Influence of Positive Stress on Nucleosome Assembly†

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ABSTRACT: Nucleosome assembly on closed circular DNA has been studied using reconstitution protocols which involve salt gradient dialysis in the presence or absence of urea. The closed circular DNA contained either +3 or −14 supercoils. The nucleosomal content of the DNA was assayed by measuring the superhelical content in the presence of topoisomerase I as a function of time and temperature and by measuring the DNA fragment size after DNase I digestion. Positively coiled DNA appears to contain an altered "open" nucleosome structure as compared to the negatively coiled DNA. Formaldehyde fixation of the reconstituted complexes was also done in an attempt to freeze the nucleosomal state prior to topoisomerase I or DNase I addition. The data from these additional experiments are consistent with a conclusion that an altered nucleosomal structure exists on positively supercoiled DNA. Control experiments are included to verify the efficiency of formaldehyde fixation in preserving the structure in the reconstituted complexes. The physiological relevance of positive stress and its effect on nucleosome structure are discussed.

The nucleosome core particle is the basic subunit of chromatin structure and consists of 145 bp of DNA coiled leftward 1.8 times around a histone core containing two each of histones H2A, H2B, H3, and H4 (Travers & Klug, 1990). This structure provides a 5-fold compaction of DNA, which can be further condensed with additional coiling to produce the necessary compaction that is observed in the nucleus. As a result, the DNA superhelix of each nucleosome should produce a −1.8 decrease in linking number (1.8 negative coils). However, due to the overwinding of the DNA helix caused by the binding interactions with the histones, each nucleosome particle stores one negative coil (Germond et al., 1975). The storage of one negative coil is a characteristic of a nucleosome. To hold the DNA in such a tight superhelix requires extremely strong interactions between the histones and DNA. Salt concentrations of 2 M NaCl are required to remove histones H3 and H4. The strength of binding is sufficient to overcome any inherent bending characteristic of the DNA to which these proteins are associated (Hayes et al., 1991). During the transcription process, RNA polymerase must disrupt these histone–DNA interactions, and as a result, it is not surprising that transcription rates *in vitro* are decreased 10-fold when histones are present (Brooks & Green, 1977; Bustin, 1978; Williamson & Felsenfield, 1978; Wasylyk & Chambon, 1979; 1980; Meneguzzi et al., 1979; O'Neill et al., 1992). However, transcription *in vivo* is not severely restricted as rates for both procaryotes and eucaryotes are similar (von Hippel et al., 1984; Cox, 1976). How does the cell circumvent these strong histone–DNA interactions?

One way to circumvent the interaction is to transiently remove the histones from the nucleosome. Providing the histones reattach soon after RNA polymerase passage, the primary packing order can still be maintained. However, to remove the core histones as a group poses a problem due to the multiple sites of interaction between the eight histones and the DNA. If the histone–histone contacts were disrupted such that the histones were released as subgroups, then the processing RNA polymerase could more efficiently disrupt the histone–DNA interactions. A second caveat to this process is that by disrupting the histone–histone interactions, the DNA is no longer in a superhelix and therefore the polymerase need no longer chase the DNA around the nucleosome. *In vivo experiments* have shown that H2A, H2B are released during transcription [review in Jackson (1990)]. The disruption of H3–H3 interactions due to ribosomal gene transcription has been observed in *in vivo* experiments and a name given for the disrupted particle, a lexosome (Johnson et al., 1979; Prior et al., 1983). What process could cause the disruption of histone–histone interactins such that subgroups of the histones could be released?

Liu and Wang (1987) proposed that during transcription positive stress is produced by a processing RNA polymerase which is prevented from rotating due to the viscous drag of its transcript. Since the polymerase must melt 10–15 bp of DNA during elongation (Gamper & Hearst, 1982), the restriction of polymerase rotation must generate one positive coil for each 10 bp of DNA transcribed (one turn of the helix). Both *in vivo* (Wu et al., 1988) and *in vitro* (Tsao et al., 1989) studies have provided support for this model. Since positive stress produces a right-handed supercoil, this tension might be expected to destabilize the histone–histone interactions within the nucleosome as this DNA is held in a left-handed supercoil. The effect of transcriptionally-induced positive stress on nucleosome structure has been tested in both *in vitro* (Pfaffle et al., 1990) and *in vivo* (Lee & Garrard, 1991) studies. In both studies, an altered nucleosome structure was generated. In particular, the *in vitro* studies (Pfaffle et al., 1990) indicated that these altered nucleosomes were unable to hold the negative coil even though the histones remained associated with the DNA. One interpretation of this result is that the histone–histone contacts have been disrupted and the superhelix dissolved. This more open state (lexosome-like) would provide a means of H2A, H2B to be released, provided an appropriate exchange factor were present to trigger the release. Is there evidence in the absence of transcription that nucleosome structure is indeed altered on positively supercoiled DNA?

