Measurement of the Frequency of Histone Displacement during the *in Vitro* Transcription of Nucleosomes: RNA Is a Competitor for These Histones†

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ABSTRACT: Transcription through tandemly arranged nucleosomes was studied to determine the frequency at which the nucleosomes would disrupt and cause displacement of the associated histones to a competitor DNA. In order to more effectively preserve topological effects, the template that was used in the *in vitro* transcription system was a large covalently, closed circular plasmid (8.9 kb). The plasmid contained two promoters for T7 RNA polymerase, each separated by 4.4 kb, and transcription was done in the presence of topoisomerase I at physiological ionic strength. Nucleosome disruption was observed at an approximate frequency of 1 in 4 nucleosomes such that after several rounds of transcription on the plasmid 80% of the nucleosomes were disrupted. Unexpectedly, all four histones were found associated with the RNA rather than the competitor DNA. The histones bound the competitor DNA only after removal of the RNA by RNase A treatment. By analyzing the topological state of the competitor DNA, it was observed that the majority of the histones that were displaced from the RNA were able to re-form nucleosomes. Additional experiments were done to determine the reasons for the preferential binding of histones to the newly synthesized RNA. It was found that the large molecular weight RNA binds histones with an approximate 100-fold greater affinity relative to DNA when at physiological ionic strength. Within the cell, this high-affinity binding would be expected to require cellular mechanisms to regulate the interaction of RNA with histones. The relatively high frequency of displacement of all four histones during transcription is higher than what is observed *in vivo* and suggests that additional factors are needed to regulate this displacement. These observations are discussed and compared with previous studies that have examined the process of transcription through nucleosomes.

Transcription within a eucaryotic cell is not a process in which RNA polymerase can freely access the DNA for either initiation or elongation of the transcript. The DNA is associated with highly basic proteins called histones which function to condense the DNA in an organized manner and to facilitate accessibility to the DNA under regulated conditions. Four of these histones, H3, H2B, H2A, H4, have a central importance in coiling the DNA into a left-handed supercoil. These proteins form an octameric complex and wrap on their outer surface 145 bp of DNA into 1.8 coils (Arents et al., 1991; Arents & Moudrianakis, 1993). This particle, termed a nucleosome, restrains the equivalence of one negative coil of DNA (Keller, 1975; Germond et al., 1975). An ordered array of these nucleosomes is the primary condensing mechanism for organizing DNA and is the major level at which RNA polymerase must initiate and elongate its RNA. Because of the highly basic character of these proteins, the binding energies with DNA are quite substantial. Sodium chloride concentrations as high as 1.2 M are required to dissociate these proteins (Simon & Felsenfeld, 1979). It perhaps then is not too surprising that several *in vitro* studies have indicated that histones substantially repress both initiation and elongation by the RNA polymerase (Morse, 1989; Felts et al., 1990; Izban & Luse, 1991; O'Neill et al., 1992), and this is an indication of the difficulty in disrupting these histone−DNA interactions. However, studies designed to examine transcriptional rates *in vivo* have provided a different observation. Transcription is extremely efficient and is an indication that cellular mechanisms must exist to facilitate transcription.

A number of *in vitro* studies have been done to determine whether transcription is facilitated by the release of histones [extensively reviewed in Thoma (1991), Kornberg and Lorch (1991, 1995), Morse (1992), van Holde et al. (1992), Felsenfeld (1992), and Adams and Workman (1993)]. Results from these studies have varied with the methodologies applied and have produced conclusions that have ranged from no histones are released to all histones are released. Some of these studies have used protocols which utilize nonphysiological ionic strengths or polyamine concentrations and have used short linear templates with DNA sequences that form very stable nucleosomes (nucleosome positioning sequences). Methods used to evaluate histone association have been indirect and have involved EMSA analysis and nuclease protection. We have readdressed this question with the following changes in the approach. The template DNA is a very large covalently closed, circular DNA with minimal nucleosome positioning sequences. Transcription is done at near-physiological ionic strength and in the presence of topoisomerase I. These combined conditions are designed to more closely mimic the state of the DNA during transcription in the cell. Topological stress induced during the transcription process (Liu & Wang, 1987) may influence the stability of the nucleosomes. These conditions are designed to preserve these transient stresses near the polymerase. Highly acetylated histones were also included in this analysis as such histones are characteristically found associated with active genes [reviewed in Gross and Garrard (1988), Csordas (1990), and Turner (1993)]. Three methods of analyses were used: (1) the histone content on the

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template and competitor DNA was determined by separating the two DNAs on a sucrose gradient and characterizing the associated histones by gel electrophoresis; (2) the ability of the histones to maintain nucleosomes on both competitor and template DNA was determined by assaying for the maintenance of negative coils in the presence of topoisomerase I; (3) the topological state of the transcriptionally competent templates was determined after isolation of the complexes on CsCl−GuHCl gradients after formaldehyde treatment. Using these approaches, we conclude that the majority of the histones are released from DNA during transcription, although repetitive transcription through the nucleosomes is required. We also observe that RNA is an extremely effective competitor for these histones.

MATERIALS AND METHODS

Preparation of Hyperacetylated and Labeled Histones. MSB cells (chicken leukemic cells transformed by Marek's virus) were grown in 10% newborn serum with medium of 1:1 Dulbecco's MEM/RPMI-1640 and supplemented with 50 mM HEPES. For labeling of cells, 1 L of cells (1×10^6/mL) was preincubated in medium lacking arginine and lysine for 30 min and then concentrated to 10 mL and incubated for 60 min with 3 mCi of [^{3}H]lysine and 1.5 mCi of [^{3}H]arginine in the presence of 5 mM sodium butyrate. For preparation of unlabeled hyperacetylated histones, cells were incubated for 24 h in growth medium containing 5 mM sodium butyrate. Histones were purified from these cells as previously described (Jackson, 1993). Briefly, the procedure involves an extraction with 2.4 M NaCl of chromatin that had been immobilized on hydroxylapatite. The extracted histones were concentrated on Amicon filters and stored in aliquots at −70 °C.

Preparation of T7 RNA Polymerase and Topoisomerase I. T7 RNA polymerase was prepared from *E. coli* strain BL21 containing plasmid pAR1219 (kindly supplied by William Studier and John Dunn, Brookhaven National Labs) which encodes T7 RNA polymerase under inducible control by the lac UV5 promoter. The enzyme was purified by the procedures of King et al. (1986) except with modifications as previously described (Pfaffle et al., 1990). The preparation had a specific activity of 1.2×10^6 units/mg with no detectable RNase or DNase activity in the preparation. One unit is defined as that amount of enzyme that will incorporate 1 nmol of CTP at 37 °C for 60 min.

Topoisomerase I was isolated from MSB cells using a modification (Pfaffle & Jackson, 1990) of the procedure of Liu and Miller (1981). The specific activity of the enzyme was 4×10^6 units/mg in which 1 unit is defined as that quantity which achieves 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C. The preparation was free of detectable RNase and DNase activity.

Preparation of DNA. The *Pvu*II-R1 fragment of plasmid T7/T3-19 which contains a T7 promoter and a multiple cloning site was inserted into the R1 site of PBR322 to obtain PBR322-T7. The PBR322-T7 was then cleaved and religated at the R1 site to obtain DIPBR322-2T7 (8916 bp) which contains two T7 promoters 180° apart promoting transcription in the same direction. A T7 promoter was also inserted into the *Xba*I−*Hin*dIII site of PIAN7 (885 bp plasmid). The DNAs were purified for these analyses by both CsCl and sucrose density gradients (Jackson, 1995). In this way, no detectable RNase or DNase activity was present, and the DNA was both covalently closed and negatively coiled. For some experiments, PIAN7 DNA was relaxed at 37 °C with topoisomerase I prior to use. The relaxation was terminated by treatment with 0.1% SDS and the DNA extracted with phenol−chloroform and ethanol-precipitated.

Reconstitution of Histone−DNA Complexes. Histones were mixed with DNA in 2 M NaCl, 40 mM Tris, 1 mM EDTA, and 5 mM 2-ME (2-mercaptoethanol), and the NaCl concentration was decreased stepwise in the same buffer as follows: 1.2, 0.6, and 0.1 M NaCl for 3 h in each step. All steps in this procedure were performed at 4 °C (Germond et al., 1975; Jorcano & Ruiz-Carrillo, 1979; Daban & Cantor, 1982; Simpson et al., 1985). Samples were then sedimented at 10000*g* for 2 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 20.0 at 260 nm (Stein, 1979).

The concentration of the reconstituted complex was kept at 200 μg/mL DNA during this protocol and adjusted to 100 μg/mL with the addition of the transcription components. No further dilutions were done to avoid dilution effects which can cause nucleosome instability at concentrations of 10 μg/mL or lower (Lilley et al., 1979; Cotton & Hamkalo, 1981; Godde & Wolffe, 1995).

Conditions for Transcription and Analysis of Complexes on Sucrose Gradients. Transcription was done at 37 °C in 100 mM NaCl, 40 mM Tris, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM 2-ME, pH 7.4, 15 units/mL Prime RNase Inhibitor (5 Prime−3 Prime, Inc.), and 0.5 mM each of ATP, GTP, CTP, and UTP. The reconstituted histone−DNA complex containing the template DNA (100 μg/mL DNA) was adjusted to the above conditions excluding the UTP and incubated for 3 min with 200 units/mg of DNA of topoisomerase I. Sufficent T7 RNA polymerase was then added to saturate all the promoters which by enzyme saturation analysis was 200 units/μg of DNA. After an additional 2 min of incubation, a sample was taken (0 time) and the remainder treated with UTP to initiate transcription. Aliquots were taken with time to characterize the topological state of the DNA. Reactions were terminated by treatment with an equal volume of 'Stop Buffer' (0.4% SDS, 20% glycerol, 50 mM Tris, 25 mM EDTA, pH 8.0, and 0.25% bromophenol blue). These samples were directly electrophoresed for RNA analysis. For analysis of DNA, samples were treated with 20 μg/mL RNase A (Pharmacia) for 30 min followed by 200 μg/mL proteinase K for 5 h at 37 °C. The samples were then heated 80 °C for 5 min before electrophoresis.

