Histone Release during Transcription: Displacement of the Two H2A−H2B Dimers in the Nucleosome Is Dependent on Different Levels of Transcription-Induced Positive Stress†

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ABSTRACT: Both indirect (transcription-induced stress) and direct effects of polymerase elongation on histone−DNA interactions were studied on closed circular DNA that was either moderately or positively coiled. The templates were reconstituted with ^{3}H-labeled H2A, H2B, H3, and H4 to form nucleosomes, and transcription was done with T7 RNA polymerase in the presence of a negatively coiled competitor DNA (reconstituted with unlabeled H3 and H4). The first of the two labeled H2A−H2B dimers readily displaced from the highly positively coiled template to the competitor even in the absence of transcription, while the indirect effect of transcription-induced stress was required for the moderately coiled template. The second labeled H2A−H2B dimer required transcription-induced stress for both moderately and highly positively coiled DNA. The displacement of the labeled H3−H4 tetramer also occurred, provided it was associated with an H2A−H2B dimer and a moderately positively coiled DNA. This displacement occurred independent of transcription-induced stress and is likely due to the direct effect of polymerase disruption of histone−DNA interactions. The inclusion of the histone chaperone, NAP1, greatly enhanced the release of both of the two H2A−H2B dimers. These observations are consistent with in vivo observations which indicate that during transcription H2A and H2B are significantly more mobile than H3 and H4 and indicate that transcription-induced positive stress is a likely cause for this selective movement.

In a eukaryotic cell, transcription is regulated by highly basic proteins called histones that not only prevent access of the RNA polymerase to promoters but also restrict elongation of the transcripts on the DNA. Two each of the histones H2A, H2B, H3, and H4 form an octameric complex in which 145 bp of DNA is wrapped on the outer surface in a 1.8 left-handed coil (*1*−*3*). This particle, termed a nucleosome, restrains the equivalence of one coil (*4*, *5*), and ordered arrays of these structures are the primary condensing mechanism for organizing DNA. Because of the strong binding energies (1.2 M NaCl is required to neutralize the binding of H3 and H4 with DNA), the initiation of RNA synthesis requires ATP-dependent chromatin remodeling proteins to disrupt nucleosomal positions and therefore provides a mechanism for the regulation of transcription by those proteins (*6*). A similar problem is encountered when transcribing through nucleosomes. There is substantial evidence from in vivo experimentation which indicates that histone movement on DNA is needed to facilitate elongation. Experiments utilizing density-labeled nucleotides or amino acids and studies utilizing FRAP (fluorescence-recovery after photobleach) technology have shown that H2A and H2B are much more mobile than H3 and H4 (*7*−*9*). The process that causes the nucleosomal disruption and the selective release of H2A and H2B remains to be determined. Since ATP-dependent chromatin remodeling complexes are required to facilitate nucleosomal restructuring and/or sliding at promoters, the question is whether a similar ATP-dependent event is required for the remodeling of nucleosomes to facilitate the elongation of transcription.

At physiological ionic strength and in the absence of DNA, H2A and H2B exist as a dimer[1] and H3 and H4 primarily as a tetramer[1] (*10*). When associated with DNA, two of these dimers interact with one tetramer and form a structure in which DNA wraps initially on the entry site dimer, followed by the centrally located tetramer, and then finally on the exit site dimer (*1*−*3*). Because of the stable state of the H3−H3 and H3−H4 interactions within the tetramer, the left-handed pitch of the DNA is defined by the surface of this tetramer (*11*, *12*). The dimer by itself is unable to hold DNA in a coiled state and in fact is readily extracted from DNA with 0.4 M NaCl (*13*). This is a salt concentration similar to what is required to extract transcription factors from DNA (*14*). Therefore if the dimer is initially placed on a moderately positively coiled DNA, it rapidly transfers (within 1 s) to a negatively coiled DNA at physiological ionic strength (*13*). The requirement of 0.8 M NaCl to extract the dimers from a nucleosome is because of their interaction with the tetramer. If this interaction were to be disrupted, the dimers would be displaceable to other DNA regions. One potential mechanism

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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; 2-ME, 2-mercaptoethanol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); SD, superhelical density; ccc, covalently closed circular; NAP1, nucleosome assembly protein-1; BSA, bovine serum albumin.

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for causing this displacement is the formation of transcription-induced positive stress (*15*). In both prokaryotic and eukaryotic systems, it has been shown that, during elongation of transcription, RNA polymerases produce positive stress in the forward direction and negative stress in their wake (*16*−*27*). The RNA transcript produces a viscous drag on the polymerase, which restricts its rotation as it elongates. As a result the DNA template is forced to rotate theoretically 360° for every 10.5 bp that is transcribed, producing substantial quantities of positive stress. This stress would tend to form right-handed supercoils and would promote unwrapping of DNA from the histones or displacement of the dimers from the tetramer. Because of the negative stress in the wake of the polymerase, the nucleosome would be expected to rapidly re-form as the displaced dimers re-associate with the tetramer on the template. Nucleosomes rapidly form on negatively coiled DNA (*28*). In this instance the RNA polymerase through the indirect effect of generating transcription-induced negative and positive stress would provide the nucleotide triphosphate-dependent process for this remodeling of nucleosomes.

Lee and Garrard (*29*) have done in vivo studies which indicate that positive stress does alter nucleosomal structure. Using a yeast strain that was ΔtopoI-top2ts and expressing *Escherichia coli* topoisomerase I, they adjusted the cells to a nonpermissive temperature, which inhibited topoisomerase II activity. The activity of *E. coli* topoisomerase I now predominated, an activity that specifically relaxes negatively coiled DNA (*20*, *30*, *31*). With the accumulation of positive stress on a transcriptionally active plasmid, they observed an increase in DNase I sensitivity, which is an indication that nucleosomal structure had been altered. We have previously observed that when in vitro transcription was done in the presence of nucleosomes on a ccc[1] DNA, topological stress was generated. This stress was observed by the accumulation of positive coils in the plasmid when transcribed in the presence of *E. coli* topoisomerase I (*32*). We observed that histones H3 and H4 tended not to release from positively stressed DNA, whereas histones H2A and H2B would displace (*33*). When transcribed in the absence of *E. coli* topoisomerase I, the H2A and H2B tended not to release unless released as a complex with the H3 and H4. The histones were seen to transfer to the RNA transcript, and it was the preference of the dimers to bind single-stranded nucleic acids that facilitated the transfer. When the histone chaperone, NAP1, was present, the transfer of histones from the template was substantially enhanced. Those studies were the first indication that transcription-induced positive stress in the template decreased affinity of the dimer for the tetramer. Because the level of positive stress varies as a function of time when transcribing in the presence of *E. coli* topoisomerase I, it is not possible to accurately measure the level of stress required to disrupt the two dimers from the tetramer. We have developed a new method to prepare substantial quantities of DNA with specific levels of positive stress. Upon transcription of this DNA in the presence or absence of RNase A (RNase A blocks formation of transcription-induced stress), we have now been able to measure more accurately the amount of positive stress required to displace the two dimers. The studies of this report indicate that the binding affinities of the two dimers to the tetramer are not equivalent when bound to positively coiled DNA. These studies also indicate that positive stress enhances the binding of the tetramer to the template as transcription proceeds. Such an enhanced binding provides a mechanism for regeneration of the nucleosome after polymerase passage when the displaced dimers reassociate with the tetramer. The general conclusion from these studies is that the remodeling of nucleosomes during transcription is an energy-dependent process and that the source of the energy is the production of positive stress through the processive action of the polymerase.

EXPERIMENTAL PROCEDURES

Preparation of Histones. Histones were purified by a modification of the procedure of Simon and Felsenfeld (*34*). 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, 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), 5 mM 2-ME,[1] and applied to a hydroxylapatite column (Bio-Rad). Histones H2A and H2B were eluted in a stepwise gradient of 0.8 to 1.1 M NaCl. Histones H3 and H4 were eluted in 2.0 M NaCl. Further purification of these histones was done by applying these fractions to a Mono S column (Pharmacia) and eluting with a gradient of 0.4 to 1.1 M NaCl. Pooled fractions were concentrated on Amicon filters and stored at −70 °C.

For preparation of DTNB-treated histones, fractions containing H3 and H4 from chick erythrocytes were adjusted to 3 mg/mL and dialyzed against 2.0 M NaCl, 30 mM KH_2P0_4 (pH 8.0) at 4 °C for 10 h to remove the 2-ME. The sample was then adjusted to 1 mM DTNB[1] from a 20 mM stock solution. After an incubation of 90 min at 23 °C, the sample was dialyzed for 10 h at 4 °C and then stored at −70 °C.

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. The label was then chased for 60 min in fresh medium before the cells were harvested. As a result the labeled histones were no longer in a newly synthesized state but contained the acetylated state of mature histones as verified by analysis on Triton−acetic acid−urea gels (*33*, *35*, *36*).

Preparation of Topoisomerases, T7 RNA Polymerase, and NAP1. Eukaryotic (MSB) topoisomerase I was isolated from MSB cells using a modification (*28*) of the procedure of Liu and Miller (*37*). One unit is defined as that quantity that achieves 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C.

Prokaryotic topoisomerase I was isolated from a clone of *E. coli* topoisomerase I (pJW312). The procedure for isolation was a modification (*32*) of the procedure of Lynn and Wang (*38*). One unit of activity is the quantity that relaxes 1 μg of DNA from −0.05 SD to −0.025 SD at 37 °C for 1 min.

T7 RNA polymerase was prepared from *E. coli* strain BL21, which contained plasmid pAR1219. The procedure for isolation was a modification (*39*) of the procedure of King et al. (*40*). One unit is defined as the amount of enzyme that will incorporate 1 nmol of CTP at 37 °C in 60 min.

