Dynamics of the Interactions of Histones H2A,H2B and H3,H4 with Torsionally Stressed DNA†

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ABSTRACT: The interactions of histones H2A,H2B and H3,H4 with closed circular DNA maintained in either a positively or negatively coiled state have been studied. The interactions were assayed by measuring the rate at which negative stress was stored in the DNA by the histones and by the salt concentration sufficient to cause dissociation on sucrose gradients. Additional experiments were performed in which DNAs of substantially different molecular weights and opposite topological states were mixed with the histones in order to study histone mobility under varied conditions. This mobility was characterized by separating the complexes on sucrose gradients and by analyzing the DNA's topological state after topoisomerase I treatment. Histones H3,H4 were found to differ substantially from histones H2A,H2B with regard to the DNA topology with which they prefer to interact. The results are consistent with a model in which transcription-induced positive stress in advance of the RNA polymerase unfolds the nucleosome to facilitate the release of H2A,H2B. The data are also consistent with a model in which histones H3,H4 remain associated with the DNA during polymerase passage and serve as a nucleation site for the reassociation of H2A,H2B. The rapid production of transcription-induced negative stress in the wake of a polymerase would have substantial importance in facilitating the reassociation of histones H2A,H2B.

Transcription through a nucleosome has been the subject of renewed interest in the light of recent experiments indicating that transcription can induce localized topological stresses in DNA. In 1987, Liu and Wang proposed that an RNA polymerase would be inhibited from rotating around a DNA helix due to the viscous drag of a growing RNA transcript. This lack of rotation would cause topological stresses in the DNA since the polymerase must separate 10–15 bp of DNA during elongation (Gamper & Hearst, 1982). An overwinding of the DNA helix (positive stress) would be observed in advance of the polymerase and an underwinding (negative stress) in its wake. *In vivo* experimental evidence in support of this model has been obtained for both eukaryotic (Brill & Sternglanz, 1988; Giaever & Wang, 1989; Ljungman & Hanawalt, 1992; Gartenberg & Wang, 1992) and prokaryotic systems (Wu et al., 1988; Pruss & Drlica, 1989). Additional support has been obtained from *in vitro* model systems (Tsao et al., 1989). Such stresses could influence the structure of a nucleosome.

A basic subunit of chromatin structure is the nucleosome, which consists of 145 bp of DNA coiled leftward 1.8 times around a histone core containing two of each of the histones H2A, H2B, H3, and H4 (Travers & Klug, 1990). If the DNA topology is analyzed after histones are removed from covalently closed, circular DNA containing a complement of nucleosomes, a correlation of one negative coil for each nucleosome is observed (Keller, 1975; Germond et al., 1975). Of particular interest is that a negative coil is stored by the histones of a nucleosome due to the leftward direction of the superhelix. Transcription-induced positive stress would tend to create a right-handed, unrestrained superhelix, which would apply disruptive pressure on the histone–histone and histone–DNA contacts of the nucleosomes. The effect of transcriptionally induced positive stress on nucleosome structure has been tested by both *in vitro* (Pfaffle et al., 1990) and *in vivo* studies (Lee & Garrard, 1991). For both studies, an altered nucleosome structure was observed. The nucleosomes were very sensitive to cleavage by micrococcal nuclease and DNase I, characteristics that have been observed in active genes *in vivo* [reviewed in Gross and Garrard (1988)]. The *in vitro* experiments (Pfaffle et al., 1990) also indicated that histones were unable to store the negative coil when excess topoisomerase I was present during transcription. That this process was dependent on transiently induced positive stress was shown by including RNase A during the transcription reaction. The storage of negative stress by the histones was then maintained, even though transcription continued to occur. The presumed free rotation of the RNA polymerase did not disrupt the storage of the negative coils. Additional experiments were performed in that report in which topoisomerase I was absent from the transcription reaction and added only immediately prior to the termination of the reaction in order to assay the DNA topology. The histones continued to store negative coils. These observations were interpreted as indicating that positive stress caused disruption of the nucleosomes and that the negative stress induced in the wake of the polymerase caused a rapid refolding of nucleosomes. That negative stress causes rapid nucleosome formation has been reported (Pfaffle & Jackson, 1990). These observations are consistent with the *in vivo* observation that DNA topology appears unaffected when transcription occurs in yeast (Pederson & Morse, 1990). The topoisomerase I concentration in these cells would appear to be insufficient to remove the transcription-induced negative coil prior to nucleosome reformation.

Rapid refolding of nucleosome structure would imply that all four histone types are present. Indeed, several *in vivo* studies have indicated that histones H2A, H2B, H3, and H4 are present on actively transcribed class II genes (Walker et al., 1990; Nacheva et al., 1989; Ericsson et al., 1990). One possible conclusion from these data is that RNA polymerase

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transcribes through DNA, without sufficient disruption of histone–DNA contacts, to cause histone release. However, it is known that when histones are present during *in vitro* experiments, transcription rates are substantially slower than the *in vivo* rates (Morse, 1989; Felts et al., 1990; Izban & Luse, 1991; O'Neill et al., 1992). A process is occurring *in vivo* that appears to facilitate transcription through nucleosomes. One possibility is that histones are transiently released during the unfolding process and rapidly reassociate after polymerase passage to reform the nucleosome. *In vivo* studies have indicated that histones H2A,H2B are transiently released during transcription (Jackson, 1990; van Holde et al., 1992). *In vitro* studies on the assembly of nucleosomes on positively coiled DNA have indicated that this stress causes disruption of the interface between H2A,H2B and H3,H4 (Jackson, 1993). Such disruptions have been observed *in vivo* on the basis of the increased accessibility of the H3-sulfhydryl (Johnson et al., 1979; Prior et al., 1983; Chen & Allfrey, 1987; Chen et al., 1990). A disrupted interface would facilitate the selective release of H2A,H2B.

In vitro studies designed to examine the question of histone release have obtained conflicting results [extensively reviewed in Thoma (1991), Kornberg and Lorch (1991), Morse (1992), van Holde et al. (1992), Wolffe (1992), Felsenfeld (1992), and Adams and Workman (1993)]. Answers to this question have ranged from no alteration in nucleosome structure to complete unfolding of the nucleosome and the release of all histones. Much of this conflict may relate to whether transcription-induced stress is produced (circular versus linear template), the effectiveness of the competitor, the character of the DNA sequence (Lorch et al., 1988), the ionic strength of the assay, the efficiency of initiation and elongation of a particular polymerase with a nucleosome, and the sensitivity of the assays. Because of these multiple variables, we have chosen to study the dynamics of histone interaction on DNAs containing moderate levels of positive or negative coils in the absence of RNA polymerase. In this way, we can study the effect of torsional stress on nucleosomal dynamics in the absence of direct RNA polymerase disruption of histone–DNA contacts. The observations of this report indicate that histones H2A,H2B are very different from histones H3,H4 with regard to their interactions with torsionally stressed DNA. The data are consistent with a model in which histones H2A,-H2B are transiently released during transcription and play a major role in initiating the reformation of nucleosome structure after polymerase passage.

MATERIALS AND METHODS

Procedures for Purification of Histones, DNA, and Topoisomerase I. Histones were purified from HTC (hepatoma tissue culture cells) using a modification of the procedure of Simon and Felsenfeld (1979). Purified nuclei were prepared by four washes of 1% Triton X-100, 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM Tris (pH 8.0). Chromatin was then prepared by one wash with 10 mM Tris/10 mM EDTA (pH 8.0), and one wash with distilled water. The chromatin was sheared by sonication, adjusted to 0.6 M NaCl/0.1 M KH_2PO_4 (pH 6.7), and applied to a hydroxylapatite column (CalBiochem). After the column was washed thoroughly, histones H2A,H2B were eluted in a stepwise gradient from 0.7 to 1.1 M NaCl, and histones H3,H4 were eluted from 1.1 to 2.0 M NaCl (0.1 M NaCl steps). After verification by SDS gel electrophoresis, fractions were combined, concentrated on Amicon filters, and stored at −70 °C.

The plasmid DNA used in these studies (pT7/T3-19, Bethesda Research Labs) was grown in HB101 at 37 °C in enriched LB broth (Maniatis et al., 1982). The bacteriophage M13mp19 was grown in TGY at 37 °C in enriched LB broth. The covalently closed supercoiled DNA was purified on CsCl density gradients and yielded DNA containing an average of 14 negative coils for T7/T3-19 and 45 negative coils for M13mp19 at 23 °C, as observed on chloroquin gels (data not shown). This DNA is referred to as S DNA in the text.