When a competitor DNA was present during the transcription, PIAN7 DNA was added at a ratio of 1:1 (w/w, competitor:template ratio) prior to treatment with topoisomerase I. Transcription was terminated by adjustment to 10 mM EDTA and the sample directly loaded on the sucrose gradient or treated with 20 μg/mL RNase A at 4 °C for 30 min before application. The 300 μL sample (30 μg of template DNA) was sedimented on 11.5 mL of 5−20% sucrose, 100 mM NaCl, 40 mM Tris, 1 mM EDTA, and 5 mM 2-ME in an SW 41Ti rotor at 35 000 rpm for 10 h. Fractions were collected (450 μL), and 30 μL was removed for treatment with 'Stop Buffer' and DNA analysis. The remainder of each fraction was treated with 5 μg of BSA which was used as carrier to ensure quantitative precipitation of the histones with the subsequent treatment of 15% TCA. After an incubation of 4 h at 4 °C, the samples were centrifuged at 20000*g* for 10 min, and the pelleted protein was sonicated into an SDS electrophoresis buffer.

Conditions for Electrophoresis. The topological state of the DNA was determined by electrophoreses on 1.0% agarose gels (Calbiochem, type C) using buffer conditions as previously described (Jackson et al., 1994). DNA was visualized by staining with ethidium bromide, photographed, and imaged on XAR-5 film for densitometric analysis.

Histones were analyzed by SDS (sodium dodecyl sulfate) gel electrophoresis using buffer conditions as previously described (Jackson et al., 1994). The quantity of radiolabeled histones that were present in the gel was determined using the fluorographic procedure of Laskey and Mills (1979). Histones were also analyzed by TAU (Triton, acetic acid, urea) gel electrophoresis using conditions as previously described (Zweidler, 1978).

CsCl Density Gradient Analysis of Cross-Linked Complexes. When formaldehyde treatment was to be applied, the preparative conditions were changed such that 'Hepes' buffer was used in place of 'Tris' at the same relative concentration (40 mM). This change did not change the quantity of RNA produced during transcription of the histone−DNA reconstitutes (data not shown). After transcription was complete, the sample was adjusted to 1% formaldehyde and incubated for 20 h at 4 °C (Jackson, 1993). The sample was then dialyzed for 6 h at 4 °C to remove the excess formaldehyde and the sample mixed with 1.4 g of CsCl, 1.5 g of GuHCl (guanidine hydrochloride), and 100 μL of 0.25 M EDTA, pH 8.0, in a final volume of 4.0 mL. In some instances 20 μg/mL RNase A was added to the sample and the sample incubated 30 min at 4 °C before addition of the salts. The samples were centrifuged to equilibrium in an SW60Ti rotor at 37 000 rpm for 72 h at 4 °C. Fractions (150 μL) were collected and dialyzed in a multiwell apparatus against 10 mM Tris, 1 mM EDTA, pH 8.0, for 6 h at 4 °C. The fractions were adjusted to 0.5% SDS, 5% glycerol, and 100 μg/mL proteinase K and incubated 37 °C for 2 days to reverse the cross-link and degrade the protein (Jackson, 1978). The samples were concentrated by ethanol precipitation and electrophoresed on agarose as previously described.

RESULTS

Histones and DNA Used for These Transcription Studies. One of the approaches that was used to evaluate the dynamics of histone mobility during transcription was to include a competitor DNA during the transcription process, separate the template DNA from competitor DNA on a sucrose gradient, and evaluate the distribution of histones on these two DNAs. This procedure required a method to increase the sensitivity of detection for the presence of histones. We chose to do this by radiolabeling the histones in MSB cells with [^{3}H]lysine, [^{3}H]arginine for a 1 h period in the presence of 5 mM sodium butyrate. Figure 1A shows a Coomassie-stained SDS gel of the purified histones H3, H2B, H2A, and H4, and Figure 1B is an autoradiograph of that gel. Figure 1C shows an autoradiograph of these histones after electrophoresis on a Triton−acetic acid−urea gel, and as expected after labeling in these conditions, these histones are highly acetylated (Ruiz-Carrillo et al., 1975; Jackson et al., 1976; Sobel et al., 1995; Riggs et al., 1977; Ip et al., 1988). These radiolabeled histones were used for the analysis of histone transfer between template and competitor DNA on the sucrose gradients. Figure 1D shows a Coomassie-stained gel of unlabeled highly acetylated histones which were prepared by a long-term treatment of cells with butyrate.

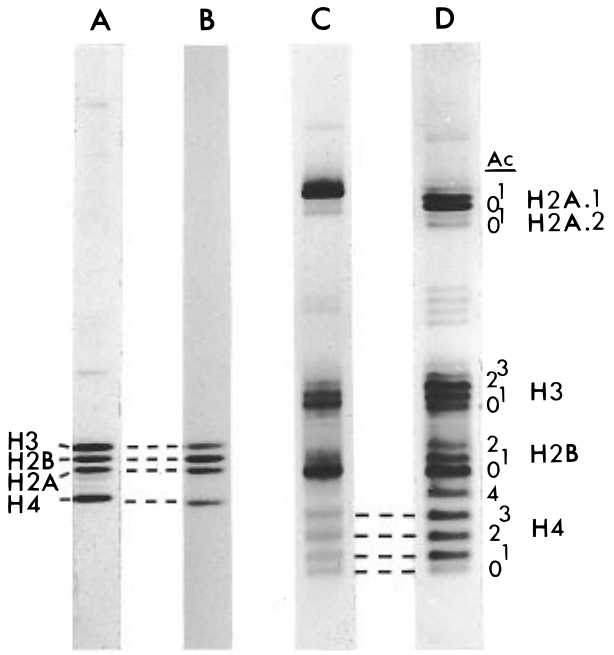

FIGURE 1: Histones used for reconstitution with DNA. Histones H3, H2B, H2A, and H4 were labeled for 30 min with [^{3}H]lysine and [^{3}H]arginine and after purification were analyzed on SDS gels. (A) Coomassie-stained gel. (B) Autoradiograph of the SDS gel. These histones were also analyzed on Triton−acetic acid−urea (TAU) gels. (C) Autoradiograph of the TAU gel. (D) Unlabeled, hyperacetylated histones analyzed on TAU gels.

These histones were used for all other experiments when an analysis on sucrose gradients was not required.

The template DNA used for these studies is a construct of PBR322 in which a T7 promoter (PBR322-T7, 4458 bp) was inserted into the R1 site. This DNA was doubled in size such that two T7 promoters transcribe in the same direction 4458 bp from one another. This increase in size improves the separation of template DNA from competitor DNA on the sucrose gradients, and the presence of the two promoters increases the efficiency of transcription in the presence of the histones.

Histones Are Unable To Maintain Negative Coils on DNA during the Transcription Process. The unlabeled, hyperacetylated histones of Figure 1D were reconstituted with DIPBR322-2T7 as described under Materials and Methods at three different histone to DNA ratios: 0.4:1, 0.6:1, and 0.8:1 (w/w, H:D). The samples were then centrifuged 2 min at 4 °C in a microfuge in order to remove insoluble histone−DNA complexes. When using hyperacetylated histones, less than 2% of the DNA is insoluble, even at the high histone to DNA ratio of 0.8:1 (data not shown). The soluble reconstituted complexes were then treated with topoisomerase I, 0.5 mM each of ATP, GTP, and CTP (no UTP) and T7 RNA polymerase over a 5 min period. Topoisomerase I removes all unrestrained negative coils in the DNA within 10 s, so that the only negative coils which remain are those retained by the histones. The estimated average number of coils which remain are shown in the 0 time points of Figure 2: 20 at a ratio of 0.4:1 (A), 30 at a ratio of 0.6:1 (B), and 40 at a ratio of 0.8:1 (C). Also during this 5 min incubation, the T7 RNA polymerase forms a ternary complex on the promoter and transcribes 8 bases before a UTP is required. As can be seen in Figure 2, the number of negative coils on the template DNA changed dramatically after the UTP was added. During the transcription, topoisomerase I removed negative coils originally retained by the histones. From a

