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Articles

Histone Release during Transcription: NAP1 Forms a Complex with H2A and H2B and Facilitates a Topologically Dependent Release of H3 and H4 from the Nucleosome†

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ABSTRACT: Transcription through a multinucleosomal template was studied to determine why histones are released to the nascent RNA. It was first determined in competition experiments between DNA and RNA that histones H2A and H2B have a 20-fold preference for binding RNA over DNA; a preference was not seen for histones H3 and H4. Histones H3 and H4 would preferentially bind RNA, provided they were in an octameric complex with H2A and H2B. In transcription studies with T7 RNA polymerase, H3 and H4 were transferred to the nascent RNA, provided the template was linear. If the DNA was topologically restrained, which is a condition that more closely maintains transcription-induced stresses, H3 and H4 would not release. Histones H3 and H4 would be released from this template when H2A and H2B were present, a release that was enhanced by the presence of nucleosome assembly protein-1 (NAP1). Since a small quantity of H2A and H2B is sufficient to facilitate this transfer, it is proposed that H2A and H2B function to repeatedly shuttle H3 and H4 from the template DNA to the RNA. Cross-linked histones (dimethylsuberimidate-cross-linked octamer) were reconstituted into nucleosomes and found to be transferred to the RNA at the same frequency as un-cross-linked histones, an indication that such large complexes can be released during transcription. Transcription was carried out in the presence of *Escherichia coli* topoisomerase I so that positive coils would accumulate on the DNA. Histones H3 and H4 would again not be transferred from this DNA, unless H2A and H2B were present. In this instance, however, when NAP1 was present, the shuttling of H3 and H4 to the RNA caused a significant depletion of H2A and H2B from the positively coiled DNA. These results are discussed with regard to current models for transcription through nucleosomes.

Transcription in a eukaryotic cell requires RNA polymerases to access DNA under conditions in which that DNA is tightly packaged into arrays of structures called nucleosomes. The basic structural unit, the nucleosome core particle, consists of 145 bp of DNA, which is tightly wrapped 1.8 times into a left-handed coil around four highly basic proteins. Two of each of these proteins, histones H2A, H2B, H3, and H4, form an octameric complex, which is the internal structure upon which the DNA is coiled. Because of the basic nature of each of these proteins, extremely strong binding energies are present between this octamer and DNA. NaCl concentrations of >0.8 M are required to displace H2A and H2B. Concentrations of >1.2 M are required to displace H3 and H4 (*1*). When the polymerase synthesizes RNA, at least

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one turn of the DNA helix (10 bases) is opened (*2*). Such intimate contact of the polymerase with DNA would prevent the interaction of histones at that site and is an indication that there are nuclear processes that disrupt these rather strong binding energies. One way of overcoming these strong binding energies is to simply remove the histones. There is substantial evidence from *in vivo* experimentation which indicates that histones do move as a result of transcription. Experiments utilizing density-labeled nucleotides or amino acids have showed that H2A and H2B tend to be more mobile than H3 and H4 (*3*, *4*). Further verification of those earlier experiments has come from studies that have used FRAP (photorecovery after bleaching) technology. Cells that express GFP-labeled H2B as compared to GFP-labeled H3 were able to more quickly disperse the bleaching effect in a process that is transcription-dependent (*5*). *In vitro* experimentation provides a means of evaluating the forces that facilitate histone movement. Particularly beneficial have been the studies with prokaryotic polymerases. These polymerases quantitatively initiate their processes on simple promoters, maintain processivity, and can be manipulated with regard to transcription rate. When it has been possible to do parallel studies with eukaryotic and prokaryotic polymerases, the results support the validity of using prokaryotic polymerases to study a eukaryotic process (see reviews in refs *6*−*10*).

A substantial part of the binding energy that stabilizes the left-handed coil of DNA in the nucleosome is the maintenance of the interaction of H2A and H2B with H3 and H4. At physiological ionic strength and in the absence of DNA, H2A and H2B exist as a dimer and H3 and H4 as a tetramer (*11*). 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 H2A−H2B dimer, followed by the centrally located H3−H4 tetramer, and then finally on the exit site H2A−H2B dimer (*12*−*14*). The centrally located tetramer is critical in establishing this structure. 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 these proteins (*15*, *16*). The subsequent binding of the two H2A−H2B dimers propagates this left-handed coil to complete the 1.8 negative coils (*17*). In the absence of H3 and H4, the H2A−H2B dimers are unable to hold a left-handed coil and are readily extracted from DNA with 0.40 M NaCl (*18*). The requirement for 0.8 M NaCl to extract H2A and H2B from a nucleosome is then a reflection of the interaction with H3 and H4. It has been proposed that one mechanism for facilitating transcription is the disruption of that interaction by the formation of transcription-induced positive stress (*8*). There is substantial evidence which shows that in both eukaryotic and prokaryotic systems, RNA polymerases produce positive stress in the forward direction and negative stress in their wake (*19*−*27*). This positive stress would tend to form right-handed supercoils and would promote the unwrapping of DNA from the nucleosome. By disrupting the interphase between the H2A−H2B dimer and the H3−H4 tetramer, the H2A−H2B dimer could then be more readily displaced from the DNA, a condition in which the RNA polymerase would then need only to disrupt interactions of the H3−H4 tetramer with DNA.

A number of *in vitro* transcription studies have been carried out on DNA templates that are either mononucleosomal or multinucleosomal. With mononucleosomes, Felsenfeld's laboratory (*28*−*31*) has observed a "spooling" process in which transcription by either SP6 RNA polymerase or RNA polymerase III displaces the octameric complex of histones retrograde to the original nucleosomal position. It is thought that this repositioning is caused by a sequential disruption of histone−DNA interactions by RNA polymerase such that the DNA behind the polymerase can reassociate with the octamer and re-establish those interactions in that same sequential manner. The octameric complex would then never fully dissociate from the template. This model also predicts that the interactions between the H2A−H2B dimers and the H3−H4 tetramer are stable during transcription. *In vitro* studies with multinucleosomal templates have not been able to determine whether retrograde repositioning does occur. Those studies have indicated, however, that histones are displaced from DNA with an average disruption frequency of one in four nucleosomes. All four histones were found on the RNA transcript (*32*, *33*). Why displacement is observed in some studies and not in others has not been clearly determined. Equally undetermined is why RNA serves as a competitor for histones or whether the interphase between the H2A−H2B dimer and the H3−H4 tetramer is disrupted during this transfer. None of these studies have attempted to characterize the state of the nucleosome when transcription is carried out under conditions that promote the presence of positive stress. The studies of this report will address these questions.

EXPERIMENTAL PROCEDURES

Preparation of Labeled Histones. MSB cells (chicken leukemic cells transformed by Marek's virus) were grown in 10% newborn calf serum with a 1:1 medium of Dulbecco's MEM and RPMI-1640 and supplemented with 50 mM Hepes. One liter of cells (1×10^6 cells/mL) was preincubated in medium lacking arginine and lysine for 30 min, then concentrated to 10 mL, and incubated with 3 mCi of [^{3}H]-lysine and 1.5 mCi of [^{3}H]arginine (Amersham) for 60 min. The label was chased for 60 min, and the cells were harvested and the histones purified by a modification (*18*) of the procedure of Simon and Felsenfeld (*1*). Briefly, 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, 0.05 M $KHPO_4$ (pH 8.0), and 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, and histones H3 and H4 were eluted from 1.1 to 2.0 M NaCl. Further purification of these histones was done by applying these fractions to a Pharmacia Mono S column and eluting with a gradient of 0.4 to 1.1 M NaCl. The purification of the histones was monitored by both SDS gel (*34*) and Triton−acetic acid−urea gel electrophoresis (*35*). Pooled fractions were concentrated on Amicon filters and stored at −70 °C. For some experiments, a cross-linked histone octamer was used. It was prepared by treatment of

[1] Abbreviations: 2-ME, 2-mercaptoethanol; SD, superhelical density; ccc, covalently closed, circular DNA; DMS, dimethylsuberimidate; NAP1, nucleosome assembly protein-1; BSA, bovine serum albumin.

the octameric complex in 2.0 M NaCl (*36*) with dimethylsuberimidate (DMS) and a subsequent reconstitution with DNA by the procedure of O'Neill *et al*. (*37*).

Preparation of Topoisomerases, T7 RNA Polymerase, and NAP1. Eukaryotic topoisomerase I was isolated from MSB cells using a modification (*38*) of the procedure of Liu and Miller (*39*). One unit is defined as that quantity that achieves 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C. This protein is termed MSB topoisomerase I.

Prokaryotic topoisomerase I was isolated from a clone of *Escherichia coli* topoisomerase I (pJW312, gift of M. Gartenberg and J. C. Wang, Harvard University, Cambridge, MA). The procedure for isolation was a modification (*18*) of the procedure of Lynn and Wang (*40*). One unit is defined as that quantity which will relax 1 μg of DNA from a superhelical density (SD) of −0.05 to −0.025 at 37 °C in 1 min.

T7 RNA polymerase was prepared from *E. coli* strain BL21, which contained plasmid pAR1219 (a gift of W. Studier and J. Dunn, Brookhaven National Laboratory, Upton, NY). The procedure for isolation was a modification (*41*) of the procedure of King *et al*. (*42*). 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 *E. coli* strain BL21, which contained plasmid pTN2 (gift of A. Kikuchi, Tokyo Institute of Technology, Tokyo, Japan). The procedure for isolation was a modification (*43*) of the procedure of Fujii-Nakata *et al*. (*44*). A weight ratio of 1:1 with the histones provided the maximum depositional activity and is the quantity that was used for these studies. When NAP1 was present in the transcription experiments, that same ratio was used relative to the quantity of histones that had been reconstituted on the DNA template. For the depositional experiments on the DNA/RNA mixture, NAP1 was preincubated with the histones for 10 min at 35 °C before addition.

