Acetylation of H4 Suppresses the Repressive Effects of the N-Termini of Histones H3/H4 and Facilitates the Formation of Positively Coiled DNA[†]

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ABSTRACT: We have studied the role of the N-termini of histones H3/H4 in the regulation of the conformational changes that occur in H3/H4 during their deposition on DNA by NAP1 (nucleosome assembly protein 1). Removal of the N-termini extensively increased the right-handed conformation of H3/H4 as assayed by the increased levels of positive coils that were formed on DNA. The osmolytes, TMAO, betaine, sarcosine, alanine, glycine, and proline to varying degrees, facilitated the formation of positive coils. The denaturant, urea (0.6 M), blocked the osmolyte effects, causing a preference of H3/H4 to form negative coils (the left-handed conformation). Acetylated H3/H4 also formed high levels of positive coils, and it is proposed that both the osmolytes and acetylation promote the formation of an α-helix in the N-termini. This structural change may ultimately explain a unique feature of transcription through nucleosomes, i.e., that H2A/H2B tends to be more mobile than H3/H4. By using combinations of H3 and H4 that were either acetylated or the N-termini removed, it was also determined that the N-terminus of H4 is primarily responsible for repressing the formation of positive coils. Additional gradient analyses indicate that NAP1 establishes an equilibrium with the H3/H4−DNA complexes. This equilibrium facilitates a histone saturation of the DNA, a unique state that promotes the right-handed conformation. NAP1 persists in the binding of the complexes through interaction with the N-terminus of H3, which may be a mechanism for subsequent remodeling of the nucleosome during transcription and replication.

Histones H3/H4 form the central component of the nucleosome. The left-handed superhelical pitch is defined by the interaction of two H3/H4 dimers through the formation of a four-helix bundle between the two H3s (*1, 2*). It is generally accepted that during replication histones H3/H4 are initially deposited by histone chaperones to begin the formation of the left-handed coil upon which two dimers of histones H2A/H2B subsequently bind to the flanking regions of the tetramer and complete the nucleosome. Histone chaperones such as CAF-1 and Asf1 have been shown to interact with a dimer of H3/H4, and it has been proposed that deposition involves the stepwise addition of two dimers to form the H3/H4 tetramer on the DNA (*3, 4*). The protein, nucleosome assembly protein (NAP1),[1] has also been found to be a highly effective chaperone for forming nucleosomes (see review in ref *5*). NAP1 preferentially exists as a dimer when at lower concentrations (*6, 7*) and binds both H3/H4 and H2A/H2B (*8, 9*). It has been found to import H2A/H2B into the nucleus and to facilitate the deposition of H2A/H2B (*10*). In vitro studies have indicated that NAP1 has a higher preference for binding H3/H4 as compared to H2A/H2B and that the N-terminus of H3 is primarily responsible for this increased affinity (*11*). NAP1 has been found as a part of remodeling complexes (*12, 13*) and is generally thought to facilitate displacement of H2A/H2B (*14, 15*) and the sliding of H3/H4 (*16*) by interaction with both sets of proteins.

We have previously observed that by adjusting the H3/H4 to NAP1 ratio subsequent deposition of the histones resulted in the formation of either negative or positive coils on DNA (*17*). When deposited as a tetramer, negative coils were formed; when deposited as a dimer, positive coils were formed. To explain this observation, we proposed that the preformed tetramer contains a left-handed pitch and maintains this pitch upon binding DNA. In order for the dimer to form a tetramer, the H3−H3 interaction of the tetramer is formed while the H3/H4 are bound to DNA. In this case, this interaction can establish either a left- or right-handed pitch. The right-handed pitch (positive coils) was found to be preferred when the histone to DNA ratio was greater than 1:1, an indication that the close proximity of H3/H4 dimers promotes this unique conformation. The removal of the N-termini appeared to facilitate this process. The relevance of this latter observation is the possibility that acetylation of the N-termini might also facilitate this process.

It is well established that histone acetylation occurs during the activation of promoters and in the elongation phase of transcription (see reviews in refs *18* and *19*). In the latter case, the processive movement of a RNA polymerase would produce topological changes that would force positive stress onto nucleosomes about to be transcribed (*20*). Nucleosomes are particularly effective in creating a viscous drag that prevents DNA rotation during transcription (*21*). We previously observed that this transcription-induced stress caused

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[1] Abbreviations: NAP1, nucleosome assembly protein 1; TAU, Triton−acetic acid−urea; 2-ME, 2-mercaptoethanol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CsCl−EtBr, cesium chloride−ethidium bromide; SD, superhelical density; ccc, circular, covalently closed.

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the release of H2A/H2B and that NAP1 facilitated this process (*22*). Histones H3/H4 would not displace from the DNA, providing this positive stress was present. A potential mechanism that would facilitate the displacement of H2A/H2B from H3/H4 would be the formation of a right-handed conformation in the H3/H4 that is induced by this positive stress. This conformation would likely disrupt the binding to H2A/H2B (*1, 2*). Since the topological stress in the wake of the polymerase would be negative, this stress would be the catalyst for the restructuring of the H3/H4 tetramer to the left-handed pitch and the subsequent rebinding of H2A/H2B. It may be possible that acetylation of the N-termini facilitates these conformational changes.

Sivolob et al. (*23*) examined the potential role of acetylation in this process. They reconstituted hyperacetylated H3/H4 or H3/H4, in which the N-termini were removed, onto minicircle DNA using a salt-jump procedure. The DNA was subsequently treated with topoisomerase I, and they observed that rather than observing minicircles with one negative coil, two minor subpopulations of minicircles were significantly increased. One population had no coils and the other had a positive coil. These observations were subsequently confirmed by Morales and Richard-Foy (*24*). From these studies it has been proposed that the acetylated tetramer constrains less DNA, i.e., the entry−exit site is more open, and the tetramer adopts a relaxed flat conformation, i.e., neither left- or right-handed (see Figure 6 of ref *23*). These observations were an indication that the N-termini may regulate the conformational state of H3/H4.

We have examined the effects of acetylation under conditions in which the deposition is done by NAP1 and under conditions in which the topological changes are characterized on larger DNAs. In this way the effects of the N-termini can be characterized in a more physiological context. As part of this study we have included the use of osmolytes to determine their effects on these conformational changes of H3/H4. Osmolytes stabilize the structure of proteins and are commonly found in organisms which exist in harsh environments (*25, 26*). We observed that osmolytes introduce secondary structure into the N-termini and as a result promote the formation of the right-handed conformation in H3/H4. This same effect was also observed with acetylated H3/H4, and we propose that this secondary structure is an α-helix in the N-termini of both H3 and H4. We also observed that the N-terminus of H4 was particularly repressive to the right-handed conformation. The N-terminus of H3 must be present to facilitate the secondary structural change in H4 that suppresses this repression.

METHODS

Preparation of Histones. Histones were purified from chick erythrocytes and MSB cells (Marek's virus transformed chick leukemia cells) using a modification of the procedure of Simon and Felsenfeld (*27*). The MSB cells had been pretreated with 8 mM sodium butyrate for 20 h prior to use. Nuclei 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_2PO_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 0.8−1.1 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 0.4−1.1 M NaCl. Pooled fractions were concentrated on Amicon filters. The Mono S column also fractionates different levels of acetylation and is the approach used to obtain the very highly acetylated H3/H4. The electrophoretic analysis for identifying levels of acetylation was as described by Zweidler (*28*) and involves TAU electrophoresis. The histone concentrations were determined with an extinction coefficient of 4.2 cm^{-1} mg^{-1} at 230 nm (*29*).

To remove the N-termini of H3 and H4, chick erythrocyte nuclei were exposed to trypsin in the presence of 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM Tris (pH 8.0), washed extensively in that buffer, and then chromatin prepared by treatment with 10 mM Tris and 10 mM EDTA. The chromatin was immobilized on a hydroxylapatite column and the histones eluted with increasing NaCl concentrations. The ΔN-H3/ΔN-H4 were further purified by application to a Mono S column. Based on mass spectroscopy the ΔN-H3 includes residues 27−135 and the ΔN-H4 includes residues 20−102 (data not shown). When nuclei are stably maintained in the presence of $MgCl_2$, fragment P1′ (residues 27−135 of H3) is preferentially produced rather than fragment P1 (residues 27−129 of H3), which is formed when chromatin is digested with trypsin (*30*).

The separation of H3 from H4 was done by an initial dialysis into 7 M urea, 40 mN H_2SO_4, and 5 mM 2-ME for 14 h at 4 °C and then an application on a Bio-Gel P-60 column. The reconstitution of the separated histones was done by adjustment to pH 8.0 with final 100 mM Tris and dialysis against 600 mM NaCl, 20 mM Tris, and 5 mM 2-ME, pH 8.0, for 14 h at 4 °C. Samples were collected and sedimented on a microfuge for 2 min to remove any potential aggregates. Minimal aggregation was detected (data not shown). Homogeneous complexes were obtained as determined on an analytical Superdex-70 column in 0.8 M NaCl (data not shown). All of the various forms of H3/H4 were stored in a buffer condition of 0.6 M NaCl, 20 mM Tris, and 5 mM 2-ME, pH 8.0 at −70 °C.