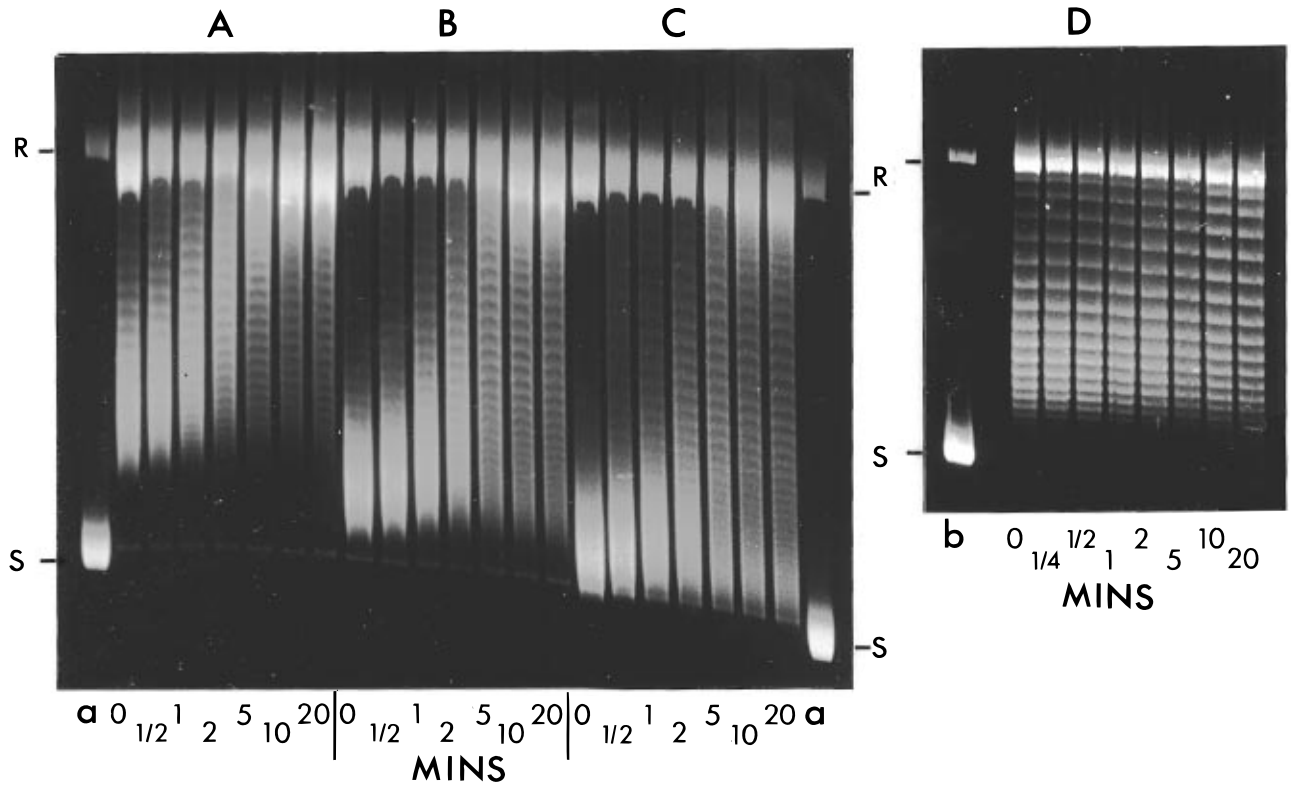

FIGURE 2: Negative coils which remain in the reconstituted template after transcription with T7 RNA polymerase. Histones were reconstituted with DNA at ratios (H:D) of 0.4:1 (panels A and D), 0.6:1 (panel B), and 0.8:1 (panel C). The DNA that was used is DIPBR322-2T7 (panels A−C) and PBR322-T7 (panel D). The 0 time lane in each panel indicates the number of negative coils maintained by the histones after treatment with topoisomerase I. The remaining time points represent the number of negative coils present during the transcription process. Lanes a and b are standards of negatively coiled DIPBR322-2T7 and PBR322-T7, respectively. S indicates the band for fully negatively coiled DNA, and R indicates the band for both nicked and covalently-closed, relaxed DNA which are not resolved on these gels.

densitometric analysis of these data, we observe that for the 10 min time point 83% of the negative coils have been lost at the 0.4:1 ratio, 70% at the 0.6:1 ratio, and 50% at the 0.8:1 ratio. The substantially lower level of disruption at the 0.8:1 ratio is probably reflective of a decreased level of initiation because of blocked promoters. The T7 promoter has the potential to position a nucleosome (Wolffe & Drew, 1989), and at the higher histone to DNA ratio, there is a greater probability for this blockage. The importance with regard to the need for two promoters on the DNA can be seen in the data of Figure 2D in which transcription was done on PBR322-T7. The PBR322-T7 had previously been reconstituted with histones at a ratio of 0.4:1, the same condition that was used with DIPBR322-2T7 in the data of Figure 2A. By a comparison of the results in Figure 2A,D, it is apparent that the DNA with the single promoter has a much decreased rate of change in the number of negative coils. By including two promoters in the template, we have increased the probability of initiation for any given plasmid. Therefore, for the reconstituted complexes with the 0.8:1 (H:D) ratio in Figure 2C, the relatively large quantity of DNA that maintains a high level of negative coils in the 20 min time point is probably DNA which contains nucleosomes that have blocked access to both promoters. Further evidence in support of this conclusion will be presented in a later section of this paper.

In order to determine the rate and level of transcription that occur in these conditions, the time points of Figure 2 were analyzed for RNA content by gel electrophoresis. Figure 3B shows the RNA produced during transcription of the reconstituted complexes for the histone to DNA ratio of 0.6:1. For comparison, Figure 3A shows the RNA produced during transcription of DIPBR322-2T7 in the absence of histones. Based on the RNA size markers shown in this figure, the rate of transcription on DNA alone is 120 bases/s (Figure 3A) and is a value consistent with other studies for these transcription conditions (Chamberlin & Ring, 1973; Bonner et al., 1994). A similar rate is also observed in Figure 3B when nucleosomes were present, except that a significantly higher level of truncated RNA fragments were produced (approximately 40%). For those remaining transcripts, the lengths were remarkably large. For example, at the 2 min time point, full-length transcripts were 14 kb in length and were produced by 1.5 rounds of transcription of the plasmid or approximately 45 nucleosomes. Even longer transcripts were generated at the 5 min time point which were not resolvable on this agarose gel. It is possible that these longer transcripts were generated from nicked DNA that was present at low levels in the preparation. It is known that when histones are reconstituted on DNA, the histones preferentially associate with negatively coiled DNA, and if nicked DNA is present, it remains free of association with the histones (Jackson et al., 1994). Since approximately 5% of the DNA is in a nicked state after completion of the reconstitution protocol, this 5% would be minimally associated with histones and could account for some of the longer transcripts. We repeated the reconstitution protocol except that in this instance the reconstituted complexes were sedimented on a 5−20% sucrose gradient to remove the nicked DNA. As shown in Figure 3D for complexes reconstituted at a ratio of 0.8:1 (H:D), the negatively coiled DNA with associated histones was easily separated from the nicked DNA. Fractions 1−6 from the gradient were then combined and the complexes transcribed with the results

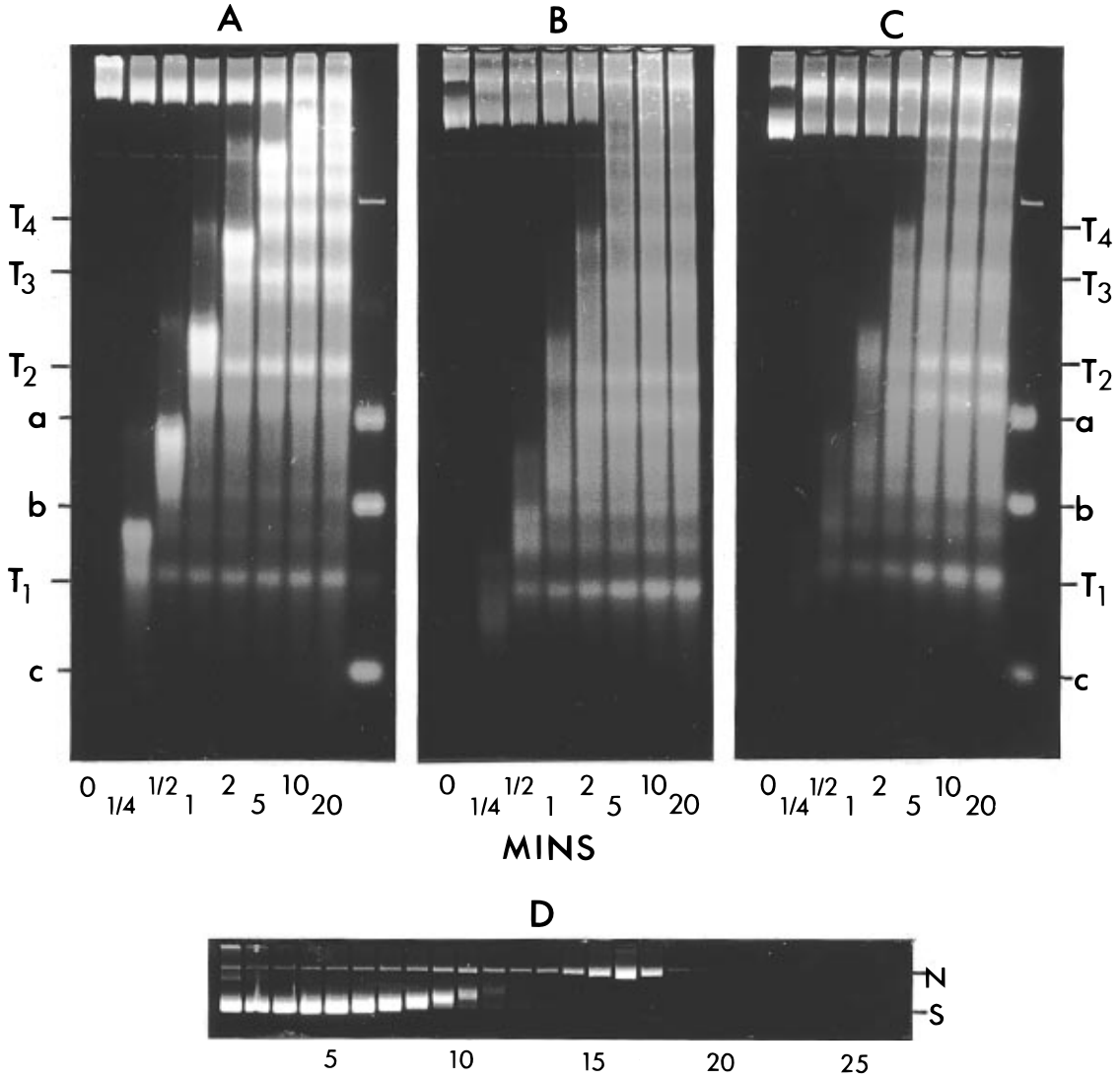

FIGURE 3: RNA that was produced during transcription on DIPBR322-2T7 DNA in the presence or absence of histones. (A) Transcription in the absence of histones. (B) Transcription of the DNA after reconstitution at a histone to DNA ratio of 0.6:1. (C) Transcription of a reconstituted sample that had been sedimented on the sucrose gradient of panel D and obtained by combining fractions 1−6. (D) Sucrose gradient showing the purification of the reconstituted sample (0.8:1, H:D) which was transcribed for the data of panel C. STD, a RNA standard created by transcription of PBR322-T7 after cleavage with the following restriction nucleases: a, 4241 bases (*Eco*RV); b, 2360 bases (*Pvu*II); c, 580 bases (*Sca*I). T_1, T_2, T_3, and T_4 mark the location for RNA terminated at a cryptic pause−termination site in the circular DNA corresponding to multiples of 4458 bp, i.e., T_1 (1270 bp), T_2 (5728 bp), T_3 (10 186 bp), and T_4 (14 644 bp). In panel D, the N and S indicate the location of nicked and covalently closed, negatively coiled DNA, respectively.

shown in Figure 3C. Full-length transcripts were produced by the polymerase, even at this higher histone to DNA ratio with an estimated 40 nucleosomes on the template DNA. The T7 RNA polymerase is capable of maintaining substantial processivity as it transcribes through these nucleosomes.