Preparation of DNA. The template DNA contained two T7 promoters tandemly arranged for transcription into 18 repeats of the 207 bp 5S RNA sequence of *Lytechinus variegatus* (*43*). This plasmid (p2T7/T3-207-18, 6055 bp) was purified on CsCl−ethidium bromide gradients to obtain negatively coiled ccc[1] DNA. This DNA was then treated with MSB topoisomerase I at 0 °C in 10 mM $MgCl_2$ and 10 mM Tris (pH 8.0) until relaxation was complete. The reaction was terminated by the addition of SDS (final concentration of 0.1%), and the DNA was extracted with a phenol/chloroform mixture and precipitated with ethanol. At this reduced temperature, ionic strength, and Mg^{2+} concentration, the helical pitch of the DNA substantially decreases (*45*, *46*). Relaxation under that condition results in a DNA that exhibits an average of 6.5 positive coils (SD = 0.011) when subsequently incubated at 35 °C under isotonic conditions [100 mM NaCl and 40 mM Tris (pH 8.0)]. These coils can be seen by gel electrophoresis and allow one to differentiate between DNA that is nicked versus that which is ccc DNA. This DNA is termed R DNA.

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 M NaCl, 50 mM Tris, 0.1 mM EDTA, and 5 mM 2-ME, and the NaCl concentration was decreased in the same buffer in increments of 1.2, 0.6, and 0.1 M for 3 h in each step (*18*, *37*). Samples were sedimented at 10000*g* for 5 min to differentiate between soluble and insoluble complexes. The histone and DNA concentrations were determined using an extinction coefficient for histones of 4.2 at 230 nm and for DNA of 20 at 260 nm (*47*). The histone:DNA ratio was maintained at 0.4:1 (w:w) for all experiments which is a condition under which minimal insoluble complexes were observed (data not shown).

Conditions for Transcription and Analysis of Complexes on Sucrose Gradients. Unless otherwise stated, transcription was carried out 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, 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 60 μg/mL under these conditions, but in the absence of UTP. 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 at either of the two promoters on the plasmid depending on the relative position of the nucleosomes. The polymerase transcribes 13 bases before a UTP is required and provides a procedure for facilitating synchronized transcription when UTP is added. For some experiments, topoisomerases and NAP1 were added at this time and the preincubation was continued for an additional 2 min. After the addition of UTP, transcription was extended for 5 min and terminated by addition of EDTA (final concentration of 10 mM). A 3-fold excess of competitor DNA (T7/T3-19, 2238 bp) was now added. The competitor is also an R DNA, which is the same topological density as the template DNA (SD = 0.011). This smaller DNA contains three coils that can be readily resolved by gel electrophoresis. After the addition of the competitor, 5 μg of RNase A was added and the incubation continued for 5 min. The samples were then concentrated over a 10 min period at 4 °C with Amicon filters, applied to 5 to 20% sucrose gradients containing 100 mM NaCl, 40 mM Tris, and 0.1 mM EDTA, and then sedimented in an SW41 Ti rotor at 40 000 rpm for 5 h at 4 °C. Fractions were collected (450 μL); 30 μL was removed and added to stop buffer [0.4% SDS, 20% glycerol, 50 mM Tris, and 25 mM EDTA (pH 8.0)] to determine the distribution of DNA. DNA electrophoresis was carried out on 1.2% agarose (Calbiochem, type C) using buffer conditions described previously (*34*). 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 acetone, dried, and dissolved into SDS electrophoresis buffer. The quantity of radiolabeled histones that were present in the PAGE gels was determined using the fluorographic procedure of Laskey and Mills (*48*).

RESULTS

Histones Used for These Studies. To increase the sensitivity of detection for the various histone types in these studies, MSB cells were radiolabeled with [^{3}H]lysine and [^{3}H]-arginine for 1 h followed by a 1 h chase. The histones were subsequently isolated and purified into separate fractions of H2A and H2B, and H3 and H4. Figure 1A shows a Triton−

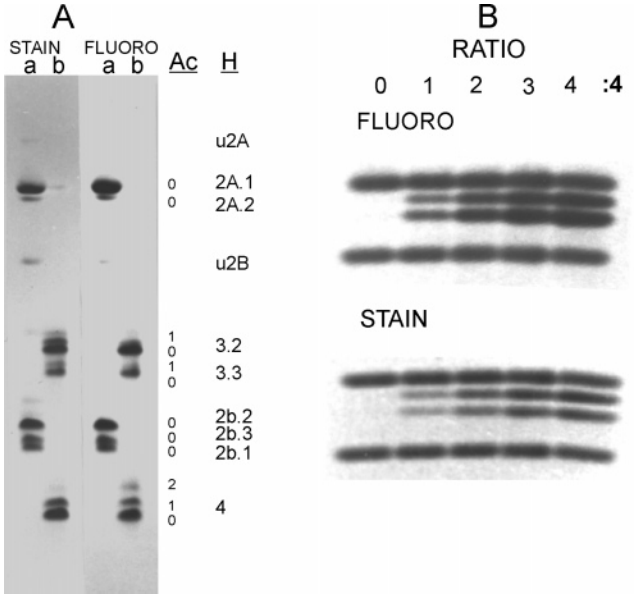

FIGURE 1: Histone used for these studies. (A) TAU analysis of purified H2A, H2B, H3, and H4. (B) SDS gel analysis of a mixture of histones H2A and H2B with histones H3 and H4 at molar ratios of 0:4, 1:4, 2:4, 3:4, and 4:4. These mixtures were reconstituted with DNA as described in Experimental Procedures. In panel A, the Ac column shows the acetylated state of the histones and the H column lists the histones in their variant forms (*35*, *49*). Lane a contained purified H2A and H2B and lane b purified H3 and H4. The panel labeled STAIN indicates the histone content based on staining with Coomassie, and the panel labeled FLUORO indicates the radiolabeled content of the histones.

acetic acid−urea gel analysis of these fractions for establishing the acetylated state of these histones. The labeled histones are at a low level of acetylation, similar to the bulk histones that are shown in the stained gel. In the following studies, we will be mixing H2A and H2B with H3 and H4 at different ratios. Figure 1B shows the stained gel and fluorogram for a mixing of these histones at molar ratios of 0:4, 1:4, 2:4, 3:4, and 4:4 (H2A and H2B to H3 and H4). The higher fluorographic intensity in H2A and H2B was accomplished by labeling with a 2-fold greater quantity of lysine than of arginine. This greater level of labeling improves the detection of H2A and H2B when they are used in limiting amounts relative to H3 and H4.

Histones H3 and H4 and Histones H2A and H2B Have Different Binding Affinities for RNA and dsDNA. We had previously observed that during transcription, all four histones would be displaced from the template DNA to the nascent RNA. Histones have a strong affinity for RNA (*32*). To understand the reasons for this strong affinity, we have carried out the following set of experiments to determine the binding characteristics of the histone subtypes for RNA and DNA. In some of these experiments, we included NAP1, which is a histone chaperone. NAP1 facilitates the deposition of histones at physiological ionic strength (*50*−*52*). To carry out these experiments, we made RNA from a plasmid that contained 18 repeats of the 207 bp 5S gene of *L. variegatus* (p2T7/T3-207-18, 6055 bp). The plasmid contains two T7 RNA polymerase promoters 39 bp apart and produces transcripts of 6022 and 5983 bases after the plasmid has been linearized with *Pvu*II. We transcribed this plasmid to produce a 10-fold greater quantity of RNA, relative to the amount of template DNA, and after terminating the transcription by

