Histone Release during Transcription: Acetylation Stabilizes the Interaction of the H2A−H2B Dimer with the H3−H4 Tetramer in Nucleosomes That Are on Highly Positively Coiled DNA†

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ABSTRACT: A high level of the post-translational modification, acetylation, is found on the N-terminal regions of the core histones H2A, H2B, H3, and H4 and is primarily located in the nucleosomes of active genes. An *in vitro* transcription system was applied, which utilizes T7 RNA polymerase and template DNAs that are either moderately or highly positively coiled, to determine whether acetylation alters the dynamics of histone displacement from these templates during transcription. To measure displacement, an excess of a competitor (negatively coiled DNA reconstituted with unlabeled H3−H4) was included during the transcription process. Acetylated but not unacetylated ^{3}H-labeled H3−H4 was found to displace with high frequency from the moderately positively coiled template. This displacement of acetylated H3−H4 was not observed when the template was highly positively coiled. Acetylated ^{3}H-labeled H2A−H2B also preferentially displaced to the competitor, but in this instance, transcription-induced stress on the highly positively coiled template was required. The histone chaperone, NAP1, was found to facilitate the displacement of both H3−H4 and H2A−H2B. Surprisingly, when acetylated H2A−H2B and acetylated H3−H4 were reconstituted together in the same nucleosomes, the displacement of acetylated H2A−H2B was much reduced during transcription. We conclude that acetylation alters nucleosome stability by enhancing displacement of H3−H4, while decreasing the displacement of H2A−H2B. These results are discussed with regard to potential *in vivo* conditions in which these observations may be relevant.

The packaging of eukaryotic DNA into chromatin creates an environment in which the transcription machinery for either initiation or elongation is severely affected. The DNA (145 bp) is wrapped in a 1.8 left-handed coil around two each of four highly basic proteins, histones H2A, H2B, H3, and H4 (*1*−*3*). The particle that is formed, termed a nucleosome, restrains the equivalence of one negative coil, and ordered arrays of these structures are the primary condensing mechanism for organizing DNA (*4*). Higher levels of compaction are achieved by the bridging of one nucleosome to another through the binding of an additional protein, histone H1. At physiological ionic strength and in the absence of DNA, H2A and H2B exist as a dimer[1] and H3 and H4 exist primarily as a tetramer (*5*). When associated with DNA, two of the dimers interact with one tetramer to form the inner core of the nucleosome. Because of the strong electrostatic interactions between the histones and DNA, salt concentrations of 0.6, 0.8, and 1.2 M NaCl are required to extract, from the chromatin histone H1, the dimer and tetramer, respectively (*6*). However, if the dimer is placed on DNA in the absence of the tetramer, the dimer is readily extractable with 0.4 M NaCl, a salt concentration that is needed to extract transcription factors (*7, 8*). The requirement for the higher salt concentration when in the presence of the tetramer is because of the strong dimer−tetramer interactions that occur within the nucleosome. The highly basic N-terminal region of each of the four core histones is the site for the strong electrostatic interactions with DNA. Post-translational modifications, which reduce the basic nature of the N-terminal regions of the histones, might be expected to weaken their interactions with DNA and increase access of the RNA polymerase to this DNA.

Acetylation of the lysines in the N-terminal regions has been found in all eukaryotes, and its presence is highly correlated with enhanced transcriptional activity (*9*−*16*). However, when considering the stability of the nucleosome, it has generally been found that the effects of acetylation are primarily observed outside the nucleosome rather than inside (*17*−*25*). Acetylation reduces nucleosome−nucleosome interactions (*26*−*29*) and the ability for both histone H1 and Mg^{2+} to create a higher-order structure (*30*, *31*). Only in low ionic strength conditions do acetylated nucleosomes have a more elongated, unfolded state as seen by electron microscopy (*32*, *33*) and gel retardation (*34*). One small effect that has been observed in isotonic conditions is the partially reduced ability for acetylated histones to maintain the single negative coil in the nucleosome, and this has been attributed

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[1] Abbreviations: dimer, a complex of one H2A histone and one H2B histone; tetramer, a complex of two H3 histones and two H4 histones; LA, low acetylation; HA, high acetylation; NAP1, nucleosome assembly protein 1; SD, superhelical density; 2-ME, 2-mercaptoethanol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); BSA, bovine serum albumin; FACT, facilitates chromatin transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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to a loosening in the interaction of the acetylated N-terminal regions of H3 and H4 with the entry and exit regions of DNA (*23*). As a result there is an increased sensitivity to thermal denaturation of the nucleosomal particle (*18*) and in the sensitivity to nucleases (*35*). This loosening in the entry/exit point may be a partial explanation for the 0−10-fold enhancement in transcription efficiency that has been seen with acetylated nucleosomes in *in vitro* experimentation using varying ionic strengths (*27*, *36*−*39*). It is also possible that a significant level of this enhancement could be due to a preferential displacement of acetylated histones. No studies have evaluated this latter possibility or whether the dimer−tetramer interaction is affected by acetylation while transcription occurs.

We have studied the stability of nucleosomes during transcription under conditions in which transcription-induced positive stress was preserved. Those experiments involved transcription with T7 RNA polymerase on template DNA that had been reconstituted with histones at low levels of acetylation. The template DNA was either moderately positively coiled (+0.01 SD) or highly positively coiled (+0.05 SD). In both prokaryotic and eukaryotic systems, it has been shown that, during the elongation phase of transcription, RNA polymerase produces positive stress in the forward direction and negative stress in its wake (*40*−*47*). Positive stress would tend to form right-handed coils in the DNA and could promote disruption of the nucleosome. We observed that when nucleosomes (containing radiolabeled histones) were present on the +0.05 SD DNA, the first dimer would readily displace from the tetramer on the template to a negatively coiled competitor (containing an unlabeled tetramer). Transcription was not required to facilitate this transfer. The displacement of the second dimer did require transcription and the presence of additional positive stress that was greater than the +0.05 SD (*48*). The tetramer could also be displaced, but it had to be bound to at least one dimer and also to a +0.01 SD template (*49*). When the dimer was absent, this lower level of positive stress was then sufficient to hold the tetramer to the template. The overall conclusion from these studies was that high levels of positive stress stabilized the tetramer on the template and facilitated displacement of the dimer. This conclusion provided a possible explanation for the *in vivo* observations that the dimer is much more mobile than the tetramer during transcription (*50*−*52*). Because genes that are highly active are also highly acetylated, we have set about to evaluate whether acetylation alters this conclusion. The studies of this paper indicate that it does. Acetylation increases the sensitivity of the tetramer to reduced levels of positive stress. The tetramer readily displaces from a +0.01 SD DNA, even in the absence of the dimer. At higher levels of positive stress (+0.05 SD), this potential for displacement is lost, but acetylation now increases the binding affinity between the tetramer and dimer. Acetylation is increasing the probability for tetramer displacement while decreasing the probability for dimer displacement.

EXPERIMENTAL PROCEDURES

Preparation of Histones. Histones were purified by a modification of the procedure of Simon and Felsenfeld (*53*). Purified 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_2P0_4 (pH 8.0), and 5 mM 2-ME, 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 these histones 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 and stored at −70 °C. For some experiments, the N-terminal regions of histones H3 and H4 were removed. This was done by trypsin treatment using a modified protocol of Ausio et al. (*54*). Briefly, micrococcal nuclease-solubilized chromatin (from chicken erythrocytes) was exposed to trypsin-coated beads. The digestion was terminated by the addition of trypsin soybean inhibitor and removal of the beads. The chromatin was immobilized on the hydroxylapatite column, and the histones were eluted with increasing NaCl concentrations. The remaining small level of contamination of H2A and H2B was removed by application to a Mono S column.

Labeled histones were prepared by incubating a concentrated solution of MSB cells (chicken leukemia cells transformed by Marek's virus) with 3 mCi ^{3}H lysine and 1.5 mCi ^{3}H arginine (Amersham) for 60 min in the presence or absence of 5 mM butyrate. The label in the cells was then chased for 60 min by suspending the cells in fresh medium (±5 mM butyrate) before the cells were harvested. As a result, the labeled histones contained either high (HA; butyrate-treated) or low (LA) levels of acetylation as verified by analysis on Triton−acetic acid−urea gels [*55*, see Figure 1 in both refs *49* (LA histones) and *56* (HA histones) for the modification levels of these histones].

Preparation of Topoisomerases, T7 RNA Polymerase, and NAP1. Eukaryotic (MSB) topoisomerase I was isolated from MSB cells using a modification (*57*) of the procedure of Liu and Miller (*58*). One unit is defined as that quantity that achieves 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C.

