Deposition of ΔN-H3/H4 on relaxed DNA with a H:D ratio of 0.6:1. (F) Deposition of ΔN-H3/H4 on relaxed DNA with a H:D ratio of 1.2:1. NAP1 was preincubated with the H3/H4 for 30 min at 23 °C at molar ratios of 4:1, 2:1, 1:1, and 0.5:1 (H3/H4 dimer:NAP1 dimer) and then added to the DNA. The 2:1 ratio is a condition in which the protein complex is primarily one H3/H4 tetramer bound to one NAP1 dimer. Similarly, the 1:1 ratio is primarily one H3/H4 dimer bound to one NAP1 dimer. For panels C and D, the H3/H4−NAP1 complexes were added to the DNA and incubated for 15 min before topoisomerase I was added. The incubation was then continued for 30 min.

that had been preincubated with topoisomerase I. This preincubation puts the DNA in a topologically relaxed state. Two different histone to DNA ratios were used, 0.6:1 (Figure 6A) and 1.2:1 (Figure 6B). As shown for the 0.6:1 ratio, negative coils were formed at all H3/H4 to NAP1 ratios. The maximum number of coils that were formed was at the 2:1 ratio (lane c), that is, two H3/H4 dimers (H3/H4 tetramer) per NAP1 dimer. Higher levels of NAP1 (lane e) tended to

reduce the number of negative coils and might be interpreted as indicating that NAP1 is not efficiently releasing all of the H3/H4 to the DNA. We have done an analysis of the sample of lane e by separating the NAP1 from the DNA on a sucrose gradient. We observed that minimal levels of H3/H4 remained on NAP1 (data not shown). The higher levels of NAP1 may be altering the conformational state of H3/H4, as it is being deposited on the DNA. This effect can be seen more clearly when the histone to DNA ratio was increased to 1.2:1 (Figure 6B). At the molar ratio of 2:1 (H3/H4 dimer:NAP1 dimer), not only negative coils (lane c) but also a small percentage of positive coils were formed. As the quantity of NAP1 was increased even further, an increasingly greater number of positive coils were established on the DNA (lane e). We interpret these observations as indicating that at the higher ratios of H3/H4 to NAP1, NAP1 tends to deposit H3/H4 primarily as a tetramer and that when NAP1 is in excess, it deposits them as a dimer. The higher histone to DNA ratio increases the probability of histone−histone interactions on the DNA, the type of interactions that were observed in Figure 4 in which higher levels of the ΔN-H3/H4 facilitated the formation of positive coils. From this analysis, we also conclude that the likely reason why there was a decrease in the number of negative coils in lane e at the 0.6:1 H:D ratio (Figure 6A) are that H3/H4 are being deposited as a dimer. This form of deposition would increase the probability for positive coils to form, but because of the lower histone to DNA ratio, the histone−histone interactions are not sufficiently established to prefer the conformational state of H3/H4 that selectively forms positive coils. The mixture of positive and negative coils reduces the overall number of supercoils on the DNA.

The experiment in Figure 3A indicated that the topological state of the DNA prior to the addition of the histones determined the extent of positive stress that could be produced on DNA. In order to determine whether a similar effect would be observed when NAP1 was involved in the deposition, the H3/H4−NAP1 complexes were added to either a −0.05 SD DNA (Figure 6C) or a +0.01 SD DNA (Figure 6D). After incubation for 15 min to allow deposition of H3/H4 onto these DNAs, topoisomerase I was then added and incubation continued for 30 min. As shown for both forms of the DNA, as the H3/H4 dimer:NAP1 dimer ratio was decreased, the topological state changed from negative to positive coils. The rate of change was significantly different, however. At the 2:1 ratio, the −0.05 SD DNA continued to remain negatively coiled in the presence of topoisomerase I (Figure 6C, lane c). For the +0.01 SD DNA, positive coils were preferentially present (Figure 6D, lane c). Negative coils could be formed on this partially positively coiled DNA, but it required a higher ratio of H3/H4 to NAP1 (Figure 6D, lane b). Therefore, both the tetramer−dimer equilibrium and the topological state of the DNA prior to H3/H4 deposition are two important factors that define the final conformational state of H3/H4. In these experiments, it should also be noted that NAP1 has reduced the cooperativity that would normally be present when H3/H4 are deposited on +0.01 SD DNA (compare with Figure 3). NAP1 is altering N-termini interactions when facilitating histone deposition.