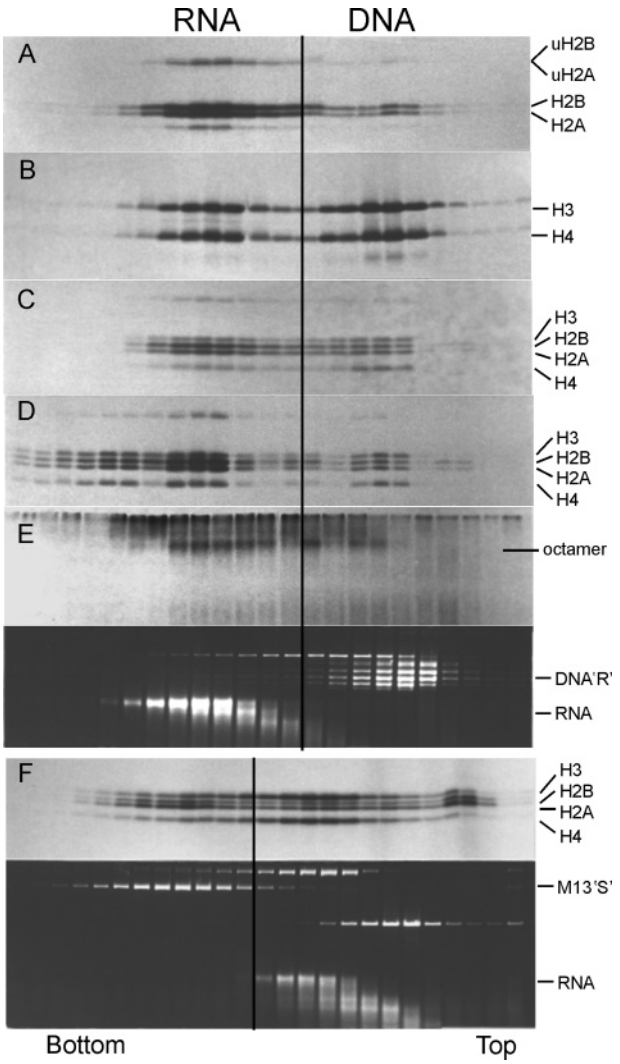

FIGURE 2: Analysis of histone binding to RNA and DNA: (A) H2A and H2B, (B) NAP1-bound H3 and H4, (C) H2A and H2B kept for 5 min with the RNA/DNA mixture followed by the addition of NAP1-bound H3 and H4 for an additional 5 min, (D) the NAP1−H2A−H2B−H3−H4 complex, and (E) the NAP1-bound octamer (cross-linked with DMS). After the histones had been added to the RNA/DNA mixture, the samples were incubated at 35 °C for 5 min and then directly applied to 5 to 20% sucrose gradients. When NAP1 is present, the histones are incubated with this protein for 10 min before being used. The panel below panel E is a representative agarose gel showing the distribution of RNA and DNA for the histone distributions of panels A−E. (F) The NAP1−H2A−H2B−H3−H4 complex was prebound to RNA by a 5 min incubation and then mixed at a ratio of 1:1 (RNA:DNA) with negatively coiled M13 DNA (S), which had been previously reconstituted with unlabeled H3 and H4 (0.4:1 histone:DNA ratio). The combined samples were incubated for a further 5 min and then applied to a sucrose gradient. The panel below panel F is the agarose gel showing the distribution of RNA and DNA for this experiment. The top of the gradient is on the right side of each panel.

addition of EDTA (final concentration of 10 mM), we added an equivalent quantity by weight (1:1 RNA:DNA) of a circular form of a smaller plasmid (T7/T3-19, 2238 bp). This DNA had been adjusted to an SD of 0.011, which equates to an average of three positive coils and is termed R DNA. To this mixture were added histones using the different conditions of panels A−E of Figure 2. After incubation for 5 min at 35 °C, the samples were applied to sucrose gradients and centrifuged at 200000*g* for 5 h to separate the RNA from DNA and to determine the distribution of the associated

histones. As shown in Figure 2A, when histones H2A and H2B were directly added to the RNA/DNA mixture, an approximate 20-fold preference for binding to the RNA was observed. We have also observed that the presence of the histone chaperone, NAP1, does not alter this preference (data not shown). Therefore, whether by directed or facilitated deposition, H2A and H2B prefer to bind RNA. We have also found that this preference is due to the single-stranded character of the RNA. This conclusion is based on subsequent experiments in which we have observed a similar preference when single-stranded M13 DNA was used in place of the RNA (data not shown). We next examined the binding of histones H3 and H4. For this experiment, NAP1 was needed to facilitate the deposition of H3 and H4 (*50*, *51*). Histones H3 and H4 were pretreated with NAP1 for 10 min and then added to the RNA/DNA mixture. As shown in Figure 2B, H3 and H4 bound both RNA and DNA equally well. When this experiment was repeated using single-stranded M13 DNA in place of the RNA, H3 and H4 were found equally on both single- and double-stranded DNA (data not shown). The preference for single-stranded character that is seen for H2A and H2B is not observed for H3 and H4.

To evaluate whether these preferences are maintained when these sets of histones are combined, the following experiments were carried out. Histones H2A and H2B were added to a RNA/DNA mixture and incubated at 35 °C for 5 min. Next, NAP1-bound H3−H4 tetramer was added, and the incubation was continued for an additional 5 min. As shown in Figure 2C, H2A, H2B, H3, and H4 were found on both RNA and DNA. There is no indication that the initial presence of H2A and H2B on the RNA has caused H3 and H4 to bind H2A and H2B and therefore show a similar preference for RNA. Rather, the opposite is observed. Histones H2A and H2B have been redistributed from the RNA to bind H3 and H4 that are on the DNA. This tendency for H2A and H2B to be redistributed from a polyanion (i.e., DNA or RNA) to a DNA that contains H3 and H4 has been described previously (*18*, *53*). In the next experiment, we premixed the H2A and H2B with H3 and H4 together in the presence of NAP1, which is a condition that stabilizes the histone octamer (*51*, *52*), and then added this sample to the DNA/RNA mixture. Both histones H3 and H4 and histones H2A and H2B were now preferentially bound to the RNA. We interpret these results as indicating that if the interphase between the H3−H4 tetramer and H2A−H2B dimer is disrupted (Figure 2C), the H3−H4 tetramer will define the location for the binding of the H2A−H2B dimer on a polyanion, even when the H2A−H2B dimer is prebound to the RNA. When the H2A−H2B dimers are within an octameric complex with the H3−H4 tetramer, the H2A−H2B dimers define the location for deposition, and that preference is RNA (Figure 2D). That the octameric complex appears to be involved is reinforced in the experiment whose results are depicted in Figure 2E. In this instance, a cross-linked octameric complex (DMS-treated) was preincubated with NAP1 prior to addition to a DNA/RNA mixture. The data indicate that this cross-linked complex also has a preference for binding RNA. These experiments have been repeated using single-stranded DNA in place of RNA, and those results also indicate that it is the single-stranded character of the RNA which is the structural characteristic causing this preferential binding (data not shown).

We next characterized the stability of the interactions of H2A and H2B with H3 and H4 when in an octameric complex on the RNA. In this experiment, the T7/T3-19 plasmid (2238 bp) was linearized with *Pvu*II and transcribed to produce a 5-fold excess of RNA relative to DNA. The size of this RNA was 2205 bases. H2A, H2B, H3 and H4 (equimolar) were preincubated with NAP1 to stabilize the octameric complex and then added to this RNA. After a 5 min incubation, the histones were now bound to the RNA. To this mixture was added negatively coiled M13 DNA (7250 bp), which had been previously reconstituted by NaCl dialysis with unlabeled H3 and H4 (see Experimental Procedures). Unlabeled H3 and H4 on the DNA provide a collection site upon which radiolabeled H2A and H2B can bind when dissociated from radiolabeled H3 and H4 that are on the RNA. After an additional incubation of 5 min, the sample was applied to a sucrose gradient, and as shown in Figure 2F, significant quantities of H2A and H2B have been transferred to the M13 DNA. Also seen on top of the gradient are significant levels of H2A and H2B. We have determined that NAP1 is distributed on top of the gradient, and it is in a complex with H2A and H2B (data not shown). Since it is known that NAP1 binds H2A and H2B more effectively than H3 and H4 and can facilitate the interaction between these two sets of proteins (*50*−*52*), we interpret these observations as indicating that NAP1 will function to shuttle H2A−H2B dimers between the H3−H4 tetramers that are on both RNA and DNA. This conclusion as well as the conclusion that an octameric complex of histones preferentially binds RNA due to the presence of H2A and H2B provides the background information that will be needed to understand histone mobility when transcription is carried out on nucleosomal templates. Since NAP1 will be used as part of these transcription studies, the next set of experiments will characterize the effects of NAP1 on nucleosome stability by measuring the size and quantity of premature transcripts that are caused by the nucleosomes.

NAP1 Is Unable To Alleviate the Premature Termination of Transcription Caused by Nucleosomes. Histones were reconstituted by NaCl dialysis onto the ccc p2T7/T3-207-18 DNA (6055 bp). The DNA was preadjusted to an SD of 0.011, which equates to an average of 6.5 positive coils (R DNA). This particular plasmid is useful for these analyses because of the tendency of nucleosomes to form precise positions on the 18 repeats of the 207 bp sequence (*54*, *55*). The ratio of histone to DNA was maintained at 0.4:1 for all experiments, which equates to an average of 12 nucleosomes for a plasmid capable of holding 30 nucleosomes. Figure 3 shows the results of a series of experiments in which DNA was reconstituted with the following ratios of histones H2A and H2B to histones H3 and H4: 0:4 (A), 1:4 (B), and 4:4 (C), keeping the histone to DNA ratio at 0.4:1 (w:w). After transcription with T7 RNA polymerase, the size and quantity of the RNA transcripts were determined. The data indicate that for all three conditions there is a tendency for the transcripts to be substantially shorter than when transcripts are produced on DNA in the absence of histones (compare to Figure 3E). Clearly, the presence of the histones has dramatically altered the processivity of the polymerase. A closer examination of these shorter transcripts reveals that there is a tendency for the polymerase to terminate in 200 bp increments. This type of distribution suggests nucleosomal

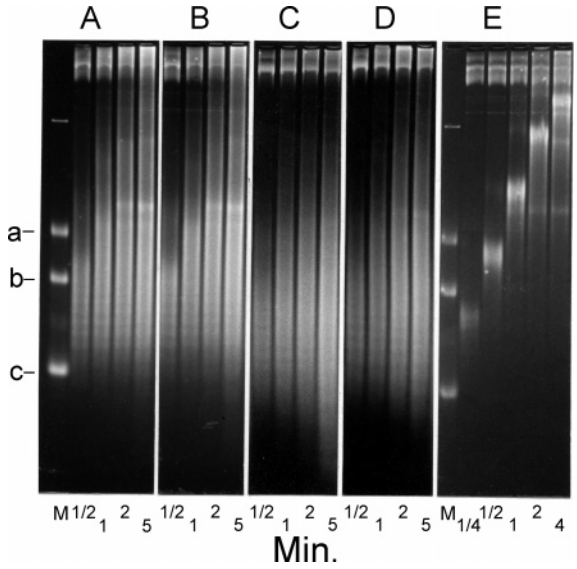

FIGURE 3: RNA produced by transcription of DNA reconstituted with H3 and H4 and increasing levels of H2A and H2B. (A) H3 and H4 alone, (B) H2A and H2B with H3 and H4 at a molar ratio of 1:4, (C) H2A and H2B with H3 and H4 at a molar ratio of 1:1, (D) same as panel C except that NAP1 was present during the transcription, and (E) no histones. The histone:DNA ratio was maintained at 0.4:1 (w:w) except for that for panel E in which no histones were present. The RNA markers (M) are 4241 (a), 2360 (b), and 580 bases (c) in length. From panel E, the transcription rate under these conditions is found to be 130 bases/s.