Evidence for Release of Histones from Actively Transcribed DNA onto RNA. Since no competitor DNA was present during the transcription of the reconstituted complexes of Figure 2, the histones are presumably still associated with the DNA, but are unable to hold the negative coils because of the disruption by T7 RNA polymerase. Perhaps these histones are transiently released from the template DNA and the loss of the negative coils is a result of topoisomerase I action before the histones are able to reassociate and hold the negative coils. It is known that nucleosome formation on a relaxed template is a time-dependent process requiring nearly 30 min to complete (Pfaffle & Jackson, 1990; Jackson, 1993). If there is a transient release, it should be possible to trap these histones on a competitor DNA. PIAN7 is a 885 bp plasmid, which is a factor of 10 smaller than the DIPBR322-2T7 DNA (8916 bp). We used this DNA as a competitor at a ratio of 1:1 (w/w) with respect to the template DNA and reconstituted the template DNA with the ^{3}H-labeled histones of Figure 1 at a histone to DNA ratio of 0.6:1. After transcription for 10 min at 37 °C, the sample was sedimented on a sucrose gradient, and fractions were collected and processed to determine the distribution of histones, DNA, and RNA within the gradient. As shown in Figure 4A, the ^{3}H-labeled histones are distributed in the region of the gradient in which the DIPBR322-2T7 DNA is located. No histones are in the region of the gradient in which the competitor DNA is present, and this is an indication that there is no transient release of histones during transcription. However, it should be noted in the data of Figure 4A that the RNA sedimented to regions of the gradient which overlap with the DIPBR322-2T7. To test for the possibility that some of the histones might be bound to the RNA, this experiment was repeated except that at the completion of the transcription reaction and before application to the sucrose gradient, the sample was incubated with

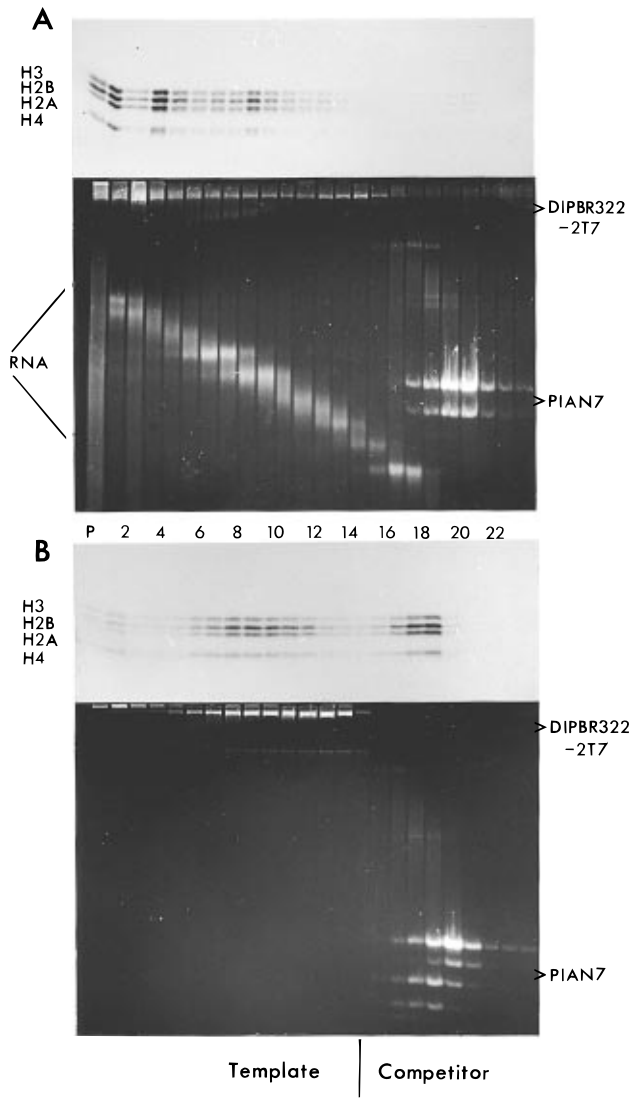

FIGURE 4: Sucrose gradient analysis of the histone distribution between template and competitor DNA after transcription with T7 RNA polymerase. (A) The reconstituted sample was transcribed for 10 min in the presence of PIAN7 DNA, adjusted to 10 mM EDTA, and directly applied to a 5−20% sucrose gradient. (B) Same as panel A except that after transcription RNase A was added and after 30 min at 4 °C the sample was applied to the gradient. The upper section of each panel is an autoradiograph of an SDS gel indicating the distribution of the histones in the gradient. The conclusion of equimolar transfer of all four histones is based on a densitometric analysis of this gel and the known specific activity of the labeled histones from Figure 1A,B. The bottom section of each panel is an ethidium bromide stained agarose gel indicating the distribution of template DNA (DIPBR322-2T7), competitor DNA (PIAN7), and RNA produced during transcription.

RNase A for 30 min at 4 °C. As shown in Figure 4B, the distribution of the histones on the gradient has changed dramatically. The competitor DNA is associated with 40% of the histones, and all four histone types are present in equimolar amounts. The removal of RNA has facilitated a transfer of all four histones to the competitor DNA. To further evaluate whether this transfer is truly a transcriptionally related process, this experiment was repeated except that this time aliquots were taken after shorter durations of transcription. As shown in Figure 5, the level of transfer to competitor was 10%, 22%, and 32% for the 1 min (A), 2 min (B), and 5 min (C) time points, respectively. This gradual increase in the transfer of histones with increasing lengths of transcription is suggestive of a displacement of

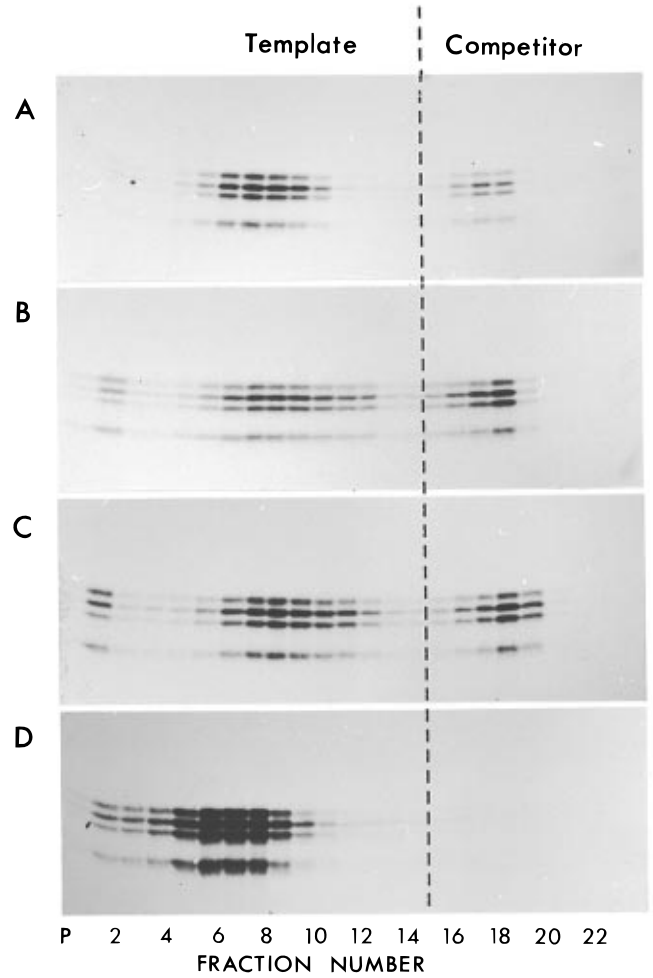

FIGURE 5: Sucrose gradient analysis of the histone distribution between template and competitor DNA with increasing durations of transcription. The reconstituted sample was transcribed in the presence of PIAN7 for 1 min (A), 2 min (B), and 5 min (C). (D) 10 min incubation in the absence of transcription. Samples were treated with RNase A before applying to the sucrose gradients as described in the legend to Figure 4.

these histones from template DNA to the RNA transcripts, a displacement that is observed indirectly by the removal of the RNA with RNase A treatment and a subsequent binding of the histones to the competitor DNA. Further support for the transcriptional dependence of this process is shown in the data of Figure 5D in which an incubation was done in the absence of UTP. No histones are found associated with the competitor DNA. We conclude that transcription is causing the loss of histones from the template DNA.

Histones Preferentially Bind RNA at Physiological Ionic Strength. The data of Figure 4A indicated that histones preferentially bound RNA during the transcription process, even when a competitor DNA was present. Does this observation reflect an actual preference for binding RNA or does it indicate the involvement of a proximity effect? A proximity effect could occur in this situation because the nascent RNA is transiently closer to the disrupted nucleosome as compared to the competitor DNA. This question can be addressed by studying the interaction of histones with a mixture of RNA and DNA. PIAN7-T7 was transcribed with T7 RNA polymerase to produce a mixture of RNA and DNA with a ratio of 5:l (w/w). The sample was reconstituted with ^{3}H-labeled histones at a ratio of 0.4:1 (H:D), and after sedimentation on a sucrose gradient, the distribution of RNA, DNA, and histones was determined. The reconstitution was done by two different protocols. In Figure 6A, the protocol