In order to determine whether the N-termini of H3/H4 were required to facilitate the action of NAP1, we repeated the experiments of Figure 6A and B with ΔN-H3/H4. At the histone to DNA ratio of 0.6:1 (Figure 6E) and in the absence of NAP1, negative coils were formed (lane a). Preincubation of the ΔN-H3/H4 with increasing amounts of NAP1 did not increase the number of these coils (lanes b−e). These results are an indication that when N-termini are absent, H3/H4 will deposit efficiently without NAP1. One might think that because the N-termini are absent, NAP1 is not binding ΔN-H3/H4 and would not be expected to affect the deposition process. However, we have done additional experimentation in which we have used a sucrose gradient to demonstrate that NAP1 binds ΔN-H3/H4 with an affinity that requires NaCl concentrations greater than 0.5 M to disrupt (data not shown). NAP1 is likely interacting with the basic regions that remain in the core domains of H3/H4. Therefore, ΔN-H3/H4 are being transferred from NAP1 to the DNA, although NAP1 is not required for efficient deposition. We next raised the histone to DNA ratio to 1.2:1 (Figure 6F), and in the absence of NAP1, negative coils were again formed (lane a). It should be noted that at this high level of ΔN-H3/H4, if these histones had been deposited on the +0.01 SD DNA, significant levels of additional positive stress would have been present following the addition of topoisomerase I (see Figure 4). Now that the DNA is in a relaxed state because of the preincubation with topoisomerase I, negative coils are preferentially formed. The default condition is to form negative coils as long as positive stress is not already in the DNA. This preference for negative coils changes rather dramatically, however, when even a small amount of NAP1 was preincubated with ΔN-H3/H4. As shown in Figure 6F (lane b), the coils were entirely positive. This 4:1 ratio (ΔN-H3/H4 dimer:NAP1 dimer) would be expected to stabilize the tetramer and therefore continue to form negative coils. That this is not the case is an indication that NAP1 tends to alter the H3−H3 interaction in the H3/H4 tetramer, a tendency that is very much accentuated when the N-termini are absent. A further analysis of this experiment indicates that as additional NAP1 is included, there is now a reduction in positive coils (lanes d and e). In order to determine why this reduction occurred, we applied a sample of Figure 6F (lane e) onto a 5−20% sucrose gradient, and as shown in Figure 7A, ∼40% of the ΔN-H3/H4 remained associated with NAP1. Because of the persistent binding of the ΔN-H3/H4 by the excess NAP1, the amount of ΔN-H3/H4 that is actually transferred to the DNA more closely approximates the 0.6:1 H:D ratio of Figure 6E. The lower amount of histone deposition would be expected to produce primarily negative coils. In summary, the results of Figure 6 are an indication that the tetramer−dimer equilibrium of ΔN-H3/H4 can also be regulated by NAP1 to form either negatively or positively coiled DNA.

NAP1 Remodels the Structural State of H3/H4 while Bound to DNA. Because NAP1 is able to affect the conformation of H3/H4 during the deposition process, would a similar change occur when the H3/H4 were pre-bound to the DNA? To test for such a conformational change, we reconstituted by NaCl stepwise dialysis both H3/H4 and ΔN-H3/H4 onto either +0.01 SD or −0.05 SD DNA at a ratio of 0.5:1 (H:D). Subsequently, the samples were incubated for 30 min with topoisomerase I in the absence and presence of NAP1. As shown in Figure 8A (lane a), ΔN-H3/H4, when associated with +0.01 SD, formed almost exclusively