To produce the positively stressed, circular DNA, the S DNA was treated with topoisomerase I at 0 °C in buffer conditions of 10 mM $MgCl_2$/10 mM TEA (pH 7.4). The angle between adjacent base pairs in DNA decreases by about 0.011 rotational degree for each degree centigrade decrease (Depew & Wang, 1975; Pulleybank et al., 1975; Wang et al., 1982a). Therefore, when the DNA is relaxed at 0 °C, the repurified DNA will contain an average of +2 coils when the temperature is readjusted to 23 °C. Additional positive coils can be introduced if the buffer contains increasing amounts of $MgCl_2$ (Anderson & Bauer, 1978). For the purposes of our analyses, we prefer T7/T13-19 DNA containing an average of +2.5 coils, which can be achieved by including 10 mM $MgCl_2$ in the buffer. This DNA is referred to as R DNA in the text. The R form of M13 was also made by this same procedure, which produced +8 coils in this larger DNA.

Topoisomerase I was isolated from a chicken leukemic cell line (MSB-1 cells) as previously described (Pfaffle & Jackson, 1990). The enzyme was free of RNase and DNase activity, with a specific activity of 4×10^6 units/mg. One unit is equivalent to the 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C.

Reconstitution of Histone–DNA Complexes. Histones were mixed with DNA in 2 M NaCl, 50 mM TEA, 1 mM EDTA, and 1 mM 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, 1976; Jorcano & Ruiz-Carrillo, 1979; Daban & Cantor, 1982; Simpson et al., 1985). Samples were sedimented at 10000*g* for 5 min to differentiate between soluble and insoluble complexes. The histone and DNA concentrations were determined using extinction coefficients for histones of 4.2 at 230 nm and for DNA of 20.0 at 260 nm (Stein, 1979).

Procedure for the Analysis of Rates of Nucleosome Formation. The reconstituted histone–DNA complexes at 100 μg/mL (DNA concentration) were treated with 400 units/μg of DNA of topoisomerase I at 23 °C. Aliquots were taken, and the reaction was terminated by the addition of an equal volume of stop buffer (0.2% SDS, 40% glycerol, 25 mM EDTA, 0.01% bromophenol blue, and 1 mg/mL proteinase K). The samples were incubated at 37 °C for 3 h, applied to a 1.5% agarose (Calbiochem, type C) gel with a running buffer of 50 mM Tris, 45 mM acetic acid, and 1.25 mM EDTA (pH 8.0), and electrophoresed for 14 h at 85 V and 4 °C. DNA was visualized by ethidium bromide staining. The gels were then photographed, and the negative was used to produce a photographic image on XAR-5 X-ray film (Kodak). In this way, the size of the image can be adjusted to maximize quantitation. The image was then scanned with a modified Gilford densitometer using Nelson analytical software to quantitate the DNA content.

Sucrose Gradient Analysis of Histone–DNA Interactions. The reconstituted complexes (100 μg of DNA in 0.5 mL) were applied to a 5–20% sucrose gradient (11 mL) containing 50 mM TEA/1 mM EDTA (pH 7.4). The gradient also contained NaCl as described in the figure legends. Centrifugation was at 35 000 rpm at 3 °C in a SW41 Ti rotor.

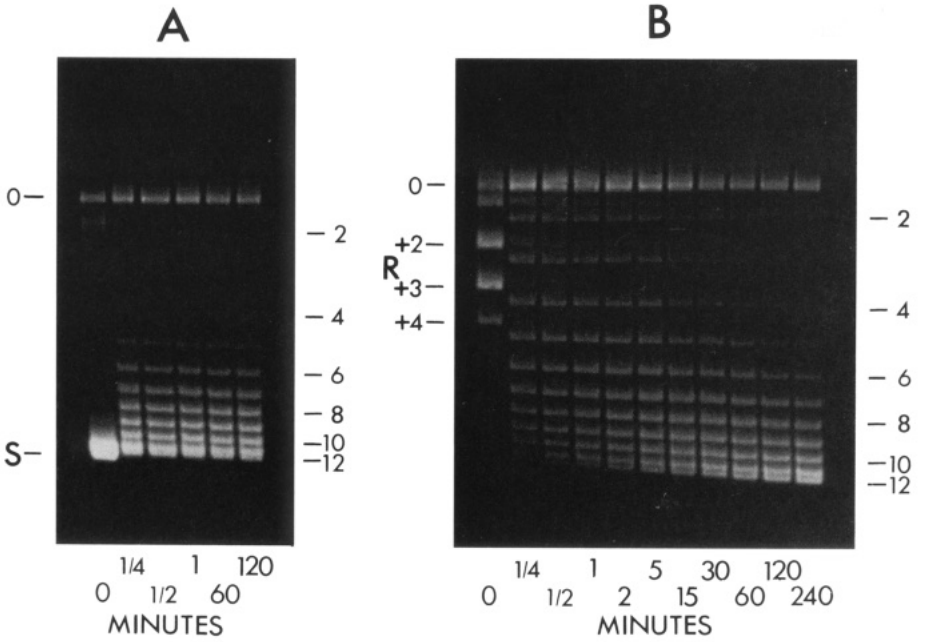

FIGURE 1: Rates of formation of stored negative stress on closed, circular DNA. Histones H2A, H2B, H3, and H4 were reconstituted at a histone to DNA ratio of 0.7:1 with two forms of DNA: S DNA, containing an average of −14 coils (A), and R DNA, containing an average of +2.5 coils (B). The reconstitution protocol was as described in Materials and Methods. Topoisomerase I (400 units/μg of DNA) was added, and samples were incubated at 23 °C. Aliquots were taken as a function of time and treated with an equal volume of an SDS-containing buffer (stop buffer). Samples were electrophoresed on 1.5% agarose gels containing 50 mM Tris, 45 mM acetic acid, and 1.25 mM EDTA (pH 8.0) at 4 °C. The gel electrophoretic conditions were established to mimic the torsional state of the DNA at 23 °C. Therefore, the −14 coils for the S DNA and the +2.5 coils for the R DNA are the torsional states of the DNA at 23 °C as represented on these gels. The electrophoretic conditions are also able to resolve positive coils from negative coils. The left sides of the panels indicate the number of positive coils, and the right sides indicate the number of negative coils. The gel band marked 0 contains nicked DNA. For the R DNA, a minor component (approximately 3%) of that band is covalently closed.

The times of centrifugation varied as indicated in the figure legends. Fractions (450 μL) were collected, and 10 μL was taken from each fraction for analysis of the DNA distribution. The remainder of each fraction was treated with 5 μg of BSA, which serves as a carrier to ensure complete precipitation of all proteins in each fraction. The samples were treated with an equal volume of 30% TCA and, after 1 h of incubation at 0 °C, centrifuged at 27000*g* for 10 min to collect the pellets. The pellets were dissolved in SDS electrophoresis buffer and applied to a PAGE–SDS gel, which consisted of a stacking gel [2.5% acrylamide, 0.13% bis(acrylamide), 125 mM Tris (pH 6.8), and 0.1% SDS] and a separating gel [18% acrylamide, 0.09% bis(acrylamide), 750 mM Tris (pH 8.8), and 0.1% SDS]. Electrophoresis was at 150 V for 24 h at 4 °C. The gel was stained with 0.1% Coomassie brilliant blue, 40% methanol, and 10% acetic acid and destained with the same in the absence of the dye. The gel was then photographed and the negative used to produce an image on X-ray film. This image was then scanned as previously described for the quantitation of DNA.

RESULTS

The Rate of Formation of Stored Negative Stress by H2A, H2B, H3, and H4 Is Dependent on the Torsional Stress in the DNA. HTC (hepatoma tissue culture) histones were isolated and separated into two components: histones H3,H4 and histones H2A,H2B. These histones were then combined in a 1:1 ratio and subsequently added to a circular plasmid DNA (T7/T3-19, 2238 bp) at a histone to DNA ratio of 0.7:1 (wt:wt). The plasmid DNA contained topological tensions of two forms: an average of either −14 coils (denoted as S in the text) or +2.5 coils (denoted as R in the text). The reconstitution protocol involved a dialysis from 2.0 to 0.1 M NaCl using intermediate steps of 1.2 and 0.6 M NaCl. Each dialysis step was for 3 h at 4 °C. The samples were then sedimented at 10000*g* for 5 min to remove insoluble complexes. The extent of insolubility under these conditions is 5% for both the R and S DNA (data not shown). The samples were then treated with excess topoisomerase I (400 units/μg of DNA) at 23 °C, and aliquots were taken with time to assay the torsional stress in the DNA.

As shown in Figure 1A, when the histones are associated with the S DNA, topoisomerase I removes unrestrained negative stress within 15 s. The negative stress that remains in the S DNA is held by the histones and is unchanged over the 2-h incubation period. The storage of this negative stress is a characteristic of nucleosomes. In contrast, when topoisomerase I is added to the R DNA (Figure 1B), a time-dependent process is observed in the formation of stored negative coils. This time-dependent process has been described previously (Jackson, 1993) and has been interpreted to indicate that nucleosome formation is inhibited in closed circular DNA due to the production of an unrestrained positive coil when each nucleosome is formed. The accumulation of these positive coils is predicted to have a destabilizing influence on further nucleosome formation. This process is not due to a lack of sufficient topoisomerase I activity, since there is more than sufficient activity to remove the unrestrained stress in the S DNA by the 15-s time point. Topoisomerase I relaxes both positive and negative coils equally well (Wang, 1985; Jackson, 1993).