Two laboratories have attempted to answer this question and have made opposite conclusions. Pfaffle and Jackson (1990) examined nucleosome assembly on covalently-closed circular DNA at physiological ionic strength using polyglutamic acid as a deposition vehicle. They concluded that nucleosome formation was prevented. Clark and Felsenfeld (1991) reconstituted histones onto highly positively coiled

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DNA by NaCl/urea dialysis and by a different set of methods and criteria concluded that normal nucleosomes were present on the DNA. It is the purpose of this report to further characterize nucleosome structure on positively coiled DNA, paying particular attention to reconstitution protocols and methods for stabilizing the structures so that they can be more effectively characterized.

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 by Simon and Felsenfield (1979). Purified nuclei were prepared by four washes of 1% Triton-X100, 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 $KHPO_4$, pH 6.7, and applied to a hydroxylapatite column (CalBiochem). After the column was thoroughly washed, core histones were eluted with 2.4 M NaCl/50 mM $KHPO_4$ and stored at −70 °C.

The plasmid DNA used in these studies (pT7/T3-19, Bethesda Research Lab) was grown in HB101 at 37 °C in enriched LB broth (Maniatis, 1982). The covalently-closed supercoiled DNA was purified on CsCl density gradients and yielded a circular DNA containing 14 negative coils at 23 °C. 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 the buffer condition of 10 mM $MgCl_2$/10 mM TEA, pH 7.4. The angle between adjacent base pairs in DNA increases by about 0.011 rotational degree for each degree centigrade decrease (Depew & Wang, 1975; Pulleybank et al., 1975; Wang et al., 1982). Therefore, when the DNA is relaxed at 0 °C, the 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 purposes of our analyses, we prefer a DNA containing +3 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.

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 100% relaxation of 0.5 μg of DNA in 30 min at 37 °C.

Reconstitution of Histone–DNA Complexes. Two reconstitution protocols were utilized in this report. The first protocol (method 1) involves a mixing of histones with DNA in 2 M NaCl, 10 mM Tris, 1 mM EDTA, and 10 mM 2-mercaptoethanol, pH 7.4, and a subsequent dialysis overnight against 5 M urea in the same buffer (Clark & Felsenfeld, 1991). The sample is then dialyzed against the same buffer except the NaCl concentration is decreased stepwise as follows: 1.2 M NaCl, 1.0 M NaCl, 0.8 M NaCl, 0.6 M NaCl for 80 min in each step. The urea is then removed by dialysis overnight against 0.6 M NaCl, 10 mM Tris, 1 mM EDTA, and 1 mM 2-mercaptoethanol. The next day the sample is dialyzed against 100 mM NaCl, 50 mM TEA, and 1 mM EDTA for 3 h. All steps in this procedure are done at 4 °C. The second protocol (method 2) involves mixing the histones with DNA in 2 M NaCl, 50 mM TEA, 1 mM EDTA, and 1 mM 2-mercaptoethanol and then decreasing the NaCl concentration stepwise in the same buffer as follows: 1.2 M NaCl, 0.6 M NaCl, 0.1 M NaCl for 3 h in each step. All steps in this procedure were done at 4 °C. The histone to DNA ratio used in these studies was 0.7:1 and is based on extinction coefficients for histones of 4.2 at 230 nm and for DNA of 20.0 at 260 nm (Stein, 1979). At this histone to DNA ratio, the reconstituted complexes are entirely soluble to ensure complete access by topoisomerase I in subsequent experiments.

Procedure for Analysis 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 either 0 or 23 °C. Aliquots are taken and the reaction terminated by 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 are incubated at 37 °C for 3 h for un-cross-linked complexes and for 2 days for cross-linked complexes (Jackson, 1978). The samples are 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. When ethidium bromide is present in the samples, the samples are extracted with an equal volume of 1-butanol prior to application on the agarose gel.

Density Gradient Analysis of Cross-Linked Complexes. The cross-linked complexes were 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 and were centrifuged to equilibrium in an SW60 Ti rotor at 40K for 70 h at 4 °C. Fractions (150 μL) were collected, and for analysis of DNA, the fractions were dialyzed in a multiwell microapparatus against 10 mM Tris/1 mM EDTA, pH 8.0, for 12 h at 4 °C and then added to "Stop" buffer. The samples were then incubated 37 °C for 2 days, and an aliquot of each sample was applied to 1.5% agarose gels. For analysis of the protein distribution on the gradients, the fractions were collected, and 5 μg of BSA was added to each. The samples were treated with 20 μL of 2-mercaptoethanol and heated at 100 °C for 30 min (Jackson, 1978, 1990). The fractions were cooled to room temperature, adjusted to 0.4 N H_2SO_4, and dialyzed against 40 mN H_2SO_4/1 mM 2-mercaptoethanol for 12 h at 4 °C. 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 in the same in the absence of the dye.