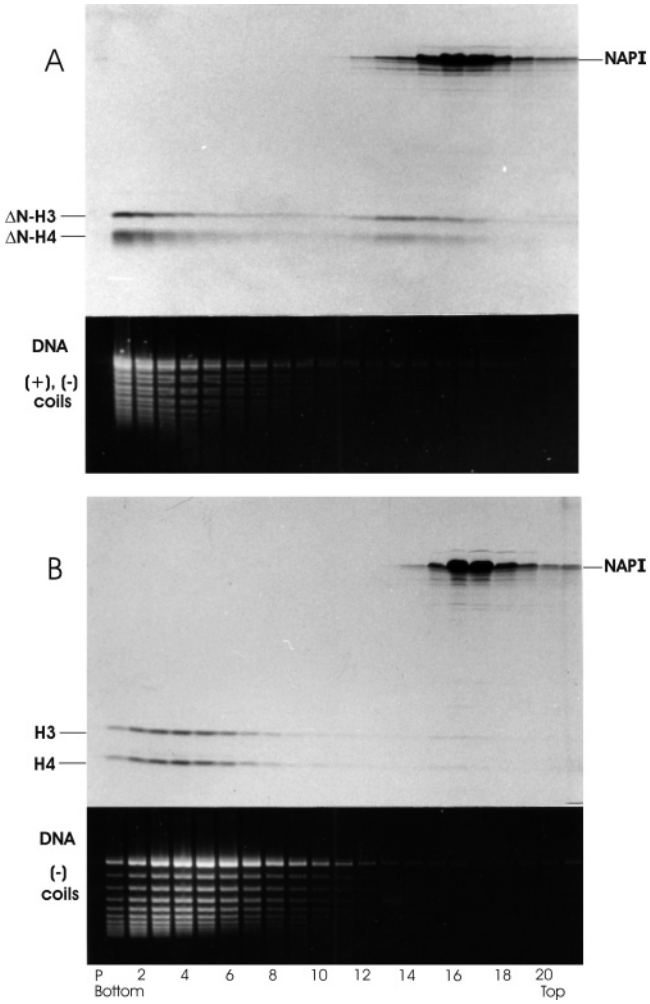

FIGURE 7: Sucrose gradient analysis of the association of H3/H4 with NAP1 and DNA. (A) The sample from Figure 6F (lane e) was applied to a 5−20% sucrose gradient, and after centrifugation, fractions were collected for SDS−PAGE analysis of protein (upper panel) and DNA (lower panel). The sample from lane e represents the deposition of the ΔN-H3/H4 dimer−NAP1 dimer complex (0.5:1) on +0.01 SD DNA (1.2:1, H:D). (B) The sample from Figure 8E (lane b) was analyzed on sucrose gradients as described for panel A. The sample from lane b represents the incubation of NAP1 (ratio 1:1, H3/H4 dimer/NAP1 dimer) with the reconstitute that contained H3/H4 (ratio 0.8:1, H:D).

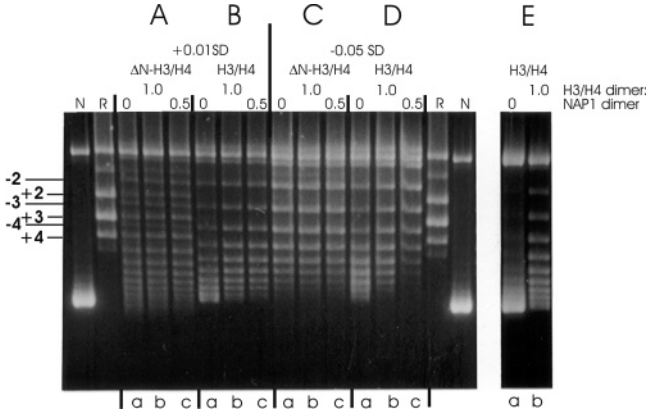

FIGURE 8: Analysis of the conformational state of ΔN-H3/H4 and H3/H4 when reconstituted with DNA and subsequently treated with NAP1. (A) ΔN-H3/H4 on +0.01SD DNA; (B) H3/H4 on +0.01 SD DNA; (C) ΔN-H3/H4 on −0.05 SD DNA; (D) H3/H4 on −0.05 SD DNA. The reconstitution was by NaCl stepwise dialysis at a 0.5:1 H:D ratio; (E) the same as D except that the ratio was increased to 0.8:1 H:D. Samples were incubated with topoisomerase I and NAPI for 30 min at 35 °C. The quantity of NAP1 that was added is indicated as the ratio of H3/H4 dimer to NAP1 dimer, although in actuality the H3/H4 on the DNA is in a tetrameric state. By using this ratio, the data in this figure (histones pre-bound) can be directly compared with Figure 6 (histones deposited). The sample in E (lane b) was applied to a sucrose gradient with the results shown in Figure 7B.