involvement in the termination. This termination, however, was also present for transcription conditions in which H3 and H4 alone were present (Figure 3A), which is a condition that produces subnucleosomal structures (*15*, *16*). If only H2A and H2B were present on the DNA, none of this premature termination would be observed (data not shown). The critical histones that cause this termination are H3 and H4, which is an observation that has been previously described in *in vitro* experimentation using both eukaryotic and prokaryotic polymerases (*56*−*58*). Figure 3D shows an experiment in which NAP1 was added to a histone−DNA reconstitute in which all four histones were present in equimolar amounts. The presence of NAP1 did not alter the inhibitory effect of these nucleosomes (compare with Figure 2C). Therefore, for the subsequent transcription studies in which we will observe that NAP1 facilitates the movement of histones, what must first occur is the disruption of histone−DNA interactions by RNA polymerase. Only then can NAP1 bind and move them. Even with substantial levels of premature termination, many of the transcripts extend to 6 kb in length. This length of transcript could be generated only by transcription around the entire circular plasmid and through its nucleosomes. What happens to the histones during this transcription can now be evaluated.

During Transcription, Histones H2A and H2B and Histones H3 and H4 Are Displaced to RNA in Association with One Another, a Transfer Facilitated by the Presence of NAP1. The data depicted in Figure 2 showed that when RNA is produced during transcription, the RNA could serve as a competitor for histones that are released during transcription. In previous studies, we have verified that during transcription, histones are displaced to the nascent transcript (*32*). This displacement was observed by destroying the RNA with RNase A in the presence of an excess of competitor DNA after transcription had been completed. Those histones that are displaced from the RNA are then found on the competitor DNA. The direct approach would have been to separate on sucrose gradients the nascent RNA from the template DNA. However, this is not possible because the sedimentation velocities of the template DNA and full-length nascent RNA are very similar (see Figure 2). An additional complication is the heterogeneous length of transcript that results from the presence of the nucleosomes. The competitor DNA (2238 bp) that is used is smaller in molecular weight than the template DNA (6055 bp) and can be separated from the template on sucrose gradients. We now applied this approach to an analysis of histone−DNA reconstitutes that contained varying ratios of histones H2A and H2B to histones H3 and H4. Using the same conditions described for panels A−C of Figure 4, we transcribed those reconstitutes for 5 min and then terminated transcription by adding EDTA (final concentration of 10 mM). At this point, a 3-fold excess of competitor DNA (R DNA) was added followed by RNase A, and the samples were incubated for an additional 5 min. The samples were then concentrated and applied to sucrose gradients. As shown in Figure 4A, when H3 and H4 were associated with the template DNA in the absence of H2A and H2B, there was no evidence of displacement of H3 and H4 to the competitor DNA. As was shown in Figure 2B, H3 and H4 bind RNA and DNA equally well, so if displacement had occurred during transcription, H3 and H4 should have been able to transfer to the RNA and subsequently to the competitor DNA after the RNA was destroyed by RNase A. These data indicate that H3 and H4 are not displaced from DNA under these conditions. As shown in Figure 4B, when H2A and H2B were present with H3 and H4 at a ratio of 1:4, there was now a detectable quantity of H3 and H4 associated with the competitor. This effect became substantially more significant when the ratio of histones H2A and H2B to histones H3 and H4 was increased to 1:1 (Figure 4C). Under this latter condition, approximately 20% of all four histones were present on the competitor. On the basis of the observation of Figure 2D in which it was observed that H2A and H2B within the octamer define the preference for binding RNA, we interpret these new observations as indicating that the histones transfer as a complex (H2A, H2B, H3, and H4) to the nascent RNA. We repeated these experiments in the presence of NAP1 to determine whether a chaperone-mediated process might enhance the release of the histones. As shown in Figure 4D with only H3 and H4 present on the template DNA, NAP1 enhanced the transfer. Approximately 15% of H3 and H4 is associated with the competitor. This transfer of H3 and H4 became even more pronounced when H2A and H2B were present at a ratio of 1:4 with respect to H3 and H4 (Figure 4E). In this instance, 35% of H3 and H4 was transferred from the template to the competitor. When the amount of H2A and H2B was increased to a ratio of 1:1 (Figure 4F), no significant increase above this 35% level was observed. A small quantity of H2A and H2B has had a significant effect on the stability of the interaction of H3 and H4 with the template DNA. Control experiments have been carried out in which UTP was omitted. No detectable transfer of H2A, H2B, H3, and H4 to the competitor DNA was observed (data not shown). We also observe in the data of panels B and E of Figure 4 that the ratio of histones H2A and H2B to histones H3 and H4

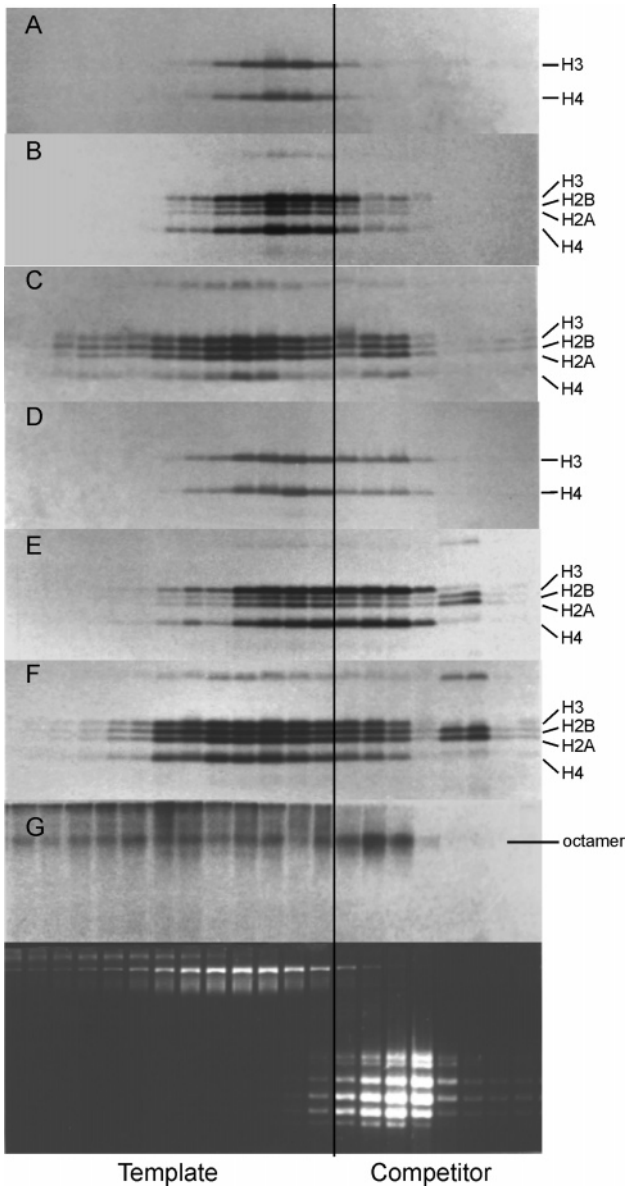

FIGURE 4: Sucrose gradient analysis of histone release during transcription. Transcription with T7 RNA polymerase was carried out on a 6055 bp plasmid that had been reconstituted with histones at a ratio of 0.4:1 (w:w) and with the following histone types: (A) H3 and H4 alone, (B) H2A and H2B with H3 and H4 at a ratio of 1:4, and (C) H2A and H2B with H3 and H4 at a ratio of 1:1. Panels D−F are the same as panels A−C, respectively, except that NAP1 was present during the transcription. (G) Cross-linked octamer reconstituted onto negatively coiled DNA (6055 bp) and exposed to MSB topoisomerase I (20 units/μg of DNA) for 2 min prior to transcription. The vertical line marks the midpoint for separation between the competitor and template DNA and is determined by agarose gel analyses of the DNA in the gradient for each panel. A representative agarose gel is shown below panel G. Transcription was carried out for 5 min at 35 °C, after which the samples were adjusted to a final EDTA concentration of 10 mM and a 3-fold excess of competitor DNA was added (2238 bp). RNase A (5 μg) was then added and the incubation extended for 5 min, after which the samples were concentrated and applied to 5 to 20% sucrose gradients.