The torsional state of the R DNA at the 15-s time point is an approximate representation of the number of stored negative coils (nucleosomes) that are present prior to the addition of topoisomerase I, which effectively removes these positive coils. The additional formation of stored negative coils during the time course may reflect the ongoing thermal flexing of the DNA which, when forming a left-handed bend,

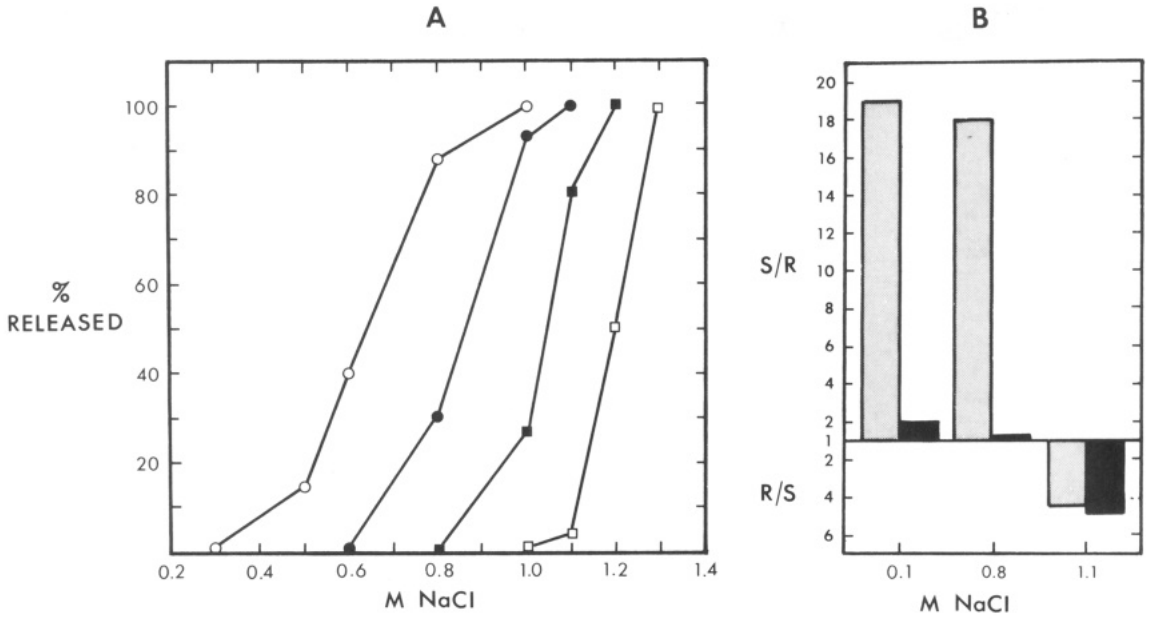

FIGURE 2: Graphical analysis of the dissociation (A) and association (B) of histones H2A, H2B, H3, and H4 when reconstituted with R and S DNA. (A) H2A,H2B dissociated from R DNA (○); H3,H4 dissociated from R DNA (□); H2A,H2B dissociated from S DNA (●); H3,H4 dissociated from S DNA (■). Histones were reconstituted with the DNA at a ratio of 0.7:1 (histone:DNA) as described in Materials and Methods, and the samples were overlayed on 5–20% sucrose gradients containing increasing concentrations of NaCl. Both samples and gradients contained 50 mM TEA/1 mM EDTA (pH 7.4). After sedimentation and collection of fractions, samples were electrophoresed to analyze the distribution of both DNA and histones on the gradient (see Figures 3 and 4). Those histones not located in the same fractions with the DNA are considered dissociated even though the rate of dissociation may vary considerably, as demonstrated by the extensive trailing of H2A,H2B and H3,H4 in Figure 4A. (B) Histones H3,H4 were reconstituted with a mixture of R and S DNA in either the presence (shaded bars) or absence (solid bars) of H2A,H2B. The ratio of H3,H4 to DNA was 0.20:1 for these experiments. This lower ratio was used to minimize the complexing of H3,H4 tetramers into octameric structures. When H2A,H2B were present, the quantity used was equivalent in weight to H3,H4. Reconstitution was by dialysis from 2.0 M NaCl to the NaCl concentration indicated on the *x*-axis of B. The samples were then applied to a 5–20% sucrose gradient containing the appropriate concentration of NaCl (see Figure 5 for an example of the raw data). Quantitation is based on a densitometric analysis of the gels that are used to determine the distribution of protein and DNA on the gradients. The ratio of S/R or R/S on the *y*-axis of B refers to the percentage of H3,H4 associated with one form of DNA divided by the percentage associated with the other form of DNA.

facilitates nucleosome formation. The corresponding unrestrained positive coils are then rapidly removed by the topoisomerase. It has been further proposed that this lack of nucleosome formation was due to the inability to form a proper interface between histones H3,H4 and histones H2A,H2B. The data supporting this proposal were the observations that an average helical pitch of 10.3 bases was found for both the R and S DNAs (Jackson, 1993). By this criterion, the histone–DNA contacts for both DNAs appear similar. The presence of this disrupted interface might be expected to increase the rate of dissociation of histones from DNA as a function of increasing NaCl concentration, since both histone–histone and histone–DNA interactions are necessary for the maintenance of a stable nucleosome. A test for this model would be an analysis of the salt solubility of histones associated with R versus S DNA.

The Dissociation of H2A, H2B, H3, and H4 from DNA in the Presence of Increasing NaCl Concentrations Is Dependent on the Torsional Stress of the DNA. Histones H3, H2B, H2A, and H4 were reconstituted on both R and S DNA as described in Materials and Methods. These complexes were then sedimented through 5–20% sucrose gradients, which contained increasing concentrations of NaCl. The results are illustrated graphically in Figure 2A, and representative gradients are shown in Figures 3 and 4. In Figure 3 the NaCl concentration is 0.6 M. Under these conditions, 40% of H2A, H2B is released from the R DNA and there is negligible release from the S DNA. As shown in Figure 2A, NaCl concentrations of 0.8 M are required to cause a significant release of H2A,H2B from S DNA (30%), and under the same conditions 87% of the H2A,H2B is released from R DNA. The positively coiled DNA (R) is a less stable substrate for the maintenance of H2A,H2B–H3,H4 and H2A,H2B–DNA interactions.

Does the positive superhelical tension that is present in the R DNA also disrupt the interactions between H3 and H4 within the tetramer and/or those with the DNA? Theoretically, since the H3,H4 tetramer is centrally located within the nucleosome, as compared to the two H2A,H2B dimers which flank the tetramer (Arents et al., 1991), the positive tension would not as adversely affect H3–H4 interactions. The interactions of H3,H4 with either R or S DNA would be expected to be similar. As shown in Figure 2A, the NaCl concentration was increased in the sucrose gradients so that the conditions for H3,H4 dissociation could be determined. To our surprise, not only was the dissociation from the two DNAs not similar, but the level of dissociation from the S DNA was substantially greater than that from the R DNA. This result is completely opposite as compared to the dissociation of H2A,H2B. Figure 4 shows the results for the sucrose gradients that contained 1.1 M NaCl. Note that for the S DNA there is a constant rate of dissociation of H3,H4 from the DNA as it is sedimented through the sucrose gradient (Figure 4A). Also note that a trailing of the H2A,H2B is observed, although its dissociation rate is more rapid than that of H3,H4. No such dissociation of H3,H4 is observed for the R DNA, nor is there any slow dissociation of H2A, H2B (Figure 4B).