For preparation of DTNB-treated H3/H4, samples were concentrated to 3 mg/mL and dialyzed against 2.0 M NaCl and 30 mM $KHPO_4$ (pH 8.0) at 4 °C for 10 h to remove the 2-ME. The sample was then adjusted to 1 mM DTNB, and after an incubation of 90 min at 23 °C, the samples were dialyzed for 10 h at 4 °C.

In order to prepare oxidized H3/H4 (a S−S bridge at cysteine 110 of H3), an aliquot of the appropriate H3/H4 was adjusted to 0.6 M NaCl/40 mM Tris, pH 8.0, and incubated for 60 min at 23 °C in 1.0 mM diamide. The sample was then dialyzed overnight to remove the excess reagent. Diamide is an effective oxidizing agent for facilitating the formation of disulfide bridges of closely aligned cysteines (*31*).

Preparation of Topoisomerase I and NAP1. Eukaryotic (MSB) topoisomerase I was isolated from MSB cells as previously described (*32*). 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 *Escherichia coli* strain BL21, which contained plasmid pTN2. The procedure for isolation was as previously described (*21*). NAP1 concentrations were determined by absorbance using a calculated molar extinction coefficient of 36100 M^{-1} cm^{-1} at 276 nm (*6*).

Preparation of DNA. The plasmid 2T7/T3-19 (2255 bp) was prepared and purified on CsCl−EtBr density gradients (*17*). The ccc plasmid is negatively coiled with an ∼−0.05 SD and is directly used for these studies. The two topological standards that were electrophoresed on the agarose gel for each analysis were made as follows: The positively coiled marker was prepared by treatment of the plasmid with topoisomerase I at 0 °C in 10 mM $MgCl_2$ and 10 mM Tris (pH 8.0). The negatively coiled marker was prepared by treatment of the DNA with topoisomerase I in 100 mM tryptophan/40 mM Tris, pH 8.0. After purification by phenol−chloroform extraction, the two DNA samples exhibit an average of 2.5 positive coils (+0.01 SD) and 2.5 negative coils (−0.01 SD), respectively. The DNA concentrations were determined using an extinction coefficient of 20 cm^{-1} mg^{-1} at 260 nm.

Reconstitution of Histone−DNA Complexes. The histone samples were adjusted to 80 μg/mL in 100 mM NaCl, 40 mM Tris, 1 mM EDTA, and 5 mM 2-ME (buffer A) and incubated for 10 min at 23 °C. NAP1 was added to 10 μL aliquots to obtain molar ratios of 4:1, 2:1, 1:1, and 0.5:1 (H3/H4 dimer:NAP1 dimer), and the samples were then incubated for 30 min at 23 °C. To these samples was added DNA (−0.05 SD) that had been preincubated with topoisomerase I (100 units/μg of DNA) for 5 min prior to use. This relaxed DNA was then added to the samples to obtain histone to DNA ratios of 0.8:1, 1.2:1, and 1.6:1 (wt:wt) in a final volume of 20 μL. The samples were incubated for 60 min at 35 °C, and the 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)]. When osmolytes were present, they were used at 0.6 M in the presence of buffer A.

The electrophoretic analysis of the 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) and at 80 V for 10 h at 4 °C (*17*). In these conditions, negative and positive coils have different mobilities. For any particular number of negative coils, the overall compaction of the DNA is less than when DNA has the same number of positive coils. The DNA helix opens up (single-strandedness) and absorbs some of the negative coils. In this way the topological state can be measured by a single electrophoresis, and a direct comparison of the number of negative and positive coils can be made. Second dimension electrophoresis in the presence of 50 μg/mL chloroquin (*17*) was done to verify each of the topological assignments given in the text. An example of such an analysis is shown in Figure 2C.

Gradient Analyses of H3/H4 and H3/H4−NAP1 Complexes. For analyses of the dimer−tetramer equilibrium of the various forms of H3/H4, 60 μg of the H3/H4 was mixed in buffer A (without 2-ME) with two standards: 30 μg of H2A/H2B and 6 μg of the appropriate oxidized H3/H4 in a total volume of 180 μL. The sample was applied to a 5−20% glycerol gradient (4.0 mL) in buffer A (without 2-ME).

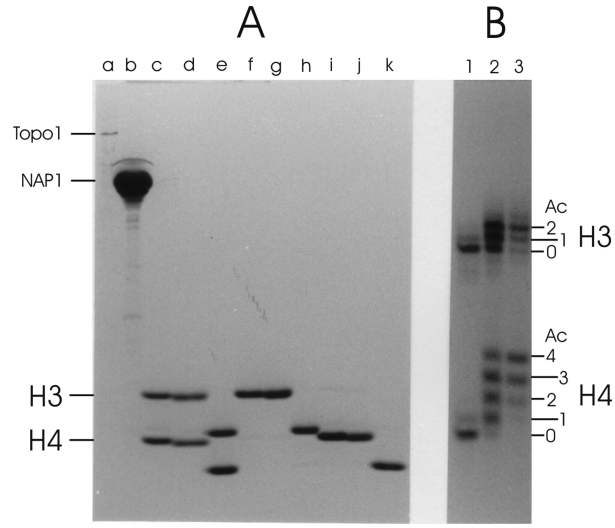

FIGURE 1: An electrophoretic analysis of the purified proteins that are used for these studies.(A) An SDS−PAGE gel of the purified proteins: a, topoisomerase I; b, NAP1; c, LA-H3/H4; d, HA-H3/H4; e, ΔN-H3/ΔN-H4; f, LA-H3; g, HA-H3; h, ΔN-H3; i, LA-H4; j, HA-H4; k, ΔN-H4. Note that the acetylated forms of H3 and H4 tend to have a slightly greater mobility. A more definitive analysis of acetylation levels was obtained by a TAU analysis. (B) TAU analysis: 1, LA-H3/H4; 2, HA-H3/H4; 3, highly purified acetylated H3/H4. The amounts of topoisomerase I, H3/H4 dimer, and NAP1 shown in panel A equate to the molar ratio of 0.01:0.5:1, respectively, which was the ratio that was used for most of the histone deposition experiments.

Centrifugation was on a SW60 rotor at 320000*g* for 24 h at 4 °C. Fractions were collected and diluted by 5× STOP buffer (without glycerol), and 50 μL aliquots were directly applied to a SDS−PAGE gel. The gel contained 18% acrylamide−0.09% bisacrylamide−0.1% SDS gel (*17*). For analysis of the H3/H4−NAP1 complexes, the samples were applied to a 5−20% sucrose gradient in buffer A and sedimented at 320000*g* for 5 h at 4 °C. For separation of free protein from protein associated with DNA, the same sucrose gradients were used, and sedimentation was for 2 h. Glycine (0.6 M) was included in the gradient where indicated in the text. Fractions were collected and analyzed on SDS−PAGE as described above.

RESULTS

The Purified Proteins for This Study. Figure 1A shows the proteins used for this study. These proteins are topoisomerase I (lane a), NAP1 (lane b), LA (low acetylated) histone H3/H4 from chicken erythrocytes (lane c), HA (high acetylated) histones H3/H4 from butyrate-treated MSB cells (lane d), ΔN-H3/ΔN-H4 (lane e), and the individually purified H3 and H4 of each version (lanes f−k). Figure 1B is a TAU gel which shows the acetylated state of the LA-H3/H4 (lane 1) and HA-H3/H4 (lane 2). A further purification of the acetylated H3/H4 was done to enrich for diacetylated H3 and tetraacetylated H4 (lane 3).