for both the competitor and template DNA is the same. For those experiments, we purposely set the ratio on the template DNA at 1:4, which is a condition in which there is not enough H2A and H2B to have one H2A−H2B dimer for each H3−H4 tetramer. A hexameric complex has a ratio of 1:2. If a hexameric complex were released from the template DNA and stably maintained on the RNA (i.e., competitor DNA), one would have expected the ratio to increase to 1:2 for the competitor and to be less than 1:4 for the template. Since this change in the ratio is not seen, H2A and H2B must be shuttling back and forth between the template DNA and RNA, displacing H3 and H4 from the DNA as transcription proceeds. It could be argued that our post-transcriptional processing, in which we use RNase A, could have caused a disruption of H2A and H2B from H3 and H4. H2A and H2B could then be uniformly redistributed on H3 and H4 of both competitor and template DNA. It is known that at physiological ionic strength the binding of H3 and H4 with H2A and H2B is dependent on the simultaneous interaction with a polyanion (*59*). However, we think that the ratios observed in panels B and E of Figure 4 occur as a result of transcription and before the RNase A treatment based on the following additional experimentation. We repeated the experiment whose results are depicted in Figure 4E except that after the addition of EDTA and before the treatment with RNase A, the sample was exposed to 1% formaldehyde to cross-link the histones to their respective polyanions. After removal of the excess fixative by dialysis, the sample was treated with RNase A and applied to a sucrose gradient containing 2 M NaCl, a condition under which un-cross-linked histones would be displaced from the template DNA. After reversal of the cross-link (*60*) and analysis of the histone distribution on the gradient, we observed a 1:4 ratio of histones H2A and H2B to histones H3 and H4 on the template DNA (data not shown). The maintenance of this ratio on the DNA is an indication that H2A and H2B must be shuttling from the RNA to the DNA in a process that is facilitated by NAP1. In the data of Figure 2F, we observed that NAP1 effectively facilitates the transfer of H2A and H2B between RNA and DNA when both polyanions contain H3 and H4. There is a detectable quantity of NAP1-bound H2A and H2B on top of the gradient that would be involved in this transfer process. A similar quantity of NAP1-bound H2A and H2B is on top of the gradient for Figure 4E, which indicates that a similar transfer process is likely occurring during transcription. As shown in Figure 3D, NAP1 was unable to repress premature termination by the RNA polymerase. NAP1 minimally alters the interactions of histones to DNA. For NAP1 to bind the histones and transfer them, the RNA polymerase must first disrupt those interactions.

In our description of this transfer process, we have focused on the transfer of the H3−H4 tetramer as a hexameric complex with one H2A−H2B dimer. It is also possible that two H2A−H2B dimers are bound to the H3−H4 tetramer in an octameric complex. To test whether an octameric complex can be displaced during transcription, we reconstituted a cross-linked octamer onto the R DNA. We found, however, that nucleosomal structure would not form on the R DNA, as can be seen in Figure 5B in which MSB topoisomerase I was added and very few negative coils were formed. When un-cross-linked histones were reconstituted on this DNA, negative coils were formed (Figure 5A). These results indicate that the presence of the partial positively coiled state in the ccc DNA prevents the wrapping of DNA around a cross-linked octamer during the reconstitution procedure. In contrast, when negatively coiled DNA (S DNA) was used with the cross-linked octamer, nucleosomal struc-

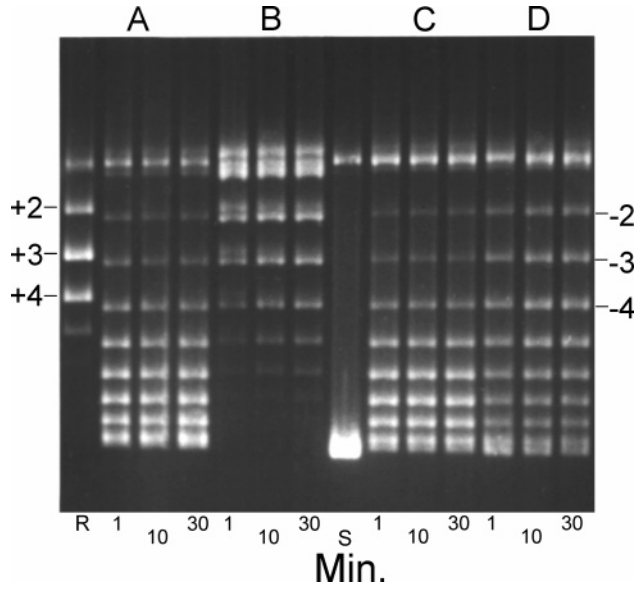

FIGURE 5: Characterization of the negative coils on ccc DNA when reconstituted with un-cross-linked and cross-linked histones. Reconstitution was by NaCl dialysis (see Experimental Procedures) onto the T7/T3-19 plasmid (2238 bp) as either R DNA, which is partially positively coiled with an SD of 0.011 (A and B), or S DNA, which is negatively coiled DNA (C and D). The histone:DNA ratio was 0.4:1 and with equimolar quantities of un-cross-linked H3, H2A, H2B, and H4 (A and C) and the cross-linked (DMS-treated) octamer (B and D). MSB topoisomerase I (200 units/μg of DNA) was added to the samples, and aliquots were taken at the indicated times.

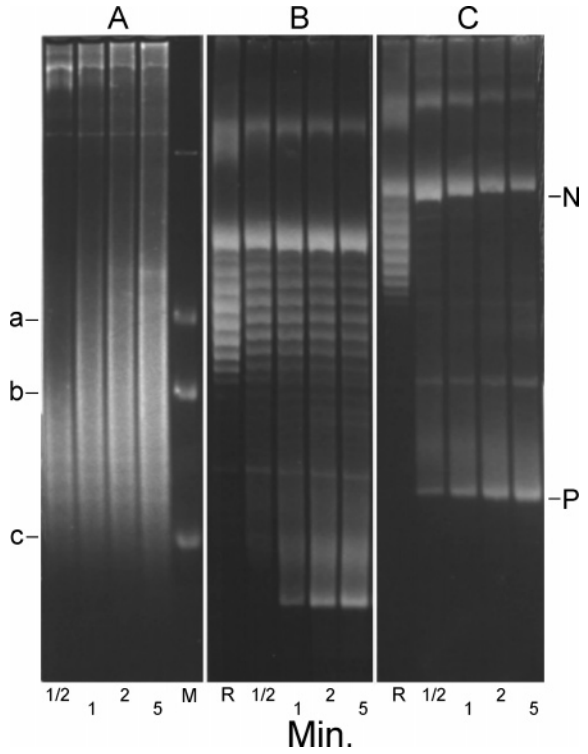

FIGURE 6: Analysis of RNA and template DNA when transcription is carried out in the presence of *E. coli* topoisomerase I. (A) RNA produced during transcription of a reconstitute containing H2A and H2B histones and H3 and H4 histones at a ratio of 1:1. (B) Topological state of the template DNA during transcription. (C) Topological state of template DNA during transcription when the DNA is reconstituted with H3 and H4 alone. The R DNA (p2T7/T3-207-18 DNA, 6055 bp) was reconstituted with histones at a ratio of 0.4:1 (H:D) and transcribed in the presence of 60 units of *E. coli* topoisomerase I/μg of DNA. Two aliquots were taken, one for RNA analysis and a second for treatment with RNase A and proteinase K prior to analysis of the template DNA. The RNA markers (M) are as described in the legend of Figure 3. The right side of the panel indicates the location for the highly positively coiled DNA (P) and nicked DNA (N).

ture was observed (Figure 5D). The number of negative coils that were maintained in the presence of MSB topoisomerase I were the same as observed when reconstitution was carried out with un-cross-linked histones (compare panels C and D). Therefore, to carry out this analysis, we reconstituted the cross-linked octamer on the ccc p2T7/T3-207-18 DNA when in the negatively coiled state and subsequently added MSB topoisomerase I to remove the unrestrained negative coils. These unrestrained negative coils must be removed because sections of the RNA transcript tend to form RNA−DNA hybrids (R loops) during transcription, which complicates the analysis (ref *61* and data not shown). Transcription was then initiated with the addition of UTP, and after incubation for 5 min, EDTA and the competitor DNA were added. After an RNase A treatment of 5 min, the sample was applied to a sucrose gradient to separate the template and competitor DNA. As shown in Figure 4G, approximately 30% of the octameric complex has transferred from the template to the competitor. This level of transfer is actually greater than the level of 20% that was observed with un-cross-linked histones (Figure 4C). We have carried out control experiments in which UTP was absent to verify that the movement of the octamer is dependent on transcription (data not shown). We conclude that since a cross-linked octamer can be displaced from the template, it is conceivable that an un-cross-linked hexameric or octameric complex could also be displaced. This conclusion is surprising as one would expect that it would be necessary to simultaneously disrupt the multiple interactions between the eight histones and DNA before displacement could occur. Since the template DNA used in these analyses was a ccc DNA, the continuously changing topological state of template DNA, as caused by the movement of RNA polymerase, may have sufficiently altered histone−DNA interactions to displace an octamer. The following studies will evaluate these topological effects.