NAP1[1] was prepared from *E. coli* strain BL21, which contained plasmid pTN2. The procedure for isolation was a modification (*32*) of the procedure of Fujii-Nakata et al. (*41*). 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. An additional 35 bp sequence containing a T7 promoter was cloned into the *Xba*I−*Hin*dIII site of plasmid 1T7/T3-19 (2238 bp) to yield a plasmid with two T7 promoters 39 bp apart and a size of 2255 bp (2T7/T3-19). A plasmid (p2T7/T3-207-18) containing 18 repeats of the 207 bp 5S RNA gene of *Lytechinus variegatus* was also used in these studies (*32*). Circular, covalently closed (ccc[1]) negatively coiled DNA was purified on CsCl−ethidium bromide density gradients for these three plasmids. In order to produce moderately positively coiled DNA, the negatively coiled DNA was treated with topoisomerase I at 0 °C in buffer conditions of 10 mM $MgCl_2$, 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 (*42*, *43*). Relaxation under this condition results in a DNA (2238 bp) that exhibits an average of 2.5 positive coils (av +0.012 SD[1]) when subsequently incubated at 35 °C under isotonic conditions. For the purpose of this study, this +0.01 SD DNA is considered to be moderately positively coiled.

Highly positively coiled template DNA was prepared as follows: The DTNB-treated H3−H4 histones which were in 2.0 M NaCl, 30 mM KH_2P0_4 (pH 8.0) were diluted 20-fold (final 150 μg/mL) in 40 mM Tris (pH 8.0) and the 1 mL sample incubated at 23 °C for 5 min. To this was added the +0.01 SD DNA at a final ratio of 0.85:1 (wt:wt) of histone to DNA. After an incubation of 10 min during which the DNA aggregated, MSB topoisomerase I (400 units) was added and the incubation continued at 35 °C for 6 h. The sample was centrifuged 10000*g* for 2 min and the pellet dissolved into 200 μL of 1.0 M NaCl, 40 mM Tris, pH 8.0 and then adjusted to 0.1% SDS. After an incubation of 35 °C for 5 min the turbid solution was centrifuged 10000*g* for 2 min to remove the SDS−histone complex. The supernatant was applied to a 5−20% sucrose gradient containing 1.0 M NaCl, 40 mM Tris (pH 8.0) and centrifuged on an SW41 Ti rotor at 35000 rpm for 18 h at 4 °C. Fractions containing the ccc positively coiled DNA were collected, washed, and concentrated on Amicon filters in order to remove the sucrose and to adjust the buffer condition to 2.0 M NaCl, 40 mM Tris (pH 8.0). To determine the superhelical density of this DNA, calibration curves were established correlating the mass density with the superhelical density (*44*). The mass density of DNA on EtBr−CsCl gradients is dependent on the level of intercalation by ethidium bromide. As the positive stress level is increased in DNA, there is less of this intercalation resulting in greater mass density for the DNA. The DNA with a superhelical density of +0.05 SD is referred to as highly positively coiled.

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, 5 mM 2-ME. The NaCl concentration was then decreased in the same buffer in three steps of 1.2 M to 0.6 M to 0.1 M for 3 h in each step (*13*, *45*). Samples were sedimented at 10000*g* for 5 min to differentiate between soluble and insoluble complexes. The histone to 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 (*46*). For an analysis of the hexamer:octamer ratio in the histone−DNA reconstitutes, a 172 bp DNA fragment from the 5S gene of *L. variegatus* was prepared by an *Eco*R1 cleavage of a plasmid (p5S172-18) containing 18 repeats of the 172 bp fragment (*47*). The 172 bp fragment was reconstituted with H3 and H4 (0.4:1, histone to DNA) and then with increasing levels of H2A and H2B. Samples were electrophoresed on 4.5% acrylamide, 0.10% bis-acrylamide in 20 mM Hepes, 1 mM EDTA, pH 8.0 at 160 V for 20 h at 4 °C.

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) and ATP, GTP, CTP, and UTP (0.8 mM each). When transcription was done in the absence of RNase A, 15 units of Prime RNase Inhibitor (Eppendorf) was included. 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 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 to 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). During a subsequent incubation of 2 min, transcription was initiated on the promoter. The polymerase transcribes 13 bases before a UTP is required and results in a synchronized initiation of transcription. With the addition of the UTP, transcription was continued for 5 min with the +0.01 SD template (1T7/T3-19) and for 10 min with the +0.05 SD template (2T7/T3-19). If NAP1 was included, it was added just prior to the UTP. Transcription was terminated by addition of EDTA (final 10 mM). If RNase A (20 μg/mL) was absent during transcription, it was now added at this time and the incubation continued for 5 min. During this incubation, histones that are displaced to the nascent RNA during transcription are now primarily displaced to the excess competitor. 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, 0.1 mM EDTA (pH 7.4) and sedimented in an SW41 Ti rotor at 40000 rpm for 5 h at 4 °C. Fractions were collected (450 μL); 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)]. DNA electrophoresis of 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 (*48*). 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 elec-

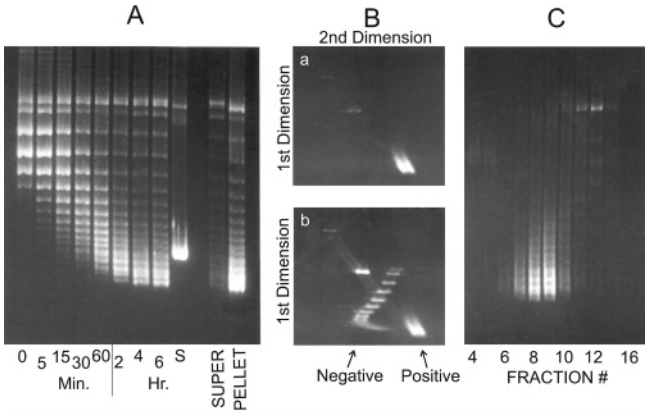

FIGURE 1: A method for the preparation of highly positively coiled DNA. (A) Time course showing the formation of positively coiled DNA after the DTNB-treated H3−H4 histones were added to +0.01 SD DNA and then incubated with topoisomerase I. The super and pellet lanes show the DNA that is present after centrifugation of the 4 h sample for 2 min in a microfuge. "S" indicates a lane showing a negatively coiled DNA standard (−0.05 SD). (B) 2-D analysis of DNA: (a) DNA in the sample; (b) analysis of the sample after mixing with a partially negatively coiled DNA. (C) Fractionation of the DNA sample on a 5−20% sucrose gradient. (See Experimental Procedures for details.)

trophoresis buffer. These samples were electrophoresed on SDS−PAGE gels, which were subsequently exposed to the fluorographic procedure of Laskey and Mills (*49*) to determine the quantity of ^{3}H-labeled histones.

RESULTS

Preparation of Positively Coiled DNA. The method that has been developed to produce large quantities of positively coiled DNA is based on the observations of Hamiche and Richard-Foy (*50*). Histones H3 and H4 when exposed to DTNB, which modifies the cysteine at position 110 of H3, tend to generate right-handed coils on DNA minicircles. If topoisomerase I is present to relax the minicircles, the subsequent denaturation of the topoisomerase and histones by treatment with SDS produces a DNA that contains positive coils. Figure 1A shows a time course in which the DTNB-treated H3−H4 histones were added to a ccc[1] DNA (1:1 wt:wt). This DNA has a topological density of +0.01 SD. After a 10 min preincubation, MSB topoisomerase I was added. As shown in this figure, the topological state of this DNA increases with time so that, by 4 h at 35 °C, the mobility of the DNA is now even greater than that of a negatively coiled DNA standard ("S", −0.05 SD). When this sample was sedimented in a microfuge for 2 min, the DNA was found in the pellet. Figure 1B (a and b) are second-dimensional gel analyses which show that this DNA is highly positively coiled. We estimate the superhelical density of this DNA to average +0.07 SD (see Experimental Procedures). The next step in this method was to dissolve the DNA pellet in 1.0 M NaCl, 40 mM Tris, pH 8.0 and adjust the sample to 0.1% SDS. The turbid SDS−histone complex was then removed by sedimentation in a microfuge for 2 min. The supernatant was then applied on a sucrose gradient, and as shown in Figure 1C, the nicked DNA can be readily removed from the ccc positively coiled DNA. The positively coiled DNA was then adjusted to 2.0 M NaCl so that this DNA could be reconstituted with the histones by NaCl dialysis (see Experimental Procedures). For the subsequent experiments of this report, it was necessary to lower the density from +0.07 to +0.05 SD. A +0.05 SD is the highest density that can be used in which relatively efficient initiation of transcription is still possible (see Figure 6). To make this DNA, the ratio of DTNB-treated H3−H4 histones to DNA was adjusted to 0.85:1 (wt:wt).