T7 RNA polymerase was prepared from *E. coli* strain BL21, which contained plasmid pAR1219. The procedure for isolation was a modification (*59*) of the procedure of King et al. (*60*). One unit is defined as the amount of enzyme that will incorporate 1 nmol of CTP at 37 °C in 60 min.

NAP1 was prepared from *Escherichia coli* strain BL21, which contained plasmid pTN2. The procedure for isolation was a modification (*61*) of the procedure of Fujii-Nakata et al. (*62*). A weight ratio of 1:1 with the histones provided the maximum depositional activity. This same ratio was used for transcription studies in which both labeled and unlabeled histones were present with NAP1.

Preparation of DNA. Circular, covalently closed (ccc) negatively coiled DNA was purified on CsCl-ethidium bromide density gradients. To produce moderately positively coiled DNA, the 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 (*63*, *64*). Relaxation under this condition results in a DNA (2238 bp, 1T7/T3−19) that exhibits an average of 2.5 positive coils (average =

+0.01 SD) when subsequently incubated at 35 °C under isotonic conditions. This DNA is considered to be moderately positively coiled.

Highly positively coiled template DNA was prepared as previously described (*48*). Briefly, DTNB-treated histones H3−H4 were added to the +0.01 SD DNA at a final ratio of 0.85:1 (wt/wt) histone/DNA. In this condition, the DNA aggregates, and when MSB topoisomerase I is added and the incubation is continued at 35 °C for 6 h, high levels of positive coils are formed. The DNA is subsequently purified on sucrose gradients. It has a superhelical density that averages at +0.05 SD and is referred to as highly positively coiled. For experiments in which this +0.05 SD DNA was used as a template for transcription, the plasmid, 2T7/T3−19 (2258 bp), was used. Its two T7 promoters are required to facilitate initiation of transcription (*48*).

Reconstitution of Histone−DNA Complexes. Reconstitutions were carried out at 4 °C by NaCl stepwise dialysis in which the histones were mixed with DNA in 2.0 M NaCl, 40 mM Tris, 0.1 mM EDTA, and 5 mM 2-ME at pH 7.4. The NaCl concentration was then decreased in the same buffer in three steps of 1.2 to 0.6 to 0.1 M for 3 h in each step (*7*, *65*). Samples were sedimented at 10000*g* for 5 min to differentiate between soluble and insoluble complexes. The histone/DNA ratio was maintained at 0.4:1 (wt/wt) for both competitor and template DNAs for all experiments. At this ratio, minimal insoluble complexes were observed (data not shown). The histone and DNA concentrations were determined using an extinction coefficient for DNA of 20 at 260 nm and 4.2 at 230 nm for histones (*66*).

Conditions for Transcription and Analysis of Complexes on Sucrose Gradients. Transcription was done at 35 °C under the isotonic conditions of 100 mM NaCl, 40 mM Tris, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-ME (pH 7.4), 15 units of Prime RNase Inhibitor (Eppendorf), and ATP, GTP, CTP, and UTP (0.8 mM each). In the initial step, the reconstituted histone−DNA complexes were adjusted to a DNA concentration of 70 μg/mL for the template and 140 μg/mL for the competitor (600 μL of total volume). This was done in the absence of UTP. The competitor is negatively coiled M13 DNA that had been reconstituted with chick erythrocyte H3 and H4 (unlabeled) at a ratio of 0.4:1 (histone/DNA). A 5-fold excess of T7 RNA polymerase was now added, which is an amount sufficient to ensure saturation of the promoters (1.6 kilounits/μg of DNA). The sample was then incubated for 2 min, during which time the polymerase transcribes 13 bases before a UTP was required. As a result, the initiation of transcription becomes synchronized. If NAP1 were to be present, it would be added after this 2 min of incubation. UTP was now added, and the transcripts were elongated for 5 min, after which transcription was terminated with the addition of EDTA (final 10 mM). RNase A (20 μg/mL) was then added, and the incubation continued for 5 min, during which time any histones that had displaced to the nascent RNA were now primarily displaced to the excess competitor (*48*, *49*). The sample was then placed on ice for 10 min and applied to a 5−20% sucrose gradient containing 100 mM NaCl, 40 mM Tris, and 0.1 mM EDTA (pH 7.4) and sedimented in an SW41 Ti rotor at 40 000 rpm for 5 h at 4 °C. Fractions were collected (450 μL); a total of 30 μL was removed and added to an equal volume of stop buffer [0.4% SDS, 20% glycerol, 50 mM Tris, and 25 mM EDTA (pH 8.0)]. Electrophoretic analysis of the DNA in these samples 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 85 V for 14 h at 4 °C (*67*). The remainder of each fraction was treated with 5 μg of BSA and then adjusted to 15% TCA. BSA serves as a carrier to facilitate quantitative precipitation of the proteins. After 4 h at 4 °C, the samples were centrifuged at 20000*g* for 10 min and the pellets were washed with 15% TCA, washed with acetone, dried, and dissolved into SDS electrophoresis buffer. These samples were electrophoresed on SDS−PAGE gels, which were subsequently exposed to the fluorographic procedure of Laskey and Mills (*68*) to determine the quantity of ^{3}H-labeled histones.

RESULTS

Acetylation Lowers the Stability of H3−H4 for the +0.01 SD DNA but Not for the +0.05 SD DNA. In our previous studies of histone movement during transcription, the emphasis was on the movement of histones that were at low levels of acetylation (referred to as LA histones). The following experiments were designed to compare LA histones with highly acetylated (HA) histones to determine whether acetylation would alter this histone movement. Our first experiment was to determine whether the modification would facilitate histone movement without the presence of transcription. Histones H2A−H2B (referred to as a dimer) and H3−H4 (referred to as a tetramer) were purified from MSB cells that had been radiolabeled with ^{3}H lysine and ^{3}H arginine either in the presence or absence of 5 mM butyrate. The butyrate treatment produces histones that are highly acetylated. The radiolabeled LA tetramers and HA tetramers were then mixed with LA dimers at a molar ratio of 1:4 (H2A−H2B/H3−H4) and reconstituted by NaCl dialysis to either +0.01 SD or +0.05 SD DNA. We used this ratio of dimer/tetramer because we had previously observed that, when both dimers are bound to a tetramer (an octameric complex), one of the two dimers was nearly completely displaced from highly positively coiled DNA (+0.05 SD) to a competitor without the need for transcription. The competitor consisted of a negatively coiled DNA (M13, 7250 bp), which had been previously reconstituted with unlabeled tetramers. The dimer forms a highly stable interaction with the tetramer when on negatively coiled DNA, and therefore, the use of this competitor is a very effective way of measuring the displacement potential of the dimer (*48*). The displacement potential for the first dimer is very high when nucleosomes are on +0.05 SD DNA. In contrast to the first dimer, the second dimer remained stably bound to the tetramer (*48*). The one dimer−one tetramer interaction has a low displacement potential. Therefore, because this 1:4 ratio produces a reconstitute in which the tetramers exist on the DNA as either tetramers with no dimers (50%), tetramers bound to one dimer (35%), or tetramers bound to two dimers (15%), the predominance of the one dimer−one tetramer complex creates a condition in which it is possible to determine whether the stability of that interaction is decreased when acetylation is present on the tetramer (*48*). After the reconstitution at this ratio was completed, we then mixed the reconstitutes with a 2-fold excess of this same competitor and incubated them at 35 °C for 10 min. After the incubation, the competitor and reconstitutes were separated on sucrose