Histones Continue To Be Displaced Even when a High Level of Transcriptionally Induced Positive Stress Is Present in the Template. We have previously described a protocol in which high levels of positive stress can be induced into a plasmid as a result of transcription (*43*). In the presence of nucleosomes and particularly H3 and H4, the globular state of the nucleosome decreases the translational flux of the transcriptionally induced negative and positive stresses around the circular DNA. If *E. coli* topoisomerase I is present during transcription, the selective removal of the negative coils results in the formation of high levels of positive stress on the DNA. Without the histones, no positive stress is induced in the plasmid (*43*). Figure 6B shows the change in the topological state of a template DNA that had been previously reconstituted with equimolar amounts of histones H2A and H2B and histones H3 and H4 prior to transcription in the presence of *E. coli* topoisomerase I. Within 1 min of transcription, a high level of positive stress has already been generated. Figure 6A shows the RNA that was produced during this transcription. RNA continues to be synthesized throughout the 5 min period despite the presence of the

positive coils. Premature termination of the transcripts continues to be observed and is an indication that the presence of the positive coils does not alter this nucleosomal effect. Figure 6C shows the high levels of positive stress that were produced when the template DNA was reconstituted with H3 and H4 alone. Promoting the formation of this positive stress is a unique characteristic of H3 and H4. Histones H2A and H2B when reconstituted on DNA by themselves are not able to do this (*43*). These observation provides further evidence of the importance of the H3−H4 tetramer in establishing the left-handed coil, hence the globular state of the nucleosome (*15*, *16*). An additional notable feature in the data depicted in Figure 6C is that the R DNA template is entirely converted to the positively coiled state (compare with Figure 6B). This effect is due to the greater accessibility of the promoters when H2A and H2B are absent (*62*). There is significant blockage of the two promoters on this template DNA by nucleosomes, even at the lower histone:DNA ratio of 0.4:1. Nevertheless, for the majority of the templates, initiation does occur and, because transcription continues to occur on the positively coiled DNA, provides an opportunity to evaluate the movement of histones in the nucleosome in the presence of this stress.

We first examined whether H3 and H4 when alone on the template would continue to remain bound to it during transcription in the presence of *E. coli* topoisomerase I. As shown in Figure 7A, H3 and H4 were not displaced. The result is similar to what was observed when this level of positive stress was not present (compare to Figure 4A). We next examined the effect of transcription on reconstitutes containing histones H2A and H2B and histones H3 and H4 at a ratio of 1:1. As shown in Figure 7B, 25% of all four histones was displaced from the template DNA to the RNA and subsequently to the competitor DNA after RNase A treatment. This level of displacement is similar to what was observed in the experiment in Figure 4C (20% displacement). Positive stress does not appear to significantly alter the frequency of histone displacement for H3 and H4 either alone or in combination with H2A and H2B. For these experiments, the rate of transcription by the T7 RNA polymerase was 130 bases/s (Figure 3E). This rapid rate could potentially cause a higher frequency of nucleosome disruption and histone displacement. We tested the effect of transcription rate in the following experiment.

Reducing the Transcription Rate Does Not Decrease the Rate of Nucleosomal Disassembly. We have previously observed that at 13 °C, T7 RNA polymerase reduces its rate of transcription 4-fold (32 bases/s under these buffer conditions). We have also observed that positive stress continues to be generated on the plasmid when transcription is carried out in the presence of *E. coli* topoisomerase I (*43*). Normally, if one were to reduce the rate of transcription at 35 °C to this level, it would not be possible to induce positive stress on the template DNA (*43*). This is because of the rapid neutralization of transcription-induced positive and negative stresses around the circular DNA. By reducing the temperature, however, one also improves the viscous state of the system (both DNA and solvent). As a result, the reduced rate at which transcription-induced stresses are generated is equally matched with a reduced rate at which these stresses

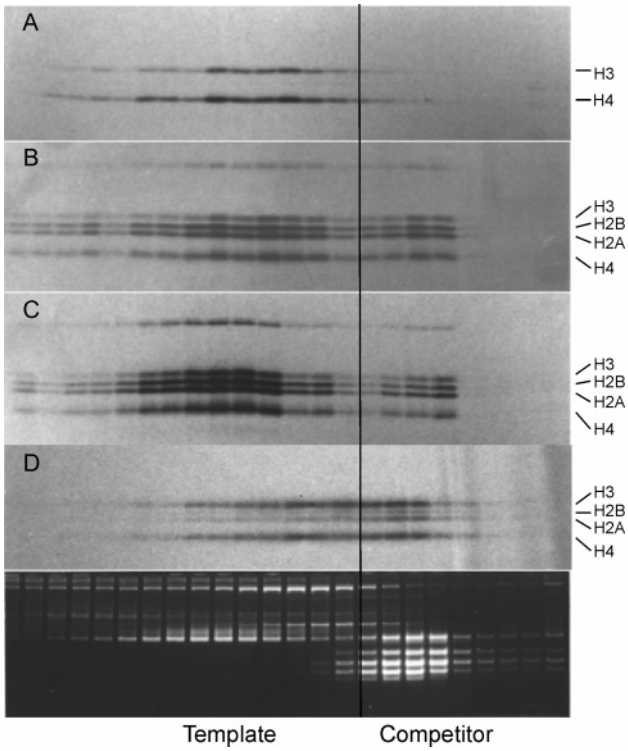

FIGURE 7: Sucrose gradient analysis of histone release during transcription in the presence of *E. coli* topoisomerase I. (A) Transcription at 35 °C for 5 min on a template DNA containing H3 and H4 alone. (B) Transcription on a template DNA containing H2A and H2B with H3 and H4 at a ratio of 1:1. (C) Same as panel B except that transcription was carried out at 13 °C for 20 min. (D) Transcription at 35 °C for 5 min of a template DNA containing H2A and H2B with H3 and H4 at a ratio of 1:4 in the presence of NAP1. After transcription, the samples were processed as described in the legend of Figure 4. The panel below panel D is a representative agarose gel showing the distribution of template and competitor DNA for the histone distributions of panels A−D.

translationally flux on the DNA. It is therefore possible to evaluate the effect of the reduced rate of transcription on nucleosomes while continuing to produce this positive stress. In this experiment, transcription was carried out for 20 min at 13 °C so that the same quantity of transcription was completed as at 35 °C for 5 min. As shown in Figure 7C, 20% of all four of the histones were displaced from the template DNA to the competitor DNA. We have also transcribed at this reduced rate in the absence of *E. coli* topoisomerase I, which is a condition under which these high levels of positive stress are absent. This same level of histone displacement was observed (data not shown). The decrease in the rate of transcription from 130 to 32 bases/s has not reduced the level of histone displacement, irrespective of the level of positive stress on the template DNA.

High Levels of Transcription-induced Positive Stress Limit the Ability of NAP1-Bound H2A and H2B To Interact with the Template DNA. From the data depicted in Figure 4E, we observed that NAP1 when bound to H2A and H2B greatly facilitated the removal of H3 and H4 from the template DNA. The ratio of histones H2A and H2B to histones H3 and H4 was 1:4 in that experiment, and we concluded that since that ratio remained the same on both the competitor and template DNA, NAP1-bound H2A and H2B were likely transferring H3 and H4 from the template DNA to the RNA as part of a hexameric or octameric

complex. We now wanted to determine whether the preservation of positive stress during the transcription process would alter this dynamic. The experiment in Figure 4E was repeated except that transcription was carried out in the presence of *E. coli* topoisomerase I. As shown in Figure 7D, a slightly different distribution of H2A and H2B relative to H3 and H4 was observed. The template is depleted of H2A and H2B as compared to the competitor. As a result, the ratio of histones H2A and H2B to histones H3 and H4 is lower in the template than in the competitor. We interpret these observations as indicating that NAP1 continues to bind H2A and H2B and facilitates the displacement of H3 and H4, similar to what was observed in Figure 4E. What is different in this instance is that NAP1-bound H2A and H2B appear to be somewhat limited in the ability to bind H3 and H4 on the template DNA. We were unable to observe this same effect when a full complement of H2A and H2B was present (Figure 7B). By reducing the content of H2A and H2B, we now can detect the effect of positive stress on the binding of H2A and H2B to H3 and H4 and to DNA. This stress is inhibiting both interactions. Since high levels of positive stress can influence these interactions, it was of interest to determine what happens when transcription is carried out in the absence of topological stress. This level of stress can be obtained by transcription on a linear template.

Histones H3 and H4 Are Displaced from the Template DNA, Even in the Absence of NAP1-bound H2A and H2B, Provided that the Template Is Topologically Unrestrained. Histones H3 and H4 were reconstituted by NaCl dialysis onto the 6055 bp DNA that had been previously linearized with *Pvu*II. The length of the transcript from the two T7 promoters of this DNA would be 6022 and 5983 bases. Subsequently, the reconstituted complex was transcribed at 35 °C for 5 min. The transcription was then terminated by treatment with EDTA followed by the addition of competitor DNA (2238 bp) and RNase A. In this instance, it was necessary to sonicate the competitor to smaller fragments in advance of the addition, because we had previously observed that as a result of transcription, the unsonicated competitor overlapped extensively on the sucrose gradients with the linearized template (data not shown). As shown in the sucrose gradient of Figure 8A, 25% of H3 and H4 have been displaced from the linear DNA to the sonicated competitor DNA after transcription. This level of displacement is occurring in the absence of NAP1, for which if a ccc DNA were used as the template, no H3 and H4 would have been displaced (compare to Figure 4A). The reason for the extensive overlap between the template and unsonicated competitor in the earlier experiment is the extensive displacement of H3 and H4 from the linear template. The substantial loss of histones causes a significant decrease in the sedimentation rate. We have previously determined that the overall rate of transcription for a linear and ccc template under these conditions is similar (data not shown). Therefore, this effect cannot be due to differences in the transcription rate. We have also observed that when the linear DNA was reconstituted with histones H2A and H2B and histones H3 and H4 (1:1 ratio), both sets of histones were displaced at that same level of 25% (data not shown). Whereas the presence of H2A and H2B greatly influenced the binding of H3 and H4 when on either a ccc DNA (Figure 4) or a highly positively stressed DNA (Figure 7), it does not do so on a linear template. We therefore conclude that the extensive release of H3 and H4 is due to the unique characteristics of a topologically unrestrained template.