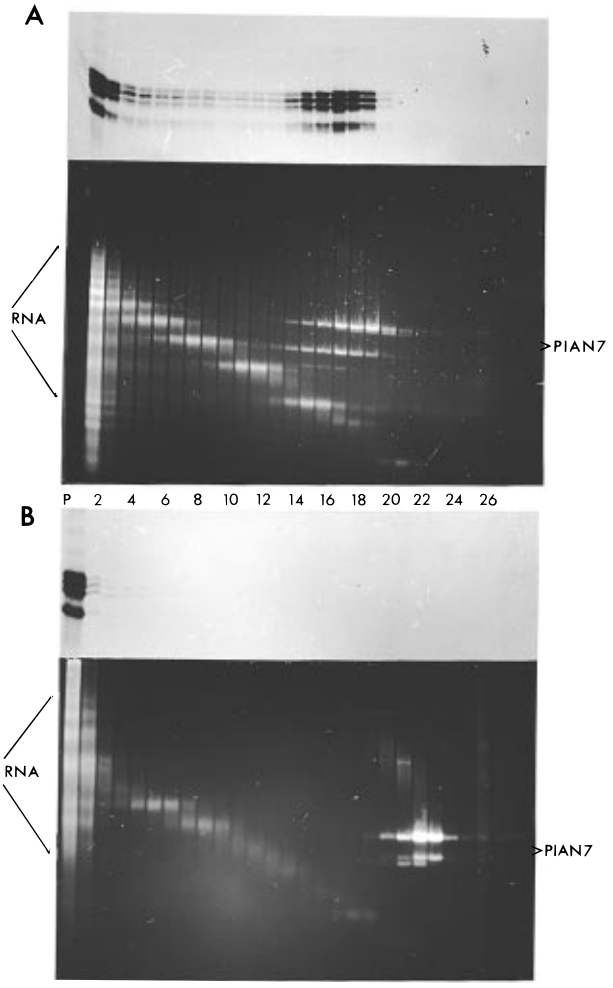

FIGURE 6: Reconstitution of histones onto a mixture of RNA and DNA. (A) Reconstituted by NaCl dialysis. (B) Reconstituted by direct addition of histone. Relaxed PIAN7-T7 DNA was transcribed in the absence of topoisomerase I until a RNA:DNA ratio of 5:1was obtained. This was added to the histones in the above reconstitution conditions to obtain a ratio of 0.4:5:1 (histone:RNA:DNA). After an incubation of 10 min at 37 °C, the samples were centrifuged at 20000*g* for 10 min to remove insoluble complexes. No insoluble complexes were detected (data not shown). The sample was then sedimented at 35 000 rpm for 10 h to separate the RNA from the DNA. The upper and lower segments of each panel show the distribution of histones and of RNA and DNA, respectively.

involved assembly of the components in 2 M NaCl (see Materials and Methods) and a slow dialysis to physiological ionic strength, and in Figure 6B the histones were rapidly added to the mixture of RNA and DNA at physiological ionic strength. When the reconstitution was done by NaCl dialysis (Figure 6A), the majority of the histones (80%) are associated with the DNA. Since there is a 5-fold higher content of RNA than DNA, the histones must have a preference for binding DNA that is estimated to be at least 25 times greater than for RNA. When the reconstitution was done by direct addition (Figure 6B), a completely opposite result is observed. The histones are found almost exclusively with the RNA at an estimated 100-fold greater affinity, as compared to the DNA. One possible interpretation of these data is that during the NaCl dialysis, the slow removal of salt allows the histones to interact and establish a nucleosomal state, a condition which increases stability of the histone−DNA interactions. When histones are added by direct addition at physiological ionic strength, the rapid binding does not favor the formation of nucleosomes before the more favored binding to the single-stranded RNA occurs. Thus, during

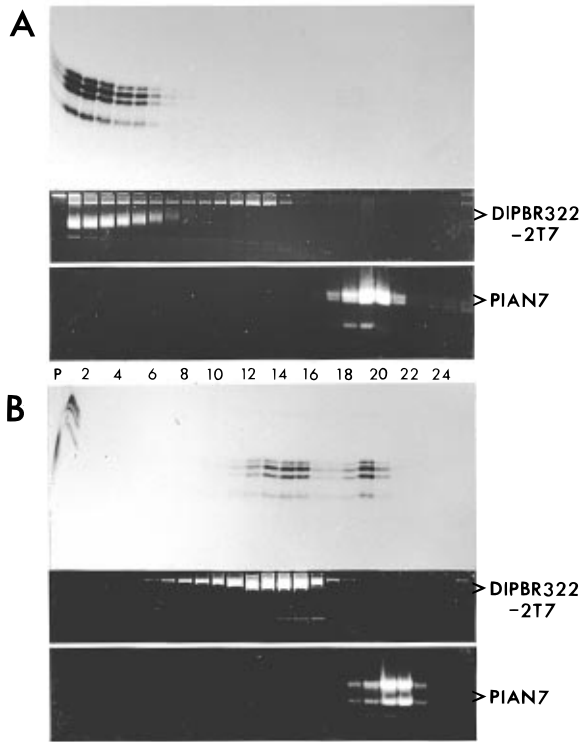

FIGURE 7: Sucrose gradient analysis of (A) the stability of histone−DNA complexes in the presence of RNA and (B) the binding preferences of histones for DIPBR322-2T7 and PIAN7. The upper segment of each panel is an autoradiograph of an SDS−PAGE gel, and the lower segment is the ethidium bromide stained agarose gel.

the transcription process, the selective binding of histones to the newly synthesized RNA is probably a reflection of an inherent structural characteristic of RNA.

RNA Is Unable To Displace Histones from DNA. If our interpretation of Figure 6 is correct, RNA should be unable to extract histones from nucleosomal DNA. If incorrect, it remains possible that the transfer of histones to RNA during transcription is substantially independent of a processive T7 RNA polymerase and is purely dependent on the avidity of histones for RNA. To test for this possibility, a transcription mix was prepared which contained all the components (template and competitor) as was described for the experiment of Figure 4A, except that UTP was excluded. To this mixture was added an excess of RNA (10:1 relative to the template DNA) which had been prepared as described for the data of Figure 6. This sample was then incubated at 37 °C for 10 min, and after removal of the added RNA by RNase A treatment, the sample was analyzed on a sucrose gradient. As shown in Figure 7A, all of the histones remain bound to the DIPBR322-2T7 DNA. If the RNA had removed any of these histones, the subsequent treatment with RNase A would have facilitated the transfer of some of those histones to the competitor DNA. Since this is not observed, the active process of transcription by the RNA polymerase is still assumed to be the causative factor for the disruption of the nucleosomes.

Histones Displaced from RNA by RNase A Treatment Bind to both Competitor and Template. To more accurately quantitate histone displacement during transcription, it is necessary to determine the level at which the histones rebind the template after displacement from the RNA by RNase A treatment. ^{3}H-Labeled histones were directly added to RNA which had been prepared as described for Figure 6B. As was shown in that figure, the histones are selectively bound

to the RNA. This solution was then added to a transcription mix which contained both competitor and template at a ratio of 1:2 (C:T). The template DNA had previously been reconstituted with unlabeled histones at a ratio of 0.2:1. This particular reconstitution was done to mimic the estimated quantity of histones which would remain after transcription for 10 min. This estimation was based on the data of Figure 2B which indicated that 70% of the negative coils are lost after 10 min of transcription for a reconstitute with a histone to DNA ratio of 0.6:1. If this loss of negative coils is a direct reflection of loss of histones from the template DNA, then 30% of the original histones (0.6:1, H:D) would remain on the template DNA with a histone to DNA ratio approximating 0.2:1. This mixture was then incubated for 10 min at 37 °C in the absence of UTP to prevent new RNA formation. RNase A was added to displace the labeled histones and allow them to distribute between the competitor and reconstituted template. As shown in the SDS gel of Figure 7B, 63% of the labeled histones are bound to the template, which is a distribution reflective of the quantity of free DNA that is present in the competitor versus the template (free DNA, 1:1.6). This observation provides an explanation for a discrepancy in the number of negative coils which remain after transcription (Figure 2B) and the quantity of histone transferred to competitor (Figure 4B). The 70% of negative coils that are lost from the template represent histones displaced to RNA. Subsequently, these histones are displaced by RNase A treatment to both competitor and template (free DNA) which yields a histone distribution in which 40% of the histones are bound to the competitor DNA and 30% are rebound to the template.

The Majority of the Transferred Histones Are Capable of Supercoiling DNA. It was of interest to determine the native state of these histones after being both displaced by the polymerase during transcription and displaced from the RNA during RNase A treatment. DIPBR322-2T7 DNA was reconstituted with histones at a ratio of 0.6:1 (H:D) and transcribed for 5 min after which EDTA was added to terminate transcription. As shown in Figure 8A, 40% of the negative coils remain on the template. Two aliquots were taken from this sample, one of which was treated with RNase A, and both were incubated 120 min at 37 °C. As shown in Figure 8A, the RNase A treatment increases the negative coils on the template from 40% back to over 70% of the original coils. Without RNase A treatment, no change is observed. We estimate that of the 60% of histones which transferred, more than half of them must be capable of forming negative coils when reassociating with the template DNA. Since no change is observed without the RNase A treatment, the RNA must be holding the histones so tenaciously that they cannot be released even after 120 min at 37 °C.