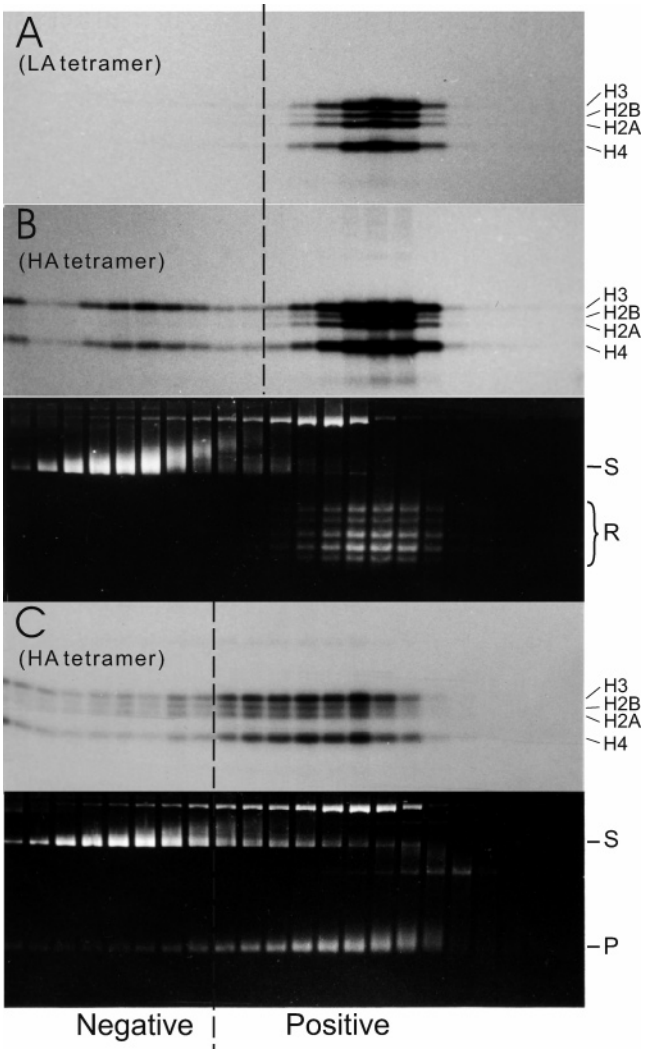

FIGURE 1: Analysis of the displacement of the LA and HA tetramer when bound to either +0.01 SD or +0.05 SD DNA. (A) LA tetramer on +0.01 SD DNA. (B) HA tetramer on +0.01 SD DNA. (C) HA tetramer on +0.05 SD DNA. The DNA was reconstituted with histone at a molar ratio 1:4 (H2A−H2B/H3−H4) by NaCl dialysis and subsequently incubated at 35 °C for 10 min with a 2-fold excess of negatively coiled M13 DNA, previously reconstituted with unlabeled H3−H4. The samples were sedimented on sucrose gradients, and the fractions were collected and analyzed to determine the distribution of protein and DNA. The dark panels below the PAGE gels of B and C are agarose gels showing the distribution of the DNA. "R" marks the location of the +0.01 SD DNA; "S" marks the M13 DNA; and "P" indicates the +0.05 SD DNA.

gradients. As shown in Figure 1A, when the LA tetramer is reconstituted to the +0.01 SD DNA and subsequently incubated with the competitor, neither the dimer nor the tetramer displaced to the competitor. Both the LA tetramer and dimer are stably maintained on the +0.01 SD DNA. We have observed a similar result when the reconstitution was done on the +0.05 SD DNA (data not shown), which is an indication that at low levels of acetylation the tetramer, when alone or in a complex with the dimer, is stably maintained on both low and high levels of positive stress. In contrast to the LA tetramer, Figure 1B shows that, when the HA tetramer was present on +0.01 SD DNA, 10% of that tetramer was displaced to the competitor. The HA tetramer is more readily displaceable from +0.01 SD DNA. As was observed for the LA tetramer, the LA dimer did not displace from the HA tetramer. The acetylated state of the tetramer has not decreased the interaction between the dimer and tetramer or the potential for the dimer−tetramer complex to remain bound to this DNA. We have also observed that, if the HA dimer is used in this analysis, a similar lack of displacement of this dimer is also observed (data not shown). Therefore, the acetylated state of the tetramer when it is free of the dimer produces the condition in which the probability for tetramer displacement is increased. Does the displacement of the HA tetramer continue to occur when on the +0.05 SD DNA? As shown in Figure 1C, this displacement was now prevented. The higher level of positive stress in the +0.05 SD DNA has enhanced the stability of the HA tetramer so that it has a stability similar to the LA tetramer when it is associated with either the +0.01 SD or +0.05 SD DNA. We conclude that acetylation has increased the probability for displacement of the tetramer at low levels of positive stress. The dimer is not similarly affected.

Transcription Extensively Enhances the Release of the HA Tetramer from +0.01 SD DNA. The data of Figure 1B indicated that the HA tetramer was less stably maintained on the +0.01 SD DNA. The question to be asked was whether transcription would amplify this characteristic of the HA tetramer. We also included NAP1 in the following experiments because we have previously observed in our transcription studies that NAP1 tends to amplify various types of histone movement (*48*, *49*). NAP1 is a histone chaperone that is known to interact with both the tetramer and dimer to facilitate both nucleosome formation (*69*, *70*) and disruption (*48*, *71*). In this way, we would be able to evaluate the effects of acetylation on a histone displacement process that might also be chaperone-mediated *in vivo*. LA dimers were mixed with LA tetramers (1:4, H2A−H2B/H3−H4), and after reconstitution on the DNA, the reconstitutes were mixed with a 2-fold excess of competitor and transcribed with T7 RNA polymerase at 35 °C for 5 min. Transcription was terminated by treatment to a final concentration of 10 mM EDTA and a subsequent addition of 5 μg of RNase A. After an additional incubation of 5 min, the samples were applied to 5−20% sucrose gradients. As shown in Figure 2A, transcription on the template containing the LA tetramers caused the displacement of 15% of the tetramers to the competitor. As was shown in Figure 1A, without transcription, none of this displacement would have occurred. When NAP1 was present during transcription (Figure 2B), 30% of the LA tetramers and nearly 100% of the LA dimers (the second dimer) were displaced to the competitor. We have previously shown that transcription on +0.01 SD DNA does not result in release of an LA tetramer except when it is bound to a dimer (*49*). The dimer has a preference for binding single-strand nucleic acids, and when the polymerase displaces the dimer from the DNA, it binds the nascent RNA while maintaining its association to the tetramer. This is why the RNase A treatment is applied immediately after transcription to displace these histones to the excess competitor. The transfer of the LA tetramer to the competitor that is observed in parts A and B of Figure 2 represents this dimer−tetramer displacement. A variation of this is observed when the HA tetramer is used. As shown in parts C and D of Figure 2, although the dimer continued to facilitate transfer of the tetramer, there was now a very high background in the transfer of the HA tetramer, 42% in the absence of NAP1

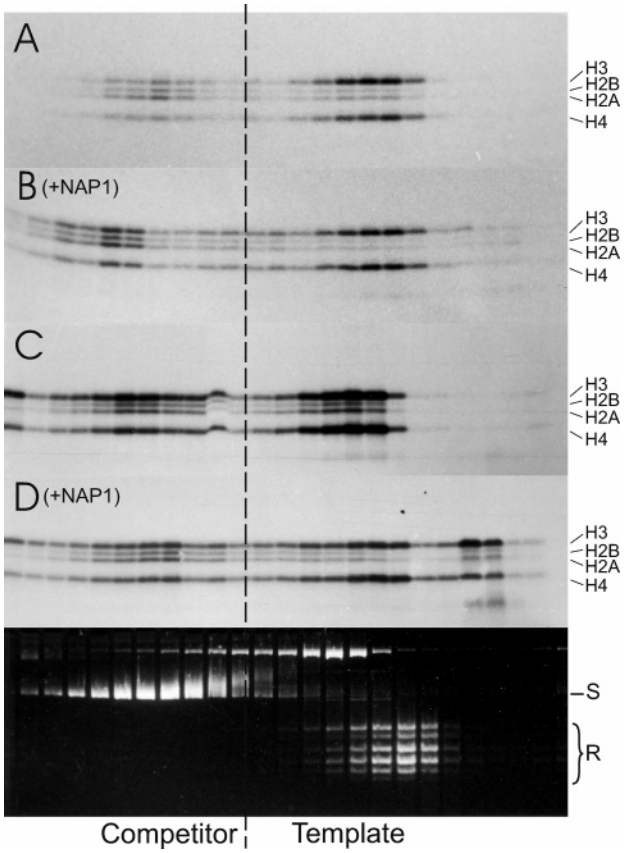

FIGURE 2: Analysis of the histones that have transferred from the template (+0.01 SD DNA) to the competitor as a result of transcription. Transcription was done on DNA reconstituted with LA tetramer and LA dimer in the absence (A) or presence (B) of NAP1. Transcription on DNA reconstituted with HA tetramer and LA dimer in the absence (C) or presence (D) of NAP1. Transcription was at 35 °C for 5 min with a reconstitute containing histones at a molar ratio of 1:4 (H2A−H2B/H3−H4). On the panel showing a representative agarose gel, "S" indicates the location of the competitor and "R" indicates the template DNA (1T7/T3−19, +0.01 SD).