NAP1 Preferentially Displaces H2A and H2B from Nucleosomes That Are on Positively Coiled DNA. To determine whether the stability of the nucleosome was dependent on the topological state of the DNA, we reconstituted by NaCl dialysis equimolar quantities of the core histones H3, H4, H2A, and H2B on DNAs containing three different topological states. The histones were prepared metabolically by labeling with ^{3}H lysine and ^{3}H arginine. The labeling was done with a 2-fold excess of ^{3}H lysine to enhance the labeling of H2A and H2B as these histones will be used at lower levels relative to H3 and H4 for some of the experiments (see Figure 1 of ref *33*). The DNA that was used contained 18 repeats of the 5S RNA gene of *L. variegatus* (p2T7/T3-207-18). This sequence strongly positions nucleosomes and therefore would form a highly stable structure (*51*, *52*). The three topological states were (A) −0.05 SD, (B) +0.01 SD, and (C) +0.05 SD. These samples were then treated with NAP1[1] (a histone chaperone) for 10 min at 35 °C and applied to a sucrose gradient. As shown in Figure 2, NAP1 did not displace histones from the −0.05 SD DNA (Figure 2A), but did preferentially displace H2A and H2B from both of the positively coiled DNAs. Approximately 20% was displaced from the +0.01 SD DNA (Figure 2B) and 45% from the +0.05 SD DNA (Figure 2C). Histones H3 and H4 were not displaced from any of these DNAs. It is known that NAP1 can interact with both the H3−H4 tetramer (henceforth referred to as tetramer[1]) and the H2A−H2B dimer (henceforth referred to as dimer[1]) and act as a depositional factor through those interactions (*53*, *54*). In this instance the positive stress has created a condition in which NAP1 displaces the dimer rather than deposits it. This displacement has occurred on what should be highly stable nucleosomes. Since NAP1 displaces 45% of the dimers from the +0.05 SD DNA and each nucleosome consists of two dimers, perhaps NAP1 has displaced only one of the two dimers and the second dimer is less affected by this level of positive stress. To test this possibility we needed to first establish a condition in which the predominate state on the DNA is a condition in which there is primarily one dimer and not two dimers bound to the tetramer (a hexamer). To determine this condition, we reconstituted a 172 bp fragment from the 5S gene of *L. variegatus* with H3 and H4 and increasing amounts of H2A and H2B. The histone−DNA complexes were then analyzed on 4.5% acrylamide gels and, as shown in Figure 3, at a 1:4 molar ratio of H2A−H2B to H3−H4 (lane 3), 50% of the tetramers were not associated with a dimer, 35% with one dimer (a hexamer) and 15% with two dimers (an octamer). As this molar ratio was changed to a 1:1 ratio (H2A−H2B to H3−H4), the histone−DNA complexes became entirely octameric. Since the 1:4 ratio predominately forms a hexamer, it is the condition that will be used for the subsequent studies. We next reconstituted the +0.05 SD DNA using this ratio and then incubated the sample with NAP1 at 35 °C for 10 min. As shown in Figure 2D, 15% of the dimers have displaced to the NAP1. If the affinity of the second dimer were equivalent to that of the

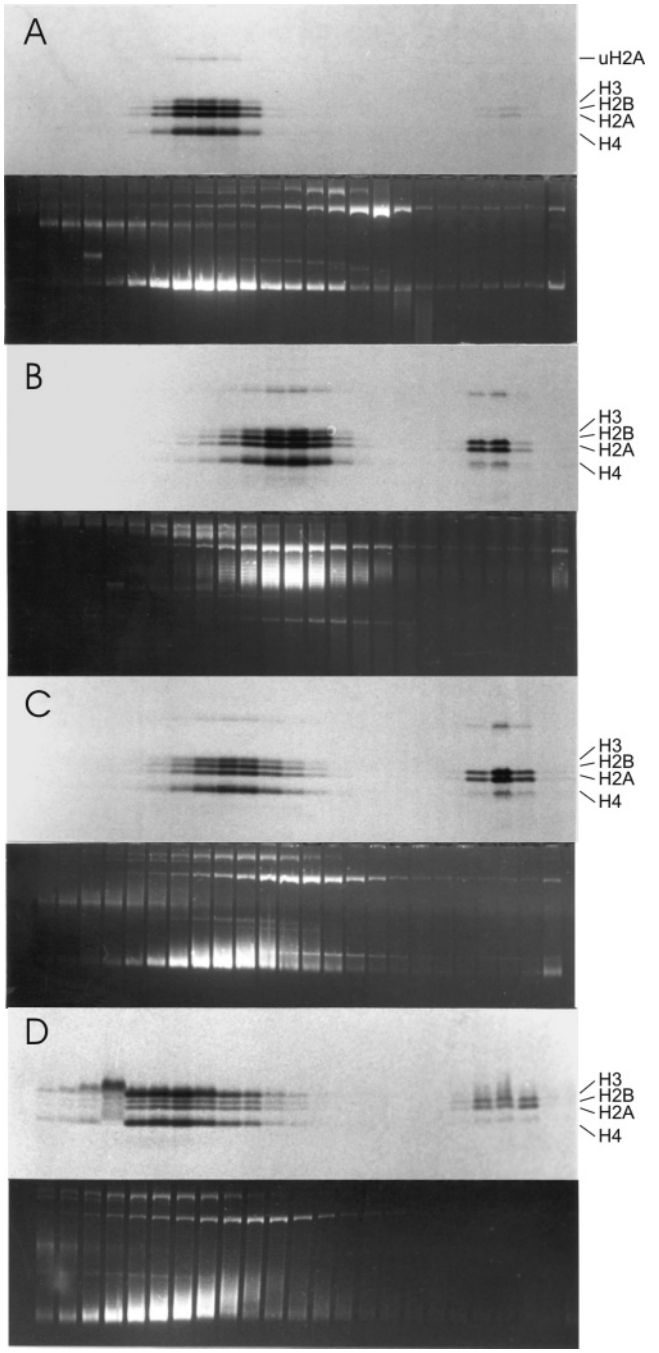

FIGURE 2: An analysis of the displacement of the dimer from DNAs of three different topological states, using NAP1. (A) Negatively coiled DNA (−0.05 SD). (B) Moderately positively coiled DNA (+0.01 SD). (C) Highly positively coiled DNA (+0.05 SD). Equimolar quantities of the histones (H2A, H2B, H3, and H4) were reconstituted by NaCl dialysis to the DNA at a ratio of 0.4:1 (histone to DNA). (D) Same as panel C except the molar ratio was 1:4 (H2A−H2B to H3−H4). After incubation of the reconstitutes with NAP1 (1:1, wt:wt, NAP1 to histone) at 35 °C for 10 min, the samples were applied to 5−20% sucrose gradients. The bottom panel for each section is an agarose gel showing the distribution of the DNA from the reconstitutes. The upper panel is an SDS acrylamide gel showing the distribution of ^{3}H-labeled histones.

first dimer, we would have expected a displacement of nearly half of the dimers. We interpret these observations as indicating that the interaction of the second dimer with the tetramer (the hexameric state) is more resistant to the disruptive effects of this level of positive stress.

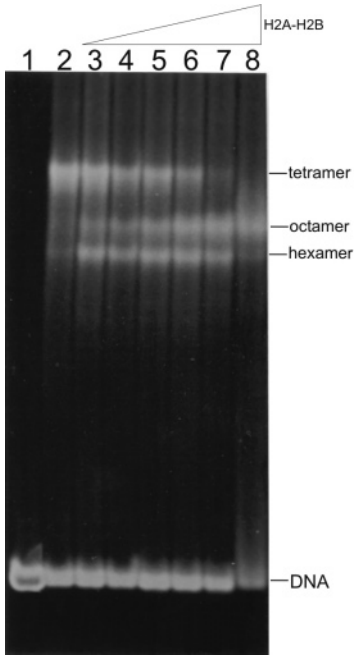

FIGURE 3: An analysis of histone−DNA complexes that are formed with increasing ratios of histones (H2A−H2B to H3−H4): lane 1, DNA alone; lane 2, H3−H4; lanes 3−8, increasing ratios of H2A−H2B to H3−H4. Ratios: lane 3, 1:4; lane 4, 1:3; lane 5, 1:2; lane 6, 1:0.75; lane 7, 1:1.5; lane 8, 1:1. The 172 bp fragment was reconstituted to the histones by salt dialysis, and the complexes were analyzed on a 4.5% acrylamide gel.

A Single H2A−H2B Dimer Preferentially Binds the H3−H4 Tetramer That Is on Negatively Coiled DNA and Not on Positively Coiled DNA. The data from Figure 2A showed that, when both of the dimers were bound to the tetramer in a nucleosome, both dimers were resistant to NAP1 displacement when on negatively coiled DNA. This observation leaves open the possibility that, during the transcription process, the formation of positive stress in front of the polymerase could displace the dimers. The negative stress in the wake of a polymerase could facilitate their rebinding to the tetramer. Such a scenario is certainly possible for the first of the two dimers. It is not as certain for the second dimer because of its stronger affinity for the tetramer on the positively coiled DNA (Figure 2D). To determine whether this second dimer would have a preference for negatively coiled DNA, the following experiment was done. Negatively coiled M13 DNA (7250 bp) was reconstituted with unlabeled H3 and H4 and the 1T7/T3 DNA (2238 bp) with ^{3}H-labeled H3 and H4. Because of the difference in molecular weight between the two DNAs, the two reconstitutes can be separated by centrifugation on sucrose gradients after they have been mixed together. The 1T7/T3 DNA was in a topological state of either +0.01 SD (Figure 4A) or +0.05 SD DNA (Figure 4B). After mixing the two reconstitutes together (1:1, wt:wt), ^{3}H-labeled H2A and H2B were added. The amount of H2A and H2B that was added gave a molar ratio of 1:8 (H2A−H2B to total H3−H4). This ratio would produce primarily a hexamer rather than an octamer on the DNA. After an incubation at 35 °C for 10 min, the reconstitutes were separated on a sucrose gradient. As shown in Figure 4A, 40% of the labeled dimers were associated with the +0.01 SD DNA. In comparison, 26% of the labeled dimers were on the +0.05 SD DNA (Figure 4B). For both