The Removal of the N-Termini from H3/H4 Extensively Increases the Formation of Positive Coils on DNA When Deposited by NAP1. We had previously reported that the ratio of H3/H4 dimer to NAP1 dimer greatly influenced the type of topological stress that was ultimately formed on DNA (*17*). This effect was particularly evident as the H3/H4 to DNA ratio was increased above 1:1 (w:w). As shown in

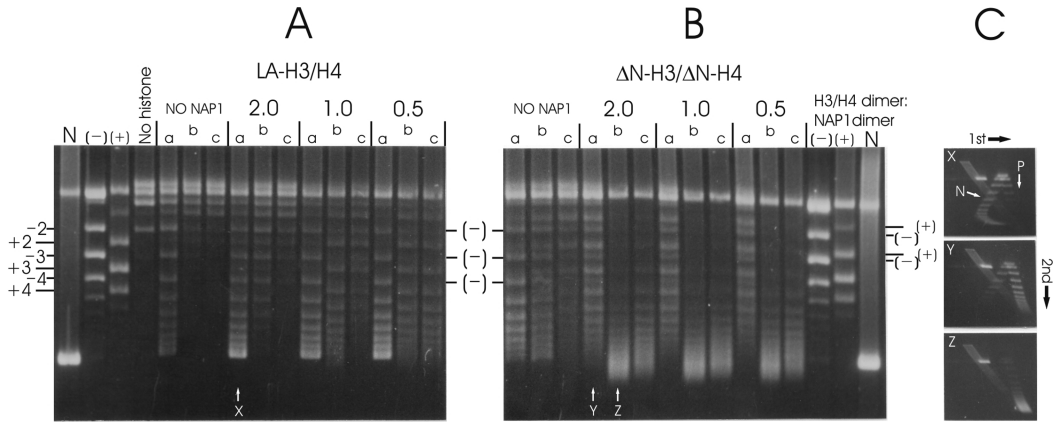

FIGURE 2: Deposition by NAP1 of LA-H3/H4 and particularly ΔN-H3/ΔN-H4 forms high levels of positively coiled DNA. (A) LA-H3/H4; (B) ΔN-H3/ΔN-H4; (C) second dimension analysis of selected samples. The selected samples were taken from the lanes marked x, y, and z in panels A and B and electrophoresed in the second dimension in the presence of chloroquin (*17*). The histones were added to NAP1 at the molar ratio 2.0, 1.0, and 0.5 (H3/H4 dimer:NAP1 dimer) and, after a 30 min incubation at 23 °C, added to DNA at a ratio (w:w, histone:DNA) of 0.8:1 (lane a), 1.2:1 (lane b), and 1.6 (lane c). The DNA had been preincubated with topoisomerase I (20 units/μg of DNA) for 5 min before addition of the histones. The incubation was continued for 60 min at 35 °C. The ionic condition for these incubations was 100 mM NaCl, 40 mM Tris, 1 mM EDTA, and 5 mM 2-ME, pH 8.0 (buffer A). Negative (−) and positive (+) electrophoretic standards and a negatively coiled DNA marker (N) are at the end of each panel.

Figure 2A, when the H3/H4 dimer:NAP1dimer ratio is 1.0 (equivalent to one H3/H4 dimer per 1 NAP1 dimer), negative coils were formed at the 0.8:1 H:D (histone to DNA) ratio (lane a). It should be noted that the identification of DNA topology is based on the DNA markers on the left side of Figure 2A. Negative coils produce partial single-strandedness in DNA which reduces the overall electrophoretic mobility of those coils compared to the same number of positive coils. These DNA markers are used throughout this report to establish the topological state of the DNA that is formed in the experimental conditions. In contrast to the 0.8:1 H:D ratio, when the ratio was increased to 1.2 (lane b), only positive coils were present. Increasing the amount of NAP1 (0.5 ratio) did not significantly increase the number of positive coils, although there was an increase in positive coils at the 1.6:1 H:D ratio (compare lane c for the 1:0 and 0.5 ratios). These results indicate that positive coils are present when the DNA is heavily saturated with H3/H4. When the ratio of H3/H4 dimer:NAP1 dimer was 2.0 (equivalent to one H3/H4 tetramer per NAP1 dimer), negative coils were again observed at the 0.8:1 H:D ratio (lane a). However, increasing the H3/H4 content (lanes b and c) prevented the formation of these coils. We have previously interpreted these observations as indicating that when deposited as a preformed tetramer the left-handed pitch is established prior to deposition and that pitch is maintained after deposition. When saturated with the tetramer, topoisomerase I is unable to efficiently interact with the DNA, causing the DNA to appear relaxed, as though no deposition had occurred (lanes b and c).When deposited as a H3/H4 dimer, the tetramer is formed on the DNA at which point either a left- or right-handed pitch can be established, the latter pitch being favored at the higher histone to DNA ratios. When this form of H3/H4 is deposited, topoisomerase I can more readily access this DNA, even at these high histone to DNA ratios. What has yet to be determined is the influence of the N-terminal regions of H3/H4 in this process.

Following the same procedure for the data of Figure 2A, ΔN-H3/ΔN-H4 were incubated with DNA in the presence of increasing amounts of NAP1. As shown in Figure 2B, the 0.8:1 H:D ratio (lane a) produced both positive and negative coils, but the higher H:D ratios produced high levels of only positive coils. It should be noted that when very high levels of positive coils are present, their electrophoretic mobility is greater than that of a similar level of negative coils (Figure 2B, lane N). At these high numbers of negative coils, any potential increase in this number causes a proportional increase in single-strandedness. As a result the number of negative coils remains relatively unchanged and the mobility remains unchanged. This upper limit of mobility is not observed for highly positively coiled DNA where single-stranded character is prevented. The mobility of the DNA is more proportional to the number of positive coils. This difference in mobility between positive and negative coils is generally sufficient for topological determination; however, we do routinely analyze these samples with a second dimension analysis in the presence of chloroquin. An example of such an analysis is in Figure 2C in which the lanes marked x, y, and z were characterized in this way. These data indicate that ΔN-H3/ΔN-H4 form high levels of positive coils.

A further analysis of the data of Figure 2B indicates that the formation of positive coils was largely independent of the amount of NAP1 that was present. Only when NAP1 was absent were negative coils observed at the higher H:D ratios (lanes b and c). Therefore, when these positive coils are observed at the 2.0 ratio (H3/H4 dimer:NAP1 dimer), a condition which should maintain a stable H3/H4 tetramer, it leaves open the possibility that removal of the N-termini has destabilized the tetramer. It is the dimer that forms positive coils. To test whether this equilibrium has been altered, glycerol gradients were applied to measure the relative "*S*" value. In order to standardize the relative positions on the gradient, histones H2A/H2B were also included. These histones form a stable dimer and at physiological ionic strength do not interact with H3/H4 (*33*). If the equilibrium for H3/H4 is shifted toward dimer, the distribution of H3/H4 will be the same as H2A/H2B. As shown in Figure 3, in both the presence (Figure 3A) and absence of the N-termini (Figure 3B), H3/H4 form a stable tetramer. Further evidence for this conclusion is that in these preparations of H3/H4 a small percentage (9%) of the

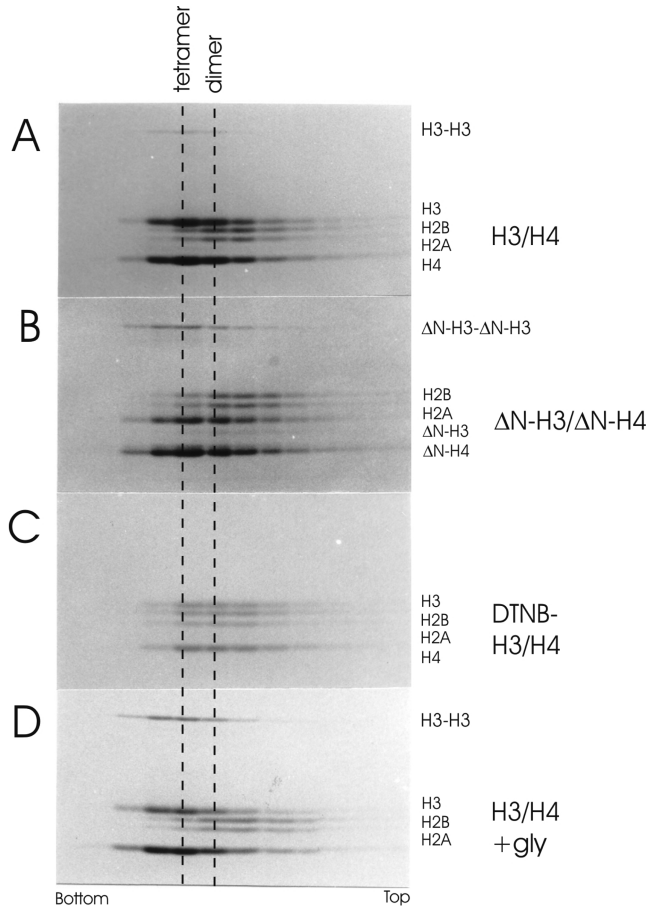

FIGURE 3: Glycerol gradient analyses which show that the tetramer is stable in the presence and absence of the N-termini of H3/H4. (A) LA-H3/H4; (B) ΔN-H3/ΔN-H4; (C) DTNB-treated H3/H4; (D) LA-H3/H4 in 0.6 M glycine. The gradients consisted of 5−20% glycerol in buffer A (without 2-ME), and the sedimentation was at 55000 rpm for 24 h at 4 °C. The 2-ME was not present in order to maintain the disulfide bridge in the small amount of H3-H3 that was included as a standard in these gradients.

oxidized version of the tetramer (H3−H3 bridged at cysteine 110) was purposely included. This form of the tetramer is not in equilibrium with the dimeric state and therefore effectively marks the expected distribution for a stable tetramer. As shown in those figures, the unoxidized H3/H4 mirrored that distribution. Also included in this figure is a condition in which cysteine 110 of H3 was modified by DTNB. We have previously shown that such a modification shifts the equilibrium heavily toward dimer (*17*). As shown in Figure 3C, the H3/H4 distribution was the same as the H2A/H2B. We conclude that the removal of the N-termini did not reduce tetramer formation and is consistent with previous observations using calorimetric and gel filtration studies (*34*). Therefore, with regard to the ΔN-H3/ΔN-H4 tetramer, it must have the capacity to either form a right-handed conformation prior to deposition by NAP1 or readily establish that conformation immediately after deposition. The N-termini are inhibitory to this transition. Are there conditions that can reduce the inhibitory effect of the N-termini? Two potential conditions that we tested were reagents that affect protein structure and histone acetylation.