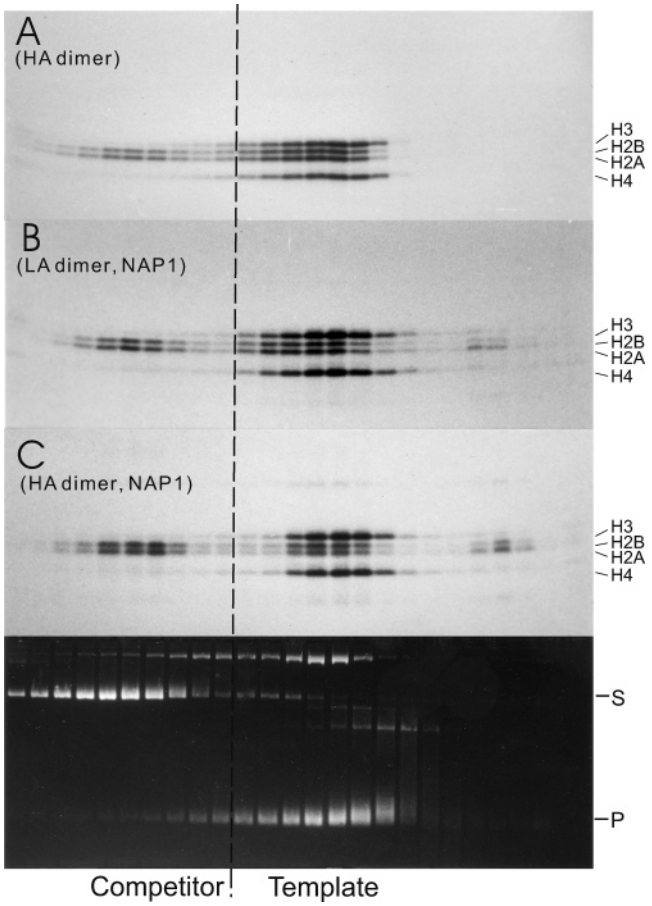

FIGURE 3: Analysis of the dissociation during transcription of the LA and HA dimers when associated with the LA tetramer on the +0.05 SD template. (A) Transcription with template DNA containing the HA dimer. (B) Transcription in the presence of NAP1 with template DNA containing the LA dimer. (C) Transcription in the presence of NAP1 with template DNA containing the HA dimer. Transcription was at 35 °C for 5 min with template DNA reconstituted with histones at a molar ratio of 1:4 (H2A−H2B/H3−H4).

(Figure 2C) and 38% in the presence of NAP1 (Figure 2D). An additional 10% of the HA tetramer is on top of the gradient bound to NAP1 (Figure 2D). This binding to NAP1 by the HA tetramer was not observed when the LA tetramer was used (compare parts B and D of Figure 2). We have done additional studies in which transcription was done on the HA tetramer in the absence of the dimer. Transfer of the HA tetramer from the template to the competitor continued to occur (data not shown). This transfer even occurred when transcription was done in the presence of RNase A, which is a condition in which transcription-induced stress would be absent (*48*). Therefore, the displacement of the HA tetramer is not from changes in the topological state of the DNA but rather because of the RNA polymerase directly disrupting histone interactions with the template. We conclude from these observations that, when the +0.01 SD template is used, RNA polymerase readily displaces the acetylated form of the tetramer without the need for an interaction with the dimer to facilitate the transfer.

The HA Tetramer Is Not Displaced from +0.05 SD DNA during Transcription, and It Also Stabilizes the Binding of the HA Dimer to the Template. The data of parts B and C of Figure 1 indicated that the HA tetramer was more stably maintained on the +0.05 SD DNA than on the +0.01 SD DNA. Would transcription increase displacement of the HA tetramer from the +0.05 SD template, as it did for the +0.01 SD template? We also observed in Figure 1C that the dimer (the second dimer) was stably maintained on the HA tetramer. Would transcription alter this stability as well? LA and HA tetramers were each mixed with the HA dimer (1:4, H2A−H2B/H3−H4), and after reconstitution on the DNA, they were transcribed for 5 min in the presence of the 2-fold excess of the competitor. As shown in the sucrose gradients of Figure 3A, no LA tetramer was found associated with the competitor, whereas 21% of the dimer (the second dimer) has transferred. Transcription on this highly positively coiled template has caused a disruption of the dimer−tetramer interaction. We have previously shown that this disruption is caused by the formation of transcription-induced positive stress, because displacement of this second dimer does not occur when RNase A is present during transcription (*48*). Positive stress greater than +0.05 SD is required to disrupt the one dimer−one tetramer interaction. As shown in Figure 4A, the HA tetramer also continued to remain resistant to displacement (compare to Figure 1C in which transcription was not present). The higher level of positive stress in the template has stabilized the HA tetramer even during transcription. Figure 4A also shows that the HA dimer was not

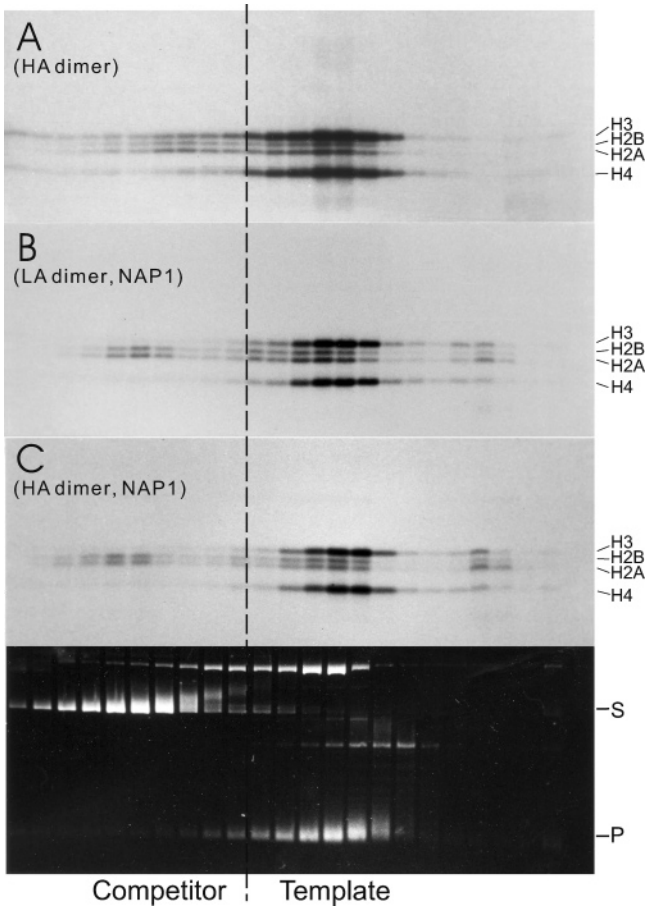

FIGURE 4: Analysis of the dissociation during transcription of the LA and HA dimers when associated with the HA tetramer on the +0.05 SD template. (A) Transcription with template DNA containing the HA dimer. (B) Transcription in the presence of NAP1 with template DNA containing the LA dimer. (C) Transcription in the presence of NAP1 with template DNA containing the HA dimer. Transcription was at 35 °C for 5 min with template DNA reconstituted with histones at a molar ratio of 1:4 (H2A−H2B/H3−H4).

quite as extensively displaced from the HA tetramer, as compared to the LA tetramer (compare to Figure 3A). It was our first indication that perhaps acetylation might possibly enhance the binding of the tetramer to the dimer. To further evaluate this possibility, we repeated these experiments in the presence of NAP1, because this chaperone tends to amplify basal levels of histone displacement (*48*, *49*). In this instance, data are shown for both the LA dimer and HA dimer. As shown in Figure 4, the first observation to be made is that the HA tetramer, whether either associated with the LA dimer (Figure 4B) or HA dimer (Figure 4C), was minimally displaced by NAP1 to the top of the gradient or to the competitor. This is in contrast to Figure 2D in which it was observed that, with a +0.01 SD template, this displacement was extensive. The higher level of positive stress in the template has limited this displacement by NAP1. The second observation to be made is that the level of LA dimer that was displaced from both forms of the tetramer was similar, 36% for the LA tetramer (Figure 3B) and 33% for the HA tetramer (Figure 4B). This result is in contrast to when the HA dimer was used. In this instance, 54% of the HA dimer was displaced from the LA tetramer (Figure 3C) and 41% from the HA tetramer (Figure 4C). It is an indication that the HA dimer has a greater displacement potential than the LA dimer when bound to either form of the tetramer. The third observation to be made is that there is a significant difference in the level of displacement of the HA dimer from the LA tetramer, as compared to the HA tetramer (54 versus 41%). The HA dimer has a lower displacement potential when bound to the HA tetramer. The acetylated state of the tetramer has largely negated the enhanced displacement potential of the HA dimer and re-established a strong dimer−tetramer interaction. With the use of NAP1 in these experiments, the differences in displacement potential for the different acetylated forms of the dimers and tetramers have become more apparent.