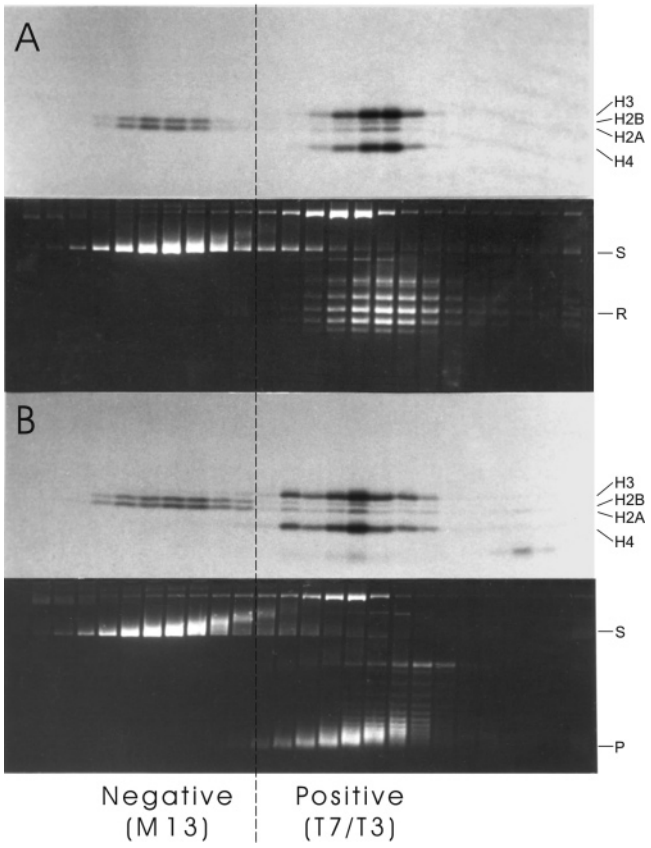

FIGURE 4: The preferential binding of labeled dimers to the unlabeled tetramers that are bound to a negatively coiled DNA. Labeled tetramers were reconstituted onto DNA (2255 bp) which was either +0.01 SD DNA (A) or +0.05 SD DNA (B). These reconstitutes were added to an equal quantity of a reconstitute of negatively coiled M13 DNA (7250 bp) containing unlabeled tetramers. To this mixture were added labeled dimers (molar ratio of 1:8, H2A−H2B to total H3−H4), and this was followed by incubation at 35 °C for 10 min. After a centrifugation on 5−20% sucrose gradients, fractions were obtained to determine the distribution of labeled histones (upper panel) and DNA (lower panel). In the panels showing the agarose gel, the +0.01 SD DNA is indicated as "R", the +0.05 SD DNA as "P", and the M13 DNA as "S".

cases the majority of the dimers were bound to the unlabeled tetramers that are on the negatively coiled M13 DNA ("S" DNA). These results suggest that under a scenario in which a single dimer is displaced from a tetramer when on either a moderately or highly positively coiled DNA, this dimer would tend to associate with the tetramer that is on the negatively coiled DNA.

NAP1 and Higher Levels of Positive Stress Facilitate the Exchange of the H2A−H2B Dimers between the H3−H4 Tetramers That Are on Negatively and Positively Coiled DNA. Negatively coiled M13 DNA was reconstituted with unlabeled H3 and H4 and +0.01 SD DNA (1T7/T3) with ^{3}H-labeled histones. The labeled histones contained a molar ratio of 1:1 (H2A−H2B to H3−H4). After reconstitution the samples were combined at a ratio of 2:1 (M13 DNA to 1T7/T3 DNA), which is a condition in which the excess negatively coiled DNA can serve as a competitor to extract the labeled dimers from the +0.01 SD DNA. After an incubation at 35 °C for 10 min in the presence of 5 mM $MgCl_2$, the sample was applied to a sucrose gradient to separate the two DNAs. (The $MgCl_2$ was included in this incubation, as the subsequent transcription experiments will require this amount of Mg^{2+}.) As shown in Figure 5A, there is no indication that the labeled dimers have been displaced from this moderately positively coiled DNA. We next included NAP1 in the incubation, and as shown in Figure 5B, 20% of these labeled dimers have now shifted to the negatively coiled DNA. This level of displacement is similar to what was observed when NAP1 displaced the labeled dimers to the top of the gradient when the competitor was absent (Figure 2B). Therefore, the competitor can associate with a dimer only after the NAPI has first displaced it from the +0.01 SD DNA. We next reconstituted the +0.05 SD DNA (1T7/T3) with the labeled histones and incubated the sample with the competitor. As shown in Figure 5C, 22% of the labeled dimers have been displaced to the competitor. In this instance the highly positively coiled state of this DNA has promoted a displacement of the labeled dimers so that the competitor can now compete even without the presence of NAP1. Since the first dimer has a greater tendency to be displaced by NAP1 when on the +0.05 SD DNA (Figure 2C), it is likely that this is the dimer that is displaced to the competitor in this experiment. The higher level of positive stress greatly weakens the interaction of this dimer with the remaining hexameric complex. Therefore, if we were to do a transcription experiment in which nucleosomes were reconstituted on the +0.05 SD DNA, there would already be a very high level of displacement of this first dimer before transcription was even initiated.

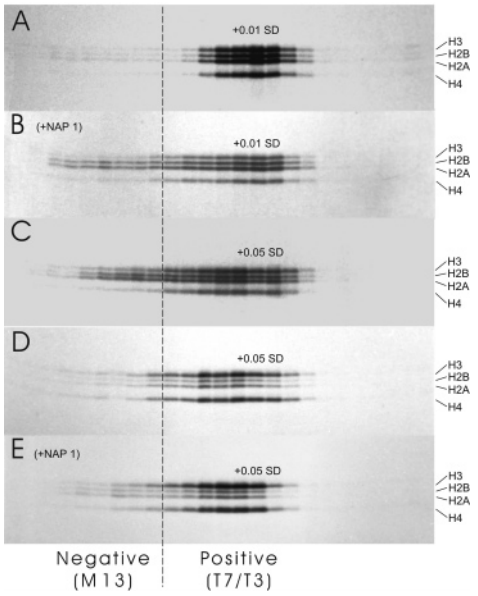

FIGURE 5: An analysis of the exchange of the dimer between the tetramers on negatively and positively coiled DNA. A 2-fold excess of negatively coiled M13 DNA, reconstituted with unlabeled H3 and H4, was added to reconstitutes of 1T7/T3 DNA with topological states of +0.01 SD (A, B) or +0.05 SD (C−E). The 1T7/T3 DNA contained ^{3}H-labeled histones with two different molar ratios of H2A−H2B to H3−H4, 1:1 (A−C) and 1:4 (D−E). All incubations were done in 5 mM $MgCl_2$ and 100 mM NaCl, 40 mM Tris, 5 mM 2-ME, pH 7.4 for 10 min at 35 °C. The incubations in panels B and E included NAP1 (1:1, NAP1: total histone). After a centrifugation on 5−20% sucrose gradients, fractions were obtained to determine the distribution of histones and DNA. Only the distribution of labeled histones is shown in the panels. The vertical line marks the point of separation between the location of the M13 DNA and the T7/T3 DNA.

From the data of Figure 2D it was observed that the second dimer was much more resistant to displacement by NAP1

Figure 6

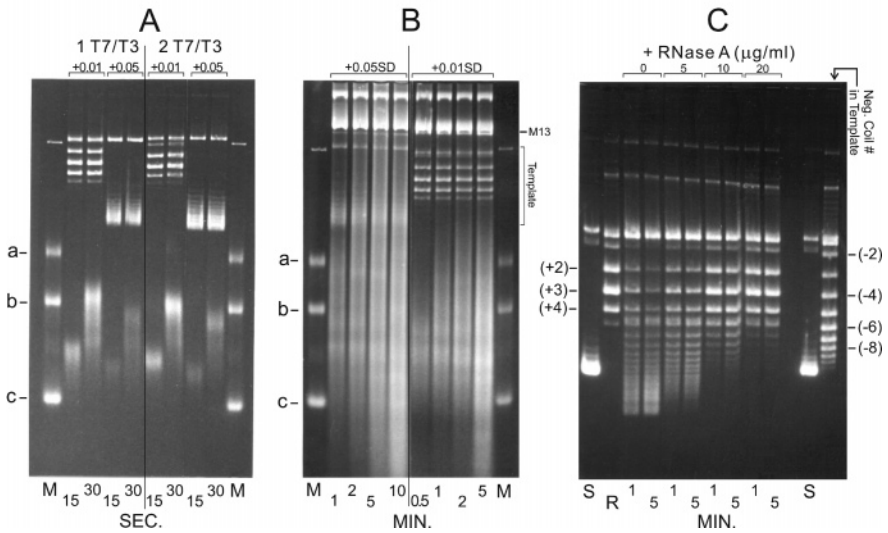

FIGURE 6: A characterization of conditions for transcription on +0.01 SD and +0.05 SD DNA. Transcription was done on two different templates, 1T7/T3 and 2T7/T3, and in two topological states, +0.01 SD and +0.05 SD. (A) Transcription in the absence of histones. (B) Transcription on histone−DNA reconstitutes of +0.01 SD (1T7/T3) DNA and of +0.05 SD (2T7/T3) DNA. A 2-fold excess of negatively coiled M13 DNA that had been reconstituted with unlabeled H3 and H4 was also present during transcription. "M" is a lane containing RNA size markers of (a) 4241 bases, (b) 2360 bases, and (c) 580 bases. (C) Transcription on a histone−DNA reconstitute of +0.01 SD (1T7/T3) DNA (indicated as "R" in the figure) in the presence of *E. coli* topoisomerase I (60 units/μg of DNA) and increasing amounts of RNase A from 0 to 20 μg/mL. (A 1 mg/mL stock solution of RNase A (Pharmacia) must be heated at 80 °C for 20 min before it can be used for these experiments because of residual DNA nicking activity that contaminates the preparation. The analysis in panel C is required to optimize RNase A levels because substantial activity is lost as a result of this heat treatment.) The histone to DNA ratio for the reconstitutes was 0.4:1 (wt:wt), and the molar histone ratio was 1:4 (H2A−H2B to H3−H4). The reconstitute was also incubated with MSB topoisomerase I and incubated at 35 °C for 60 min in order to determine the "negative coil # on the template" that is being held by the histones at this histone to DNA ratio. "S" indicates a lane containing 1T7/T3 in a topological state of −0.05 SD.

when it was on +0.05 SD DNA. We next evaluated the stability of this second dimer by reconstituting the +0.05 SD DNA at a ratio of 1:4 (H2A−H2B to H3−H4) and then incubating the sample with the 2-fold excess of competitor. As shown in Figure 5D, minimal levels of the labeled dimers (5%) were transferred to the negatively coiled DNA. When NAP1 was included (Figure 5E), this displacement increased to only 10%. These observations indicate that, under conditions in which the dimer is primarily interacting with the tetramer in a hexameric state, this dimer is quite resistant to displacement even when associated with a highly positively coiled DNA. The results of Figure 5D and Figure 5E will serve as control experiments for our subsequent transcription studies. By reconstituting DNA at this 1:4 ratio (H2A−H2B to H3−H4), we will be able to clearly detect the effect of transcription alone on the movement of these histones in the presence of positive stress.