Analysis of Digestion Fragments Produced by DNase I. One hundred-microliter aliquots of the histone–DNA complexes (200 μg/mL DNA) in 100 mM NaCl, 50 mM TEA, 4 mM $MgCl_2$, and 1 mM EDTA, pH 8.0, were treated with increasing quantities of DNase I (Pharmacia) at either 0 °C or 23 °C. The reactions were terminated by the addition of an equal volume of 0.5% SDS, 25 mM EDTA, 50 mM Tris, pH 8.0, and 1 mg/mL proteinase K. Samples were incubated 37 °C for 12 h for un-cross-linked complexes and for 2 days for cross-linked complexes. The DNA was precipitated by the addition of 2.5 volumes of ethanol and redissolved in formamide. Samples were heated at 100 °C for 2 min prior

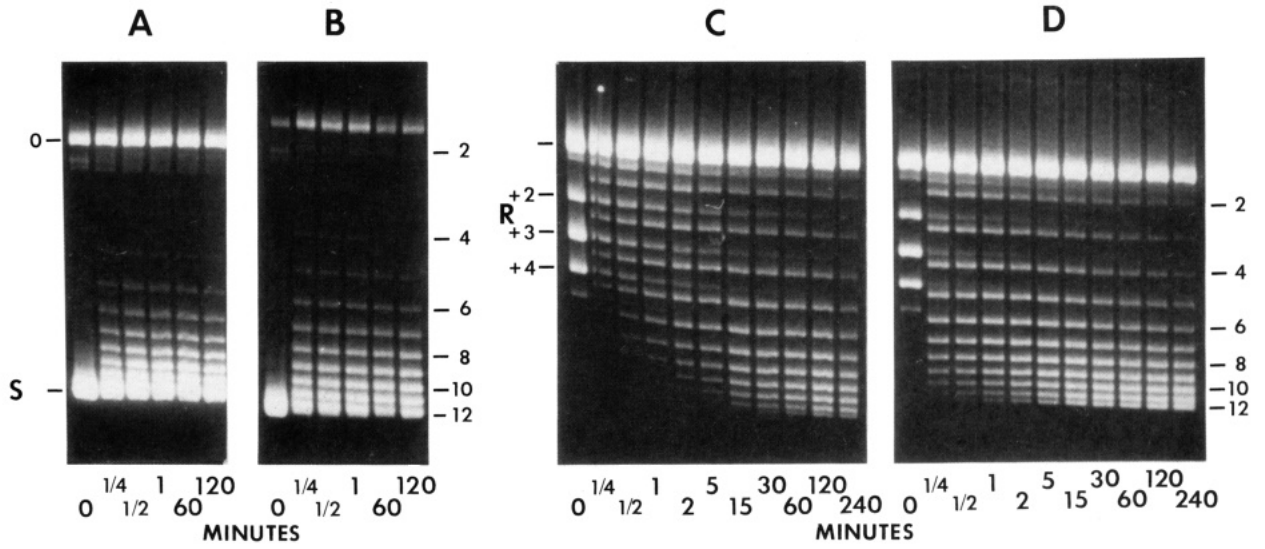

FIGURE 1: Rates of formation of stored negative stress on closed circular DNA. The time courses were done at either 0 °C (A and C) or 23 °C (B and D) with two forms of closed circular DNA: "S" DNA containing –14 coils (A and B) and "R" DNA containing +3 coils (C and D). For these assays, the addition of 400 units/μg of DNA of topoisomerase I initiates the time course. The reactions are terminated by the addition of an SDS-containing buffer. The initial supercoiled states of the two forms of DNA used in this study represent an average number at 23 °C in 100 mM NaCl, 50 mM TEA, and 1 mM EDTA, pH 7.4. The gel electrophoretic conditions are established to mimic the topological condition of the DNA at 23 °C and also to resolve both positively and negatively coiled DNA such that a direct measurement can be done in the conversion between the two states by the action of topoisomerase I. The left side of panel C indicates the mobility of the positive supercoils, and the right side of panels B and D indicates the negative supercoils. The gel band marked "0" contains primarily nicked DNA and a minor component (approximately 3%) of the DNA that is covalently-closed but with no supercoils as assayed on chloroquin gels (data not shown). Procedures for preparation of the two superhelical forms of the DNA are as described under Materials and Methods. The term "coils" refers to the superhelical tension that is present on the DNA helix. Therefore, –14 coils refers to a –14 decrease in linking number (number of times the DNA strands wrap around each other). This deficit in linking number will be exhibited in the overall circular DNA molecule by the formation of –14 supercoils in which the DNA will turn leftward in a multiple figure-eight configuration. When positive stress is present, this superhelical configuration will turn rightward.

to application on a PAGE gel consisting of 12.5% acrylamide, 0.125% bis(acrylamide), 7 M urea, 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA, pH 8.0. The temperature on the gel was maintained at 55 °C by electrophoresis at 800 V for 5 h, after which the gel was stained with ethidium bromide.