The N-Terminal Sequences of the HA Tetramer Facilitate the Preferential Binding of the HA Dimer to the +0.05 SD DNA. The previous transcription experiments indicated that the HA dimer has a greater affinity for the HA tetramer than for the LA tetramer. To determine whether this affinity represents an intrinsic property of the histone−DNA complexes and is not dependent upon the action of either NAP1 or a RNA polymerase, the following experiment was done. HA tetramer (parts A−C of Figure 5) and LA tetramer (Figure 5D) were each mixed with HA dimer (ratio of 1:4, H2A−H2B/H3−H4) and then reconstituted by NaCl dialysis onto a equal mixture of +0.01 SD and +0.05 SD DNA. The samples were then applied to a 5−20% sucrose gradient to separate the two DNAs. As shown in Figure 5A, the two DNAs were completely separated even though their molecular weights are the same. The reason for this separation is that the +0.05 SD DNA is more highly coiled and the HA tetramer has an intrinsically much higher affinity for this DNA than for the +0.01 SD DNA. This conclusion is based on the data of Figure 5B in which fractions from this gradient were treated with MSB topoisomerase I to determine the amount of histone that was bound to these DNAs based on the number of negative coils being held by them. The +0.05 SD DNA contained the majority of the negative coils. Figure 5C shows the distribution of the histones for this gradient. The HA tetramer is distributed preferentially on the +0.05 SD DNA as expected, but also distributed on this DNA was the HA dimer (the second dimer). This is in contrast to the LA tetramer (Figure 5D) in which the HA dimer was preferentially associated with the +0.01 SD DNA. This latter observation is to be expected because the maintenance of the interaction between the dimer and tetramer would require the formation of negative coils (*1*−*3*). It is much easier to form negative coils on the +0.01 SD DNA during the reconstitution procedure than on the +0.05 SD DNA. It is very surprising then that the HA tetramer was able to maintain the interaction with the HA dimer when bound to the +0.05 SD DNA. It is an indication that the HA tetramer has a unique capability of maintaining the HA dimer on highly positively coiled DNA and is the likely reason that this dimer is more resistant to displacement by transcription.

The N-terminal regions of the tetramer are the location for the acetylation and therefore are presumably responsible for this enhanced binding. To verify that this is the case, the N-terminal regions were removed by trypsin treatment and the modified tetramer was reconstituted with the HA dimer (ratio of 1:8, H2A−H2B/trypsin-treated H3−H4) followed by reconstitution onto a mixture of +0.01 and +0.05 SD DNA. As shown in Figure 5E, the tetramer continued to preferentially bind the +0.05 SD DNA, but the HA dimer

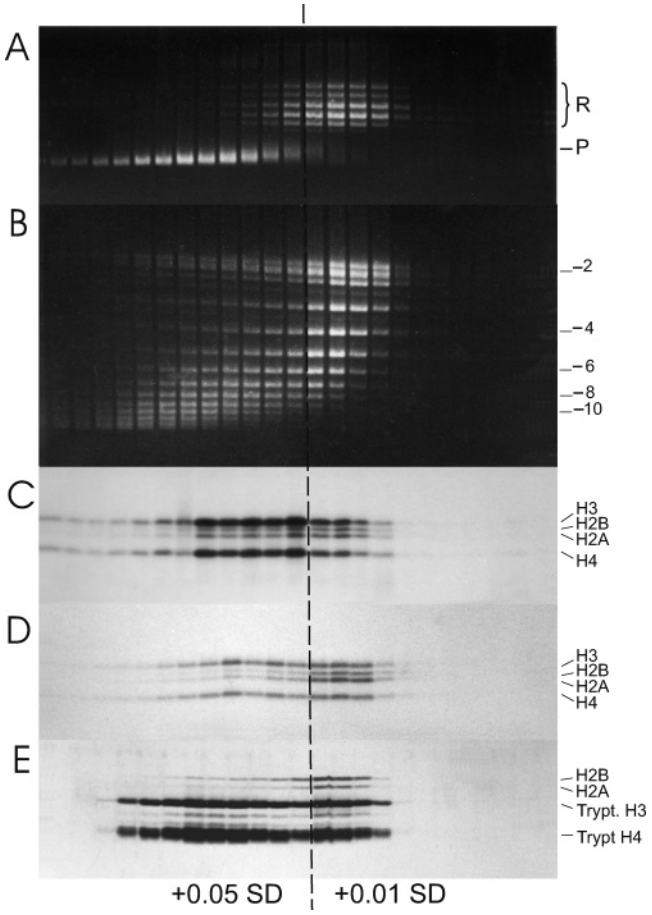

FIGURE 5: Analysis of the dimer−tetramer interaction based on affinity for +0.01 SD DNA versus +0.05 SD DNA. A 1:1 mixture of +0.01 SD and +0.05 SD DNA was reconstituted by NaCl dialysis with histones at a ratio of 1:4 (H2A−H2B/H3−H4). The two forms of DNA were then separated on 5−20% sucrose gradients, and the protein and DNA were analyzed. (A) Agarose gel showing the separation of the +0.01 SD DNA "R" and +0.05 SD DNA "P" after reconstitution with HA tetramer and HA dimer. (B) Agarose gel analysis of the fractions after treatment with MSB topoisomerase I (20 units/μg of DNA) at 35 °C for 60 min. The numbers on the left of the panel indicate the number of negative coils maintained by the histones. (C) SDS− PAGE analysis of the distribution of the HA tetramer and HA dimer from the DNA analysis of A. (D) SDS−PAGE analysis of the LA tetramer and HA dimer after reconstitution on the DNA mixture and separation on sucrose gradients. (E) SDS−PAGE analysis of trypsin-treated tetramer and HA dimer after reconstitution on the DNA mixture and separation on sucrose gradients.

preferentially bound the +0.01 SD DNA. We conclude that the preferential binding of the tetramer to the +0.05 SD DNA is not dependent upon their N-terminal regions; however, the increased binding of the HA dimer to the HA tetramer is dependent upon the acetylation of those regions.

The Enhanced Binding of the HA Tetramer to the HA Dimer Requires the Interaction with DNA in Isotonic Conditions. There have been several previous studies that have measured the dimer−tetramer interaction when in high-salt solutions for acetylated and unacetylated histones. In one study, acetylation was thought to subtly increase stability (*72*), and in another, acetylation was thought to subtly decrease stability (*73*). To simulate the loss of charge by acetylation, a study was done on the core histones in the absence of their N-terminal regions. Removal of those regions had a minimal effect on the stability of the dimer−tetramer interactions (*74*). The results of those studies are relevant to our observations from the previous experiments (Figures 3−5), and because of the variable observations that have been reported for those studies, we re-evaluated the stability of the dimer−tetramer interaction in high-salt solutions. These results can be summarized in the data of Figure 6 in which HA tetramer (Figure 6A) and LA tetramer (Figure 6B) were each mixed with HA dimer (ratio of 1:4, H2A−H2B/H3−H4) and then applied to a Superdex-75 column in 1.0 M NaCl. The HA dimer (the second dimer) appears not to bind as tightly to the HA tetramer because its average fractional distribution is fraction 5.5 (marked by arrow) compared to fraction 4.5 when associated with the LA tetramer. The HA dimer when applied to this column in the absence of the tetramer distributes at fraction 6.0 (Figure 6C). Because it is known that lowering the salt concentration decreases the stability of the dimer−tetramer interaction (*5*), we have done additional experiments to determine the salt concentration that will result in a fractional distribution of 5.5 for the HA dimer−LA tetramer interaction. We found this salt concentration to be 0.8 M NaCl, which is an indication that the LA tetramer forms a more stable interaction with the HA dimer at 1.0 M NaCl (data not shown). The enhanced binding of the HA tetramer to the HA dimer that was observed in Figure 5C is not observable in these high-salt solutions.

The following experiment was done to determine whether DNA or its topological state is the important factor that allows acetylation to stabilize the dimer−tetramer interaction. HA tetramer (parts A and C of Figure 7) and LA tetramer (parts B and D of Figure 7) were each mixed with HA dimer (ratio of 1:4, H2A−H2B/H3−H4) and then reconstituted by NaCl dialysis onto either linear DNA (parts A and B of Figure 7) or the +0.05 SD DNA (parts C and D of Figure 7). The samples were then applied to 5−20% sucrose gradients containing 0.7 M NaCl. In this concentration of NaCl, the dimer begins to displace from the DNA, and therefore, the level of displacement can be used as a measure of the stability of the dimer−tetramer interaction. As shown in Figure 7A (note arrows), when the HA tetramer was bound to linear DNA, a small percentage of the tetramer displaced to the top of the gradient. This displacement was not observed when the HA tetramer is bound to the +0.05 SD DNA (Figure 7C). This susceptibility to displacement was not observed for the LA tetramer when on either +0.01 SD DNA (Figure 7B) or +0.05 SD DNA (Figure 7D). These results are again an indication that the HA tetramer is more prone to dissociation when bound to a DNA that is not highly positively coiled. If we now consider the affinity of the HA dimer (the second dimer) to these DNAs, we observe that the HA dimer has the same affinity for either the LA or HA tetramer when on the linear DNA (compare parts A and B of Figure 7). In contrast to the linear DNA, the HA dimer is much more readily displaced from the +0.05 SD DNA for both the HA and LA tetramers. This result would be expected, because of the high level of positive stress that destabilizes the dimer−tetramer interaction. Now, if we more closely compare the affinity of the HA dimer to the HA and LA tetramers when on the +0.05 SD DNA, it can be seen that the HA tetramer has a slightly weaker affinity for the HA dimer. This dimer trails more extensively toward the top of the gradient (compare parts C and D of Figure 7).