The Presence of Glycine Promotes the Formation of Positive Stress. Osmolytes function to stabilize protein structure in organisms which exist in environments that tend to cause protein instability (*25, 26*). This instability generally involves changes in hydrogen bonding which will destabilize α-helical structure. Glycine-based forms of these osmolytes are often involved and are present in molar concentrations. It has been proposed that the N-termini of the histones have the potential to form α-helical structure, particularly when acetylated (*35, 36*), and these osmolytes could facilitate a similar formation. We tested the effects of glycine by depositing LA-H3/H4 on DNA using the 0.5:1 ratio of H3/H4 dimer to NAP1 dimer. As shown in Figure 4A, at a concentration of 0.6 M, the level of positive stress that was formed at the 1.2:1 H:D ratio (lane b) is equivalent to the level of stress that was observed when the N-termini were absent (Figure 2B, lane b). Even at a ratio of 0.8:1 H:D (lane a), both positive and negative coils were present, which is again similar to conditions in which the N-termini were removed (Figure 2B, lane a). Raising the glycine concentration beyond 0.6 M did not effectively increase the amount of positive stress that was formed. The formation of positive coils was dependent on the presence of NAP1 as minimal levels of these coils were observed at any histone to DNA ratio when this protein was absent (first three lanes of Figure 4B).

In order to clarify whether the glycine effect is specific to the N-termini, we repeated this experiment with ΔN-H3/ΔN-H4. As shown in Figure 4B, the quantity of positive stress that was formed was the same as was observed when glycine was absent (compare to Figure 2B). In particular, if there were changes in the core regions that would further promote positive stress, the 0.8:1 H:D ratio (lane a) should have shown an increase in this stress. This increase was not observed. The osmolyte effect appears to be specific to conditions in which the N-termini are present. Figure 4B also shows that in the absence of NAP1 minimal positive coils were formed by ΔN-H3/ΔN-H4. Whether the N-termini were present or absent, the formation of positive stress on DNA, even in the presence of glycine, requires histone deposition by NAP1.

Since this effect is dependent on NAP1 deposition, it is possible that glycine has altered the H3−H3 interface sufficient to shift the equilibrium from tetramer to dimer. We tested this possibility by applying the LA-H3/H4 to a glycerol gradient in the presence of glycine. As shown in Figure 3D, the H3/H4 tetramer was stable.

The Presence of Glycine Does Not Alter the H3/H4−NAP1 Interaction. This dependence on NAP1 to form positive coils leaves open another possibility. Glycine may directly affect the structure of NAP1 and, in this instance, enhance its ability to bind the dimeric rather than the tetrameric form of H3/H4. With this scenario not all the H3/H4 would be expected to bind NAP1 if the ratio were greater than 1:1 (H3/H4 dimer:NAP1 dimer). We tested this possibility by applying to sucrose gradients complexes of H3/H4−NAP1 that were formed at different ratios, initially in the absence of glycine. As shown in Figure 5, all of the H3/H4 were bound to NAP1 at both ratios of 1:1 (panel C) and 2:1 (panel D). If any H3/H4 was not bound to NAP1, it would be on top of the gradient (panel B) and separated from the NAP1 dimer (panel A). This experiment was then repeated in the presence of 0.6 M glycine. As shown in Figure 5F and 5G for those same H3/H4 to NAP1 ratios, there was no release of H3/H4 from NAP1. Additional gradients are shown which further

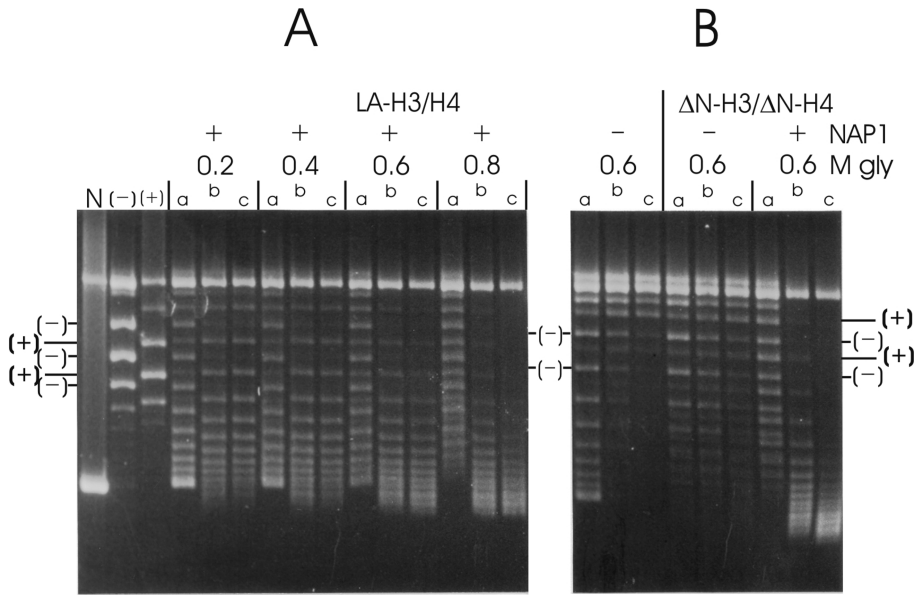

FIGURE 4: The presence of glycine increases the formation of positive coils by LA-H3/H4. (A) LA-H3/H4 were deposited on DNA at a molar ratio of 0.5:1 (H3/H4 dimer/NAP1 dimer) and in the presence of 0.2, 0.4, 0.6, and 0.8 M glycine/buffer A. (B) Deposition in the presence (+) and absence (−) of NAP1 in 0.6 M glycine/buffer A with LA-H3/H4 and ΔN-H3/ΔN-H4. The histone to DNA ratios were 0.8:1 (lane a), 1.2:1 (lane b), and 1.6:1 (lane c).

evaluate the binding characteristic of NAP1 in the presence of glycine. At a ratio of 4:1, which is equivalent to the binding of two tetramers to one NAP1 dimer, no unbound H3/H4 was still observed. The sedimentation rate has substantially increased with the addition of another H3/H4 tetramer. A similar result has been obtained in the absence of glycine (data not shown). These results are an indication that NAP1 is able to bind a wide range of H3/H4 complexes. It has been reported that at a minimum one H3/H4 dimer binds one NAP1 dimer (*11*). Figure 5E shows the results of a gradient analysis of the H3/H4−NAP1 complexes at the 0.5:1 ratio. At this ratio there is one H3/H4 dimer per NAP1 dimer and an additional NAP1 dimer that remains unbound. As shown in Figure 5E, half of the NAP1 was bound to the H3/H4 and the remaining half was not. A similar result has been observed when glycine was absent (data not shown). We interpret these observations as indicating that glycine does not alter the interaction between H3/H4 and NAP1 for a wide range of histone to NAP1 ratios.

The Effects of Different Osmolytes Are Indicative of an α-Helical Involvement of the N-Termini in the Formation of Positive Stress. In order to further characterize the effects of osmolytes on the N-termini of H3/H4, we have examined several additional osmolytes that stabilize α-helical structure to varying degrees. Proline is weaker in this regard whereas TMAO is stronger. Alanine, sarcosine, and betaine are intermediate (*37*). As shown in Figure 6A, when LA-H3/H4 were deposited by NAP1 onto DNA in the presence of 0.6 M of these osmolytes, varying levels of positive coils were observed. Proline contained the lowest level whereas the remaining four were very effective. Particularly for TMAO, even the 0.8:1 H:D ratio (lane a) contained almost exclusively positive coils. It should be noted that in some of these conditions the level of positive stress is reduced at the highest histone to DNA ratio (compare lanes b and c). This reduction is likely due to a reduction in topoisomerase I activity, possibly because of a more enhanced level of saturation by the histones on the DNA. Even when the structural state of H3/H4 promotes the formation of positive coils, it is possible to oversaturate the DNA and inhibit topoisomerase I activity.