The experiments of Figure 5 involved incubations that included $MgCl_2$. This divalent cation is required for polymerase activity and has the potential to stabilize nucleosome structure through the formation of a compact state by higher-order interactions (*55*). To determine whether this higher-order structure might affect the stability of the histone−DNA complexes, we repeated the experiments of Figure 5, but without the presence of this cation. We observed no difference in the level of histone displacement (data not shown). The presence of Mg^{2+} does not appear to alter nucleosome stability in these conditions and is likely due to the low histone to DNA ratio (0.4:1) that is used in this study.

Initiation of Transcription on Highly Positively Coiled DNA Is Repressed, but Can Be Enhanced by Increasing the Number of T7 Promoters and Length of Time for Transcription. Figure 6 shows an analysis of RNA that is produced from +0.01 and +0.05 SD DNA on a plasmid that contains one T7 promoter (1T7/T3, 2238 bp) or two tandem T7 promoters (2T7/T3, 2255 bp). In Figure 6A the DNA alone was transcribed, and it can be seen that the initiation of RNA synthesis for the +0.05 SD DNA is very repressed for both the one and two promoter plasmids. However, the presence of the second promoter for both the templates did increase promoter efficiency by 2-fold. We have increased the number of tandem promoters beyond two and did not observe a significant increase beyond this 2-fold increase (data not shown). The data of Figure 6A also show that the rate of transcription on the +0.05 SD DNA was reduced by 25% (81 b/s for +0.01 SD and 61 b/s for +0.05 SD DNA). Therefore, in order to produce relatively equivalent levels of transcription on these DNAs, we have used the following conditions. For transcription on reconstitutes of +0.01 SD DNA, the DNA contained the single promoter (1T7/T3) and the transcription was for 5 min. For transcription on reconstitutes of +0.05 SD DNA, the DNA contained two promoters (2T7/T3) and the length of transcription was increased to 10 min. The DNAs were reconstituted with ^{3}H-labeled histones at a ratio of 1:4 (H2A−H2B to H3−H4). The transcription experiments also included a 2-fold excess of the M13 DNA reconstituted with unlabeled H3 and H4. This competitor serves as a sink to which the labeled dimer will bind when displaced from the template.

Figure 6B shows the RNA that was produced when the +0.01 and +0.05 SD reconstitutes are transcribed in these conditions. The length of the RNA from both reconstitutes extended to greater than 4 kb, which indicates that a significant number of polymerases are able to complete two

rounds of transcription on the template. However, a substantial level of premature termination was also observed for both reconstitutes. This is expected as the presence of histones on DNA has been shown to disrupt the transcription of both prokaryotic and eukaryotic polymerases (*56*−*58*). A high level of positive stress in the template (+0.05 SD DNA) does not appear to change this level of premature termination. Since the amount of RNA that was produced at the 10 min point of the +0.05 SD reconstitute is similar to that produced at the 5 min point of the +0.01 SD reconstitute, we will now be able to compare the type and quantity of the histones that are transferred from these templates to the competitor DNA.

The Higher the Level of Positive Stress in the Template, the Less the Dissociation of H3−*H4 and the Greater the Dissociation of H2A*−*H2B during Transcription.* We had previously determined that, during transcription, significant levels of the dimer in a complex with the tetramer were displaced from the template DNA (+0.01 SD) and preferentially bound to the nascent RNA (*33*). Those experiments also indicated that it was the dimer that caused this binding due to its 20-fold preference for binding single- rather than double-stranded nucleic acid. In the absence of the dimer, the tetramer was resistant to release from the template, provided the template was not linear (*33*). In order to observe this transfer, both RNase A and an excess of a competitor DNA were added after transcription was completed. With the destruction of the RNA, the histones transferred to the competitor DNA, which could then be separated on sucrose gradients from the template DNA. In those experiments the competitor DNA was also a +0.01 SD DNA. This competitor DNA had not been reconstituted with unlabeled H3 and H4. The reason for this is that we wanted to observe the transfer of the labeled tetramer and the need for the labeled dimer to be associated with it to facilitate the transfer. If unlabeled H3 and H4 had been on the competitor for those experiments, we would not have been able to know whether the labeled dimer remained bound to the labeled tetramer or had transferred to the unlabeled tetramer. Having evaluated the conditions that bring about the displacement of the tetramer from the template by the dimer, we can now evaluate more quantitatively the levels of positive stress that disrupt the interaction between the tetramer and the dimer. To do this, the transcription protocol was changed only with regard to the use of a competitor DNA that was negatively coiled and also associated with unlabeled H3 and H4. If the labeled dimer were to be released from the labeled tetramer while on these templates, this labeled dimer would tend to selectively transfer to the unlabeled H3−H4 tetramer of the competitor (Figure 4). Because the competitor is negatively coiled, the dimer would be stably maintained on the unlabeled tetramer, even in the presence of NAP1 (Figure 2A). Since we have previously observed that the tetramer does not release from +0.01 SD DNA during transcription unless a single dimer is present to facilitate the transfer (*33*), any labeled tetramer that would be found on the competitor must have been transferred from the template as a hexamer with the labeled dimer. By knowing the molar ratio of labeled H2A and H2B to labeled H3 and H4 on the competitor, we can determine the percentage of labeled dimer that transferred to the unlabeled tetramer or transferred as a hexameric complex with the labeled tetramer.

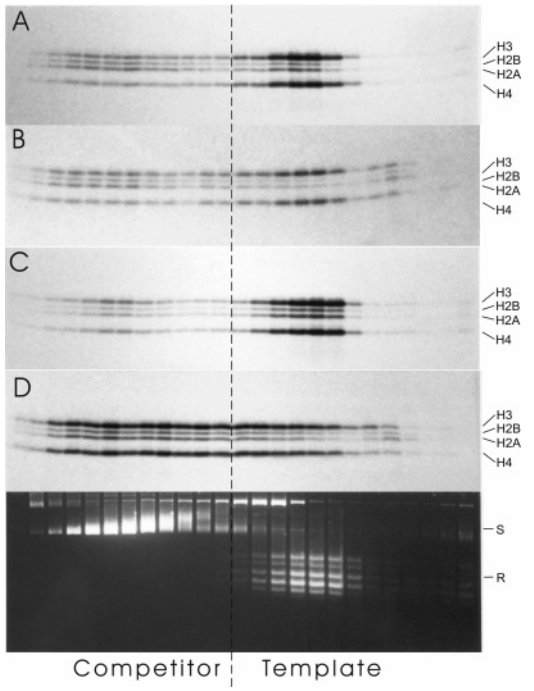

FIGURE 7: An analysis of the histones that have transferred from the template (+0.01 SD DNA) to the competitor as a result of transcription: (A) transcription in the absence of NAP1; (B) transcription in the presence of NAP1; (C) transcription in the presence of RNase A; (D) transcription in the presence of RNase A and NAP1. Transcription was at 35 °C for 5 min with a reconstitute containing histones at a molar ratio of 1:4 (H2A−H2B to H3−H4). On the panel showing a representative agarose gel, "S" indicates the location of the negatively coiled M13 DNA and "R" indicates the 1T7/T3 DNA (+0.01 SD).

Figure 7A (+0.01 SD DNA) and Figure 8A (+0.05 SD DNA) show the distribution of histones after transcription for 5 and 10 min, respectively (time points of Figure 6B). Approximately, 50% of the labeled dimers have transferred to the competitor in both cases. There is a striking difference, however, in the relative amounts of the labeled tetramers that have transferred, 15% for +0.01 SD DNA and 3% for the +0.05 SD DNA. Since the templates were reconstituted with a molar ratio of 1:4 (H2A−H2B to H3−H4), any transfer of a labeled tetramer would have had to involve primarily one dimer (a hexamer), which gives a molar ratio of 1:2 (H2A−H2B to H3−H4). In Figure 7A the radiographic intensity of the histone bands on the competitor indicate a molar ratio of 1:1. Therefore, half of the labeled dimers that transferred from the +0.01 SD DNA must have been associated with the labeled tetramers. The other half (25% of the dimers) transferred as dimers to the unlabeled tetramers of the competitor. This is in contrast to the +0.05 SD DNA in which the majority of the labeled dimers have transferred to the competitor without the labeled tetramers. We next repeated this experiment but now included the histone chaperone, NAP1. As shown in Figure 7B (+0.01 SD DNA) and Figure 8B (+0.05 SD DNA), the transfer of the labeled dimers was very extensive, 95% for +0.01 SD DNA and 85% for +0.05 SD DNA. A significant increase in the relative displacement of the labeled tetramers was also observed, 30% for the +0.01 SD DNA and 7% for the +0.05 SD DNA. Just as was observed in the absence of NAP1 for the +0.01 SD DNA, the radiographic intensities of the histone bands on the competitor DNA show a molar ratio of

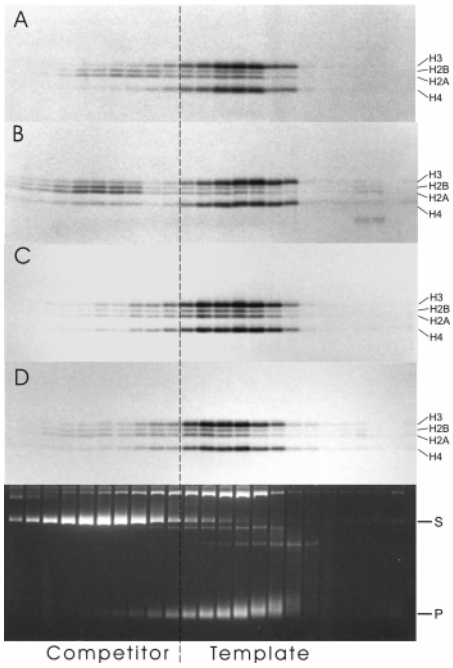

FIGURE 8: An analysis of the histones that have transferred from the template (+0.05 SD DNA) to the competitor as a result of transcription: (A) transcription in the absence of NAP1; (B) transcription in the presence of NAP1; (C) transcription in the presence of RNase A; (D) transcription in the presence of RNase A and NAP1. Transcription was for 10 min at 35 °C with a reconstitute containing histones at a molar ratio of 1:4 (H2A−H2B to H3−H4). On the panel showing a representative agarose gel, "S" indicates the location of negatively coiled M13 DNA and "P" indicates the 2T7/T3 DNA (+0.05 SD).