RESULTS

Histone–DNA Complexes Are Prevented from Storing Negative Stress When Positive Stress Is Present. HTC (hepatoma tissue culture) core histones (H3, H2B, H2A, H4) were mixed with a circular plasmid DNA (T7/T3-19, 2238 bp) containing either an average of –14 coils (denoted as "S" in the figures) or +3 coils (denoted as "R" in the figures). The reconstitution protocol (method 1) is as described by Clark and Felsenfield (1991) and involves a stepwise dialysis from 2.0 to 0.6 M NaCl in the presence of 5 M urea (see Materials and Methods). Subsequently, in sequential order the urea and 0.6 M NaCl are removed by dialysis. The final condition is 100 mM NaCl, 50 mM TEA, and 1 mM EDTA (pH 7.4), and the histone to DNA ratio is 0.7. The samples were then treated with topoisomerase I at either 0 °C or 23 °C, and aliquots were taken with time to assay the torsional stress within the circular DNA. As shown in Figure 1, when the histones are associated with the "S" DNA (heretofore referred to as S–H complexes), topoisomerase I removes unrestrained torsional stress within 15 s. The level of stored negative stress (nucleosomal DNA) averages to approximately –11 coils. The rate and extent of relaxation by the topoisomerase are the same for both temperatures (Figure 1A,B). Incubation times of up to 2 h do not change the extent of restrained stress that is seen at the earliest time point (15 s). In contrast, when this process is applied to the "R" DNA (heretofore referred to as R–H complexes), the formation of stored negative coils occurs in a time- and temperature-dependent process. At the 15-s time point, the number of negative coils in the R–H complexes at 0 °C (Figure 1C) is substantially less than in the R–H complexes at 23 °C (Figure 1D). In both cases, the number of negative coils is significantly less than the 15-s time point for the S–H complexes (Figure 1A,B). These results suggest that the histones in the R–H complexes are unable to efficiently store negative stress, which is a characteristic of a nucleosome. By definition, a nucleosome stores one negative coil. The formation of 11 nucleosomes (–11 supercoils) would of necessity generate +14 coils on the "R" DNA (the "R" DNA contains initially +3 coils). The rapid relaxation by the topoisomerase I should have removed the +14 unrestrained coils such that at the 15-s time point the number of negative coils present should have been the same as for the S–H complexes (Figure 1A,B). The time-dependent formation of these negative coils on the R–H complexes is suggestive of a time-dependent formation of nucleosome structure. The temperature dependence for this process is similar to that observed when reconstitution was done at physiological ionic strength using polyglutamic acid as the deposition vehicle (Pfaffle & Jackson, 1990). In that study, we proposed that this time dependence was due to the dynamic bending and flexing of the DNA in solution. When bent into a left-handed coil, the associated histones are able to establish histone–histone contacts which preserve the coil. When topoisomerase I is present, it can subsequently relax the corresponding positive coils produced as the nucleosome is formed. Without topoisomerase I, the further production of nucleosome structure would be prevented as the right-handed unrestrained positive coils would predominate. Such a process would be expected to be temperature dependent and is interpreted as the probable cause of the temperature dependence observed with this reconstitution protocol.

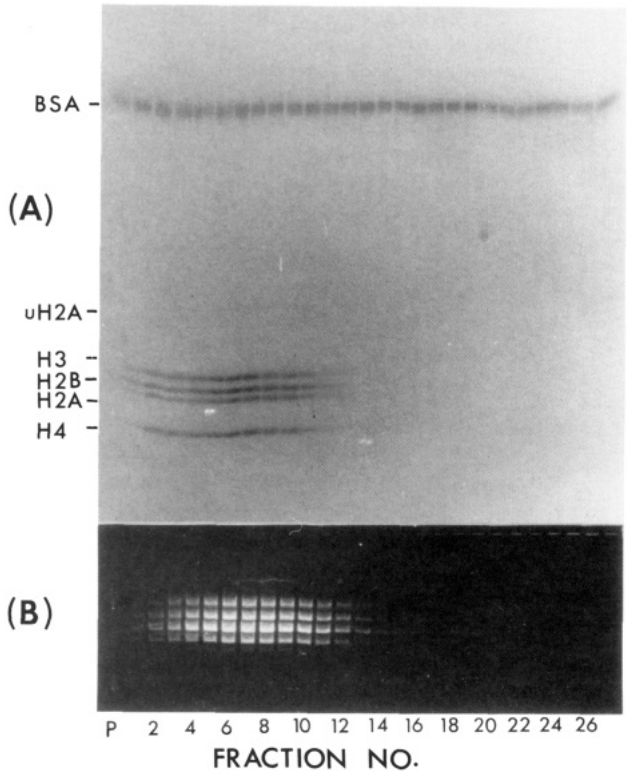

FIGURE 2: Sucrose gradient analysis of R–H complexes indicating that histones are associated with DNA. The R–H complexes were sedimented on a 5–20% sucrose gradient containing 0.3 M NaCl, 50 mM TEA, and 1 mM EDTA, pH 7.4, at 35 000 rpm for 9 h and 3 °C in an SW41 Ti rotor. Both the fractions and the potential pellet (P) were collected, and an aliquot was taken from each for analysis of DNA content by agarose gel electrophoresis (B). The remainder of each fraction was treated with 5 μg of BSA, which is used as carrier, and the proteins precipitated with a final 15% TCA. The pelleted protein was then dissolved into an SDS electrophoresis buffer and applied to an SDS–polyacrylamide gel (A).

The time-dependent formation of nucleosomes observed on the "R" DNA could be due to a weak association of histones to this DNA. Figure 2 shows a sucrose gradient that contained 0.3 M NaCl in which the R–H complexes were sedimented and both histones and DNA analyzed in each fraction. All four core histones are found in equimolar amounts associated with the "R" DNA. If a weak association had occurred, histones would be observed in fractions 24–26. The time-dependent formation would appear to be due to histone–DNA interactions which are restricted from forming negative coils on DNA that is positively coiled.