In contrast to the stabilizing effects of osmolytes, the denaturant, urea, would be expected to have a destabilizing effect. A depositional analysis was done in the presence of 0.6 M urea, and as shown in Figure 6A, the topological state of the coils was now primarily negative. Urea promotes negative coils and the osmolytes promote positive coils. We further evaluated these opposing effects by combining urea with the glycine, both at 0.6 M. As shown in Figure 6B when in the presence of NAP1, urea significantly decreased the formation of positive coils that would normally be formed by the LA-H3/H4 (compare lanes b and c with those same lanes in Figure 4A). When NAP1 was absent, no change in the topological distribution was observed (compare to Figure 4B). This latter observation is an indication that NAP1 must be present to facilitate the repressive effects of urea. In order to determine whether this effect could be attributed to a loss in secondary structure in the N-termini, this experiment was repeated with the ΔN-H3/ΔN-H4. As shown in Figure 6B, whether in the presence or absence of NAP1, urea did not significantly alter the topological state of the DNA (compare lanes b and c to those same lanes in Figure 4B). The presence of urea is having minimal effects on the ΔN-H3/ΔN-H4, but extensive effects on the LA H3/H4. The denaturating effects of the urea appear to be directed toward the N-termini.

A possible explanation for the effect that glycine has on the N-termini is that the N-termini are displaced from the DNA, thus simulating conditions in which the N-termini are absent. This displacement could be as a result of changes in apparent ionic strength, although this effect would not likely be significant as glycine is a zwitterion. Nevertheless, we tested for this contribution by examining the effects that ionic strength would have on this process. The upper limit in which topoisomerase I is active is 0.3 M NaCl and is the condition that was used. As shown in Figure 6C, no supercoils were observed at any histone to DNA ratio for both LA-H3/H4

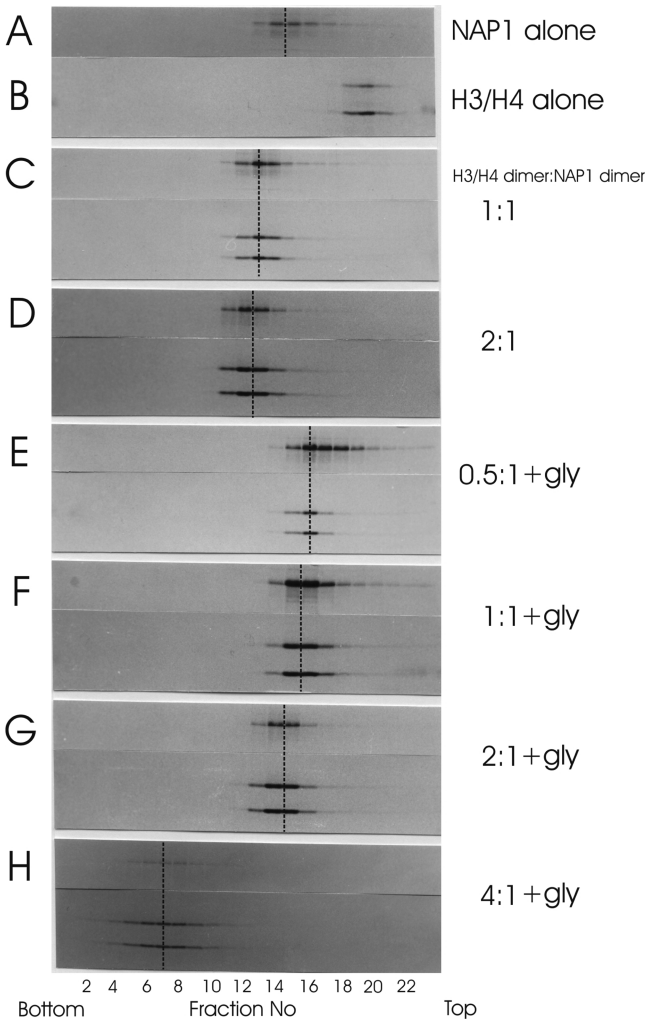

FIGURE 5: Sucrose gradient analyses which show that 0.6 M glycine does not alter the interaction of H3/H4 with NAP1. (A) NAP1 alone; (B) LA-H3/H4 alone. Panels C−H show the analyses of different ratios of H3/H4 dimer:NAP1 dimer. The ratios are (C) 1:1, (D) 2:1, (E) 0.5:1, (F) 1:1, (G) 2:1, and (H) 4:1. For panels C and D, the gradients consisted of 5−20% sucrose in buffer A; for E−H, the gradients contained 5−20% sucrose in 0.6 M glycine/buffer A. The slower sedimentation rate in panels E−H is due to the change in viscosity from the presence of glycine.

and ΔN-H3/ΔN-H4. This ionic strength is inhibitory to deposition by NAP1, which is an indication that the osmolytes must introduce unique structural effects on the N-termini that are unrelated to the shielding of the charged lysines and arginines. This secondary effect may be the introduction of an α-helical state to the N-termini.

Acetylation of the N-Termini of H3/H4 Enhances the Formation of Positive Stress. The acetylation of histones increases the propensity for the N-termini to form an α-helical state (*36*). If osmolytes facilitate this same condition, then one would predict that the HA-H3/H4 would produce that same high level of positive stress, in this case independent of the presence of glycine. HA-H3/H4 were preincubated with increasing levels of NAP1 and subsequently with DNA. As shown in Figure 7A, high levels of positive stress were indeed observed. This is particularly apparent at the 0.5 and 1.0 ratios (H3/H4 dimer:NAP1 dimer) and at the 1.2:1 H:D ratio (lane b). When compared with similar conditions for the LA-H3/H4 (Figure 2A), the striking effect of the acetylation becomes apparent. The HA-H3/H4 that was used in this analysis had the acetylation level shown in Figure 1B (lane 2). We next tested the highly enriched fraction (Figure 1B, lane 3) to determine whether a further increase in positive stress would be observed. The results were identical to those shown in Figure 7A (data not shown), which is an indication that very high levels of acetylation are not needed to observe this effect on DNA topology. Figure 7B shows that when 0.6 M glycine was included, there was an additional increase in positive coils, particularly at the 2.0 ratio (H3/H4 dimer:NAP1 dimer), but generally the overall pattern was similar to Figure 7A. We interpret these observations as indicating that acetylation causes secondary structure changes in the N-termini that are similar to those that are induced by glycine. This structure is likely to be α-helical for both conditions.

The N-Terminus of H4 Are Responsible for the Repressive Effects That Acetylation Will Suppress. There are two N-termini in the H3/H4 dimer, and it is unclear whether both are needed to inhibit the formation of positive coils. We attempted to address this question by purifying the individual histones and then reconstituting them in various combinations. As shown in Figure 8A, the LA-H3/ΔN-H4 combination very effectively formed positive coils whereas the ΔN-H3/LA-H4 combination did not. This effect was apparent whether or not 0.6 M glycine was present. These results are an indication that it is the N-terminus of H4 that is repressive to the right-handed conformation of H3/H4. We similarly assayed the combination of LA-H3/HA-H4 and HA-H3/LA-H4 (Figure 8B). The combination containing the acetylated H4 formed significantly more positive coils than the combination containing acetylated H3. The data of Figure 8B also indicate that when glycine was present, the number of positive coils produced by the LA-H3/HA-H4 was greatly enhanced. This latter observation is an indication that when acetylation of H4 facilitates the formation of positive coils, the unacetylated N-terminus of H3 inhibits this process, an inhibition that can be overcome by the osmolyte. The secondary structure of the N-terminus of H3 is also of importance. This importance is further demonstrated when considering the observed persistence of negative coils in the presence of glycine with the ΔN-H3/LA-H4 for all three histone to DNA ratios (Figure 8A). As was shown in Figure 4A, the presence of glycine would have produced high levels of positive coils when the N-terminus of H3 is present. Since its absence blocks the formation of positive coils (Figure 8A), we interpret these observations as indicating that the N-terminus of H3 must be present and must undergo the secondary structural change brought about either by the osmolytes or by acetylation in order to facilitate the secondary structure change in the N-terminus of H4. Ultimately, it is the structural change in the N-terminus of H4 that permits the right-handed conformation for H3/H4. Only when the N-terminus of H4 is missing, then and only then can this conformation be formed in the absence of the N-terminus of H3. By itself, the N-terminus of H3 is not inhibitory to the right-handed conformation. It becomes inhibitory and a regulator of that conformation only when the N-terminus of H4 is present.