Histones H3,H4 Selectively Associate with R DNA under Conditions of 1.1 M NaCl. If we are correct in our assessment that the H2A,H2B interactions with H3,H4 are more stable when the DNA is negatively coiled, then it is possible that the maintenance of this interaction could adversely affect the

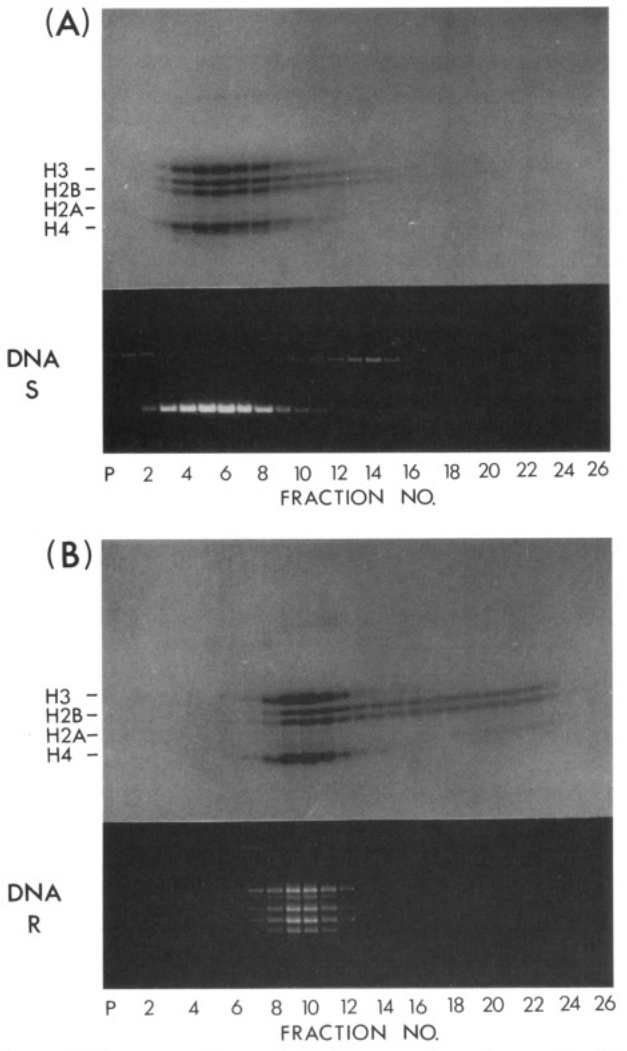

FIGURE 3: Sucrose gradient analysis of histone release from torsionally stressed DNA in 0.6 M NaCl. Histones H2A, H2B, H3, and H4 were reconstituted at a histone to DNA ratio of 0.7:1 with S DNA (A) and R DNA (B). The samples were then applied to a 5–20% sucrose gradient containing 0.6 M NaCl, 50 mM TEA, and 1 mM EDTA (pH 7.4) and sedimented at 35 000 rpm for 9 h at 3 °C. The fractions (450 μL) were collected from the bottom of the tube, and a 10-μL aliquot was taken for analysis of the DNA distribution by agarose gel electrophoresis (bottom gel of each panel). A pellet (P) may also be present, and this was characterized by resuspension in 450 μL of the 20% sucrose solution and analysis on the agarose gels. The remainder of each fraction was then treated with 5 μg of BSA, which serves as carrier, and the protein was precipitated with a final concentration of 15% TCA. The pelleted protein was then dissolved into an SDS electrophoresis buffer and applied to an SDS–polyacrylamide gel (upper gel of each panel).

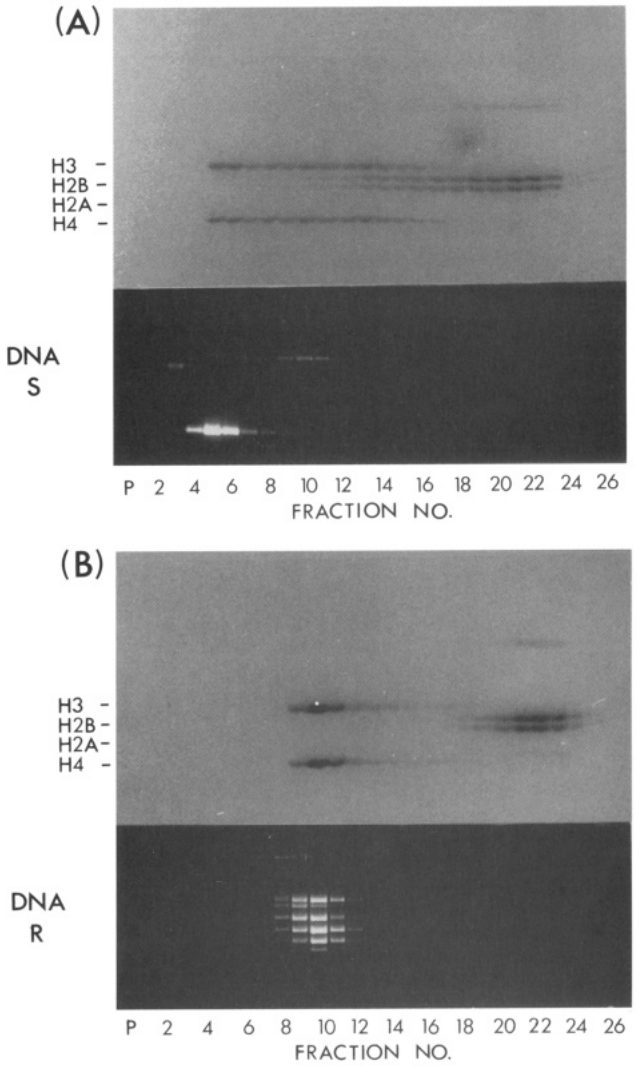

FIGURE 4: Sucrose gradient analysis of histone release from torsionally stressed DNA in 1.1 M NaCl: (A) histone–S DNA complexes; (B) histone–R DNA complexes. Procedures are as described in the legend to Figure 3, except that the sucrose gradients were done in 1.1 M NaCl and the length of centrifugation was increased to 18 h.

ability of H3,H4 to interact with DNA, since H2A,H2B no longer interact with DNA at this NaCl concentration. It may be a situation of a large protein complex with minimal sites of interaction with DNA. A second possibility is that there is an inherent structural characteristic of the R DNA that increases its interaction with H3,H4. To differentiate between these two possibilities, mixing experiments were performed in which S DNA in the form of replicative M13mp19 DNA (7250 bp) was mixed with R DNA in the form of the standard T7/T3-19 plasmid (2238 bp). To this mixture was added H3,H4 at a ratio of 0.2:1 (histone:DNA) in the presence of 2.0 M NaCl. The sample was then dialyzed to 1.1 M NaCl over a 3-h period and sedimented on a 5–20% sucrose gradient containing 1.1 M NaCl. Because of the difference in molecular weights between these two DNAs, it is possible to determine on these gradients whether the H3, H4 histones are preferentially associated with either DNA. As shown in Figure 5A, H3,H4 have a 5-fold preference for association with the R DNA (also see Figure 2B for graphical analysis). Since this preference for R DNA is observed in the absence of H2A,H2B, it must be concluded that an inherent characteristic of the R DNA is the cause of the selectivity.

What is this inherent characteristic? Since high NaCl concentrations would be expected to decrease the helical periodicity of the DNA, which in turn would reduce the overall positive stress that was initially in the R DNA, the altered pitch rather than the positive stress could be the the reason for the specificity. The S DNA would be expected to be more restricted from generating this altered pitch due to the concurrent production of higher numbers of negative coils. This question can be resolved by repeating the mixing experiment and this time dialyzing to a NaCl concentration of 0.1 M. In this situation, the altered helical pitch will have been removed and the positive torsional stress increased. If the preference is maintained, the moderate positive stress in the plasmid would be the likely cause of the selective binding. A stepwise dialysis from 2.0 to 0.1 M NaCl was performed as described in Materials and Methods, and the sample was applied to a sucrose gradient containing 0.1 M NaCl. The results of this experiment are shown in Figure 5B. Histones