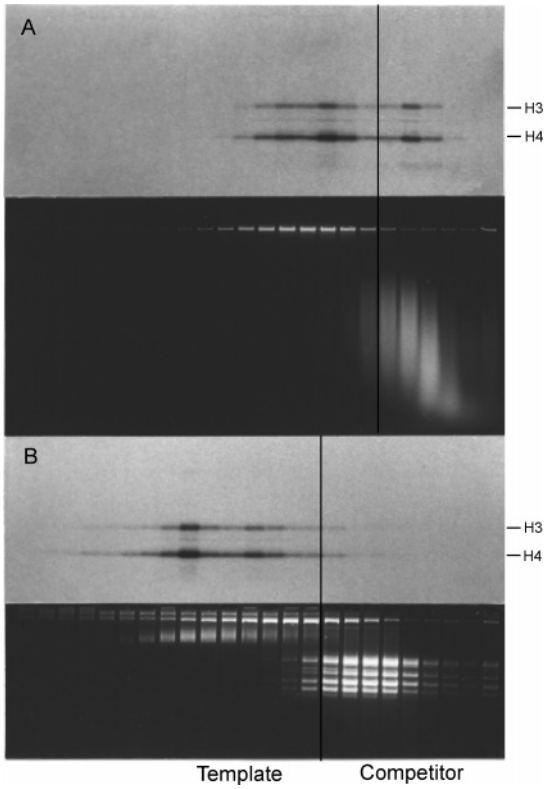

FIGURE 8: Sucrose gradient analysis of H3 and H4 release during transcription on DNA with varying topological conditions. (A) Transcription on a linear template. (B) Transcription on a ccc template in the presence of MSB topoisomerase I (200 units/μg). The histone:DNA ratio was 0.4:1. For panel A, the competitor DNA was sonicated prior to addition. Control experiments have been carried out to show that both sonicated and unsonicated competitor DNA compete equivalently for the histones released from the RNA during RNase A treatment (data not shown). After transcription for 5 min, the samples were processed as described in the legend of Figure 4.

We next evaluated the level of unrestrained stress that was required to promote this displacement. The experiment in Figure 4A was repeated in which a ccc DNA containing H3 and H4 was transcribed, except that in this instance the transcription was carried out in the presence of 200 units/μg of DNA of MSB topoisomerase I. This level of activity is sufficient to relax all DNA in the sample within 10 s at 35 °C. As shown in the sucrose gradient of Figure 8B, H3 and H4 did not transfer to the competitor DNA. The high level of topoisomerase I activity was not able to remove enough of the transcription-induced stress to mimic the unrestrained state of a linear DNA. Given the rate of transcription for this polymerase under these conditions (130 bases/s), theoretically 13 positive and negative coils would be generated every second, a rate that is substantially faster then the rate of relaxation that is provided by even this excess quantity of topoisomerase. The transcription-induced stresses are likely facilitating the maintenance of H3 and H4 on the DNA.

DISCUSSION

A model is shown in Figure 9 that summarizes the results of this study. The model is based on the observation that

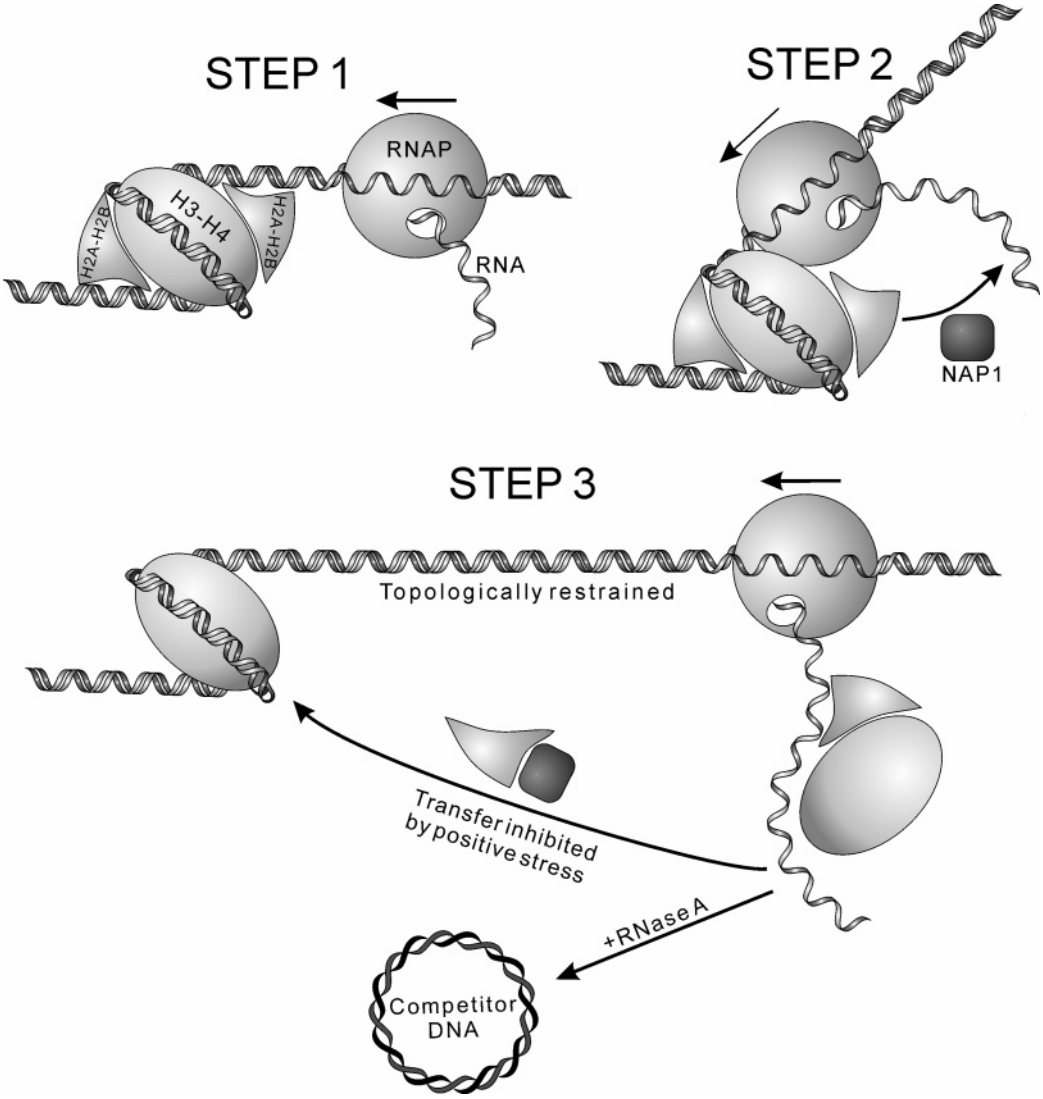

FIGURE 9: Model for the spooling of histones to RNA during transcription. In step 1, the RNA polymerase (RNAP) is shown advancing toward a nucleosome. In step 2, the RNA polymerase has disrupted the interaction of DNA with the entry site H2A−H2B dimer. The curved arrow indicates that the entry site H2A−H2B dimer now establishes interactions with the nascent RNA, interactions that are facilitated by first binding to NAP1. At this step, there is occasional premature termination as the polymerase attempts to advance through the rest of the nucleosome. The H3−H4 tetramer is primarily responsible for this premature termination. In step 3, the polymerase has successfully disrupted all the histone−DNA interactions and the complex of H2A, H2B, H3, and H4 has spooled to the RNA. Spooling refers to the sequential disruption of histone−DNA interactions and a sequential formation of interactions with RNA. The RNA polymerase is then shown to transcribe toward another H3−H4 tetramer that is bound to the template DNA. This DNA is in a topologically restrained state, and therefore, the H3−H4 tetramer will not be displaced to the nascent RNA when that region of DNA is transcribed. To return to step 1, the H2A−H2B dimer must be transferred from RNA to the H3−H4 tetramer. Binding to NAP1 facilitates this trasfer. With the addition of the H2A−H2B dimer, the RNA polymerase can now displace the H3−H4 tetramer as shown in step 2. Step 3 also shows that the transfer of the H2A−H2B dimer to the H3−H4 tetramer is significantly inhibited when the template DNA is in a high positively coiled state. Positive stress limits the cycling back to step 1. Step 3 also shows the experimental approach that is used to assay the histones on the RNA. RNase A is added after transcription has been completed so that the histones can be transferred to the excess competitor DNA. This DNA can then be separated from the template DNA using sucrose gradients.

H2A and H2B, and not H3 and H4, have a preference for binding RNA and that when bound together in an octameric complex, the complex will be transferred preferentially to RNA (Figure 2). Therefore, the model shows RNA polymerase displacing all four histones of the nucleosome to the RNA. That there is an absolute requirement for the presence of H2A and H2B to facilitate this process was shown in the transcription experiments in which H2A and H2B were present with H3 and H4 at a ratio of 1:4. In the presence of NAP1, 35% of the H3 and H4 were released (Figure 4E). When H2A and H2B were absent, H3 and H4 tended not to be released (Figure 4A,D). Substantial shuttling of NAP1-bound H2A and H2B between the template DNA and the nascent RNA would have been required to displace 35% of the H3 and H4. The model shows this shuttling process in step 3 and also indicates that displacement of H3 and H4 is dependent on the topological state of the DNA. High positive stress is shown to inhibit this transfer process and is based on the experiment in which *E. coli* topoisomerase I was present during transcription and the quantity of H2A and H2B on the template DNA was significantly reduced (Figure 7D).