1:1 (compare Figure 7A and Figure 7B). NAP1 has increased both transfer processes equally well. Half of the labeled dimers have transferred as dimers and the other half as a hexameric complex with the labeled tetramers. NAP1 also did this for the +0.05 SD DNA, for there was now a detectable transfer of the labeled tetramers. However, the major effect of NAP1 was to enhance the release of the labeled dimers from the labeled tetramers on the +0.05 SD DNA. We conclude that high levels of positive stress impede the displacement of the tetramers during transcription. As a result the labeled dimers are displaced as dimers rather than as a complex with the labeled tetramers. Both of these processes are facilitated by NAP1.

Transcription-Induced Stress Is Required To Displace the Second H2A−H2B Dimer from the H3−H4 Tetramer. During transcription the nascent transcript influences the topological state of the template DNA. The inability of the polymerase to efficiently rotate on the template due to the viscous drag of the transcript forces the DNA to form positive coils in the forward direction and negative coils in the wake of the polymerase (*16*−*27*). The experiments of Figure 7A,B and Figure 8A,B would contain such topological effects, even in the presence of templates which were already positively coiled. These additional coils are transient since these plasmids are circular and therefore the negative and positive stress would tend to be neutralized as they translationally move around the DNA. When nucleosomes are on the DNA and in particular the tetramer, the rate of translational movement along the DNA is much reduced. The viscous drag of the nucleosomes slows the rotation of the DNA (*32*). Therefore, as long as the polymerase is transcribing, a transient increase in positive stress would be present. However, if RNase A is present during transcription, the polymerase now freely rotates and this transient increase in stress would be prevented. Figure 6C shows the effect of RNase A on the formation of transcription-induced stress. In this experiment the histone−DNA reconstitute was transcribed in the presence of *E. coli* topoisomerase I. Because this topoisomerase selectively removes negative coils (*30*), the presence of transcription-induced stress is indicated by the accumulation of additional positive coils in the template (*17*). As shown in Figure 6C, when no RNase A was present, high levels of positive stress were observed even within 1 min of transcription, but when increasing amounts of RNase A were included, the induced positive stress decreased to a point at which, in 20 μg/mL RNase A, no induced positive stress was formed. This loss of induced stress was not due to loss of T7 RNA polymerase activity for it has been shown that this polymerase is unaffected by RNase A (*24*, *39*). It should be noted in Figure 6C (no RNase A) that there was considerable heterogeneity in the levels of induced positive stress. This is in part caused by the heterogeneity in the number of negative coils that are stored by the histones in the plasmid molecules (Figure 6C). Also the number of positive coils that were formed in the presence of *E. coli* topoisomerase I do not indicate the actual levels of positive stress that were transiently present. In the absence of this topoisomerase, negative coils would also be present and these would continually neutralize the induced positive stress on the ccc DNA. Therefore, the experiment of Figure 6C only indicates that transcription-induced stress is present, not the actual level of the stress. This conclusion is the basis for our approach to using templates that contain preformed levels of positive stress. By including RNase A, it is possible to evaluate specifically the role of the polymerase itself in the disruption of histone−DNA interactions in templates that contain those specific levels of preformed stress. When RNase A is present during transcription, no additional transcription-induced stress is formed.

We repeated the experiments of Figure 7A and Figure 8A except that RNase A was included during transcription. As shown in Figure 7C (+0.01 SD DNA) and Figure 8C (+0.05 SD DNA), the transfer of the labeled dimers was substantially decreased, 25% for +0.01 SD DNA and 7% for +0.05 SD DNA. The amounts of the labeled tetramers that were displaced to the competitor were 12% for +0.01 SD DNA and 3% for +0.05 SD DNA. This level of displacement of the labeled tetramers was similar to what was observed when transcription was done in the absence of RNase A (Figure 7A and Figure 8A). On the basis of the histone band intensities in Figure 7C, the lower level of the labeled dimers that is now observed on the competitor equates to a molar ratio of 1:2 (H2A−H2B to H3−H4). This is the ratio that would be expected if one labeled dimer had transferred to the competitor in a complex with one labeled tetramer (a hexamer). The presence of the RNase A has stopped the displacement of the labeled dimer from the labeled tetramer, but not the transfer of the labeled hexameric complex. A confirmation of this conclusion can be seen in the transcription on the +0.05 SD DNA. The +0.05 SD DNA would normally have extensive displacement of the labeled dimers away from the labeled tetramers (Figure 8A). As shown in Figure 8C, minimal amounts of the labeled dimers were

released in the presence of RNase A. We conclude that transcription-induced positive stress is required to disrupt the dimer−tetramer interaction within the hexamer. Direct polymerase action alone can only disrupt the hexameric interaction with DNA, which can be seen on the +0.01 SD DNA, but not on the +0.05 SD DNA. The strong binding of the tetramer to the +0.05 SD DNA negates the disruptive effect of the polymerase.

From the data of Figure 7B it was observed that the presence of NAP1 both increased the release of the labeled dimers from the labeled tetramers and increased the release of the labeled hexameric complexes from the +0.01 SD DNA. This was a condition in which transcription-induced positive stress was present. Now with the observation that the polymerase by itself can only facilitate the release of the hexameric complex, one would expect that if NAP1 were now included with the RNase A, the release of the hexamer would become very extensive for the +0.01 SD DNA, but not for the +0.05 SD DNA. As shown in Figure 7D for the +0.01 SD DNA, the majority of the labeled tetramers have indeed transferred with the labeled dimers to the competitor DNA. As shown in Figure 8D for the +0.05 SD DNA, minimal release of either the labeled tetramers or dimers was observed. We conclude that the combination of NAP1 and polymerase is unable to sufficiently disrupt the tetramer interaction with the +0.05 SD template to facilitate their release. The tetramer forms a very stable interaction with the +0.05 SD DNA. We also conclude that NAP1 is unable to disrupt the interaction of the second labeled dimer with the labeled tetramer for either template when transcription-induced stress is absent.

Transcription-Induced Stress Will Displace the First H2A−H2B Dimer from the Nucleosome. In Figure 5A and Figure 5C it was observed that when both labeled dimers were associated with the labeled tetramer to form nucleosomes on either the +0.01 or +0.05 SD DNA, the addition of competitor caused extensive displacement of the first dimer from the +0.05 SD DNA (Figure 5C), but not from the +0.01 SD DNA (Figure 5A). Because of the lower background of displacement on the +0.01 SD DNA, it is possible to examine the effects of transcription when both dimers are present. Figure 9 shows transcription in the absence (Figure 9A) and presence of RNase A (Figure 9B), using the +0.01 SD DNA that had been reconstituted with histones at a ratio of 1:1 (H2A−H2B to H3−H4). When RNase A was absent, 65% of the labeled dimers readily displaced to the competitor. This depletion of the dimers from the template can be seen even in the coomassie-stained gel (Figure 9A). Figure 9A also shows that 25% of the labeled tetramers transferred to the competitor. This level of transfer was higher than the 12% displacement of the labeled tetramers that was observed when a ratio of 1:4 (H2A−H2B to H3−H4) was used (Figure 7A). The additional labeled dimer has facilitated a greater displacement of the labeled tetramers. Because a much higher level of the labeled dimers were displaced from the template than were the labeled tetramers, we conclude that the majority of these labeled dimers were transferred to the unlabeled tetramers of the competitor. These dimers would primarily be the first dimer. We next repeated this experiment in the presence of RNase A. As shown in Figure 9B, minimal transfer of either the labeled dimers or labeled tetramers was observed. This result

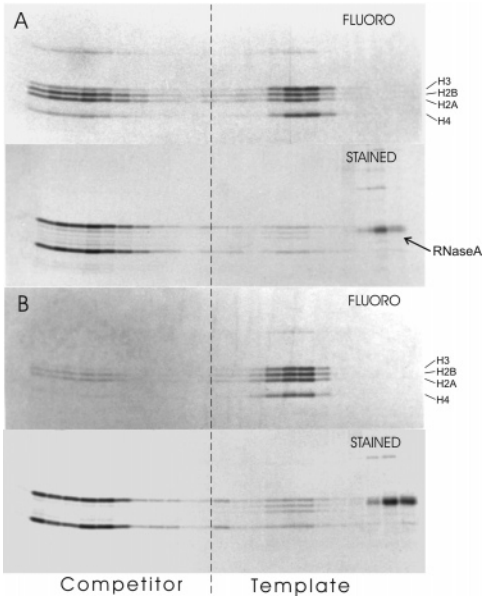

FIGURE 9: An analysis of histones that have transferred during transcription on +0.01 SD DNA reconstituted with histones at a molar ratio of 1:1 (H2A−H2B to H3−H4): (A) transcription in the absence of RNase A; (B) transcription in the presence of RNase A. Transcription is for 5 min at 35 °C. The upper gel for each panel is the autoradiogram (FLUORO), and the bottom gel is a coomassie-stained picture (STAINED). The stained gel is shown to illustrate that it is possible to see the depletion of H2A−H2B (transfer of the first dimer) from the +0.01 SD DNA to the 2-fold excess of competitor (M13 DNA-unlabeled tetramer) for panel A, but not for panel B. The presence of RNase A in the stained gel of panel A is due to the posttranscriptional addition of RNase A. This is done in order to displace histones to the competitor that had initially bound the nascent RNA as result of transcription (*33*).

is very much different from what was observed when we applied these same conditions using reconstitutes that contained a 1:4 ratio (H2A−H2B to H3−H4). In that case we observed that 12% of the labeled tetramers displaced to the competitor (Figure 7C). The presence of the additional labeled dimer to form a complete nucleosome has created a condition in which the labeled tetramers are not as readily displaced from the template. The polymerase as it moves through the nucleosomes is unable to as efficiently disrupt the histone−DNA interactions of an octameric complex as compared to a hexameric complex when transcription-induced stress is absent.