In the previous transcription studies (Figures 4 and 5), competitor DNA was present during the transcription process, yet based on the selective binding of the histones to RNA, one might predict that the competitor DNA need not be present except during the RNase A treatment to visualize the histone transfer. At that time, the histones can transfer to both template and competitor DNA equally well. To test this prediction, reconstituted complexes (0.6:1, H:D) were prepared and transcribed for 10 min. As shown in Figure 8B, 70% of the negative coils are removed after this length of transcription. The transcription was then terminated by addition of EDTA, and increasing quantities of PIAN7 were added to separate aliquots. RNase A was then added and

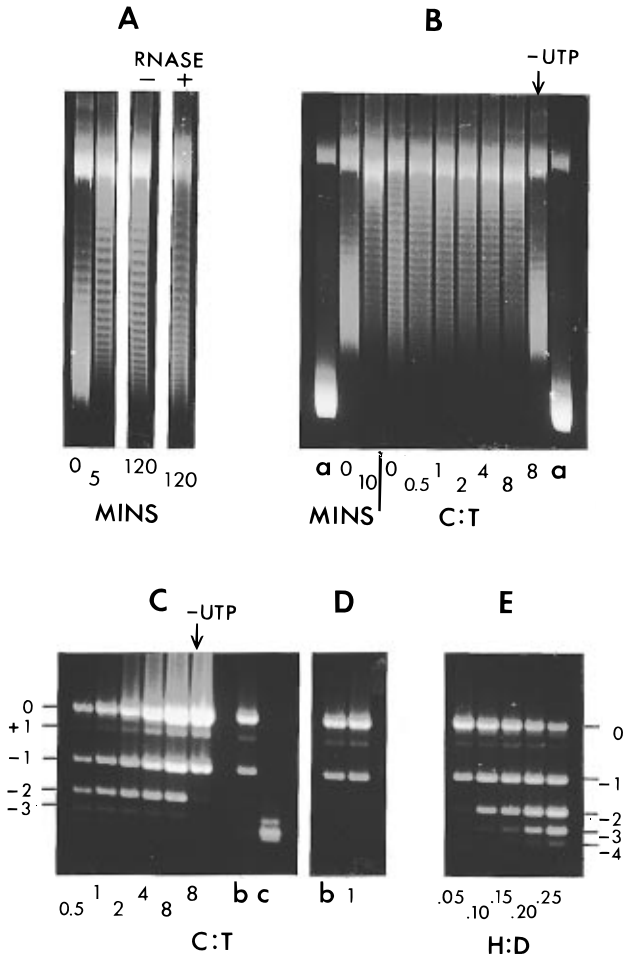

FIGURE 8: Effect of RNase A treatment on the topological state of the template and competitor DNA. (A) After transcription for 5 min at 37 °C, the sample was adjusted to a final concentration of 10 mM EDTA and incubated with and without RNase A for 120 min. (B) After transcription for 10 min, separate aliquots were treated with RNase A in the presence of increasing quantities of competitor DNA (PIAN7) to obtain a competitor to template ratio (C:T) between 0 and 8. The competitor was pretreated with topoisomerase I just prior to addition, so that the DNA was in a relaxed state prior to exposure with the transcription mix. After an incubation of 60 min at 37 °C, the reaction was terminated by treatment with 'Stop Buffer'. The lanes in panel B show the topological state of the template DNA (DIPBR322-2T7). The lane marked −UTP represents an incubation with competitor DNA in which transcription had not occurred. (C) The topological state of the competitor DNA (PIAN7) at the different C:T ratios for the experiment of panel B. (D) Same as for panel B except that RNase A was not present during the incubation. Data for only the C:T ratio of 1:1 are shown. (E) Histones were reconstituted by NaCl dialysis with PIAN7 DNA at ratios of (0.05−0.25):1 (H:D) to obtain the standards for comparison with panel C. Lanes a and c are mobility markers of the negatively coiled DNAs DIPBR322-2T7 and PIAN7, respectively. Lane b is PIAN7 DNA that had been relaxed by topoisomerase I. The numbers on the sides of panels C and E are the number of negative coils present on the PIAN7 DNA.

the incubation continued for 60 min. As shown in Figure 8B, increasing the competitor to template ratio (C:T) decreases the number of negative coils that are regenerated on the template DNA. At a ratio of 8:1, the number of negative coils on the template DNA is nearly the same as were present after the initial 10 min of transcription. Figure 8C shows the topological state of the competitor DNA for each of these samples. When compared to the relaxed state of the PIAN7 DNA (lane b), there is a proportional increase in the quantity of the −2 negative coil on the competitor DNA. These results are interpreted as indicating that the

majority of histones have transferred from the RNA to the competitor when used at these higher ratios. These data also indicate that the transfer of histones to RNA is transcription-dependent. This conclusion is based on the control of Figure 8B,C in which UTP was excluded in a sample containing a C:T ratio of 8:1. There is no release of histones from the template (compare to the 0 time point in Figure 8B) nor is there any formation of negative coils in the competitor (Figure 8C). To demonstrate the importance of RNase A treatment in facilitating the transfer from the RNA, the data of Figure 8D are also shown. In this instance, an aliquot of the 10 min transcription mix was incubated with the competitor for 60 min in the absence of RNase A. No increase in negative coils on the PIAN7 DNA is observed when compared to the relaxed DNA control (lane b). In addition, we have repeated the experiment of Figure 4B in which we characterized the transfer of radiolabeled histones to competitor. In this instance, the competitor was added after transcription rather than before. After RNase A treatment and separation on a sucrose gradient, 40% of the radiolabeled histones transferred to the competitor DNA (data not shown). Since this is the same level of transfer that was observed in Figure 4B, we conclude that it does not matter when the competitor is added. The RNA is an exclusive sink for the displaced histones.

We estimated from the data of Figure 8A that more than half the histones which transferred to the template from the RNA were able to form negative coils. The data of Figure 8C may also be used to provide a more accurate assessment of the native character of these transferred histones. The competitor DNA is smaller, and it is, therefore, easier to separate and quantitate the negative coils. Previous data had indicated that at a competitor to template ratio of 1:1, histones will transfer equally well to both the competitor and the free DNA of the template (Figure 7B). If 70% of the histones are displaced from the template to the RNA (Figure 8B, 10 min time point), 40% of the histones will transfer to the competitor in the presence of RNase A (Figure 4B). Since the original histone to DNA ratio on the template was 0.6:1, 40% of 0.6 or 0.24:1 is the predicted histone to DNA ratio that is present on the competitor. It is now necessary to determine the number of negative coils that would be expected for such a ratio, if all the histones maintained their native state. Standards were prepared, as shown in Figure 8E, in which histones were reconstituted with DNA at ratios of 0.05:1 to 0.25:1 (H:D), and these were treated with topoisomerase I at 37 °C for 60 min. It is assumed that the number of negative coils on the DNA standards represents 100% supercoiling activity by those histones. By comparing these data with the data of Figure 8D at the competitor to template ratio of 1:1, it can be seen that the number and quantity of negative coils are equivalent to a ratio of 0.15:1 in the standards. An estimated 0.15/0.24 or 63% of the transferred histones are capable of re-forming negative coils.

Isolation and Characterization of the Active Transcription Complex. It would be useful to have an alternative approach to evaluate the quantity of histone which remains on the DNA after transcription. Even more useful would be a method which could trap the transcription complex with its associated RNA polymerase, RNA, and template in order to evaluate the nucleosomal state of only those templates that are in an active state of transcription. We have previously utilized formaldehyde cross-linking to characterize nucleosomal structure on topologically stressed DNAs (Jackson, 1993, 1995). This procedure is also useful for trapping

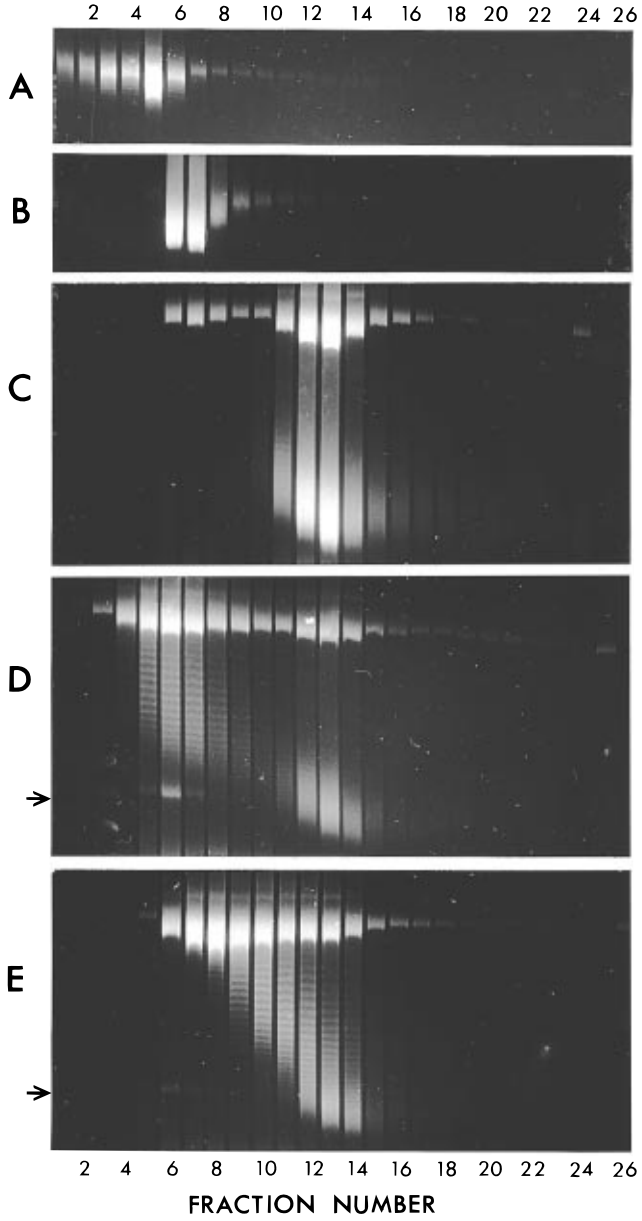

FIGURE 9: Isolation of transcription complexes on CsCl−GuHCl gradients. (A and B) DIPBR322-2T7 DNA was transcribed for 5 min followed by treatment with 1% formaldehyde and centrifugation to equilibrium on the density gradients. (A) Without RNase A treatment before the centrifugation. (B) With RNase A treatment before the centrifugation. (C−E) DIPBR322-2T7 DNA was reconstituted with histones at a ratio of 0.8:1 (H:D) and incubated for 10 min in transcription conditions without (C) and with (D) UTP. The samples were then processed as described for panel A. (E) Same as panel D except that the sample was treated with RNase A prior to centrifugation in the gradient. The arrows on the left side of panels D and E mark the location of a DNA (10 kb) that was included in the gradient as a reference point to indicate the region of the gradient in which naked DNA is located (fractions 6 and 7).