positive coils, even in the absence of NAP1. In contrast, when these same histones were associated with the −0.05 SD DNA (Figure 8C, lane a), negative coils were formed. The topological state of the DNA during deposition is again defining the conformational state of these histones. Adding increasing amounts of NAP1 did not alter this distribution for either the +0.01 SD or −0.05 SD DNA (Figure 8A and C, lanes b and c). Once ΔN-H3/H4 are bound to DNA, NAP1 cannot extract these histones or change their conformation. We then repeated this experiment with H3/H4, and as shown for both +0.01 SD DNA (Figure 8B, lane a) and −0.05 SD DNA (Figure 8D, lane a), negative coils were maintained by these histones. The N-termini clearly produce a condition in which negative coils are preferred, even on the +0.01 SD DNA. It should also be noted that the negative coils that were formed have a Gaussian distribution, which is significantly different from the cooperativity that was observed in Figure 3B. The stepwise removal of NaCl is similar to the deposition process by NAP1. Both these depositional processes prevent the cooperativity that is facilitated by the N-termini, and as expected, the complexes are completely soluble (data not shown). We next determined whether the inclusion of NAP1 would alter this number of negative coils. As shown in Figure 8B and D (compare lanes a with lanes c), the number of negative coils on the DNA was reduced significantly for both the +0.01 SD and −0.05 DNA. In this instance, NAP1 is interacting with H3/H4, and it is likely interacting with the N-termini. This reduction in negative coils could be a result of two possibilities. Either NAP1 has displaced H3/H4, or it has facilitated their conformational change on the DNA. In order to differentiate between these two possibilities, we did the following experiment. We repeated the reconstitution with the −0.05 SD DNA, except that the histone to DNA ratio was increased to 0.8:1. This sample was then treated with topoisomerase I in the presence or absence of NAP1, and as shown in Figure 8E, an estimated 40% of the original negative coils (lane a) were missing when NAP1 was present (lane b). Note that if one compares the number of coils that are present at the 0.5:1 H:D ratio (Figure 8D, lane a) with the number of negative coils that remain after NAP1 treatment of the reconstitute at the 0.8:1 H:D ratio (Figure 8E, lane b), these numbers are very similar. Therefore, NAP1 is reducing the number of negative coils to an equivalent 0.5:1 (H:D) ratio. This reduction could indicate that ∼40% of the H3/H4 were displaced to the NAP1. To determine whether this transfer had occurred, we applied the sample in Figure 8E (lane b) to a 5−20% sucrose gradient, and as shown in Figure 7B, only ∼3% of the H3/H4 were associated with the NAP1. Clearly, NAP1 is not displacing sufficient H3/H4 to explain the loss of negative coils. NAP1 must be facilitating a conformational change in H3/H4 through a process similar to what was observed when excess NAP1 was used to deposit H3/H4 on DNA (Figure 6A, lane e). By interaction with the N-termini, NAP1 is facilitating a conformational change in some of the H3/

H4, as if they had been initially deposited as an H3/H4 dimer. As shown with the ΔN-H3/H4, such a change cannot occur without the N-termini. When the N-termini are missing, NAP1 can only interact with the basic regions of the core domains. These domains are interacting with DNA and are no longer available for interaction with NAP1.

DISCUSSION

At physiological ionic strength, H3/H4 establishes a tetramer−dimer equilibrium in which the primary state of the H3/H4 is tetrameric (*7*−*9*). The molecular size analysis of the DTNB-treated H3/H4 indicated that the modification of cysteine 110 shifted this equilibrium toward the dimer (Figure 1). An IAA modification would not do this. The EMSA analysis demonstrated that this tendency to form a dimer was also observable on the 172 bp DNA (Figure 2). When examining the effects of these modifications on the supercoiling activity of H3/H4, it was observed that positive coils were formed by the dimeric state and negative coils by the tetrameric state (Figure 3). This tendency for H3/H4 dimers to form positive coils was further observed in the experiments with NAP1. At the lower ratios of H3/H4 to NAP1, which are conditions that enhance interactions of one H3/H4 dimer with one NAP1 dimer (*20*), positive coils were efficiently formed on relaxed DNA, providing the histone to DNA ratio was also high (1.2:1, H:D). This requirement of a higher level of histones to produce positive stress was also observed when ΔN-H3/H4 were deposited on +0.01 SD DNA (Figure 4). It is an indication that protein−protein interactions are needed to facilitate the formation of these positive coils.