DISCUSSION

Figure 10 summarizes the results of this study. This figure focuses on the transcription studies in which the template DNA was reconstituted with the histones using a molar ratio of 1:4 (H2A−H2B to H3−H4). This is a condition in which half of the tetramers were associated with dimers, the primary complex being hexameric (one dimer to one tetramer). It was necessary to transcribe in these conditions as we observed that when both the labeled dimers were present on the +0.05 SD DNA (1:1, H2A−H2B to H3−H4), the first of the two dimers would readily exchange to NAP1 (Figure 2C) or to a negatively coiled competitor DNA containing unlabeled H3 and H4 (Figure 5C). Therefore the first dimer appears to exchange with a level of positive stress of +0.05 SD with no need for transcription. The actual level of positive stress is likely to be greater than +0.05 SD because of the

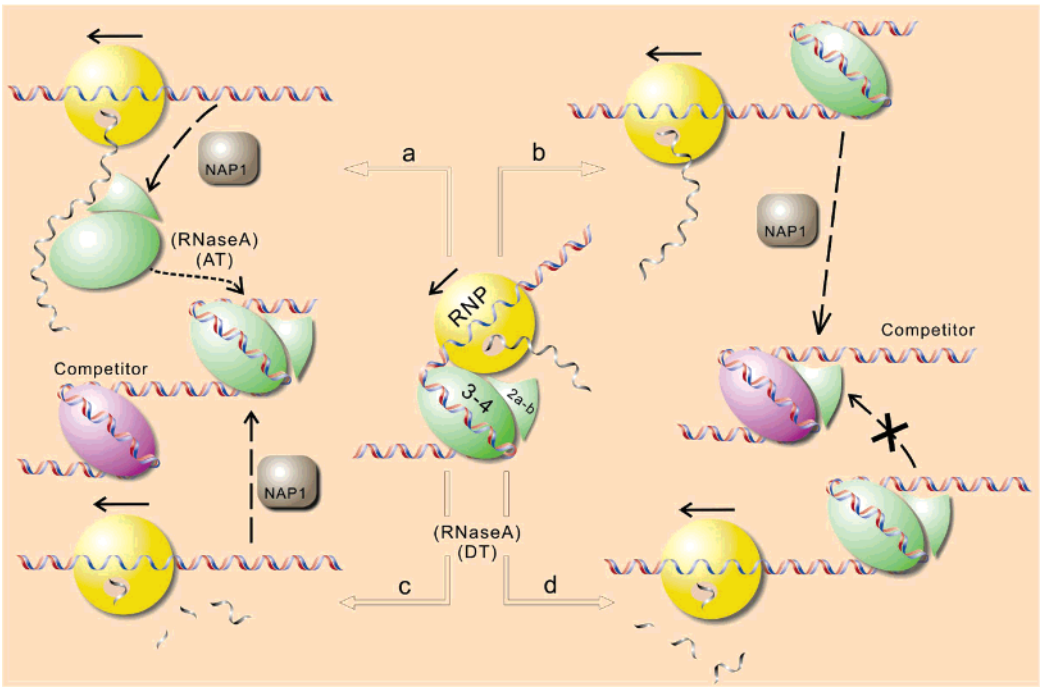

FIGURE 10: Summary of transcription studies on histone−DNA complexes. A labeled tetramer (3-4, in green) is shown bound to template DNA and one labeled dimer (2a-b, in green) and what happens as transcription occurs by T7 RNA polymerase (RNP, in yellow). Process "a" illustrates displacement of the labeled dimer−tetramer complex (hexamer) from the template DNA (+0.01 SD DNA) to the nascent RNA transcript. After transcription is complete, RNase A is added (AT, after transcription) to remove the RNA and the complex is shown to transfer to the competitor DNA. Process "b" illustrates displacement of the labeled dimer from the labeled tetramer and its transfer to the unlabeled tetramer (in purple) on the competitor DNA. This process occurs for both the +0.01 SD and +0.05 SD DNA. Transcription-induced stress is present for both process "a" and process "b". Process "c" (+RNase A) illustrates the displacement of the labeled hexamer directly from template (+0.01 SD DNA) to the competitor DNA. This direct transfer likely occurs, because RNase A is present to destroy the RNA as transcription proceeds (DT, during transcription). No transcription-induced stress is formed when RNase A is present. Process "d" is the same as process "c" except that no histones are displaced to the competitor. This happens under conditions in which the template is highly positively coiled (+0.05 SD DNA). NAP1 is shown to facilitate processes "a", "b", and "c", but not "d".

tetramer. A tetramer wraps the DNA in a left-handed direction (holding a partial negative coil), which in turn causes the formation of additional unrestrained positive stress in the ccc DNA. In a reconstitute with a 0.4:1 ratio (histone to DNA), the level of positive stress on the +0.05 SD DNA would increase to between +0.06 to +0.07 SD and is the level of stress likely responsible for displacement of the first dimer from the hexamer. However, Figure 10 shows that to displace the second dimer from the hexamer, polymerase action was required (process "b"). This polymerase action involved the indirect production of transcription-induced stress, because when RNase A was present during transcription on the +0.05 SD DNA (process "d"), the second dimer would not transfer to the competitor. Stress greater than +0.07 SD must be required to remove the second dimer. In contrast to the dimers, the tetramer is not displaced from the +0.05 SD DNA either in the presence (process "d") or absence (process "b") of RNase A. Therefore the +0.06 to +0.07 SD in that DNA is sufficient to prevent the polymerase from directly competing with the hexamer for binding to this DNA. No additional transcription-induced stress is required. When transcription was done on the +0.01 SD DNA, the release of the second dimer (process "b") also occurred, but was equally matched by process "a" in which the dimer released from the template in a complex with the tetramer and was transferred to the nascent RNA. Because the +0.01 SD DNA was also reconstituted at a 0.4:1 ratio (histone to DNA), the actual positive stress in this DNA would be between +0.02 to +0.03 SD. We interpret this observation as indicating that the polymerase is unable to generate sufficient transcription-induced positive stress above this level to cause process "b" to be dominant. The limiting factor for generating higher levels of induced positive stress is the circular nature of the plasmid. When RNase A was present during transcription on the +0.01 SD DNA (process "c"), the complete absence of induced stress created a condition in which the second dimer could only be released as a hexameric complex with the tetramer. Both processes "a" and "c" are occurring on the +0.01 SD DNA and must be caused by the direct action of the polymerase in disrupting histone−DNA interactions. Processes "a" and "c" cannot occur on the +0.05 SD DNA, because of the affinity of the tetramer for that DNA. This observation is consistent with our previous studies in which we observed that H3 and H4 have a strong affinity for positively coiled DNA (*44*).

We attempted to verify that these general principles also apply to nucleosomes. A nucleosome contains two dimers associated with the tetramer. This could be done on the +0.01 SD DNA, as the level of positive stress resident in this DNA was not sufficient to displace the first dimer (Figure 5A). We observed that transcription on nucleosomes caused a displacement of the first dimer (Figure 9A) and that, when transcribed in the presence of RNase A, the first dimer would not displace (Figure 9B). Therefore, process "b" is applicable since the first dimer is displaced by transcription-induced stress. In those experiments we also observed that the transfer of the tetramer was increased from 12% (Figure 7A) when a single dimer was present to 25% (Figure 9A) when both

dimers were present. This increased rate of transfer would be expected because as soon as the first dimer is displaced from the tetramer (process "b"), the remaining tetramer is in a hexameric complex with the second dimer. The larger quantity of hexamer that remains on the template can be readily displaced by the direct effect of polymerase action. Process "a" then becomes extensive on the +0.01 SD DNA. It is unclear how often process "a" occurs under in vivo conditions. Process "a" is dependent on the accessibility of the nascent RNA. Posttranscriptional processing of the RNA may limit this accessibility so that the dimer is unable to transfer the tetramer. Also the negative coils in the wake of the polymerase would normally be expected to reestablish the interaction of the displaced dimers with the tetramer. Because we used an excess of a negatively coiled competitor DNA that contained unlabeled tetramers, the displaced dimer was unavailable to reassociate with the hexamer to reestablish an octameric state as the polymerase completes its transcription through the nucleosomes. Under in vivo conditions such a depletion of the dimer would not occur. In essence our competitor simulates the process in which the dimer rebinds the tetramer on the negatively coiled DNA in the wake of the polymerase. The importance of the rebinding of this first dimer can be seen in that the octameric state is quite resistant to displacement by the polymerase when the RNA transcript is missing (Figure 9B). Process "a" must occur to some extent in vivo, however, since it has been observed that the nonreplicative variant of H3 is frequently associated with active genes, replacing the replicative one (*59*). In summary, our observations are consistent with a model in which positive stress in advance of a polymerase displaces the dimers from the nucleosome and the negative stress in its wake has the potential to rapidly re-form it with the reassociation of the dimers to the tetramer (*15*).