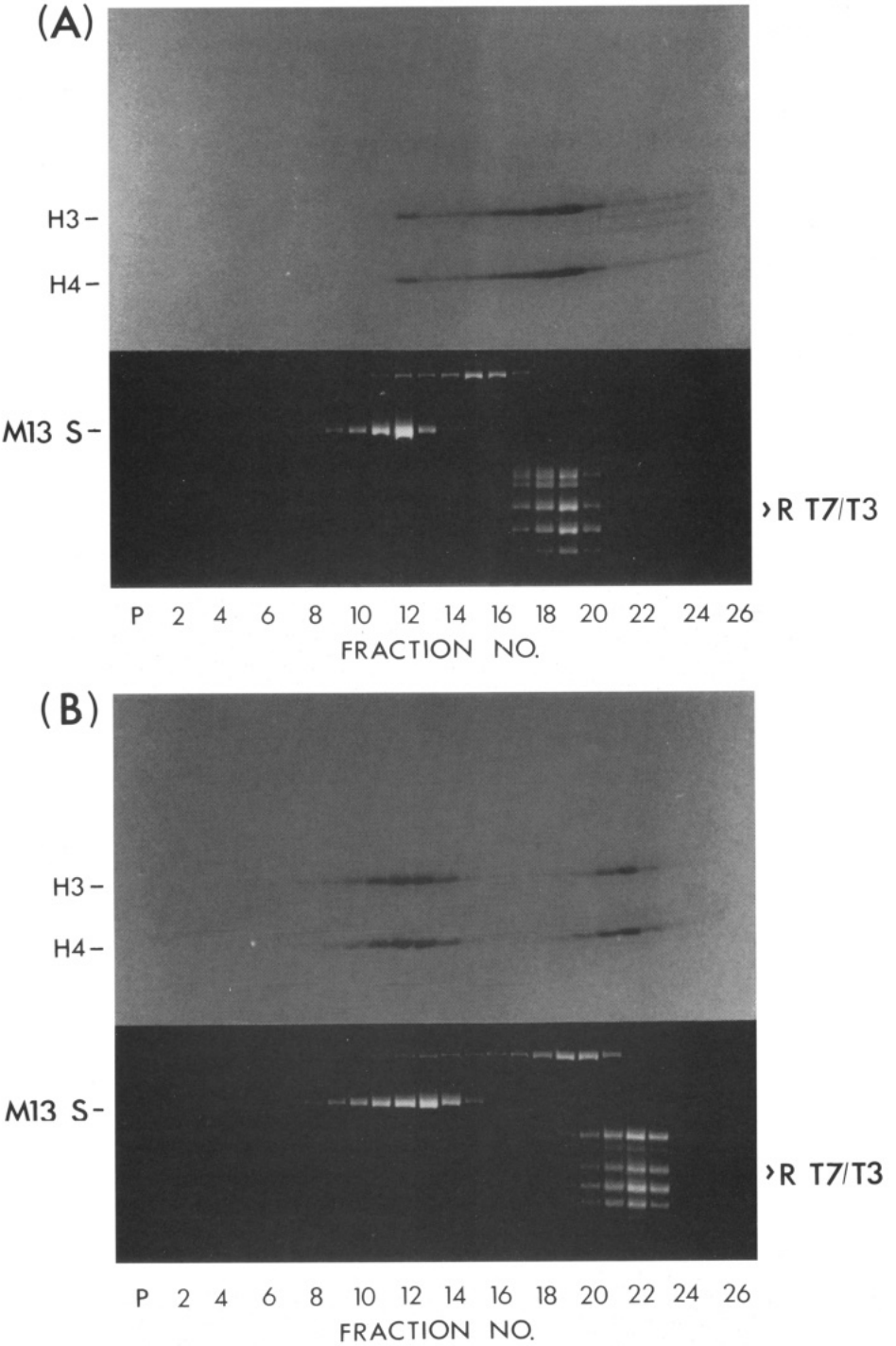

FIGURE 5: Sucrose gradient analysis of H3,H4 association with R versus S DNA: (A) reconstituted complexes sedimented on a 5–20% sucrose gradient containing 1.1 M NaCl; (B) reconstituted complexes sedimented on a 5–20% sucrose gradient containing 0.1 M NaCl. The reconstitution was done by adding H3,H4 to an equal mixture of M13mp19 S DNA and T7/T3-19 R DNA at a ratio of 0.2:1 (histone:DNA) in a NaCl concentration of 2.0 M. The sample was then dialyzed to 1.1 M for 3 h at 4 °C and applied to a sucrose gradient (A). For B, the NaCl dialysis was continued from 1.1 to 0.6 to 0.1 M NaCl for another 6-h period. This sample was then applied to the sucrose gradient (B). Sedimentation conditions were 35 000 rpm at 3 °C for 9 h (A) and for 5 h (B) in an SW41Ti rotor. The analysis of the fractions was as described in the legend for Figure 3. The gel on the bottom of each panel indicates the location of the S and R DNA in the gradients. The upper gel of each panel shows the distribution of histones.

H3,H4 no longer show the preference for R DNA and instead show a 2-fold preference for the S DNA (see quantitation of data in Figure 2B). As also shown in Figure 2B, a 1.2-fold preference for S DNA is observed at an intermediate ionic strength of 0.8 M NaCl. These results indicate that there must be a transitional point between 0.8 and 1.1 M NaCl, at which either an altered pitch in the DNA is established or a conformational change in H3,H4 is produced that favors H3, H4–R DNA interactions.

The Presence of Histones H2A,H2B Extensively Increases the Selectivity of Histones H3,H4 for Association with S DNA. From the data of Figure 3, it was observed that, at a NaCl concentration of 0.6 M, H2A,H2B was readily released from H3,H4 when associated with R DNA, but did not release when S DNA was used. Presumably, the negative coils in S DNA stabilize the H3,H4 interaction with H2A,H2B. If this assumption is correct, then the inclusion of H2A,H2B with H3,H4 in a competition experiment between R and S DNA

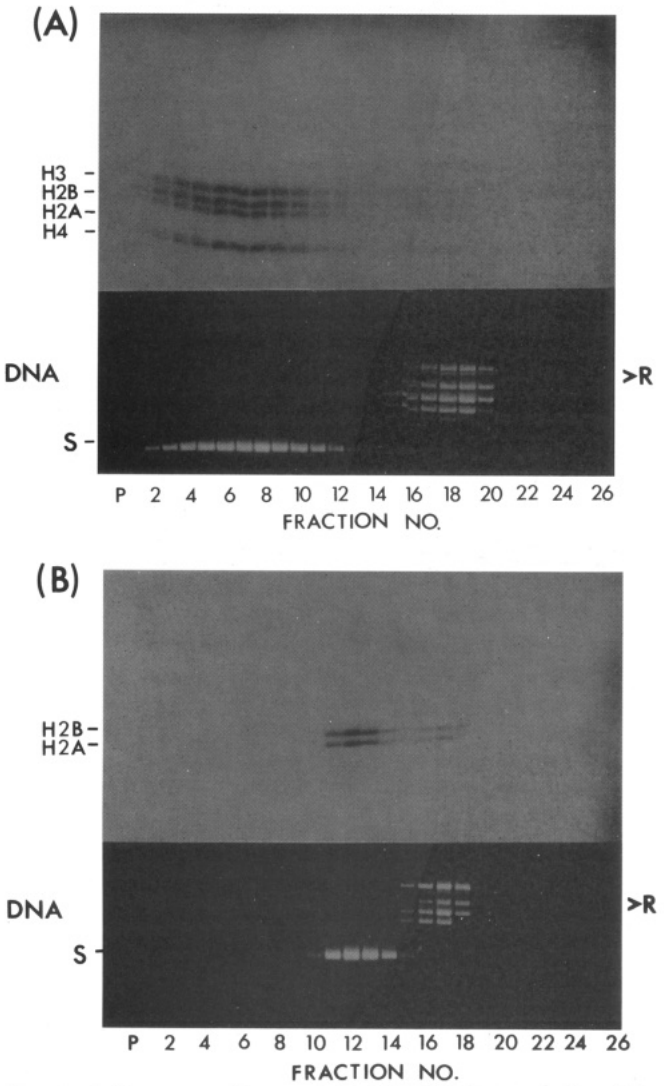

FIGURE 6: Sucrose gradient analysis of H2A,H2B association with R and S DNA when H3,H4 are present (A) and absent (B). Equal quantities (wt:wt) of H3, H4, H2A, and H2B were added to a mixture of R and S DNA (both DNAs were the T7/T3-19 plasmid) at a ratio of 0.4:1 (histone:DNA). (B) Histones H2A,H2B were added to a mixture of R and S DNA at a ratio of 0.2:1 (histone:DNA). Both samples were then dialyzed stepwise from 2.0 to 0.1 M NaCl and applied to a 5–20% sucrose gradient. Sedimentation was at 35 000 rpm at 3 °C for 8 h (A) and for 9 h (B). The analysis of the fractions was as described in the legend for Figure 3.

could result in the selective binding of all four histones to the S DNA. Equal quantities of R and S DNA were mixed together. In this instance, both DNAs were composed of the T7/T3-19 plasmid. Histones H3, H2B, H2A, and H4 were added at a ratio of 0.4:1 (histone:DNA) in the presence of 2.0 M NaCl. The sample was then subsequently dialyzed stepwise to 0.1 M NaCl and applied to a 5–20% sucrose gradient containing 0.1 M NaCl. As shown in Figure 6A, all four histones are selectively associated with the S DNA. The R DNA sediments at a rate typical of DNA that is not associated with histones. Figure 2B graphically illustrates the selectivity observed in this experiment, which was found to be 19-fold.

In order to establish where in the dialysis protocol this enrichment was established, further competition experiments were performed. As shown in Figure 2B, if the dialysis protocol was terminated at 1.1 M NaCl, H3,H4 were 4.5-fold enriched for selective binding to R DNA. The presence of H2A,H2B does not interfere with the selectivity of H3,H4 for R DNA, as these histones bind DNA very weakly at this ionic strength. When the dialysis protocol was terminated at 0.8 M NaCl, H3,H4 were no longer associated with the R DNA but were now selectively associated with the S DNA (Figure 2B). The level of enrichment is 18-fold. Seventy percent of the H2A,-H2B was associated with the S DNA as would be expected since, at 0.8 M NaCl, this percentage of H2A,H2B binds S DNA (Figure 2A). At 0.8 M NaCl, 13% of H2A,H2B will bind the R DNA (Figure 2A). These data indicate that, during the stepwise dialysis protocol from 2.0 to 0.1 M NaCl, there is a transitional process between 1.1 and 0.8 M NaCl in which the H3,H4 are released from the R DNA and transferred to the S DNA. The major driving force for this transition is the presence of H2A,H2B.