A Persistent Interaction of NAP1 with the N-Termini of H3/H4 Establishes a Condition of Histone Saturation on the DNA While Maintaining the Solubility of the Complex. The previous observations indicated that the ability to form highly

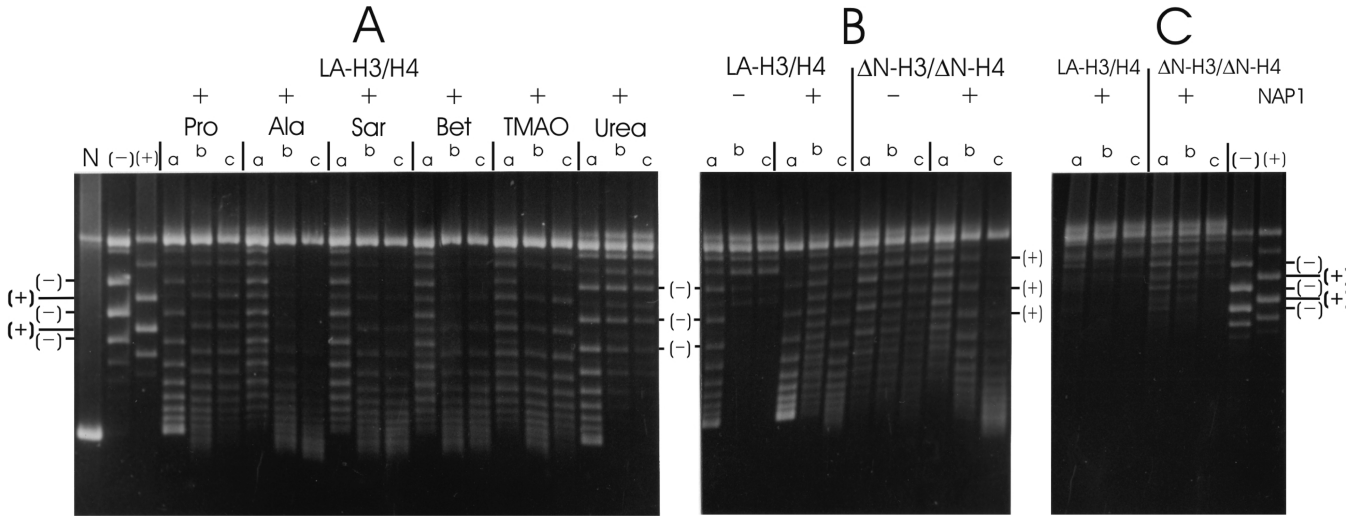

FIGURE 6: A variety of osmolytes facilitate the formation of positive coils on DNA by LA-H3/H4. (A) Deposition by NAP1 in the presence of 0.6 M proline (Pro), alanine (Ala), sarcosine (Sar), betaine (Bet), trimethylamine *N*-oxide (TMAO), and urea. (B) Deposition in the presence (+) and absence (−) of NAP1 in the buffer condition of 0.6 M glycine/0.6 M urea/buffer A. (C) Deposition by NAP1 in the presence of 0.3 M NaCl/40 mM Tris/1 mM EDTA/5 mM 2-ME. The molar ratio of H3/H4 dimer:NAP1 dimer that was used was 0.5:1. The histone to DNA ratios were 0.8:1 (lane a), 1.2:1 (lane b), and 1.6:1 (lane c).

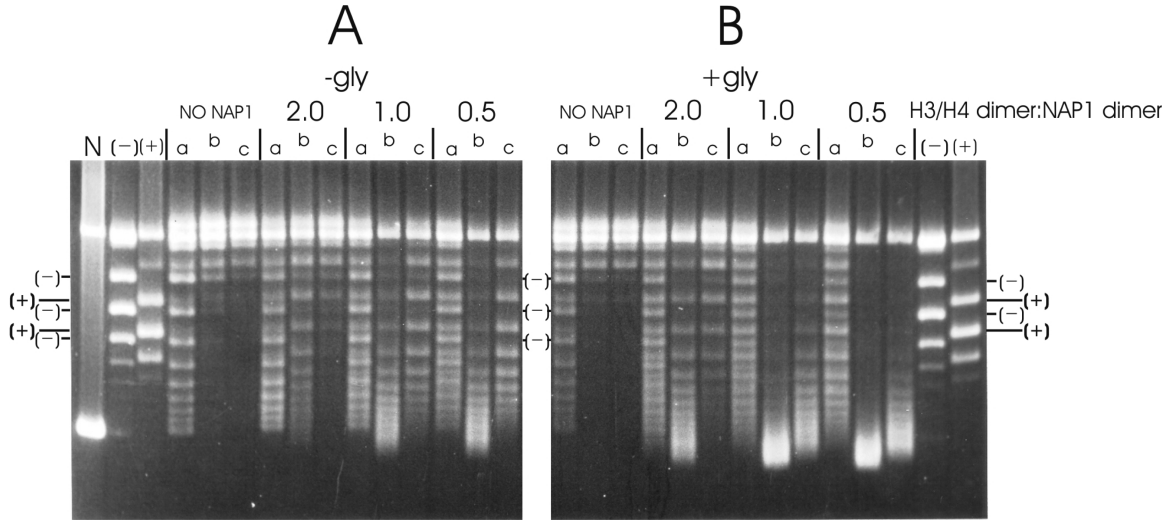

FIGURE 7: The HA-H3/H4 form a high level of positive coils which is independent of the presence of glycine. (A) Deposition by NAP1 in buffer A. (B) Deposition in 0.6 M glycine/buffer A. The histones were added to NAP1 at the molar ratio of 2.0, 1.0, and 0.5 (H3/H4 dimer:NAP1 dimer) and, after a 30 min incubation at 23 °C, added to DNA at a ratio (w:w) of 0.8:1 (lane a), 1.2:1 (lane b), and 1.6 (lane c).

positively coiled DNA is dependent on high histone to DNA ratios. These ratios would be expected to be sufficient enough to cause total aggregation and therefore prevent conformational changes of both proteins and DNA. Figure 9A shows that this is indeed the case. When the LA-H3/H4 were added to DNA at a 1.6:1 H:D ratio, the complex was found in the pelleted fraction. The presence of 0.6 M glycine did not increase the solubility of the aggregate. However, if NAP1 was present in either condition, the complex was in the supernatant for both conditions. This experiment was repeated with the ΔN-H3/ΔN-H4. As shown in Figure 9A, the presence of NAP1 was no longer an absolute requirement for solubility of the histone−DNA complex. Removal of the N-termini has significantly increased the solubility. It should be noted that the DNA in this soluble fraction was primarily negatively coiled. However, when NAP1 was present during the deposition, those soluble complexes now contained exclusively positively coiled DNA. We interpret these data as indicating that at these higher histone to DNA ratios the N-termini promote aggregation. NAP1 prevents this aggregation by interacting with them. As a result positive coils have the potential to form, providing the N-termini are further suppressed either by their removal or by altering their secondary structure by the inclusion of osmolytes in the medium.

This increase in solubility at the high histone to DNA ratios may be due to the persistent interaction of NAP1 with H3/H4, and as a result the excess histones are prevented from binding DNA. In order to determine whether this is the case, the H3/H4−DNA complexes were prepared in 0.6 M glycine using the 1.6:1 H:D ratio and then separated from unassociated proteins using 5−20% sucrose gradients. As shown in Figure 9B (panel a), approximately 30% of the H3/H4 did remain associated with NAP1 at the top of the gradient. As a result, the DNA is saturated with LA-H3/H4 at a level that approximates a 1:1 ratio (H:D). By the persistent binding to the excess H3/H4, NAP1 has maintained the solubility of the H3/H4−DNA complex. This figure also shows that a

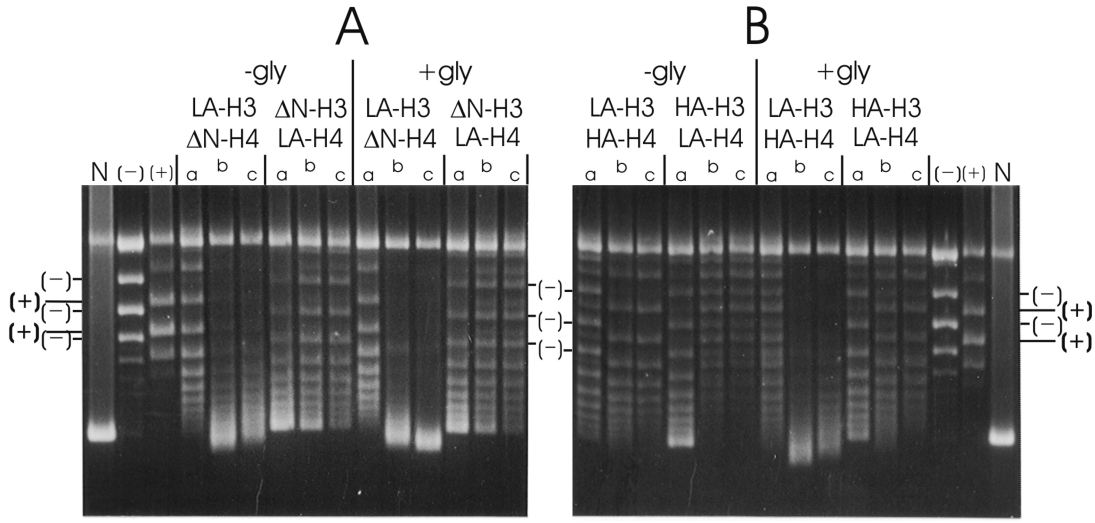

FIGURE 8: ΔN-H4 and HA-H4 histones are required to form the positive coils. (A) Different combinations of LA-H3, ΔN-H3, LA-H4, and ΔN-H4 were reconstituted and then deposited with NAP1. (B) Different combinations of LA-H3, HA-H3, LA-H4, and HA-H4 were reconstituted and then deposited with NAP1. The deposition was done in the presence (+gly) and absence (−gly) of 0.6 M glycine in buffer A. The molar ratio of H3/H4 dimer:NAP1 dimer that was used was 0.5:1. The histone to DNA ratios were 0.8:1 (lane a), 1.2:1 (lane b), and 1.6:1 (lane c).