transcription complexes. DIPBR322-2T7 DNA was transcribed for 5 min with T7 RNA polymerase and treated with 1% formaldehyde for 20 h, and after removal of the excess reagent, the sample was split into two fractions, one of which was incubated with RNase A for 30 min. Both samples were then centrifuged to equilibrium in CsCl−GuHCl gradients, and the distribution of DNA was determined by agarose gel electrophoresis as shown in Figure 9A,B. In the absence of RNase A treatment, the DNA is distributed in fractions 1−6 (Figure 9A) and after RNase A treatment in fractions 6 and 7 (Figure 9B). RNase A has removed the RNA from the template, RNA which had been cross-linked to the template

by way of the polymerase. Free RNA will normally distribute in fractions 1−3, and T7 RNA polymerase cross-links to DNA (data not shown). The formaldehyde treatment has stabilized the transcription complex. This approach was then applied to template DNA which had been reconstituted with histones at a ratio of 0.8:1 (H:D). Figure 9C,D shows the distribution of the template DNA after an incubation of 10 min with and without transcription. Without transcription (Figure 9C), the DNA is distributed in fractions 12−14 and is an indication of the quantity of histone that was cross-linked to the DNA. With transcription (Figure 9D), the template DNA is now distributed into two separate regions of the gradient. Fractions 12−14 contain the highly negatively coiled DNA, and fractions 5−7 contain DNA with a much lower number of negative coils. This increased density for the DNA may result from two components. It may be caused by the lower number of histones associated with the DNA, as reflected by the lower number of negative coils, and/or by the presence of RNA cross-linked to the DNA by way of the polymerase. Figure 9E shows the results when the transcribed template was treated with RNase A after fixation with formaldehyde. The DNA with the lower number of negative coils is no longer distributed in fractions 5−7, but is now distributed in fractions 9−12. A major cause for the altered density is the presence of RNA. The DNA that is present in fractions 5−7 of Figure 9D is DNA that was trapped in active transcription. The reduced number of negative coils in that DNA is an independent verification that transcription is the causative factor for the loss of the negative coils. From these data, we are also able to determine the actual percentage of template DNA that was active. At a histone to DNA ratio of 0.8:1 for the 10 min time point, 52% of the templates were active. This value is determined by a comparison of DNA content (topologically restrained) in fractions 5−7 and fractions 12−14. This conclusion is consistent with our previous interpretation that at this histone to DNA ratio, initiation by the polymerase is restricted and is the cause for the biphasic distribution of negative coils that was seen in Figure 2C after 10 min of transcription. Additional information is obtainable from the data of Figure 9E. The difference in density of the template DNA is directly proportional to the number of negative coils that are present on the DNA. Since the RNase A treatment has removed the RNA, this difference in density must be due to differences in the quantity of histone cross-linked to the DNA. Because of this proportional relationship, the number of negative coils that are present after transcription (Figure 2) must be a fairly accurate reflection of the quantity of histones which remain on the template DNA.

DISCUSSION

The goal of this study was to determine, by a direct analysis of competitor and template DNA, the quantity of histone that is displaced during transcription at physiological ionic strength using a topologically restrained template. The results indicate that substantial displacement occurs. This conclusion is based on the inability of the histones to maintain the negative coils on the DNA during the transcription process (Figure 2) and on the presence of histones on the competitor DNA (Figures 4 and 5). By using formaldehyde cross-linking, we were able to trap the transcription complex with its associated T7 RNA polymerase and RNA and demonstrate directly that those templates which are transcribing are the templates with the reduced number of negative coils (Figure 9D). From the data of Figure 9E, we observed a close relationship between the number of negative coils and histone content. This result indicates that the number of negative coils in the template DNA of Figure 2 is an accurate reflection of the quantity of histone present on the template during transcription. Therefore, by comparing values for the transcription rate (Figure 3) with values for the number of histones present on the active reconstituted template (Figures 2, 4, 5, and 9D), it is possible to estimate the frequency at which disruption occurs on the nucleosomes. This calculation is also based on the assumption that on average only one of two promoters within the DIPBR322-2T7 template DNA is available for initiation in the reconstitute with the 0.4:1 (H:D) ratio. This assumption is experimentally verified by the low level of initiation that occurs on a plasmid with a single promoter (Figure 2D). From a densitometric analysis of Figure 2, transcription for 2 min disrupts 43% of the estimated 20 nucleosomes originally present (Figure 2A, 0 time point) after 1.5 rounds of transcription (Figure 3). This corresponds to 1 nucleosome disrupted per 3.5 nucleosomes transcribed. If this calculation is applied to the reconstitute with the 0.6:1 ratio, the estimate is 1 per 4.5 nucleosomes. These numbers may be an underestimation because of the extensive truncation of the transcripts (40%) that occurs during transcription (Figure 3). However, if we assume that the T7 RNA polymerase can rapidly reinitiate on a promoter and transcribe again, this underestimation may not be significant. We have done control experiments to test for reinitiation and have found this to be the case (data not shown). In this calculation, we have also assumed that one nucleosome holds the equivalent of one negative coil. Norton et al. (1989) have observed that hyperacetylated histones form a nucleosome in which a 0.8 negative coil is maintained. If this is the case in our study, then the number of nucleosomes initially present would be 25 rather than 20, but since our calculation is based on percent disruption, the calculated frequency of the disruption remains unchanged. Based on these considerations, we estimate that on average 1 of 4 nucleosomes is disrupted sufficiently to displace the histones from the DNA with a single passage of the T7 RNA polymerase. Because hyperacetylated histones were used in this study in order to more closely mimic the modified state of histones associated with active genes, the frequency of disruption could possibly be influenced by the level of this modification. However, we have found that histones with low levels of acetylation, as obtainable from chick erythrocytes, continue to maintain the same frequency of disruption (data not shown). In summary, we are not observing an all or none process with respect to histone release during transcription, irrespective of the acetylated state of the histones.

These data indicate that there is considerable stability to the nucleosome during transcription and are therefore supportive of previous *in vitro* observations in which similar conclusions were made (Losa & Brown, 1987; Kirov el al., 1992; O'Neill et al., 1993; Protacio & Widom, 1996). It has also been reported that during transcription of a single nucleosome, the histones will transfer to the retrograde region of the template (Clark & Felsenfeld, 1992; Studitsky et al., 1994, 1995). In our study, it is unknown whether such a transfer occurs for the three of four nucleosomes which do not displace from DNA in a single passage of the polymerase. As the template in our study has a tandem array of nucleosomes, retrograde transfer might be expected to be more severly retarded, since such transfer would be very

dependent on the efficiency of transfer of adjacent nucleosomes in the tandem array. This would be expected to be more of a problem at the higher histone to DNA ratios. However, as the number of displaced histones increases after repeated passages of the polymerase, retrograde transfer may become a more common event for the remaining histones on the template.

These data also indicate that there is instability in the nucleosome during transcription, which is particularly evident after repeated rounds of transcription on the plasmid. These results are therefore consistent with the *in vitro* results made by several other laboratories that have observed this instability (Lorch et al., 1987; O'Donohue et al., 1994; Gallego et al., 1995). These observations may have an analogy to the *in vivo* observations indicating that the frequency of transcription correlates with general depletion of histone content (Karpov et al., 1984). *In vivo* experiments designed to examine the dynamics of histone movement in the nucleus have shown, however, that H3,H4 is generally very resistant to release, in contrast to H2A,H2B (Jackson, 1990). Thus, the frequency of disruption of H3,H4 that is observed in our study is likely to be much higher than what occurs *in vivo*. A possible factor which may regulate nucleosome disruption is the rate of transcription. The eucaryotic transcription rate is between 20 and 40 bases/s (Cox, 1976; Sehgal et al., 1976; Ucker & Yamamoto, 1984; Shermoen & O'Farrell, 1992), which is 3−5-fold slower than the 120 bases/s that was observed with T7 RNA polymerase in this report. These more rapid rates may not allow successful re-formation of histone−DNA interactions prior to histone disruption from DNA. It is quite remarkable that even with this more rapid rate of transcription the majority of the nascent transcripts (60%) are full-length, measuring several kilobases in length. Even for those transcripts which are truncated, the sizes of the transcripts are between 2 and 10 kb (Figure 3). These sites of truncation are unrelated to sequence-directed termination sites that are in the plasmid (compare Figure 3A with Figure 3B,C) and therefore are sites directed by nucleosome location. For the majority of the nucleosomes, however, transcription occurs efficiently and is an indication that these structures are not immovable objects. Additional factors that may be involved in limiting disruption are proteins such as histone H1 (Srebreva & Zlatanova, 1992) or the HMG proteins (Bustin & Reeves, 1996) which were not included in our study. The level of transcription-induced stress that is obtainable in these *in vitro* conditions may also be a limiting factor. We had previously observed that histones H3,H4 preferentially bound highly positively coiled DNA as compared to negatively coiled DNA and hypothesized that this tight binding might facilitate maintenance of H3,H4 on the template DNA during transcription (Jackson, 1995). The neutralization of positive and negative stresses around the plasmid may limit the positive stress obtainable in even the large covalently closed plasmid that was used in this study. It may also be necessary to manipulate topoisomerase I content. Experiments are in progress to evaluate these factors.

A major inconsistency in our study when compared to what has been observed *in vivo* is the lack of a selective displacement during transcription of histones H2A,H2B relative to H3,H4 (Jackson, 1990). However, it should be noted that H2A,H2B exclusively associate with DNA containing H3,H4 (Brooks & Jackson, 1994; Jackson et al., 1994) and since the competitor DNA lacks these histones one would not necessarily expect to observe a selective release of those histones. Based on studies which have characterized transcription on nucleosomes depleted of H2A,-H2B, such a depletion might be expected to enhance transcription (Baer & Rhodes, 1983; Gonzalez & Pallacian, 1989; Puerta et al., 1993; Hansen & Wolffe, 1994). It is also unclear in our experiments whether the four histones which are transferred to the RNA are transferred as an intact octameric complex. The octamer is not stable at physiological ionic strengths when not associated with a polyanion (Feng et al., 1993), and unless there is facilitated transfer from one polyanion to another, the octamer is not likely to remain intact.