Histones H2A,H2B Preferentially Associate with Negatively Coiled DNA. In order to evaluate the characteristic of H2A,H2B that causes the transfer of H3,H4 from R to S DNA, a reconstitution was performed in which H2A,H2B were mixed with a 1:1 ratio of R and S DNA. The histone to DNA ratio was 0.2:1 (wt:wt), and both forms of the DNA were the T7/T3-19 plasmid. Reconstitution was by dialysis from 2.0 to 0.1 M NaCl as previously described, and the sample was applied to a 5–20% sucrose gradient containing 0.1 M NaCl. As shown in Figure 6B, H2A,H2B have a 10-fold preference for association with the S form of the plasmid. These data indicate that H2A,H2B play an active rather than a passive role in the transfer of H3,H4 from R to S DNA. In terms of the model for an *in vivo* transcription process, the release of H2A,H2B from an unfolded nucleosome that is caused by transcription-induced positive stress would be reversed very rapidly when negative stress is present after polymerase passage. The H2A,H2B would actively rebind the negatively coiled DNA to establish interactions with H3,H4 and the DNA.

The Presence of H2A,H2B Extensively Increases the Selectivity of Histones H3,H4 for the Binding and Storage of Negative Stress on S Rather Than R DNA. With these sucrose gradient analyses, the data indicate that H3,H4 will maintain a 2-fold specificity for S DNA when the dialysis is allowed to continue to 0.1 M NaCl. If H2A,H2B are also present, this specificity is increased to 19-fold. A second method is available that can independently verify this specificity and also directly analyze the specificity at different histone to DNA ratios. In this instance, the topological state of the DNA in the complexes is evaluated. The presence of negative coils after treatment with topoisomerase I is an indicator of native histone–DNA interactions. M13 S DNA and T7/T3-19 R DNA were mixed at a ratio of 1:1 (wt:wt), and individual aliquots were then treated with increasing quantities of histones H3, H2B, H2A, and H4 in the presence of 2.0 M NaCl. The stepwise dialysis protocol was then applied to produce a final NaCl concentration of 0.1 M. The samples were sedimented at 10000*g* for 5 min to remove aggregated material. Approximately 5% of the reconstituted complexes is insoluble at the higher histone to DNA ratios (data not shown). This aggregated material must be removed since topoisomerase I cannot efficiently access insoluble material.

Topoisomerase I was then added to the aliquots, and the samples were incubated for 2 h at 23 °C. As shown in Figure 7A, as the histone to DNA ratio increases, the M13 S DNA rapidly increases in the number of stored negative coils. At a ratio of 0.4 (Figure 7A, lane 4), the S DNA contains the maximum number of negative coils. In contrast, the T7/T3-19 R DNA shows an average of only three negative coils at the 0.4:1 ratio, which progressively increases in number as

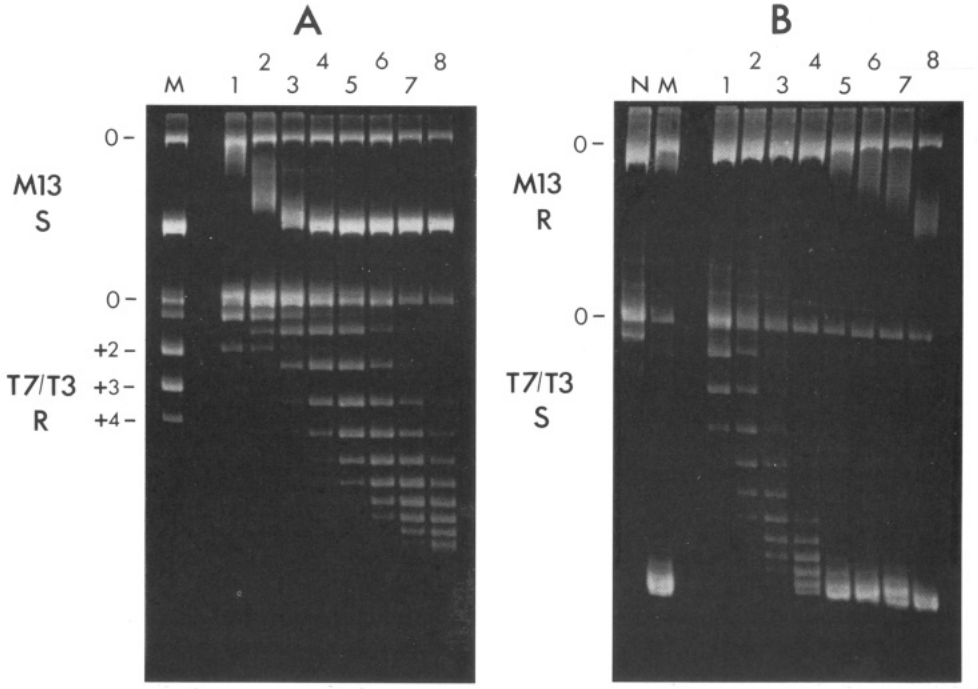

FIGURE 7: Analysis of the stored negative stress established when histones H2A, H2B, H3, and H4 are added to a mixture of R and S DNA at increasing histone to DNA ratios. (A) Histones H2A, H2B, H3, and H4 were reconstituted as described in Materials and Methods with a mixture of M13mp19 S DNA and T7/T3-19 R DNA. (B) Histones H2A, H2B, H3, and H4 were reconstituted with a mixture of M13mp19 R DNA and T7/T3-19 S DNA. The histone to DNA ratios were (1) 0.1; (2) 0.2; (3) 0.3; (4) 0.4; (5) 0.5; (6) 0.6; (7) 0.7; and (8) 0.8. After reconstitution, the samples were sedimented at 10000*g* for 5 min. Topoisomerase I (100 units/μg of DNA) was added to each supernatant and incubated at 23 °C for 2 h. Stop buffer was added and samples were electrophoresed on 1.5% agarose gels. M indicates the state of the DNA prior to topoisomerase I addition. N indicates the state of the DNA after topoisomerase I addition when histones are absent.

the histone to DNA ratio increases. The data indicate that there is a transition during which the histones will initially associate with the S DNA, and only after a full complement of histones has bound this DNA will the R DNA serve as a site for histone interaction. Of additional interest is the observation that once the full complement of histones has associated with the S DNA, additional histones do not bind as evidenced by the continued solubility of the complexes. If the R DNA were absent in this experiment, substantial quantities of the S DNA would have been insoluble at histone to DNA ratios greater than 1:1 (data not shown).

These experiments have not addressed the possibility that sequence variation between M13mp19 and T7/T3-19 DNA may be the cause of the specificity. This possibility can be tested by reversing the form of the DNA. In this instance, we used T7/T3-19 S DNA and mixed it with M13mp19 R DNA. As shown in Figure 7B, this S DNA stores negative coils at a histone to DNA ratio similar to that observed for the M13mp19 S DNA in the data of Figure 7A. The M13mp19 R DNA also shows a lag in the formation of negative coils similar to that observed for the T7/T3-19 R DNA of Figure 7A. Whether in the sucrose gradient analyses or in the analyses of stored negative coils (Figure 7A,B), the driving force for histone interaction is the topological state of the DNA.

A similar analysis can be performed with H3,H4 alone. Several investigators have shown that H3,H4 in the absence of H2A,H2B are able to store negative coils on a closed circular DNA (Camerini-Otero et al., 1976, 1977; Oudet et al., 1977). The retention of these coils is therefore a marker for H3,H4 interaction with DNA. We repeated the experiment of Figure 7, except that we did not include H2A,H2B. In this way, we could confirm the conclusion of the sucrose gradient analyses that H3,H4 bind S DNA with only 2-fold specificity. Increasing quantities of H3,H4 were mixed with aliquots of a mixture of M13mp19 S and T7/T3-19 R DNA, and after stepwise reconstitution to a NaCl concentration of 0.1 M, the aliquots were sedimented at 10000*g* for 5 min. Topoisomerase I was then added to the supernatants, which contained the soluble complexes, and they were incubated for 2 h at 23 °C. The DNAs were analyzed on agarose gels with the results shown in Figure 8A.

As increasing quantities of H3,H4 are added to the DNAs, an increasing number of stored negative coils are observed for both the R and S DNAs. The S DNA maintains a slightly higher percentage of negative coils. For example, at an H3, H4 to DNA ratio of 0.4:1 (Figure 8A, lane 4), approximately 70% of the total possible negative coils is stored in the M13mp19 S DNA. An average of 4.5 out of a possible 14 negative coils or approximately 30% of the negative coils in the T7/T3-19 R DNA is stored by H3,H4. These observations further support the sucrose gradient analyses, which indicated that H3,H4 have only an approximately 2-fold specificity for S DNA at 0.1 M NaCl. It should also be noted that at high H3,H4 to DNA ratios (Figure 8A, lanes 7 and 8) both R and S DNA are missing from the supernatants. As shown in Figure 8B, if we examine the DNA that is present in the pellets produced by centrifugation at 10000*g* for 5 min, we observe that both R and S DNA are present (Figure 8B, lanes 5–8). At H3,H4 to DNA ratios greater than 0.6:1, extensive aggregation occurs, which is in contrast to when total core histones are used in which negligible aggregation occurred (Figure 7). The presence of H2A,H2B greatly diminishes this aggregation process. The importance of H2A,H2B in complementing H3,H4 will be shown further when we examine H3,H4 interactions with either R or S DNA separately (*vide infra*).