small percentage of NAP1 continued to bind the H3/H4−DNA complex. To evaluate whether NAP1 is bound to the N-termini, this experiment was repeated with the ΔN-H3/ΔN-H4. As shown in Figure 9B (panel b), NAP1 was no longer associated with the H3/H4−DNA complex. This persistent binding could be a result of the saturated level of H3/H4 that is on the DNA and/or the presence of glycine, which is promoting the structural change in the N-termini. To evaluate whether this is the case, we repeated the analysis with the LA-H3/H4 and in this instance used a subsaturating histone to DNA ratio (0.8:1, H:D) and did not include glycine. This condition will form specifically negative coils (Figure 2A, lane a). As shown in Figure 9B (panel c), a small percentage of NAP1 continued to remain associated with the complex. We interpret these observations as indicating that it is an intrinsic property of some of the NAP1 to remain bound to the N-termini after deposition, irrespective of the secondary structure in those N-termini. We estimate that the amount of NAP1 that binds the complex is equivalent to 8 H3/H4 dimers per dimer of NAP1.

We next determined which of the N-termini of H3/H4 were associated with the NAP1 in the H3/H4−DNA complex. The ΔN-H3/LA-H4 and LA-H3/ΔN-H4 combinations were deposited by NAP1 in the presence of 0.6 M glycine at the 1.6:1 (H:D) ratio, and as shown in Figure 9B, NAP1 did not bind when the N-terminus of H3 was absent (panel d), but when it was present (panel e), NAP1 did bind the complex. This persistent binding of NAP1 to the H3/H4 complex is through the N-terminus of H3.

DISCUSSION

Figure 10 summarizes some of the major observations of this study. The N-termini of H3/H4 were found to be inhibitory to the conformational change that facilitates the formation of positively coiled DNA. When the N-termini are absent, this conformational change is quite efficient (Figure 2B), and when present, inefficient (Figure 2A). It is therefore of significance that a particular environment can suppress the effects of the N-termini. The osmolytes appear to be particularly effective in this (Figure 6). The data of Figures 3 and 5 showed that glycine did not alter the dimer−tetramer equilibrium or the interaction of H3/H4 with NAP1, effects which could have enhanced the formation of positive stress. The presence of glycine did not alter the level of positive stress that was formed by ΔN-H3/ΔN-H4, an indication that this osmolyte effect is directed toward the N-termini (Figures 2 and 4). The data of Figure 7 showed that acetylation of the N-termini largely removed the need for the osmolyte, an indication that both conditions promote the same secondary structure in the N-termini of H3/H4. The data of Figure 8A showed that just the removal of the N-terminus of H4 was sufficient to promote the positive coils. The N-terminus of H3 did not block this process. One might then have predicted that the inducing of secondary structure in the N-terminus of H4 by glycine in the histone combination of ΔN-H3/LA-H4 should have also formed positive coils. The opposite effect was observed. The N-terminus of H3 must be needed to facilitate the effects of the osmolyte on histone H4. It is playing an important regulatory role on the secondary structure of the N-terminus of H4. At this point it is difficult to ascribe a specific model for this process as experimental data that defines sites of interaction between DNA and the N-termini have not been determined for these conditions. Potentially, this effect could be through direct interaction between the two N-termini or indirectly by competition for common DNA binding sites either within a particular tetramer or between adjacent tetramers.

In order to form the highly positively coiled DNA, histone to DNA ratios greater than 0.8:1 were required. As shown in Figure 9A, the ratio of 1.6:1 caused complete aggregation except when NAP1 was present. The cause of this aggregation was the presence of the N-termini, since the ΔN-H3/ΔN-H4 were substantially soluble even in the absence of NAP1. Solubility was not the factor that facilitated the formation of positive coils. The ΔN-H3/ΔN-H4 formed negatively coiled DNA in the absence of NAP1. NAP1 is needed to produce the positive coils. The sucrose gradient analysis of Figure 9B showed that when an excess of LA-

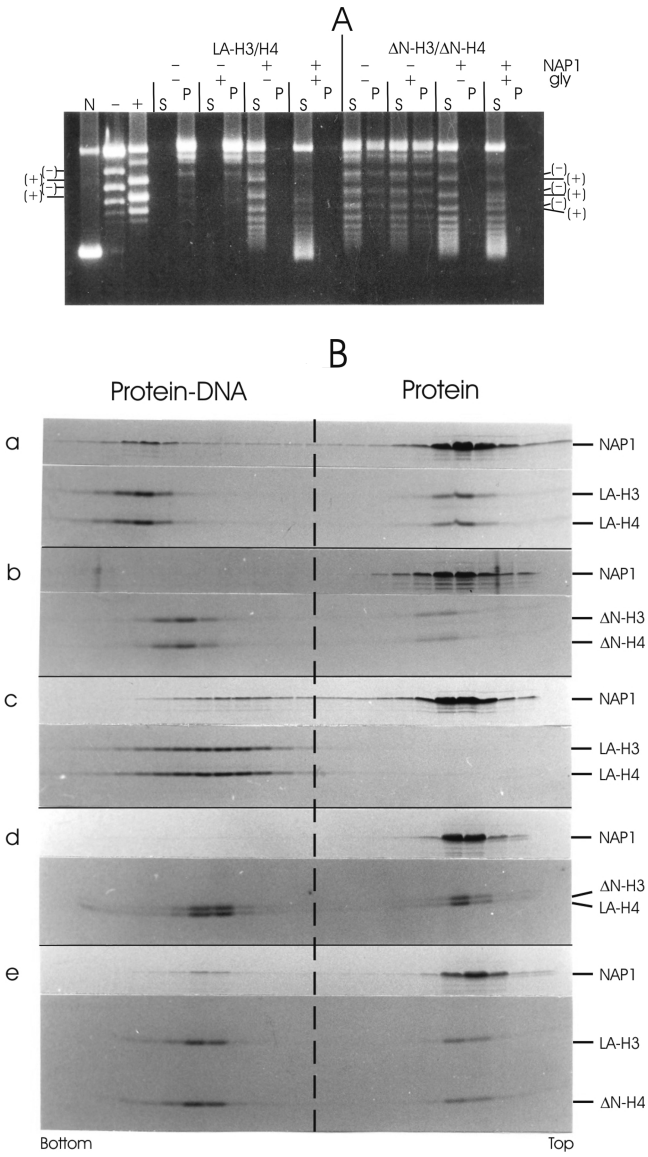

FIGURE 9: The solubility of the H3/H4−DNA complexes at high histone to DNA ratios (1.6:1) is because of persistent interaction of NAP1 with H3/H4. (A) Solubility of the complexes in the presence and absence of 0.6 M glycine and NAP1 (0.5:1 H3/H4 dimer:NAP1 dimer). The H3/H4−DNA complexes were prepared as described in the legend to Figure 2 and centrifuged for 4 min in a microfuge to obtain the soluble fraction (S) and the aggregated fraction in the pellet (P). (B) The H3/H4−DNA complexes that were made by NAP1 deposition were applied to 5−20% sucrose gradients. Panel a, LA-H3/H4 at a 1.6:1 (H:D) ratio with glycine; panel b, ΔN-H3/ΔN-H4 at a 1.6:1 (H:D) ratio with glycine; panel c, LA-H3/H4 at a 0.8:1 (H:D) ratio without glycine; panel d, ΔN-H3/LA-H4 at a 1.6:1 (H:D) ratio with glycine; panel e, LA-H3/ΔN-H4 at a 1.6:1 (H:D) ratio with glycine.