The data of Figure 6 indicated that histones preferentially bind single-stranded RNA as compared to double-stranded DNA at physiological ionic strength. We estimate this preference to be at least 100-fold. It is therefore not surprising that during the transcription reaction histones are displaced to the RNA rather than the competitor DNA. It is therefore important in any study of this type to evaluate the integrity and quantity of RNA that is synthesized when evaluating the mobility of histones. Our data indicate that the histones can be displaced from the RNA by RNase A treatment and that 63% of these histones are capable of reforming nucleosomes (Figure 8). Nelson et al. (1979, 1981) described an observation that *Drosophilia* embryo extracts contained a factor which served as a deposition vehicle for nucleosome formation on DNA. They found this factor to be RNA and in subsequent experimentation indicated that generic single-strand RNA of large molecular weight was sufficient for this activity. Our observations are consistent with these observations and indicate further that RNA degradation during the incubation is required to facilitate the release. Gallego et al. (1995) reported that during transcription of a small template containing a single nucleosome, histones were displaced to competitor DNA and did not interact with the RNA. In their experiment, the RNA that was synthesized was 221 bases in length which from our observations (data not shown) and those of Nelson et al. (1981) is not of sufficient length to be an effective competitor. The question then needs to be addressed as to whether this high affinity of histones for RNA has physiological relevance. Since nascent RNA is very rapidly packaged by heterogeneous nuclear RNA binding proteins (hnRNP) in *in vivo* conditions (Dreyfuss et al., 1993), these proteins would be expected to prevent histone interaction with RNA. We have observed that NaCl concentrations greater than 0.7 M are required to displace H3,H4 from the RNA (data not shown). It is also known that at NaCl concentrations of 0.7 M, 50% of the hnRNP proteins are displaced and another 30% are removed at 1.0 M NaCl (Pandolfo et al., 1987). Therefore, H3,H4 could compete effectively with these proteins for RNA, but only transiently so because of the excess of these hnRNP proteins in the nucleus. The histones would be expected to be rapidly displaced back to the double-stranded DNA for which these hnRNP proteins are unable to compete. The treatment with RNase A in our studies is a simulation of this potential displacement which has allowed us to evaluate the ability of the displaced histones to reform nucleosomes. It is also known that the distribution of the hnRNPs is nonrandom because of considerable sequence specificity (Matunis et al., 1993; Burch & Dreyfuss, 1994). Particular regions of the RNA would be expected to have a greater susceptibility to histone interaction. Since the *in vivo* studies have suggested that histones H3,H4 are minimally displaced during transcription (Jackson, 1990), this transfer

to the RNA is not expected to be extensive. Whether RNA might serve as a transient storage vehicle for newly synthesized histones remains to be determined.

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REFERENCES

Adams, C. C., & Workman, J. L. (1993) *Cell 72*, 305−308.
Arents, G., & Moudrianakis, E. N. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10489−10493.
Arents, G., Burlingame, R. W., Wang, B., Love, W. E., & Moudrianakis, E. N. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10148−10152.
Baer, R. W., & Rhodes, D. (1983) *Nature 31*, 482−488.
Bonner, G., Lafer, E. M., & Sousa, R. (1994) *J. Biol. Chem. 269*, 25120−25128.
Brooks, W., & Jackson, V. (1994) *J. Biol. Chem. 269*, 18155−18166.
Burch, C. G., & Dreyfuss, G. (1994) *Science 265*, 615−621.
Bustin, M., & Reeves, R. (1996) *Prog. Nucleic Acid Res. Mol. Biol. 54*, 35−100.
Chamberlin, M., & Ring, J. (1973) *J. Biol. Chem. 248*, 2245−2250.
Clark, D. J., & Felsenfeld, G. (1992) *Cell 71*, 11−22.
Cotton, R. W., & Hamkalo, B. A. (1981) *Nucleic Acids Res. 9*, 445−458.
Cox, R. F. (1976) *Cell 7*, 455−465.
Csordas, A. (1990) *Biochem. J. 265*, 23−38.
Daban, J. R., & Cantor, C. R. (1982) *J. Mol. Biol. 156*, 749−769.
Dreyfuss, G., Matunis, M. J., Pinol-Roma, S., & Burd, C. G. (1993) *Annu. Rev. Biochem. 62*, 289−322.
Felsenfeld, G. (1992) *Nature 355*, 219−224.
Felts, S. J., Weil, P. A., & Chalkley, R. (1990) *Mol. Cell. Biol. 10*, 2390−2401.
Feng, H. P., Scherl, D. S., & Widom, J. (1993) *Biochemistry 32*, 7824−7831.
Gallego, F., Fernandez-Busquets, X., & Daban, J. R. (1995) *Biochemistry 34*, 6711−6719.
Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M., & Chambon, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1843−1847.
Godde, J. S., & Wolffe, A. P. (1995) *J. Biol. Chem. 270*, 27399−27402.
Gonzalez, P. J., & Pallacian, E. (1989) *J. Biol. Chem. 264*, 18457−18462.
Gross, D. S., & Garrard, W. T. (1988) *Annu. Rev. Biochem. 57*, 159−198.
Hansen, J. C., & Wolffe, A. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2339−2343.
Ip, T. Y., Jackson, V., Meier, J., & Chalkley, R. (1988) *J. Biol. Chem. 263*, 14044−14052.
Izban, M. G., & Luse, D. S. (1991) *Genes Dev. 5*, 683−696.
Jackson, S., Brooks, W., & Jackson, V. (1994) *Biochemistry 33*, 5392−5403.
Jackson, V. (1978) *Cell 15*, 945−954.
Jackson, V. (1990) *Biochemistry 29*, 719−731.
Jackson, V. (1993) *Biochemistry 32*, 5901−5912.
Jackson, V. (1995) *Biochemistry 34*, 10607−10619.
Jackson, V., Shires, A., Tanphaichitr, N., & Chalkley, R. (1976) *J. Mol. Biol. 104*, 471−483.
Jorcano, J. L., & Ruiz-Carrillo, A. (1979) *Biochemistry 18*, 108−113.
Karpov, V. L., Preobrazhenskaya, O. V., & Mirzabekov, A. D. (1984) *Cell 36*, 423−431.
Keller, W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4876−4880.
King, G. C., Martin, C. T., Pham, T. T., & Coleman, J. E. (1986) *Biochemistry 25*, 36−40.
Kirov, N., Tsaneva, I., Einbinder, E., & Tsanev, R. (1992) *EMBO J. 11*, 1941−1947.
Kornberg, R. D., & Lorch, Y. (1991) *Cell 67*, 833−836.
Kornberg, R., & Lorch, Y. (1995) *Curr. Opin. Cell Biol. 7*, 371−375.
Laskey, R. A., & Mills, A. D. (1979) *Eur. J. Biochem. 56*, 335−342.
Lilley, D. M. J., Jacobs, M. F., & Houghton, M. (1979) *Nucleic Acids Res. 7*, 377−399.
Liu, L. F., & Miller, K. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3487−3491.
Liu, L. F., & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7024−7027.
Lorch, Y., LaPointe, J. W., & Kornberg, R. D. (1987) *Cell 49*, 203−210.
Losa, R., & Brown, D. D. (1987) *Cell 50*, 801−808.
Matunis, E. L., Matunis, M. J., & Dreyfuss, G. (1993) *J. Cell Biol. 121*, 219−228.
Morse, R. H. (1989) *EMBO J. 8*, 2343−2351.
Morse, R. H. (1992) *Trends Biochem. Sci. 17*, 23−26.
Nelson, T., Hsieh, T., & Brutlag, D. (1979) *Proc. Natl. Acad Sci. U.S.A. 76*, 5510−5514.
Nelson, T., Wiegland, R., & Brutlag, D. (1981) *Biochemistry 20*, 2594−2601.
Norton, V. G., Imai, B. S., Yau, P., & Bradbury, E. M. (1989) *Cell 57*, 449−457.
O'Donohue, M. F., Duband-Goulet, I., Hamiche, A., & Prunell, A. (1994) *Nucleic Acids Res. 22*, 937−945.
O'Neill, T. E., Roberge, M., & Bradbury, E. M. (1992) *J. Mol. Biol. 223*, 67−78.
O'Neill, T. E., Smith, J. G., & Bradbury, E. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6203− 6207.
Pandolfo, M., Valentini, O., Biamonti, G., Rossi, P., & Riva, S. (1987) *Eur. J. Biochem. 162*, 213−220.
Pfaffle, P., & Jackson, V. (1990) *J. Biol. Chem. 265*, 16821−16829.
Pfaffle, P., Gerlach, V., Bunzel, L., & Jackson, V. (1990) *J. Biol. Chem. 265*, 16830−16840.
Protacio, R. U., & Widom, J. (1996) *J. Mol. Biol. 256*, 458−472.
Puerta, C., Hernandez, F., Gutierrez, C., Pineiro, M., Lopez-Alarcon, L., & Palacian, E. (1993) *J. Biol. Chem. 268*, 26663−26667.
Riggs, M. G., Whittaker, R. G., Neumann, J. R., & Ingram, V. (1977) *Nature (London) 268*, 462−464.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. J. (1975) *Science 190*, 117−128.
Sehgal, P., Derman, E., Molloy, G. R., Tamm, I., & Darnell, J. E. (1976) *Science 194*, 431−433.
Shermoen, A. W., & O'Farrell, P. H. (1992) *Cell 67*, 303−310.
Simon, R. H., & Felsenfeld, G. (1979) *Nucleic Acids Res. 6*, 689−696.
Simpson, R. T., Thomas, F., & Brubaker, J. M. (1985) *Cell 42*, 799−808.
Sobel, R. E., Cook, R. G., Perry, C. A., Annunziato, A. T., & Allis, C. D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1237−1241.
Srebreva, L. N., & Zlatanova, J. S. (1992) *Mol. Cell. Biochem. 110*, 91−100.
Stein, A. (1979) *J. Mol. Biol. 130*, 103−134.
Studitsky, V. M., Clark, D. J., & Felsenfeld, G. (1994) *Cell 76*, 371−382.
Studitsky, V. M., Clark, D. J., & Felsenfeld, G. (1995) *Cell 83*, 19−27.
Thoma, F. (1991) *Trends Genet. 7*, 175−177.
Turner, B. M. (1993) *Cell 75*, 5−8.
Ucker, D. S., & Yamamoto, K. R. (1984) *J. Biol. Chem 259*, 7416−7420.
van Holde, K. E., Lohr, D. E., & Robert, C. (1992) *J. Biol. Chem. 267*, 2837−2840.
Wolffe, A. P., & Drew, H. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9817−9821.
Zweidler, A. (1978) *Methods Cell Biol. 18*, 223−233.

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