Histones H2A,H2B Are Necessary To Promote the Complete Storage of Negative Coils on R DNA, but Not S DNA. To examine further the role of H3,H4 in establishing

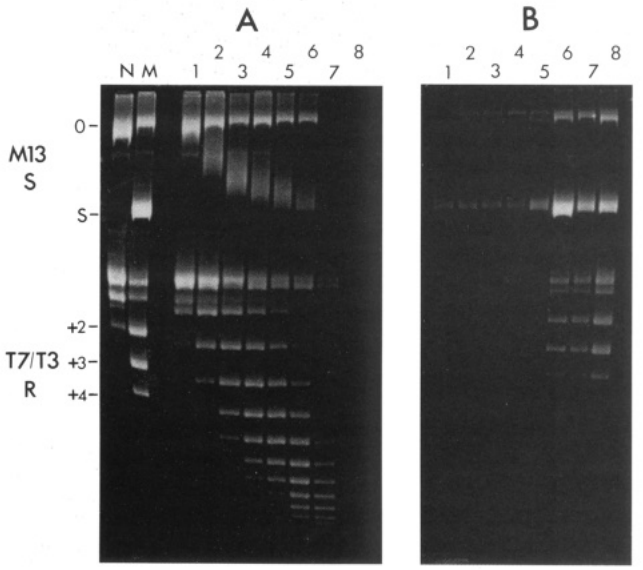

FIGURE 8: Analysis of the stored negative stress established when histones H3,H4 are added to a mixture of R and S DNA. Histones H3,H4 were reconstituted as described in Materials and Methods with a mixture of M13mp19 S DNA and T7/T3-19 R DNA. The histone to DNA ratios were (1) 0.1; (2) 0.2; (3) 0.3; (4) 0.4; (5) 0.5; (6) 0.6; (7) 0.7; and (8) 0.8. After reconstitution, the samples were sedimented at 10000*g* for 5 min. The pellets were redissolved in an equivalent volume and treated with stop buffer. These samples were then applied to 1.5% agarose gels and are shown in panel B. The supernatants were treated with topoisomerase I (100 units/μg of DNA) and incubated at 23 °C for 2 h. Stop buffer was added, and samples were applied to a 1.5% agarose gel as shown in panel A. M indicates the state of the DNA prior to topoisomerase I addition. N indicates the state of the DNA after topoisomerase I addition when histones are absent.

nucleosome structure, we reconstituted H3,H4 onto the T7/T3-19 plasmid in either the R or S torsional state. After dialysis to 0.1 M NaCl, the samples were sedimented at 10000*g* for 5 min. No aggregate material was observed at the lower histone to DNA ratio, but at the 0.7:1 ratio, 60% of the R DNA and 10% of the S DNA aggregated (data not shown). Whatever the characteristic that causes the aggregation, it is amplified when the reconstitution is on the R DNA. The soluble complexes were next treated with an excess of topoisomerase I (400 units/μg of DNA), and aliquots were taken with time to determine the topological state of the DNA. As shown in Figure 9A,C for the S DNA, topoisomerase I has removed unconstrained negative stress within 15 s to reveal the ability of H3,H4 to store the remaining negative stress. An average of five negative coils is stored at a histone to DNA ratio of 0.35:1 (Figure 9A) and ten negative coils at 0.7:1 (Figure 9C). The level of stored negative coils does not change over the 2-h time period, which indicates that H3,H4 stably maintain these negative coils. At the 0.7:1 ratio, H3,H4 store negative coils as efficiently as if one-half of this H3,H4 had been replaced by an equal complement of H2A,H2B (compare Figures 1A and 9C). It is well-established that octameric complexes of H3,H4 can be produced at higher histone to DNA ratios, which can further stabilize the negative stress in these complexes (Camerini-Otero et al., 1976, 1977; Oudet et al., 1977; Bina-Stein & Simpson, 1977; Klevan et al., 1978; Simon et al., 1978; Stockley & Thomas, 1979; Read & Crane-Robinson, 1985). Of importance is the stability of the negative coils and the solubility of the S DNA complexes in the absence of H2A,H2B.

When topoisomerase I is added to R DNA, a quite different result is observed. At a histone to DNA ratio of 0.35:1 (Figure 9B), the number of stored negative coils observed at the 15-s time point averages four, which increases to five over a 2-h period. The state of the stored negative coils in the R DNA appears quite similar to that the S DNA at this ratio (Figure 9A). However, at the histone to DNA ratio of 0.7:1 (Figure 9D), those R DNA complexes that are soluble do not eventually produce ten negative coils. Rather, the number of coils produced after a 2-h period averages seven. Doubling the amount of H3,H4 does not double the number of negative coils that is on the R DNA. At the higher histone to DNA ratio, the H3,H4 interaction with R DNA has substantially changed with respect to both the solubility and the number of stored negative coils.

DISCUSSION

A number of *in vivo* studies have indicated that nucleosome structure is altered during transcription [reviewed in Igo-Kemenes et al. (1982) and Gross and Garrard (1988)]. One aspect of this alteration is the observation that H2A,H2B are selectively released during transcription [reviewed in Jackson (1990) and van Holde et al. (1992)]. A corollary to this observation is that H3,H4 are not released during the transcription process, but remain associated with the DNA to serve as nucleation sites for the rebinding of H2A,H2B after polymerase passage. The importance of H3,H4 in establishing the position of a nucleosome on the DNA has been reported (Dong & van Holde, 1991). *In vitro* studies have shown that transcription is enhanced by the removal of H2A,H2B (Gonzalez & Palacian, 1989) and that nucleosomes that are associated with RNA polymerase are depleted of H2A,H2B (Baer & Rhodes, 1983). Another example of the importance of removing H2A,H2B from H3,H4 to permit accessibility to the DNA has been shown in experiments in which TFIIIA was unable to bind to the 5S RNA gene in the presence of H2A,H2B (Hayes & Wolffe, 1992). The nucleosome would need to unfold to promote the disruption of the interface between H2A,H2B and H3,H4. One series of events that could promote this unfolding is the production of transcription-induced positive stress in advance of a transcribing RNA polymerase (Liu & Wang, 1987; Tsao et al., 1989). We have observed by *in vitro* studies that such stresses do disrupt nucleosome structure (Pfaffle et al., 1990; Jackson, 1993). This same disruption by transcription-induced positive stress has been observed *in vivo* (Lee & Garrard, 1991). In like manner, Tsao et al. (1989) have shown that transcription-induced negative stress is produced in the wake of a translating RNA polymerase. These negative coils might greatly influence the rate at which a nucleosome is reformed. We have observed in *in vitro* experiments that negative stress causes extremely rapid formation of nucleosome structure (Pfaffle & Jackson, 1990). The potential significance of the data described in this article relates to these transcriptionally induced stresses.

The data of Figures 3 and 2A indicate that H2A,H2B are more weakly associated with H3,H4 when those histones are associated with a DNA that contains positive coils (R DNA) rather than negative coils (S DNA). The data of Figure 6B indicate that H2A,H2B have a 10-fold specificity for S DNA rather than R DNA. When H3,H4 are also present, this specificity increases to 19-fold (Figure 6A). Relating this information to transcription-induced stresses, the positive stress in advance of the polymerase could promote disruption of the H2A,H2B interface with H3,H4, such that H2A,H2B are released from the nucleosome. The transcription-induced negative stress generated after RNA polymerase passage would then enhance the reformation of a folded nucleosome due to the preference of H2A,H2B for negatively coiled DNA. This