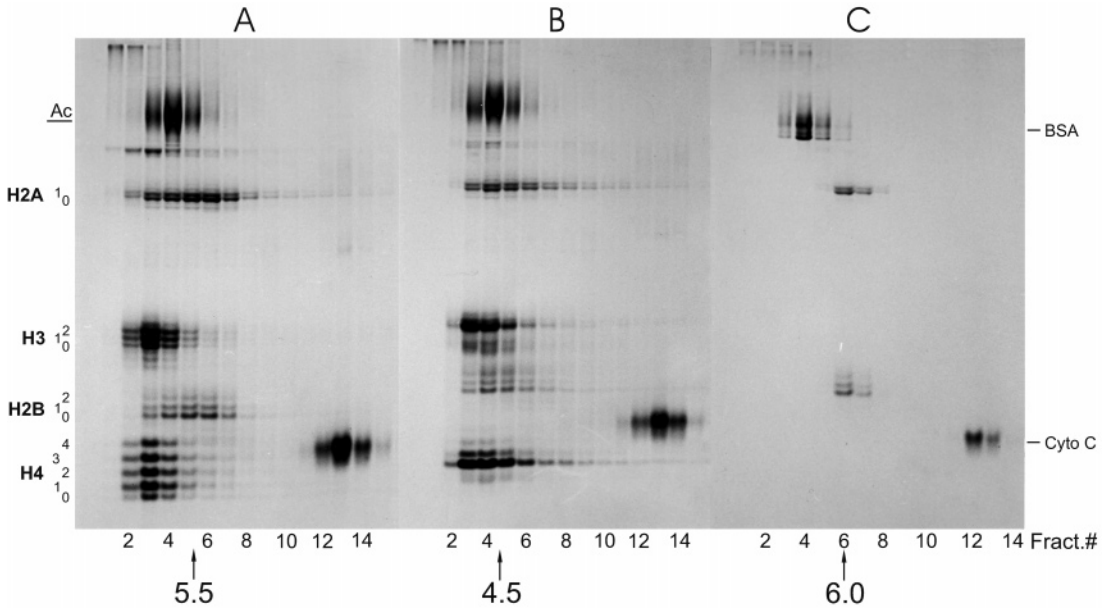

FIGURE 6: Analysis of the dimer−tetramer interaction in 1.0 M NaCl using a molecular-sieve column. (A) Distribution of the HA tetramer and HA dimer after being mixed at a ratio of 1:4 (H2A−H2B/H3−H4) and analyzed on a (Pharmacia) Superdex 75 column in 1.0 M NaCl, 40 mM Tris, and 5 mM 2-ME at pH 8.0. (B) Same as A except that the LA tetramer was mixed with the HA dimer. (C) Distribution of the HA dimer alone after analysis on the column. The distribution of histones was determined using Triton−acetic acid−urea gels. BSA (66 kDa, fraction 4) and cytochrome *c* (12.4 kDa, fraction 13) were mixed with the histone samples prior to application on the column and serve as markers that facilitate a comparison of A to C. The arrows indicate the average distribution for the HA dimer in each of the panels. The HA histones were from butyrate-treated MSB cells, and the LA tetramer was from chicken erythrocytes.

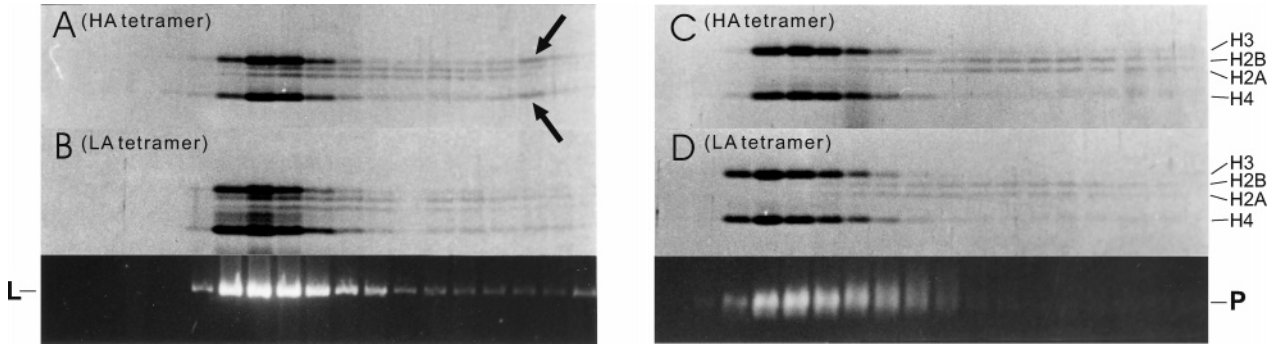

FIGURE 7: Analysis of the salt sensitivity of the HA dimer when associated with either the LA or HA tetramer and either linear or +0.05 SD DNA. (A) HA tetramer on linear DNA. (B) LA tetramer on linear DNA. (C) HA tetramer on +0.05 SD DNA. (D) LA tetramer on +0.05 SD DNA. The HA dimer was mixed with either the HA tetramer or LA tetramer (ratio 1:4, H2A−H2B/H3−H4) and then reconstituted by NaCl dialysis on either linear DNA or the +0.05 SD. The linear DNA was prepared by pretreatment of 1T7/T3−19 with *Pvu* II. The samples were slowly adjusted with continuous mixing to a concentration of 0.5 M NaCl and then applied to 5−20% sucrose gradients containing 0.7 M NaCl, 40 mM Tris, and 0.5 mM EDTA at pH 8.0. The dark panels at the bottom are representative agarose gels showing the distribution of the linear DNA "L" and positively coiled DNA "P". The arrows in A indicate the location of displaced HA H3 and H4.

This weaker affinity is again different from the results of Figure 5, in which it was observed that the HA tetramer bound the HA dimer more strongly and not more weakly. We conclude that the elevated salt concentrations, independent of whether the histones are bound to DNA, alter this enhanced binding of the HA dimer to the HA tetramer. This enhanced binding can only be observed in isotonic conditions when acetylated histones are bound to highly positively coiled DNA.

The Additional HA Dimer (the First Dimer) Is Also Stabilized on the HA Tetramer when Associated with the +0.05 SD DNA. To this point, we have focused our experimentation on the effects of acetylation on the dimer−tetramer interaction as defined by one dimer and one tetramer (referred to as the second dimer). Because the tetramer is interacting with two dimers in the nucleosome, it was necessary to evaluate whether this tendency of the HA tetramer to bind the second dimer more tightly also occurs for the first dimer. We have previously shown that, when both dimers are bound to the tetramer when on +0.05 SD DNA, the first dimer is easily displaced by NAP1 without the need for transcription to facilitate the displacement (*48*). For the second dimer to be removed by NAP1, transcription and transcription-induced positive stress were required. The transcription experiments of Figures 3 and 4 are an example of this displacement of the second dimer. Therefore, we did the following experiment to evaluate the affinity of the first HA dimer to the tetramer. HA tetramer and LA tetramer were each mixed with the HA dimer (molar ratio of 1:1, H2A−H2B/H3−H4) and then reconstituted by NaCl dialysis with the +0.05 SD DNA. At this ratio, two dimers would be associated with each tetramer. Subsequently, the samples were incubated for 10 min at 35 °C with NAP1 and then applied to 5−20% sucrose gradients. The histone distribu-