The Lack of Nucleosome Formation on the Positively Stressed DNA Is Not Due to a Rate-Limiting Activity of Topoisomerase I. The conclusion that the R–H complexes are predominantly not in a nucleosome structure is dependent on a demonstration that topoisomerase I activity is not rate-limiting. "S" DNA was treated with topoisomerase I under the same conditions as described for the experiment of Figure 1. As shown in Figure 3B for the incubation at 23 °C, the –14 coils are removed before the 5-s time point is taken. When the incubation is done at 0 °C (Figure 3A), the –14 coils are removed within 10 s. The nicking–closing activity of the enzyme decreases 2-fold between the two temperatures. On the basis of these observations, the 15-s time points of Figure 1C,D for the R–H complexes would have been of sufficient duration to ensure complete relaxation by the topoisomerase, the same complete relaxation that is observed for the S–H complexes of Figure 1A,B. Second, as shown in the data of Figure 3, the closing activity of the enzyme is not sufficiently

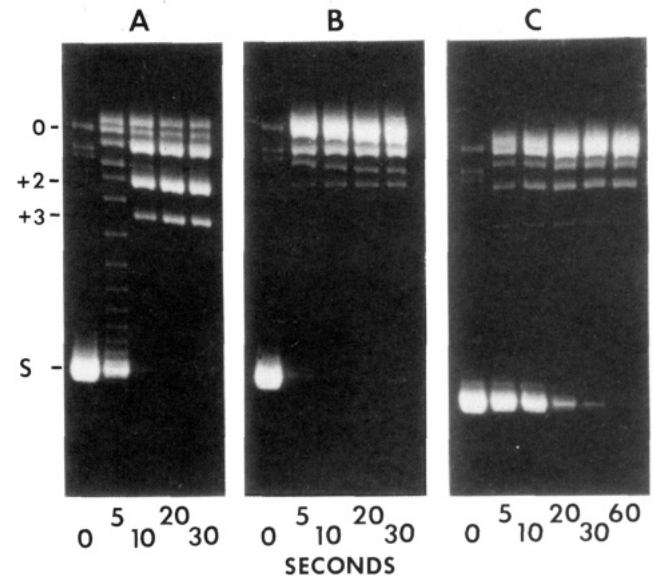

FIGURE 3: Rates of topoisomerase I activity on the "S" DNA. Rate of relaxation at 0 °C (A) and 23 °C (B) with the same topoisomerase I activities as were used for the experiments of Figure 1. (C) Rate of relaxation at 23 °C with a 10-fold decrease in topoisomerase activity. When relaxation of DNA topology occurs at 0 °C, an average of +2 supercoils are present in the DNA as analyzed on this gel system for which the buffer system is adjusted to indicate the condition of the DNA when at 23 °C. Therefore, when relaxation at 23 °C is shown in panels B and C, the topology of the relaxed DNA has an average of 0 supercoils.

rapid to produce intermediate levels of torsional stress. When the DNA is nicked by the enzyme, whether at 0 °C (Figure 3A) or at 23 °C (Figure 3B), the torsional stress is released before the closing occurs. To further demonstrate this point, "S" DNA was treated with a 10-fold lower concentration of topoisomerase I than was used for the data for Figure 3B. As shown in Figure 3C, there are no intermediate topological forms of the DNA between the –14 coils and the relaxed (0 coils). Therefore, in the observations of Figure 1, the time-dependent formation of negative coils is not due to a rate-limiting process whereby the closing reaction of the enzyme is producing unrestrained topological isomers of the histone–DNA complexes.

This experiment, however, does not take into account a unique characteristic of topoisomerase I. This characteristic is the slow rate of nicking–closing activity that occurs when the DNA substrate is not in a highly supercoiled state. An illustration of this point is shown in the data of Figure 4B. "R" DNA was treated with topoisomerase I under identical conditions to that of Figure 3. The relaxation of the "R" DNA at 23 °C (Figure 4B) is not complete until 20 s has elapsed, which is approximately 5-fold slower than the relaxation rate for "S" DNA. A similar decreased rate is observed when incubated at 0 °C (Figure 4A). This effect has been reported previously (Muller, 1985; Camilloni et al., 1988) and is due to a selective cleavage activity of the enzyme for highly supercoiled DNA. Therefore, in the data of Figure 1C,D, if the quantity of unrestrained stress is minimal in the R–H complexes, the topoisomerase I would appear to be rate-limiting. However, the lack of unrestrained positive coils which causes the apparent rate-limiting character is actually due to an inability of the histones to store negative stress. The stored negative stress is required to produce the unrestrained positive coils in the closed circular DNA.

The data of Figure 3 indicate that the removal of unrestrained negative coils by topoisomerase I is very efficient.