For H2A and H2B to facilitate the release of H3 and H4 during transcription, a transient formation of either a hex-

americ or octameric complex with H3 and H4 would be required. That such a large complex could be displaced was confirmed when transcription was carried out on DNA reconstituted with a cross-linked octamer. The cross-linked octamer was displaced at a level similar to that of un-cross-linked histones (Figure 4G). O'Neill *et al*. (*37*) have also observed that T7 RNA polymerase will transcribe through cross-linked octamers when assembled into a multinucleosomal template. It is very surprising that an octamer can be displaced because of its multiple interactions with the DNA. Perhaps if those interactions were displaced rapidly (virtually instantaneously), the complex could be released to the RNA before those interactions with the template DNA were re-established. If this scenario were correct, one would expect that by reducing the rate of transcription, the displacement of histones would proportionately decrease. We tested this possibility by reducing the transcription rate from 130 to 32 bases/s and found that all four histones continued to be displaced at the same relative frequency (compare panels B and C of Figure 7). Another possible explanation is a facilitated transfer of the histone complex from DNA to RNA. In this scenario, the interactions between histone and DNA are sequentially disrupted by the polymerase and sequentially re-established with the nascent transcript. Felsenfeld's laboratory has observed that transcription on a mononucleosomal template with SP6 RNA polymerase (*28*−*30*) or RNA polymerase III (*31*) caused a "spooling" process in which the histones in the nucleosome were displaced retrograde with respect to the original nucleosomal position. This displacement is thought to result from a disruption of interactions between histones and DNA by the polymerase as it enters the nucleosome. Those same histone interactions are then re-established with DNA behind the polymerase, and as additional interactions between the octamer and DNA are disrupted, the octamer is eventually relocated retrograde to the original nucleosomal position. On the basis of the nucleosomal structure, one would expect that the initial interactions that are disrupted as a polymerase enters a nucleosome would be the entry site H2A−H2B dimer (*12*−*14*). It may be more than coincidental that the first histones that are displaced from DNA are the ones that have specificity for RNA. This interpretation also provides an explanation for what is observed with H3 and H4. Since H3 and H4 do not have a preference for binding RNA and tend not to be released from DNA, the preferential displacement to RNA of a hexameric or octameric complex may involve a similar spooling mechanism in which histone interactions with DNA are sequentially displaced and simultaneously regenerated with RNA rather than with DNA. This facilitated transfer may also be an explanation for the stability of the interaction between the H2A−H2B dimer and the H3−H4 tetramer. Feng *et al*. (*59*) have shown that at physiological ionic strength, a facilitated transfer between two polyanions would be required to maintain those interactions. A direct transfer from DNA to RNA would provide an explanation for the stable transfer.

We have previously observed that the average frequency of nucleosomal disruption was one of four nucleosomes (*32*). The frequency of disruption in this study is consistent with those observations. Therefore, the displacement of histones to RNA is not the favored process. Why are the majority of nucleosomes stable to the action of the polymerase? One possibility is that they are only perceived to be stable. If in step 2 of the model in Figure 9, the H2A−H2B dimers are displaced not only from the DNA but also from the H3−H4 tetramer, the H3−H4 tetramer would then tend not to be released from the DNA. The displaced H2A−H2B dimers would temporarily bind the nascent RNA, but quickly reassociate with the H3−H4 tetramer after the polymerase has passed through. The nucleosome would then have re-formed with a perception that it is stably maintained. The RNA/DNA mixing experiment of Figure 2C showed that H2A and H2B do have a stronger preference for binding H3 and H4 than for RNA. Kireeva *et al*. (*63*) have obtained experimental evidence of such a scenario. They observed by electrophoretic mobility shift assays that RNA polymerase II displaced H2A and H2B from H3 and H4 when linear mononucleosomal templates were used. Even though it is unclear from those experiments why displaced H2A and H2B would not rebind H3 and H4 after transcription was terminated, these results do indicate that transcription can facilitate the displacement of H2A and H2B from H3 and H4. We have additional evidence of this type of displacement, which will be the subject of a separate report.

It is also possible that the perceived stability of the nucleosome may involve a spooling on the DNA template as described by Felsenfeld's laboratory. Two separate laboratories have observed that T7 RNA polymerase can cause spooling on a linear mononucleosomal template similar to what has been seen with SP6 and RNA polymerase III (*64*, *65*). However, the observation that RNA appears to be involved in studies that involve multinucleosomal templates may be informative (*32*, *33*). The retrograde repositioning of ordered arrays of nucleosomes could be self-limiting and provide an environment in which repositioning to the RNA becomes more significant. The histone:DNA ratio of this report was 0.4:1, which produces an average of 12 nucleosomes in a plasmid capable of holding 30 nucleosomes. This lower ratio is required to provide conditions under which the majority of the plasmids can be initiated by the polymerase at either of the two promoters. If conditions were such that a full complement of nucleosomes were present, as would be present *in vivo*, the displacement to RNA would potentially become a more predominant mechanism for facilitating transcription through nucleosomes.

We have chosen NAP1 as the histone chaperone for these studies. NAP1 is highly ubiquitous in the nucleus, has a preference for binding H2A and H2B (*50*), and works effectively as a deposition factor for the formation of nucleosomes by interaction with H2A, H2B, H3, and H4 as part of an octameric complex (*51*, *52*). It has also been implicated in the transport of H2A and H2B into the nucleus (*66*). It has been shown to facilitate transcription factor binding by disruption of the octamer through the binding of H2A and H2B (*67*) and has also been observed to interact with p300 as a component of promoter remodeling complexes (*68*, *69*). Our observation that NAP1 is able to shuttle H2A and H2B as well as a complex of H2A, H2B, H3, and H4 between RNA and DNA is consistent with the known characteristics of this protein. Orphanides *et al*. (*70*, *71*) have shown that transcription through nucleosomes with RNA polymerase II is greatly facilitated by a complex of two proteins called FACT (facilitates chromatin transcription). These proteins preferentially bind H2A and H2B, and this

binding is thought to be an important component of the mechanism that facilitates transcription. Since NAP1 has a binding characteristic similar to that of FACT and yet is present in a much higher abundance, it may play a similar role not only in the potential remodeling of nucleosomes at promoters but also in the remodeling that may occur during transcription elongation for the three forms of RNA polymerases.

These studies have indicated that H3 and H4 tend not to be displaced from DNA unless H2A and H2B are present. However, there is one condition under which displacement does occur. This condition is when the template DNA is linear. The template loses 25% of H3 and H4 after transcription for 5 min (Figure 8A). Since linear templates are topologically unrestrained, we attempted to mimic this level of unrestrained stress by adding excess MSB topoisomerase I to a circular DNA template. We did not observe release of H3 and H4 during transcription (Figure 8B). Our interpretation of these observations is that transcription-induced stresses continue to be present and are probably a reflection of the rapid rate at which T7 RNA polymerase transcribes (130 bases/s). How these stresses function to promote the maintenance of H3 and H4 on the DNA is difficult to define. Because the template is a circular DNA, the generation of these stresses would tend to cause the DNA to coil on top of itself, and as a result, if H3 and H4 were being released, instead of binding the RNA, it could possibly be released to another portion of the template DNA. With a linear DNA, the DNA would not be expected to loop back on itself, thus limiting the possibility of transfer within the template. Additional experimentation will be required to determine whether the transcriptionally induced stresses are playing a direct or indirect role. It is tempting, however, to speculate that they are playing a direct role and that there are *in vivo* conditions that can mimic a linear template. Such an example might be the ribosomal genes in which multiple arrays of polymerases are active on the template. The negative stress in the wake of the forward polymerase would equally neutralize the positive stress of the following polymerase. The greater the frequency of transcription initiation, the more effective would be this neutralization. This neutralization would likely be more effective than having even an excess of topoisomerase I. The DNA would tend to appear to be topologically unrestrained, causing a depletion of all four histones as a result of transcription, a depletion that has been observed for ribosomal genes (*72*−*74*).

We have previously observed that nucleosomes, and most particularly the presence of H3 and H4, facilitate the maintenance of transcription-induced stresses so that high levels of positive stress accumulate (*43*). This occurs when transcription is carried out in the presence of *E. coli* topoisomerase I, which selectively removes the transcription-induced negative coils (*75*−*77*). The data depicted in Figure 6 show the formation of these positive coils and also indicate that RNA synthesis continued to occur even when positive stress was present. In the data depicted in Figure 7D, it was observed that this positively coiled template was depleted of H2A and H2B. We have interpreted these results as an indication that the NAP1−H2A−H2B complex is not able to effectively rebind H3 and H4 on this DNA. In earlier studies, we have reported that H2A and H2B extensively dissociate from H3 and H4 in 0.6 M NaCl when on positively coiled DNA, a dissociation that does not occur with negatively coiled DNA (*34*). Prunell's laboratory (*78*, *79*) has also observed using gel retardation analysis with positively coiled DNA minicircles, that H2A and H2B do not bind H3 and H4. They have proposed that a chiral transition occurs, in which the H3−H4 tetramer switches from a left-handed to a right-handed pitch. In support of these observations, Hamiche and Richard-Foy (*80*) have shown that modification of cysteine 110 of H3 with DTNB causes the tetramer to stably maintain this right-handed form. They have also observed that the presence of H2A and H2B, during assembly onto negatively coiled DNA, prevented H3 and H4 from staying in this altered state. Therefore, the inability of the NAP1−H2A−H2B complex to efficiently recycle back to a positively coiled template during transcription is an observation that is consistent with an altered state for H3 and H4 and is supportive of models that predict such disruption of the H2A−H2B interphase with H3 and H4 (*8*). Further studies will be required to determine whether this chiral transition is involved.

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