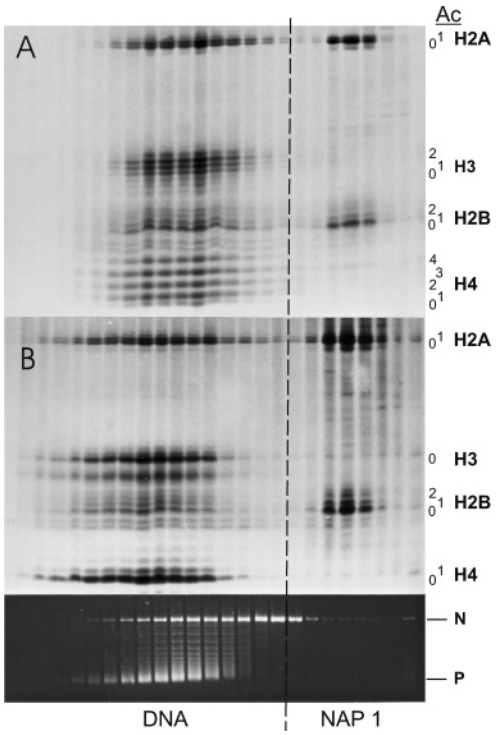

FIGURE 8: Analysis of the displacement by NAP1 of the HA dimers when two dimers are associated with each tetramer, either the HA tetramer or LA tetramer. (A) HA tetramer. (B) LA tetramer. The +0.05 SD DNA was reconstituted by NaCl dialysis with the dimer and tetramer at a ratio of 1:1 (H2A−H2B/H3−H4) and then incubated with NAP1 (1:1, wt/wt, NAP1/histone) at 35 °C for 10 min. The samples were applied to sucrose gradients, and the fractions were characterized for protein on TAU gels and for DNA on agarose gels. N, nicked DNA; P, highly positively coiled DNA. The HA histones were from butyrate-treated MSB cells, and the LA tetramer was from chicken erythrocytes.

tions were then determined on Triton−acetic acid−urea (TAU) gels to identify the state of the histone modifications. As shown in Figure 8, 30% of the HA dimer was displaced by the NAP1 to the top of the gradient for the HA tetramer (Figure 8A) as compared to 45% for the LA tetramer (Figure 8B). Because the second dimer is not displaceable by NAP1 (*48*), the 45% of the dimer that is displaced from the LA tetramer must be nearly all of the first dimer. The 30% displacement from the HA tetramer is an indication that this tetramer tends to bind the first dimer more tightly, similar to its binding to the second dimer. We conclude that the HA tetramer has an enhanced binding for both the first and second HA dimer. There is additional information that can be obtained from the data of Figure 8. Because a TAU analysis was done of the histones, we also observed that NAP1 tends to displace and bind that portion of the HA dimer (both H2A and H2B), which is less acetylated for both the HA tetramer (Figure 8A) and LA tetramer (Figure 8B). This result is the opposite of what was observed in the transcription experiments of Figures 3 and 4, in which it was observed that NAP1 tended to displace the acetylated dimer (HA dimer). Is this preferential displacement of the unacetylated dimer caused by how the dimer interacts with the tetramer or by a specificity of NAP1 for binding unacetylated dimers?

NAP1 Preferentially Binds Unacetylated Dimers. A 2-fold molar excess of the HA dimer was incubated with NAP1 at 35 °C for 10 min and then applied to a 5−20% sucrose gradient. As shown in Figure 9A, NAP1 has a slight preference for binding the unacetylated H2A and H2B. Therefore, the preferential displacement of the unacetylated dimers that was observed in parts A and B of Figure 8 is consistent with the preference of NAP1 for such dimers. This result also implies that, for NAP1 to preferentially displace the HA dimer during transcription (Figures 3 and 4), the transcription process itself must preferentially weaken the interaction of that dimer with the tetramer and the +0.05 SD DNA.

Transcription Is Required To Facilitate the Displacement of the HA Tetramer from the +0.01 SD DNA by NAP1. The experiment of Figure 2D indicated that NAP1 displaced 10% of the HA tetramer to the top of the gradient. To determine whether transcription was required for this particular dis-

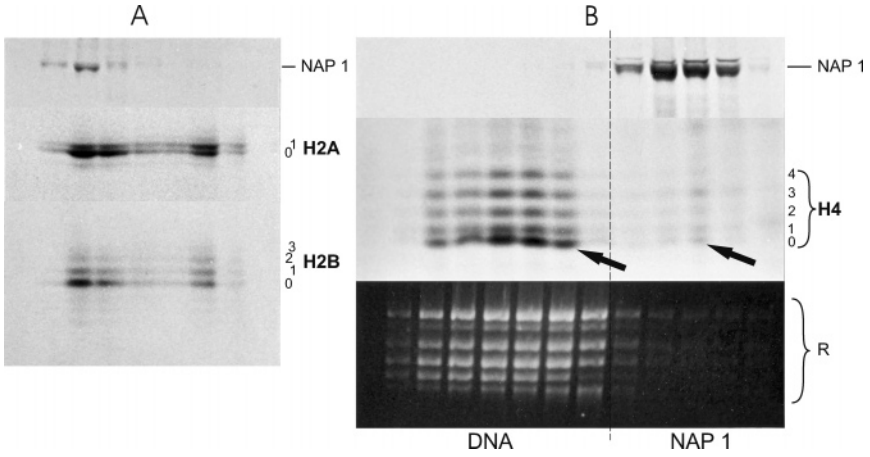

FIGURE 9: Analysis of the binding of NAP1 to HA and LA histones. (A) HA dimer was incubated with NAP1 (molar ratio 1:2, NAP1/H2A−H2B) at 35 °C for 10 min and then sedimented on a 5−20% sucrose gradient in 100 mM NaCl, 40 mM Tris, and 0.5 mM DTT at pH 8.0 and 55 000 rpm for 5 h at 4 °C in a SW60Ti rotor. The fractions were analyzed on TAU gels, and both the acetylated levels of H2A and H2B are shown. Silver staining was used for the histones, and Coomassie staining was used for NAP1. NAP1 stains poorly with silver staining. (B) HA tetramer and LA tetramer were each reconstituted on +0.01 SD DNA by NaCl dialysis, and equal quantities of the two reconstitutes were mixed together, followed by a incubation with NAP1 (1:1, NAP1/histone) at 35 °C for 10 min. After sedimentation on a sucrose gradient, the fractions were analyzed on TAU gels. Only the acetylated levels of H4 are shown in the panel, because the acetylated forms of H3 are poorly resolved. The arrows in B indicate the H4 band that is not acetylated (0H4), which primarily comes from the LA tetramer reconstitute. The HA tetramer was from butyrate-treated MSB cells, and the LA tetramer was from chicken erythrocytes.

placement, the following experiment was done. HA tetramers and LA tetramers were each reconstituted on +0.01 SD DNA by NaCl dialysis. Then, equal quantities of both samples were combined and incubated with NAP1 at 35 °C for 10 min. The sample was then applied to a 5−20% sucrose gradient, and the fractions were analyzed on TAU gels. Figure 9B shows the distribution of histone H4 throughout the gradient. Less than 2% of the H4 was displaced by the NAP1, but of that which was displaced, the acetylated forms were much preferred (note the small amount of 0H4 that is displaced by NAP1). We conclude that the 10% of the HA tetramer that was displaced by NAP1 to the top of the gradient in the transcription experiment of Figure 2D must be a result of the direct action of the RNA polymerase in displacing this tetramer. It is a result of an inherent instability in the interaction of the acetylated tetramer with a DNA that has a low level of positive stress (+0.01 SD). Without transcription, one can still observe a tendency for the acetylated tetramer (2%) to be preferentially displaced by NAP1.

DISCUSSION

The transcription experiments that have been done in this study have focused on evaluating the stability of one dimer interacting with one tetramer. The reason for this is that we have previously observed that, when both dimers were interacting with the tetramer (a full nucleosome), one of the two dimers (the first dimer) would readily displace from a +0.05 SD DNA when exposed to either NAP1 or a competitor that was in the form of a DNA−tetramer complex (*48*). Transcription was not required. The second dimer was much more resistant to displacement. Its displacement to the competitor required the formation of transcription-induced stress, because it would not displace if RNase A were present during transcription. This was particularly apparent when the template was highly positively coiled (+0.05 SD). RNase A prevents the formation of transcription-induced stress (*48*). When a +0.01 SD template was used, this second dimer would displace as a result of transcription-induced stress but was equally matched by a process in which this dimer would maintain its interaction with the tetramer and facilitate the displacement of the entire hexameric complex to the nascent RNA. To displace these histones from the RNA to the competitor, RNase A was added after transcription and before the analysis on the sucrose gradients. The experiments of Figure 2 show this transfer of the hexameric complex, which occurs for both the LA and HA tetramer when in the presence or absence of NAP1. The acetylated state of the histones does not alter this process. What is significantly altered is the surprisingly extensive displacement of the HA tetramer to both the competitor and NAP1. The experiment of Figure 9B showed that NAP1 minimally displaced the HA tetramer when transcription was absent. The direct action of the RNA polymerase in disrupting histone−DNA interactions is the likely reason that NAP1 can bind and displace the HA tetramer. NAP1 has four highly acidic regions distributed throughout the protein that have been shown to be responsible for the preferential binding to the N-terminal regions of the H3−H4 tetramer (*75*). It remains to be determined why the acetylated version of the N-terminals are more prone to the interaction with NAP1 when the tetramer is on +0.01 SD DNA but not on +0.05 SD DNA.