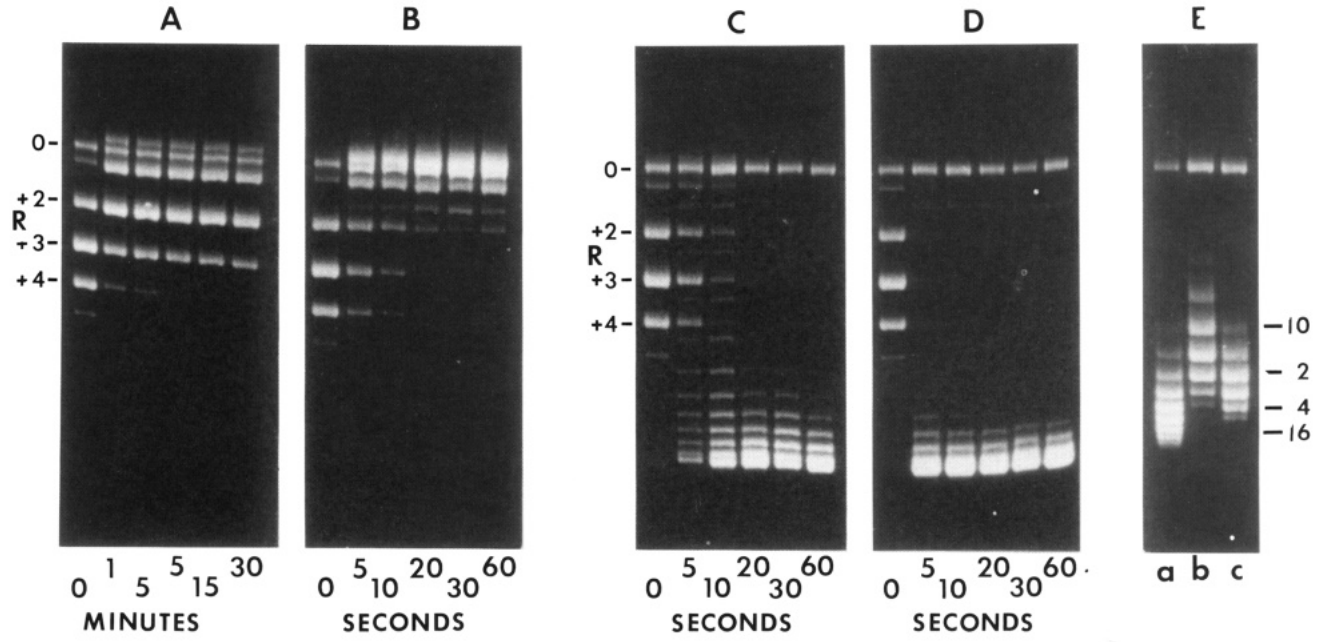

FIGURE 4: Rates of topoisomerase I activity on "R" DNA in the presence (C and D) or absence (A and B) of ethidium bromide. Rates of relaxation at 0 °C (A and C) and 23 °C (B and D) with the same topoisomerase I activities as were used for the experiments of Figure 1. The method of producing the "R" DNA containing the +3 supercoiled DNA involves incubation of DNA at 0 °C in the presence of 10 mM $MgCl_2$ (see Materials and Methods). When this DNA is now incubated at 0 °C with topoisomerase I in the absence of $MgCl_2$, the DNA topology changes from +3 to +2 as observed on these gels. The actual topological change the topoisomerase would see if at 0 °C is from +1 to 0. Ethidium bromide (5 μg/mL) was present during the relaxation of the DNA in panels C and D. The intercalation creates positive supercoils which will be removed from the DNA at a rate dependent on the ability of the enzyme to remove positive coils. After each time point is terminated by addition of SDS, the ethidium bromide is removed (see Materials and Methods) to allow a normal DNA helix to reestablish. The DNA now contains a negative supercoil in which there was once a positive coil. The rate of cleavage by topoisomerase I is based on the rate at which this negatively supercoiled DNA is observed on these gels. Panel E is a 3 μg/mL chloroquine gel which is used to establish the number of negative coils in the "S" DNA (a) and is seen to be −14. The gel is also used to determine the number of positive coils induced by the ethidium bromide when incubated with the "R" DNA at 0 °C (b) and 23 °C (c). The samples for the chloroquine analysis are taken from the 60-s time point of panels C and D and indicate that the ethidium bromide has introduced an average of +11 coils in the DNA at 0 °C and an average of +12.5 coils at 23 °C.

Even though it is known that topoisomerase I relaxes both negatively and positively coiled DNA equally well (Wang, 1985), it is necessary to demonstrate in these experimental protocols that relaxation of unrestrained positive coils is also not rate-limiting. "R" DNA was treated with 5 μg/mL ethidium bromide and subsequently incubated with topoisomerase I. This concentration of ethidium bromide is sufficient to generate approximately +11 coils at 0 °C and +12.5 coils at 23 °C due to the intercalation with the DNA (chloroquine gel of Figure 4E). The "R" DNA was then incubated with topoisomerase I at either 0 °C (Figure 4C) or 23 °C (Figure 4D). At both temperature conditions, the topoisomerase I activity can be seen to relax the positive coils with a rate similar to the relaxation of negative coils (Figure 3A,B). It should be noted in this experiment that the relaxation is demonstrated by the formation of negative coils in the DNA as a result of the removal of ethidium bromide prior to electrophoresis. Therefore, if +14 coils of positive unrestrained stress were present in the R–H complexes of Figure 1C,D, the quantities of topoisomerase I used in these experiments would be more than sufficient to remove them within the 15-s time point.