The cross-linking experiments (Figure 5) indicated that the protein−protein interactions that were involved in forming the supercoils were H3−H3, H3−H4, and H4−H4, through the N-termini but perhaps more significantly between the core regions of the histones. Nonspecific interactions in the aggregates may be present, and they could be the source of the cross-linked dimers, although we do not think that this is likely. These aggregates are dynamic and form very high levels of positive supercoils during the 4 h incubation with topoisomerase I. Formaldehyde bridges distances of 2 Å, which indicates that there are very intimate interactions between the proteins. If these cross-linked dimers are describing the interactions that form the supercoil, then it is possible to propose a potential structural state for these complexes. A stacking model has been described for the formation of positive coils for the archaeal histones HMfB and HMfA (*70* and see Figure 3 of ref *71*). It has been shown for these histones that at low protein to DNA ratios, the DNA is negatively coiled, and at high ratios, the DNA is positively coiled. This effect is very similar to our results with ΔN-H3/H4 (Figure 4). Marc et al. (*71*) have also shown by mutagenesis of the α2 and α3 helix of the 4-helix bundle in a $(HMfB-HMfB)_2$ tetramer, which is homologous to the H3−H3 interface in the H3/H4 tetramer, that disruption of the interface caused the formation of positive stress at a much lower protein to DNA ratio. Their interpretation of this observation was that the shift toward a more dimeric state of the $(HMfB-HMfB)_2$ tetramer was the source of the increased positive stress. The continual stacking of multiple dimers by interaction through their altered 4-helix bundles is promoting an extended right-handed pitch in the DNA. When considering H3/H4, we propose that the H3−H3 interface of the tetramer is in a left-handed pitch and when deposited as such will form negatively coiled DNA. A superhelical pitch cannot be established on DNA without the H3−H3 interaction of an H3/H4 tetramer. When H3/H4 dimers are involved, this H3−H3 interface is established on the DNA. In this scenario, the 4-helix bundle is being established at a point where it can take either a right or a left-handed conformation, depending on the existing topological state of the DNA. The data of Figures 2A and 6D demonstrated the importance of the topological state of the DNA prior to the addition of histones. The third and fourth H3/H4 dimers now bind by way of H4−H4 interactions to the newly reconstituted tetramer to further propagate the positive coils. It is of interest to point out that when an H4−H4 interaction is established, its own unique 4-helix bundle could be formed. It is another example of the remarkable similarities between eukaryotic and archaeal histones. For this scenario to occur, however, NAP1 or similar chaperones will need to be present to suppress the tendency for the N-termini of H3/H4 to promote the formation of the left-handed form of the tetramer (Figures 3B and 8B). The archaeal histones do not have these N-termini (*70*).

The experiments of Figure 4 in which the N-termini were removed indicated that it is the N-termini that are largely responsible for the cooperativity that was observed in Figure 2. It is known that the N-termini of H3/H4 are required to facilitate internucleosomal interactions within a chromatin fiber (*5*, *64*−*66*). It is therefore understandable why a preferential distribution of H3/H4 would form on select plasmids and aggregation would occur. That higher-order interactions are involved is further validated in that the complexes formed on the 172 bp fragments remained soluble. We have repeated these experiments with the DTNB-treated H3/H4 using the well-established protocol of NaCl stepwise dialysis. As expected, positive coils were formed; however, no cooperativity was observed (data not shown), nor was there any aggregation. Instead, a Gaussian distribution of positive coils was observed that was identical to the data in Figure 4, in which DTNB-treated ΔN-H3/H4 were directly added to DNA in isotonic conditions. These observations indicate that when deposition vehicles such as NAP1 or NaCl stepwise dialysis are used (Figures 6 and 8), the N-termini are less effective in facilitating this cooperativity. It is known that the N-termini of H3/H4 adopt significant amounts of α-helical structure when bound to DNA (*72*, *73*), and it may be that these chaperones are preventing protein−protein interactions by influencing their helical state. How extensively these cooperative interactions actually occur *in vivo* remains undefined because most H3/H4 deposition is expected to occur through the chaperone activities of CAF-1 and Asf1, both of which are proposed to deposit H3/H4 as dimers, while interacting with their N-termini (*12*−*14*). However, because NAP1 has been found to be a component of chromatin remodeling complexes (*25*−*31*), a part of its function may be to disrupt higher-order cooperative interactions that are facilitated by the N-termini.