There is a variation with respect to how nucleosomes respond to transcription and that is when RNase A is present (process "c" and "d"). All four histones types would not displace from the +0.01 SD template (Figure 9B). When only one dimer was present (Figure 7C), the hexamer transferred to the competitor (process "c"). Since the nascent RNA is not present to facilitate displacement of the histones, why does the hexamer displace more readily to a competitor DNA than the octamer? Perhaps as the polymerase transcribes the nucleosome, the additional interaction of this dimer with the tetramer more effectively facilitates a spooling process in which the polymerase displaces histone interactions with DNA in a sequential manner. Those interactions are then sequentially reestablished retrograde to the original position on the template rather than the whole complex transferring to a competitor. Felsenfeld's laboratory (*60*−*63*) has suggested such a spooling process based on transcription studies with SP6 RNA polymerase and RNA polymerase III, using small linear templates. However, they also observed that 50% of all four histones would transfer from the nucleosome of a linear template when an excess of a competitor DNA was present (*61*). Several other investigators have reported a similar histone transfer from a linear template to a competitor (*64*−*66*). We have also observed this transfer from a linear template, even when RNase A was present during transcription to continually remove the RNA transcript (data not shown). Therefore, the additional dimer does prevent displacement of the hexamer from the +0.01 SD DNA (Figure 9B), but not from linear DNA. What is it about the +0.01 SD DNA that enhances the ability of the additional dimer to stabilize the hexamer? Since RNase A was present during the transcription (Figure 9B), transcription-induced stress would be absent, just as if transcription occurred on a linear template. Perhaps the resident stress within the +0.01 SD DNA is sufficient to enhance the binding of the tetramer so that, when combined with the presence of the two H2A−H2B dimers, a threshold of stability is achieved. If this conclusion is correct, we would predict that, in the presence of RNase A, the tetramer (in the absence of both dimers) would readily release from either the linear DNA or the +0.01 SD DNA. This is what we have observed (data not shown). The resident level of positive stress within the +0.01 SD DNA is insufficient to hold the tetramer on the DNA when the two dimers are absent. If we now repeat this experiment but in the absence of RNase A, we would predict that the increased level of transcription-induced positive stress on the +0.01 SD DNA would substantially enhance the binding to the tetramer. This is what we have observed (*33*). The two dimers are not required to hold the tetramer on the DNA during transcription. Even the nascent RNA that is formed during this transcription is unable to compete for the tetramer. Transcription-induced positive stress is all that is needed to strengthen the binding of the tetramer to the +0.01 SD template. Therefore, under conditions in which transcription-induced stress is absent, both the dimers as well as the residual +0.01 SD must be present to negate the disruptive effects of a polymerase. Process "d" and not process "c" actually applies for nucleosomes on a moderately positively coiled ccc template, and for a linear template the reverse is true. At first glance it might appear that this residual stress is very small (one positive coil per 1000 bp). However, this DNA has been reconstituted at a 0.4:1 ratio (histone to DNA) and the actual level of stress is likely to be between +0.02 to +0.03 SD. Can these observations be applied to an in vivo transcription process? One possibility is that during early stages of transcription, perhaps immediately after initiation, when low levels of positive stress are present, the polymerase would not be able to displace the octameric complex of histones. The first dimer would tend not to release at this low level of stress. This scenario would be possible only if access to the nascent RNA by the dimer was limited (as if RNase A were present). Otherwise one of the two dimers would tend to transfer the tetramer to this RNA (*33*). Very early stages of transcription would contain short transcripts. As transcription continues, high levels of transcription-induced positive stress would rapidly appear and the two dimers would be displaced from the tetramer. These dimers would not be available to facilitate transfer of this tightly bound tetramer from the template to the RNA. The tetramer will not displace from a highly positively coiled template, even in the presence of extensive amounts of nascent RNA. Whether by either low or high positive stress, the tetramer is firmly maintained on the template.

In vivo experimentation has shown that H2A and H2B are substantially more mobile during transcription than H3 and H4 (*7*−*9*). The desired state of the nucleosome is to keep the tetramer on the template and if possible facilitate transcription by displacement of the dimers. Positive stress facilitates both of them. Why is it that the preferred state in

the cell is to limit the displacement of the tetramer? Perhaps the tetramer serves as a nucleation site for rapid re-formation of the nucleosome after the polymerase has passed. The tetramer is generally thought to serve a similar function as a nucleation site for the binding of the dimers during nucleosome assembly at the replication fork (*11*, *67*−*69*). The transient opening and rapid reclosing of the nucleosome would be an efficient mechanism for maintaining the actively transcribed DNA in a continuously packaged condition. It has also been proposed that the covalent modifications of H3 and H4 can serve as a molecular marker for regulating transcription (*70*−*71*). Limiting the displacement of the tetramer would be a necessary prerequisite for such regulation.

Kireeva et al. (*72*) have examined RNA polymerase II transcription on a linear template containing a single nucleosome. Although transcription was extensively inhibited at physiological ionic strength, they were able to observe by EMSA assays that some of the templates lost one of the two dimers. Baer and Rhodes (*73*) have observed that RNA polymerase II does preferentially bind transcriptionally active mononucleosomes that are deficient in one of the two dimers. These observations indicate that the direct action of a large polymerase may be sufficient to displace a dimer. However, in the nucleus of a cell, transcription occurs on extended lengths of DNA. Transcription-induced stress would be present and would likely cause displacement of the first dimer prior to its contact with the large polymerase. With the size of the eukaryotic polymerase and its likely association with nuclear substructures, this induced stress would be substantial, even after initiation of transcription when the length of the transcript is relatively short (*74*, *75*). This scenario assumes that topoisomerases are unable to rapidly remove the induced stress. Kouzine et al. (*27*) have studied the far upstream element (FUSE) of the c-myc promoter when centrally located between the promoters of divergently transcribing T3 and T7 RNA polymerases. They observed that when topoisomerase was included during transcription, the formation of transcription-induced non-B DNA structure in this element continued to occur. Theoretically, a negative and positive coil would be formed for every 10.5 bp of DNA that is transcribed. Therefore, one would expect the maintenance of a large level of this induced stress, despite the continual action of topoisomerases.

Figure 10 also shows that NAP1 facilitates histone displacement. NAP1 is an abundant histone chaperone in the cell (*76*, *77*). Defects in NAP1 alter expression of 10% of the genes in yeast (*78*). NAP1 has been shown to work effectively as a deposition factor for the formation of nucleosomes by interaction with the dimer and tetramer separately or as part of an octameric complex (*53*, *79*). It has been shown that NAP1 has a greater preference for the binding of the tetramer as compared to the dimer (*54*), although it has been implicated in the transport of the dimer into the nucleus (*80*, *81*). It has been recently reported that NAP1 can displace the dimer from a nucleosomal particle and will also induce nucleosomal sliding through the displacement and reassociation of the dimer (*77*). It has been observed that this action of NAP1 is greatly enhanced when coupled with ATP-dependent promoter remodeling complexes (*82*−*85*), and it has been proposed that the general remodeling of nucleosomes at promoters is dependent on displacement of the dimer (*86*). In a rather similar nucleotide triphosphate-dependent process, our observations indicate that the polymerase either indirectly (transcription-induced stress) or directly (competition for DNA binding) remodels nucleosomes creating conditions in which NAP1 can bind and displace a dimer or its hexameric complex with the tetramer. Orphanides et al. (*87*) have shown that the movement through nucleosomes of RNA polymerase II is greatly facilitated by a complex of two proteins called FACT (facilitates chromatin transcription). FACT has also been shown to have a histone chaperone activity similar to that of NAP1 (*88*). These proteins also bind H2A and H2B and have been shown in in vitro transcription studies with RNA polymerase II to enhance the release of one of the dimers (*89*). NAP1 may function in a similar manner, and because of its abundance, this chaperone could provide a more general function of facilitating promoter remodeling and transcription elongation for all three forms of RNA polymerase.

These experiments indicate that the first dimer displaces from the nucleosome at a lower level of positive stress as compared to the second dimer. Given what appears to be a 2-fold symmetry for the nucleosome, it is not immediately evident why these dimers should have different stabilities. Godfrey et al. (*10*) have shown that the K_a for the binding of both the first and second dimers to the tetramer when these proteins are free in solution is similar. We have done additional experimentation in which linear DNA was reconstituted with the histones at molar ratios of 1:1 or 0.5:1 (H2A−H2B to H3−H4). When exposed to 0.6 M NaCl on a sucrose gradient, less than 10% of the H2A and H2B was extracted for both the 1:1 and 0.5:1 ratios (data not shown). The equivalent level of dissociation for the two dimers is consistent with a similar K_a for the two dimers even when bound to DNA. However, if the +0.05 SD DNA is used, we observed that 65% of the H2A and H2B was released from the 1:1 reconstitute whereas 25% was released from the 0.5:1 reconstitute (data not shown). The positively coiled DNA has not only decreased the interaction of the two dimers with the tetramer but also has created a condition in which the association constant for the two dimers is significantly different. What could be the source for such a difference? A close examination of the nucleosome structure does indicate that there are subtle asymmetries for all four of the core histones and that one of these locations is the major site for interaction between the dimers and the tetramer, and that is at Y-83 (H2B), Y-88 (H4), and Y-72 (H4) (see Figure 5, ref *3*). How positive stress amplifies this asymmetry remains to be determined.

Ultimately, the second dimer can be displaced from the tetramer, but in this instance polymerase action and the presence of transcription-induced stress is required. It has been observed that treatment of the tetramer with DTNB to modify the single cysteine of H3 (C-110) causes the tetramer to undergo a chiral transition in which the DNA forms a right-handed supercoil (*50*). It is thought that this bulky adduct when bound to the two cysteines at the H3−H3 interphase disrupts that interphase. The method that we have used to produce the positively coiled templates used in these studies is an indication of such a transition. We have also observed that DTNB-treated H3 and H4 maintain a dimeric rather than a tetrameric state in 2.0 M NaCl, a further indication of the disruption of the H3−H3 interphase (data

not shown). Prunell's laboratory (*90*, *91*) has shown that when positively coiled minicircles were used, this chiral transition was observed in the absence of a modifying agent. They also observed that when H2A and H2B were present, this chiral transition was prevented. When considering the structure of the nucleosome, it can be seen that the presence of a dimer would prevent the tetramer from shifting into a right-handed pitch (*1*−*3*). One possibility regarding why the second dimer is displaced only after polymerase action is that this action is required to force the chiral transition which in turn would force displacement of the second dimer. The presence of positive stress provides the background that facilitates the transition. On the other hand, it may be just the increase in positive stress from the transcription itself that disrupts the second dimer−tetramer interphase without the transition. The chiral transition is not likely to be involved in the release of the first dimer, as both dimers would be expected to release at the same level of positive stress. This is clearly not the case. Further studies will be required to determine whether a chiral transition of the tetramer is involved in the release of the second dimer.

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