The Presence of Histone Does Not Alter Topoisomerase I Activity. The experiments described for Figures 3 and 4 do not consider the possibility that topoisomerase I binds histones and hence the presence of the histones would alter the activity of the enzyme. Mixing experiments were done in which supercoiled M13 (7250 bp) was mixed with equal quantities of either "S" DNA (Figure 5A), S–H complexes at a histone to DNA ratio of 0.7:1 (Figure 5B), or R–H complexes at a histone to DNA ratio of 0.5:1 (Figure 5C). The purpose for the mixing was to determine whether the rate of cleavage of M13 DNA was affected by the presence of the histones that were associated with the "S" or "R" DNA. To increase our sensitivity for measuring topoisomerase I inhibition, the enzyme content was decreased 50-fold relative to that which was used for the data of Figure 1. As shown in Figure 5A, 5 min is required to complete the cleavage of M13 DNA when the naked "S" DNA is present as competitor to compete for the binding of the enzyme. When the histones are present with the "S" and "R" DNA, the rate of cleavage of M13 actually increases in proportion to the histone content (Figure 5B,C). Therefore, it can be seen that for the S–H complexes with a histone to DNA ratio of 0.7:1, only 30 s is required for M13 cleavage (Figure 5B). For the R–H complexes in which data are shown for a lower histone to DNA ratio of 0.5:1, 1 min is required for M13 cleavage (Figure 5C). We interpret these data to indicate that those regions of DNA which are complexed with histones are restricted from interactions with topoisomerase I. Only regions of DNA free of histone are available for topoisomerase I cleavage. With lower histone to DNA ratios, these regions are increased and can compete more efficiently with the M13 DNA. These observations indicate that topoisomerase I is not inhibited by histones for either "S" or "R" DNA. These observations also indicate that it is important to complete these studies at suboptimal histone to DNA ratios to ensure that sufficient free DNA is present to allow for relaxation of unrestrained topological stresses. It is for this reason a histone to DNA ratio of 0.7:1 is used.

The Observed Lack of Nucleosome Structure on Positively Stressed DNA Is Independent of Reconstitution Protocol.