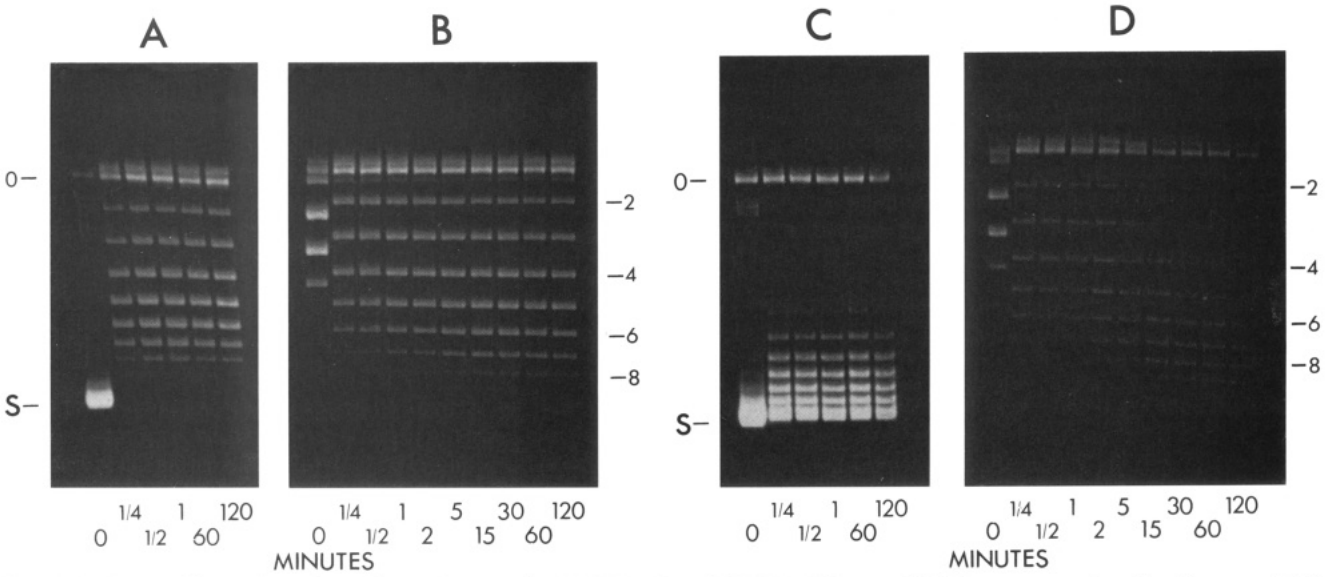

FIGURE 9: Rates of formation of stored negative stress by H3,H4 on R and S DNA. Histones H3,H4 were reconstituted at histone to DNA ratios of 0.35:1 (A and B) and 0.7:1 (C and D) with two forms of the T7/T3-19 plasmid: S DNA (A and C) and R DNA (B and D). After reconstitution, samples were sedimented at 10000*g* for 5 min, and the supernatants were treated with topoisomerase I (400 units/μg of DNA) at 23 °C. Aliquots were taken as a function of time, treated with stop buffer, and electrophoresed on 1.5% agarose gels. The lower quantity of DNA in panel D is due to the removal by centrifugation of the extensive aggregates that are present on R DNA at the H3,H4 to DNA ratio of 0.7:1.

scenario would not necessarily be a requirement for transcription through a nucleosome. O'Neill et al. (1993) have shown that transcription can occur on DNA reconstituted with a cross-linked octameric complex of H3, H2B, H2A, and H4. The scenario may represent a specific situation in which transcription-induced stress increases transcription efficiency by the transient release of H2A,H2B.

From the data of Figure 1B, it was observed that an average of five stored negative coils are formed at the 15-s time point when total core histones are reconstituted on the R DNA. We interpret these observations to indicate that the five resulting unrestrained positive coils plus the average of three positive coils already present in the R DNA are able to prevent the storage of additional negative coils. For the remaining histones that are on the DNA, their distribution must be of a form that facilitates further nucleosome formation when the positive stress is removed by the topoisomerase as the incubation continues. When histones H3,H4 are reconstituted on R DNA in the absence of H2A,H2B, a different pattern emerges. At the lower ratio of 0.35:1, H3,H4 alone efficiently store those five acceptable negative coils (Figure 9B, 15-s time point). At the ratio of 0.7:1, the additional H3,H4 interact with the R DNA to generate structures that aggregate and are unable to efficiently form stored negative coils after topoisomerase addition (Figure 9D). When H2A,H2B are substituted for the extra complement of H3,H4, this aggregation is prevented and the storage of negative stress is complete (compare Figures 1B and 9D). These data indicate that H2A,H2B are needed to promote the efficient storage of negative coils on R DNA. This is a condition that is not required for S DNA.

For the mixing experiment of Figure 8, it was observed that both R and S DNA aggregated at similar histone to DNA ratios when H3,H4 were present. Yet the data of Figure 9 indicate that when H3,H4 are reconstituted on R and S DNA separately, the S DNA remains more soluble. Why the difference? We propose that the difference is due to the 2-fold selectivity that H3,H4 have for the S DNA when both S and R DNA are mixed together. Therefore, as the histone to DNA ratio is increased, the S DNA tends to saturate slightly faster than the R DNA, resulting in a preferential overabundance of H3,H4 on the S DNA. The overabundance results in aggregation. Again, it is of interest that if H2A,H2B are present in the same mixing experiment (Figure 7), the S DNA will not become insoluble after being preferentially saturated with the core histones. Rather, the excess histones directly transfer to the R DNA. It is the presence of H2A,H2B that maintains the solubility and facilitates the transfer of excess H3,H4.

The data of Figure 9 indicate that two H3,H4 tetramers could produce a pseudonucleosome when negatively coiled DNA is present in the nucleus of a cell. The formation of these pseudonucleosomes by *in vitro* reconstitution has been reported by several laboratories (Camerini-Otero et al., 1976, 1977; Oudet et al., 1977; Bina-Stein & Simpson, 1977; Klevan et al., 1978; Simon et al., 1978; Stockley & Thomas, 1979; Read & Crane-Robinson, 1985). Such structures, however, have not been observed *in vivo* and apparently are not allowed. How could these structures be avoided? Histones H3,H4 store negative coils very efficiently on S DNA at even high histone to DNA ratios and form complexes with DNA that are as soluble as when H2A,H2B are present (compare Figures 1A and 9C). In the competition experiments, one might then predict that H3,H4 would preferentially bind S DNA as compared to R DNA at a level that is similar to that observed when H2A,H2B are present. However, at 0.1 M NaCl, H3,H4 have only a 2-fold selectivity for S DNA, as compared to the 19-fold selectivity when H2A,H2B are present (Figure 2B). The lack of such specificity with H3,H4 alone suggests that a characteristic of R DNA enhances interaction with those particular histones. This, in particular, is the situation in a salt concentration of 1.1 M, at which H3,H4 bind R DNA with a 5-fold preference (Figure 2B).

The data of Figure 5 have been interpreted to indicate that this binding preference could be due to a decrease in the helical pitch of the DNA. Such a decrease in helical pitch may transiently occur during transcription. McClellan and Lilley (1991) have reported that helical pitch changes are produced by positive stress. These changes can be at least partially translated through the DNA of a nucleosome (Feigon & Kearns, 1979; Wang et al., 1982b; Askikawa et al., 1983;

Schurr & Schurr, 1985). Therefore, under conditions of high positive stress induced by transcription, H3,H4 release could be kept to a minimum by this enhanced binding. It is this enhanced binding that may be the answer to the question. This lack of release provides a mechanism to limit the intranuclear pools of free H3,H4. In this way, the negative coils produced in the wake of an RNA polymerase would be reconstituted by reassociation with two H2A,H2B dimers rather than another H3,H4 tetramer. Such a scenario would also enhance the maintenance of nucleosome phasing by the preservation of H3,H4 interactions with DNA.

A potential problem exists when we consider the synthesis of new H3,H4. These histones would minimally compete with both new and old H2A,H2B for the negative coils produced during transcription. The presence of replication-specific deposition factors for newly synthesized H3,H4 would provide the cell with an efficient way to direct H3,H4 to replicative DNA, a process observed *in vivo* (Jackson, 1990) and *in vitro* (Smith & Stillman, 1989, 1991). Such a process would eliminate any further possibility of H3,H4 interacting with the negatively coiled DNA produced by transcription. As shown in Figure 9C, H3,H4 at a histone to DNA ratio of 0.7:1 store negative coils very effectively for DNA already negatively coiled. The reconstituted complexes are in a soluble state. The opposite situation is observed for R DNA (Figure 9D). Therefore, during replication, if one assumes that the newly replicated DNA strands are maintained in a relaxed state due to the discontinuous nature of replication, the formation of pseudonucleosomes consisting of two H3,H4 tetramers would be greatly restricted. The inclusion of H2A,H2B is preferred when nucleosome formation must begin with a DNA that is not negatively coiled (compare Figures 1B and 9D).

The enhanced binding of H3,H4 on R DNA is also of significance if one considers that, during the transcription process, there is the transient formation of an RNA–DNA duplex of 10–15 bp (Gamper & Hearst, 1982). Such duplexes form an A helix, which increases the helical pitch to 11 bp/turn (Krylov et al., 1989). Dunn and Griffith (1980) have reported that nucleosomes cannot form on this hybrid. Presumably, either the helical pitch or the general stiffness (Levitt, 1978) of this hybrid excludes histone interaction, as such hybrids appear to associate very weakly with histones (Dunn & Griffith, 1980; Kunkel & Martinson, 1981; Hovatter & Martinson, 1987). At the same time, the site at which this hybrid is generated is in intimate contact with a disruptive RNA polymerase. Maintenance of the H3,H4 position would be dependent on the preservation and perhaps enhancement of binding to regions of DNA proximal to the hybrid site.

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