In the experiment of Figure 8A, it was observed that NAP1 showed a slight preference for the displacement of unacetylated dimers from both the LA and HA tetramers when on +0.05 SD DNA. This result is probably an indication that the acidic domains of NAP1 are responsible for this selection as well. We have previously observed that the acetylated and unacetylated dimers are equally extractable at 0.4 M NaCl (*7*; data not shown), and Mutskov et al. (*24*) have observed that the cross-linking of the N-terminals of unacetylated and acetylated histones is the same even when transcription factors are bound to the nucleosomes. The ability for NAP1 to preferentially displace unacetylated dimers may be an indication that the N-terminal regions (acetylated or not) are equally accessible to NAP1 and that NAP1 simply prefers the unacetylated versions because of enhanced electrostatic interactions. Under transcription conditions, however, this preference is not observed. The HA dimer and not the LA dimer was preferentially displaced by NAP1 (Figures 3 and 4). Clearly, NAP1 is not determining what form of the dimer is to be released during transcription. The transcription process itself is doing this. Therefore, irrespective of the histone chaperone that is used, it is likely that the HA dimer would be preferentially displaced. This point is of relevance regarding the histone chaperone, FACT. This chaperone is a RNA polymerase II elongation factor that binds the dimer with an affinity similar to NAP1 and has been shown to facilitate the displacement of the dimer during transcription (*76*). A similar preference for displacement of an HA dimer by this chaperone might be anticipated.

The results of Figure 5C indicated that the HA dimer continued to bind the +0.05 SD DNA, as long as it was associated with the HA tetramer. This level of positive stress would be expected to prevent dimer−tetramer interactions, which is the situation for the LA tetramer (Figure 5D). The acetylated N-terminal regions of the tetramer are stabilizing the dimer−tetramer interaction. The consequence of this increased stability is that the transcription process does not as efficiently displace the HA dimer from the HA tetramer when on the highly positively coiled DNA (Figure 4C). The experiments of Figures 6 and 7 showed that this stabilization was not observed when the dimer−tetramer interactions were characterized in high-salt solutions. Rather, there was a slight tendency for the HA tetramer to have a weaker interaction with the HA dimer. This unique stability is only observable in isotonic conditions, which may indicate a possible role of electrostatic interactions in that stability. We propose that the C-terminal of H2A has a potential role in this stabilization. Eickbush et al. (*77*) have shown that, when the C-terminal region of H2A was removed, the dimer−tetramer interaction was significantly weakened. In the nucleosome structure, it can be seen that this region interacts with H3 close to the entry/exit sites of the DNA, and as a result, the N-terminal region of H3 is in very close proximity (*1*−*3*). Sung and Dixon (*78*) proposed many years ago that the acetylated N-terminal regions could form a α-helical structure, and there is experimental evidence that strongly supports such an effect (*72*, *79*−*81*). A structural alteration of this type could facilitate an interaction with the C-terminal region of H2A, stabilizing the interaction between the dimer and tetramer. There are several minor isoforms of H2A that primarily vary in their C-terminal regions (*82*). The isoform, H2ABbd, is truncated at the C-terminal domain and forms

unstable nucleosomal particles (*83*). Both the plant H2As (*84*) and macroH2A (*85*, *86*) have extended lengths in the C-terminal region and form very stable nucleosomes. The isoform, H2A.X, has a slightly longer C-terminal region and low diffusion mobility in the nucleus (*87*), an indication of a more stable nucleosomal structure. The isoform, H2A.Z, has a C-terminal end of similar length to H2A but is variable in sequence. This variation has been observed to cause potential destabilization of the nucleosome (*88*, *89*) or stabilization (*90*, *91*) depending upon conditions of preparation and measurement. It is enriched in promoter regions, consistent with a function to facilitate the opening of nucleosomes (*92*−*94*). It is known that, of the two rates of histone acetylation [$t_{1/2}$ of ∼5 versus 30 min (*95*−*97*)], the rapid rate is highly correlated with genes that are actively transcribing (*13*). The results of our study indicate that perhaps acetylation of the tetramer may be yet another possible mechanism in which the C-terminal of H2A could stabilize the dimer−tetramer interaction on a more rapid basis reflective of the rapid on and off rate in which acetylation occurs on active genes.

Many studies have been done to enrich for actively transcribed chromatin. An analysis of the modification state for the histones has generally found that all four of the core histones are enriched in high levels of acetylation (*11*−*16*). One implication from these studies is that, when H3 and H4 are highly acetylated on a gene, that same gene would include highly acetylated H2A and H2B. In one particular study of note, Myers et al. (*98*) did observe that, when antibodies to tetra/triacetylated H2B or H4 were used in a CHIP assay to characterize the associated histones and DNA sequences of chicken erythrocyte mononucleosomes, HA H2B and HA H4 tended to cohabit the same nucleosome. Although that study did not directly address the possibility that HA H2B might exist independent of HA H3 and HA H4, their data would support such a possibility based on the observation that antibodies toward HA H4 were able to pull out a much greater enrichment of HA H2B than the antibodies of HA H2B for HA H4 (see Figures 3 and 4 of ref *98*). They also observed that HA H3 and HA H4 were found in both highly active genes (β globin) as well as on a housekeeping gene, GAPDH. Interestingly, HA H2B was absent from this housekeeping gene. Therefore, their study leaves open the possibility that the level of transcriptional activity creates conditions in which the acetylated state of the dimer may not always correlate with that of the tetramer. In another study, Hebbes et al. (*99*) observed that the β-globin gene of 5 day (poised chromatin) and 15 day (active chromatin) chick embryo erythrocytes showed similar levels of hyperacetylation and DNase I sensitivity. Schubeler et al. (*100*) observed that this effect was particularly apparent for histone H4 but that active transcription was required to produce much higher levels of H3 acetylation. These studies indicate that there is a basal level of hyperacetylation, which produces DNase I sensitivity and represents the "poised state", and an even higher level of acetylation that represents the active transcriptional state. A prime candidate for causing this higher rate of acetylation would be complexes similar to the elongator complex, which is associated with RNA polymerase II (*101*, *102*). This complex specifically acetylates histone H3. Specificity of this nature may be responsible for the differential rates of acetylation that have been observed between the dimer and tetramer (*96*).

In vivo conditions that would tend to promote dimer displacement are those that produce transcription-induced positive stress and would be present in all transcription conditions except those in which the genes were very small or the rate of transcription initiation was exceedingly high. Examples of these conditions would be the 5S gene and the ribosomal genes, respectively. In the latter case, the high frequency of initiation creates a condition in which the tandemly arrayed RNA polymerases create transcription-induced stress that is rapidly neutralized by the action of the adjacent polymerase. The tetramer that is associated with highly active ribosomal genes is highly acetylated (*12*), and with the low level of positive stress, the polymerases would tend to displace these histones from DNA, similar to what was seen in parts C and D of Figure 2. It has been found in *in vivo* studies that such genes are generally depleted of histones, which would greatly facilitate the transcription process (*103*−*105*). For genes in which the rate of transcription initiation is less, positive stress would begin to be substantial and prevent the displacement of the HA tetramer. Because of the high level of acetylation for both the dimer and tetramer, transcription is facilitated without the need for displacement of the dimer. Puerta et al. (*37*, *38*) have observed increased efficiency of transcription with HA dimers and HA tetramers, and Protacio et al. (*39*) have also observed that the rate of transcription for the HA tetramer is similar to a tetramer without their N-terminal regions. For genes in which the frequency of initiation is even less, perhaps some housekeeping genes, it may not be possible to maintain the highly acetylated state. The turnover of acetylation becomes predominant, lowering the level of histone acetylation and potential efficiency of transcription through the nucleosomes. To enhance transcription efficiency for the infrequent initiation, displacement of the dimer occurs and is most effective when the tetramer is unacetylated and the dimer is acetylated. Such displacement of the dimer has been observed in the exchange of newly synthesized dimers with the dimers of transcriptionally active chromatin (*106*, *107*). These observations also may have relevance to chromatin remodeling at promoters. Remodeling complexes frequently utilize acetylation to facilitate transcription factor access to a promoter (*108*−*111*). This may be done either by a translational sliding of the histones within the nucleosome or by a direct displacement of the dimer (*112*−*119*). Perhaps those complexes, which extensively utilize acetylation of the tetramer, will tend to cause sliding, whereas those conditions that are less dependent upon acetylation would tend to cause displacement of the dimer. With the use of an appropriate competitor, it should be possible to clarify the role of acetylation at this level of gene regulation as well.

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