H3/H4 were present, an equilibrium was established in which the DNA was saturated with histones and the excess histones remained associated with the NAP1. This saturated state, which approximates a 1:1 histone to DNA ratio, appears to be of importance in forming positive coils. We have previously proposed (*17*) that this effect may be due to an interaction between the H4s of adjacent H3/H4 dimers, a pattern that is similar to the four-helix bundle that is formed between adjacent dimers of the archaeal histones HMfA and HMfB (*38*). Positive coils tend to also form with these histones when at higher histone to DNA ratios. These histones lack the N-termini of H3/H4 and therefore are not

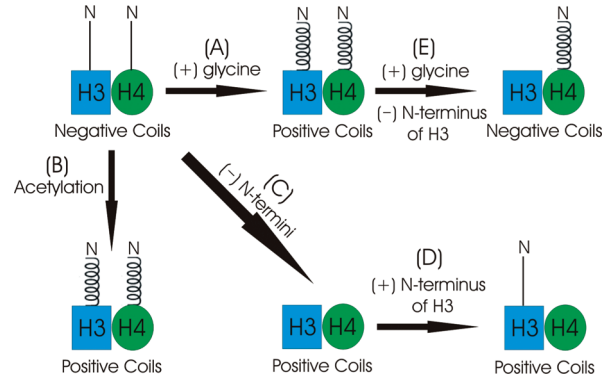

FIGURE 10: NAP1-mediated deposition of H3/H4 forms negative or positive coils dependent on the conformational state of the N-termini of H3/H4. The addition of glycine (A) or the presence of acetylation (B) produces a helical state in the N-termini that forms positive coils. This effect can be simulated by removal of the N-termini for both H3 and H4 (C). The inclusion of the N-terminus of H3 (D) does not prevent the formation of positive coils, but the inclusion of the N-terminus of H4 does (E). The N-terminus of H4 is repressive even in the presence of glycine, an indication that the N-terminus of H3 needs to be present to block this repression.

subject to the repression by these sequences of the right-handed conformation. A potential explanation for these observations is that adjacent H4s of two H3/H4 dimers can more effectively interact when their N-termini contain secondary structure. It is at this level that the N-terminus of H3 may be most relevant in promoting interaction between adjacent H4s.

The data of Figure 9B indicated that significant quantities of NAP1 remained bound to the histone−DNA complex and that this binding was to the N-terminus of H3. McBryant et al. (*11*) have similarly observed by using GST-fused peptides of the N-termini that NAP1 preferentially associates with the N-terminus of H3. This persistent interaction is not dependent on whether the DNA is saturated with histones and forming positive coils. When subsaturating levels of H3/H4 were deposited by NAP1 in the absence of glycine (Figure 9B, panel c), a condition that forms negative coils, NAP1 continued to maintain interaction with the N-terminus of H3. This persistent binding occurs independent of whether H3/H4 are in a right- or left-handed conformation. In a previous report (*17*) we showed that NAP1 would not bind histone−DNA complexes when those complexes were prepared in advance using a reconstitution protocol that involves NaCl dialysis. NAP1 did remodel those complexes, reducing the number of negative coils, but would not persistently bind them. This persistence in binding is a characteristic of the deposition process. The binding of NAP1 could provide a partial explanation for why the N-terminus of H3 promotes the formation of positive coils when the N-terminus of H4 is present. It would be of interest to determine whether this persistence in binding is a general phenomenon of histone chaperones, potentially serving as sites for continuous nucleosome remodeling after replication when epigenetic information is being established.

We have used stabilizing osmolytes to facilitate a structural change in the N-termini of H3 and H4. Protecting osmolytes push the folding equilibrium of a protein toward the native state. The osmolytes that we used in our study were TMAO, betaine, sarcosine, alanine, glycine, and proline. It has been reported that their relative efficiency in the stabilization of

a protein follows that general order (*37*). Proline is a weak stabilizer but is particularly effective in limiting aggregation (*39*). TMAO is a strong stabilizer, and it has been proposed that this stabilization is dependent on the number of methyl groups in the osmolyte of which TMAO has the largest number (*37*). By decreasing the extent of osmolyte interaction with the protein backbone, the water activity around the backbone is altered and the backbone is stabilized (*26, 37*). TMAO has been used in a number of studies to characterize the folding and unfolding properties of proteins and is considered to be very effective in establishing the native state (*40*). In Figures 5 and 6 we showed that when these different osmolytes were present, their efficiency in forming positively coiled DNA with LA-H3/H4 correlated with their known ability to stabilize protein structure. The data of Figure 6B showed that when both 0.6 M urea and 0.6 M glycine were present, urea prevented the formation of positive coils that would normally be present. This effect was particularly evident for the LA-H3/H4 and not the ΔN-H3/ΔN-H4. We propose that this effect is an indication that urea has altered the structure in the N-termini that was initially induced by the glycine. Urea promotes the formation of left-handed coils. The osmolyte effect could be due to an increase in the ionic strength of the medium, and as a result there is a reduction in the interaction of the N-termini with DNA, mimicking conditions in which the N-termini are missing, i.e., ΔN-H3/ΔN-H4. However, Figure 6C showed that 0.3 M NaCl prevents proper deposition of either H3/H4 or ΔN-H3/ΔN-H4. We have determined that this effect is due to the inability for NAP1 to function effectively as a deposition vehicle, and as a result the binding of H3/H4 to DNA is disordered (data not shown). Osmolytes preserve this function for NAP1, an indication that changes in ionic strength are not likely to be involved. The charged lysines and arginines of the N-termini remain available to maintain their interaction with DNA, not unlike the charged groups that continue to remain, even after acetylation of some of the lysines. There are three arginines in histone H4: R3, R17, and R19. The critical factor appears to be the secondary structure of the N-termini, which must significantly alter how those charged groups interact with DNA. This alteration is sufficient to establish a condition in which the N-termini appear to be missing.

We propose that the secondary structural change in the N-termini is the formation of α-helical structure. In 1970 Sung and Dixon (*35*) first proposed that the N-termini of histones could potentially form an α-helix, and it has been proposed that this structure, particularly for H3 and H4, facilitates accessibility of the acetylated sites to serve as a histone code for bromo-domain-binding proteins (*41*). Wang et al. (*36*) have observed by CD analysis of acetylated and unacetylated nucleosomes a significant 2−3% increase in ellipticity at 282.5 nm for the acetylated nucleosome. They also observed that the acetylated N-terminal peptide of H4 (resid 1−23) had a greater propensity to form an α-helix when placed in 90% TFE, a known α-helix stabilizer. In that analysis the α-helical state was observed to increase exponentially as the acetylation level of the peptide was increased. Using several structural prediction methods, residues 10−20 were predicted to have a particular propensity toward this structure (*36*). This region includes K12 and K16, and it has been shown by a microarray analysis of a K16R mutant that this site is of particular importance in establishing potential conditions for transcription (*42*). Acetylation of this single site has also been shown to decrease higher order chromatin structure and enhance ACF-mediated nucleosome sliding (*43*). It is also known that newly synthesized H4 is specifically acetylated at K5 and K12 and may have a major role in the deposition process (*44*). A "zip" model for the acetylation of the N-termini of H4 has been proposed based on the observation that the order of modification begins with K16 and in consecutive order is followed by K12, K8, and K5 (*45, 46*). Moderate levels of acetylation would emphasize the modification of K16 and K12. Our observations that high levels of acetylation (the tetraacetylated state) produce the same level of conformational change in H3/H4 as the moderate level of acetylation, i.e., HA-H3/H4, is an indication that K12 and K16 may be of particular importance in facilitating the helical state of the N-terminus.

Numerous studies have been done to determine the effects of histone acetylation on nuclear function (see reviews in refs *18* and *19*). Acetylation has been observed to alter both higher order structure and nucleosome stability. The N-terminus of H4 has been shown to be of particular importance with regard to self-association and higher order structure (*47, 48*). In particular, Wang and Hayes (*48*) have shown that with K to Q mutations of the four relevant lysines, K5, K8, K12, and K16, that the cation-dependent, self-association of the histone−DNA complexes was greatly diminished. The N-terminus of H4 interacts between nucleosomes, and the reduction of charge by acetylation and the subsequent α-helical state destabilize this interaction. Similarly, studies on the thermal stability of nucleosomes have shown that it is the acetylated N-terminus of H4 that is primarily responsible for their decreased thermal stability (*49*). This instability appears to be located at the entry−exit points of the nucleosome as the linking number of acetylated nucleosomes is reduced by 20% (*50*). The N-termini of H3/H4 have a major role in stabilizing the nucleosome at that region (*1, 2*). These changes do not imply lack of interaction, however. Mutskov et al. (*51*) have observed that even though nucleosomes that are assembled on DNA containing GAL-4 sites are more accessible to the transcription factor when acetylated histones are present, they also observed by UV cross-linking that the N-termini persist in their interaction with DNA, even in the presence of GAL4. This persistence of interaction must be quite dynamic in order to facilitate an average reduction of 20% in the linking number. Therefore, when considering transcription through nucleosomes that contain acetylated H3/H4, the transcription-induced positive stress that is formed by RNA polymerase would be expected to have a greater propensity to open this entry−exit region. Subsequently, those same acetylated N-termini which would continue to interact with the DNA would then facilitate the right-handed conformation of H3/H4 that is promoted by this induced positive stress. Such a conformational change is not conducive to a stable interaction with H2A/H2B. This scenario may in part explain the in vivo observations which indicate that H2A/H2B is much more mobile during transcription than H3/H4 (*52, 53*). All of this is because of the secondary structural changes induced by acetylation of the N-termini of H3/H4.

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