Two other studies of relevance have been done to characterize the role of the N-termini in DNA supercoiling (*74*, *75*). In both studies, H3/H4 were reconstituted onto minicircle DNA at low histone to DNA ratios. As a result, one negative coil was formed on the DNA. When both

studies removed the N-termini, the number of coils that were observed averaged close to zero. Both studies concluded that the N-termini stabilize the left-handed conformation and that a more right-handed conformation produced a neutral coiling. It is likely that the positive coils we have observed in our study are a result of histone−histone interactions that are being facilitated at our higher histone to DNA ratios and our larger DNA. Such conditions facilitate the propagation of positive coils on the DNA, so much so that an equivalent amount of H3/H4 will form the same number of either positive or negative coils. The superhelical pitch of the left-handed versus the right-handed conformations appears to be very similar.

Because these studies were done in the absence of H2A/H2B, the question may be asked as to the relevance of the H3/H4 interactions that have been described in this article. One possible example of relevance is the process of chromatin remodeling at promoters. Remodeling complexes that function by displacement of H2A/H2B, would leave H3/H4 available to be reordered by histone chaperones such as NAP1 (*25*−*31*). From *in vitro* studies, Park et al. (*36*) have reported that NAP1 facilitates the displacement of H2A/H2B and the sliding of H3/H4. A possible scenario could be that with the displacement of the H2A/H2B dimers, the adjacent H3/H4 tetramers slide together as a result of NAP1's interaction with their N-termini. The importance of the N-termini in this reordering was shown in the experiments in Figure 8, in which it was observed that when ΔN-H3/H4 were bound to DNA, NAP1 could not change their conformational state. In this scenario, intimate interactions between tetramers could only be sustained if both were either left-handed or right-handed. Induced topological stresses, which could be either negative or positive, would make either of the preferred conformations possible. With the reintroduction of H2A/H2B and the binding of H2B to H4, the H4−H4 interaction would be disrupted and normal nucleosomal spacing re-established. If the topological stress were positive, the right-handed conformation of H3/H4 would be the preferred state. In this state, the DNA tends to be in a more open condition, as shown by the persistent accessiblity to topoisomerase I (Figure 3A). Changes in DNA topology have been observed for some remodeling complexes (*76*, *77*; see reviews in refs *33* and *78*), which provides some relevance to this scenario.

We have previously reported that NAP1 greatly enhances the displacement of H2A/H2B during transcription of nucleosomes (*32*). Positive stress of +0.05 SD was effective in displacing one of the H2A/H2B dimers to NAP1, but the second H2A/H2B dimer required additional transcription-induced positive stress as well as the active action of RNA polymerase to be displaced. These new results now provide a possible explanation for those earlier observations. The displacement of the N-termini from DNA by the action of both RNA polymerase and NAP1 would establish a condition in which H3/H4 could undergo a conformational change to the right-handed state. However, the H2A/H2B dimer, which remains bound, blocks this transition. A right-handed conformation would not be compatible with the binding of H2A/H2B (*1*−*3*). By raising the positive stress level greater than +0.05 SD through the additional transcription-induced positive stress, H2A/H2B are no longer able to block this transition and are subsequently displaced to NAP1. NAP1 is both displacing the H2A/H2B and changing the conformation of H3/H4. Because transcription-induced negative stress is also produced in the wake of the polymerase, NAP1 would be expected to facilitate the reformation of the left-handed conformation of the H3/H4 and the subsequent rebinding of the displaced H2A/H2B dimers. If this scenario is correct, these conformational changes of H3/H4 are likely to have a major role in facilitating transcription through nucleosomes.

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