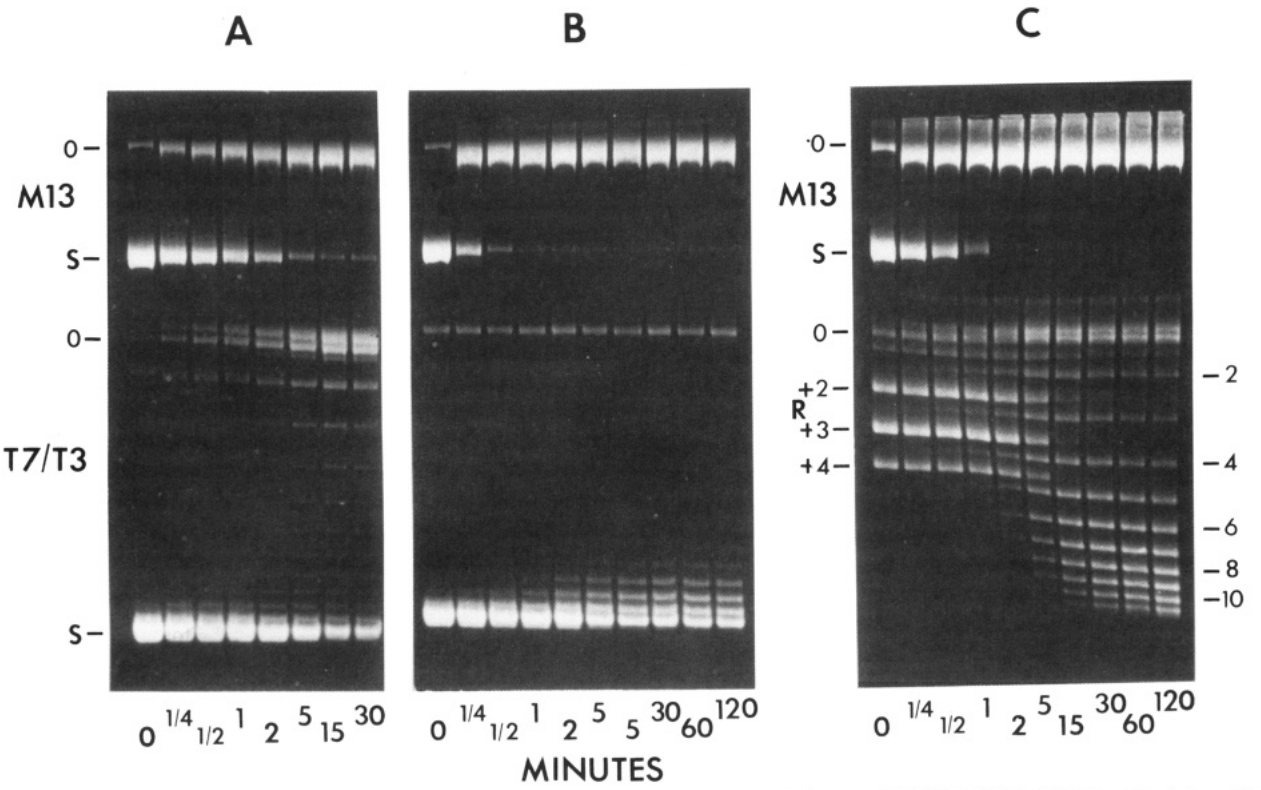

FIGURE 5: Assays to determine the effect of histones on topoisomerase I activity. (A) Supercoiled M13 DNA (7250 bp, labeled as "S" on the upper half of each panel) was added to equivalent quantities (w/w) of the T7/T3-19 plasmid DNA (labeled as "S" on the lower half of panel A). Topoisomerase I (8 units/μg of DNA) was added and the sample incubated 23 °C for the indicated times. The "0" position on the upper portion of the panels marks the location of the relaxed M13 DNA. (B) Same as (A) except the competitor was the T7/T3-19 S–H complex with a histone to DNA ratio of 0.7:1. (C) Same as (A) except the competitor was the T7/T3-19 R–H complex with a histone to DNA ratio of 0.5:1. For panel A, a more rapid rate of M13 DNA relaxation relative to the T7/T3-19 DNA is observed and is due to the larger size of M13 (hence lower molar content) for which less cleavage by topoisomerase I is required to relax a M13 molecule.

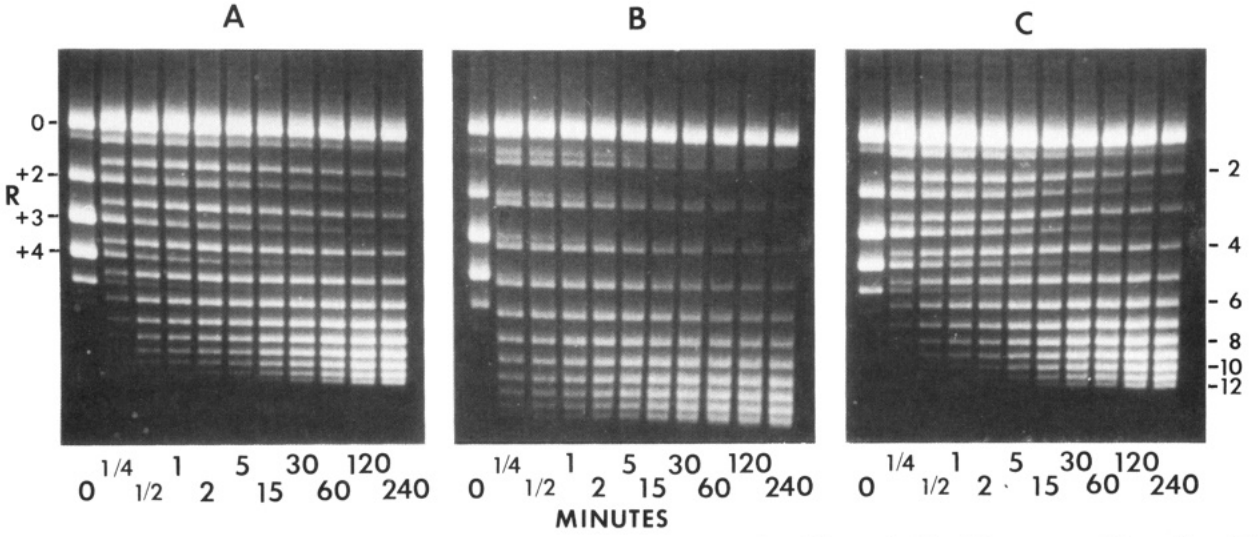

FIGURE 6: Rates of formation of stored negative stress on R–H complexes reconstituted by method 2. Time courses of formation at 0 °C (A) and 23 °C (B). Except for the reconstitution protocol, all conditions are the same as described for Figure 1. (C) R–H complexes were incubated for 60 min at 23 °C in the absence of topoisomerase I. The sample was then reequilibrated to 0 °C for 10 min followed by the addition of topoisomerase I. The time course was maintained at 0 °C.

The reconstitution protocol (method 1) that was used for the experiment of Figure 1 is a commonly used method for reconstitution of nucleosomes. However, because urea is present as a part of the protocol, the lack of structures that can be defined as nucleosomes on the R–H complexes may be due to a previously unobserved denaturation effect which can only be observed in these types of experiments. There is a second commonly used method for reconstitution which does not include urea in the protocol (Germond et al., 1975, 1976; Jorcano & Ruiz-Carrillo, 1979; Daban & Cantor, 1982; Simpson et al., 1985). This method (method 2) involves a stepwise dialysis from 2.0 M NaCl to 1.2 M NaCl to 0.6 M NaCl and finally to 100 mM NaCl. This procedure was applied to generate reconstituted R–H complexes which were then treated with topoisomerase I at either 0 °C (Figure 6A) or 23 °C (Figure 6B). The rate of formation of stored negative stress is observed to be similar to the rate that was observed in the previous reconstitution protocol (compare with Figure

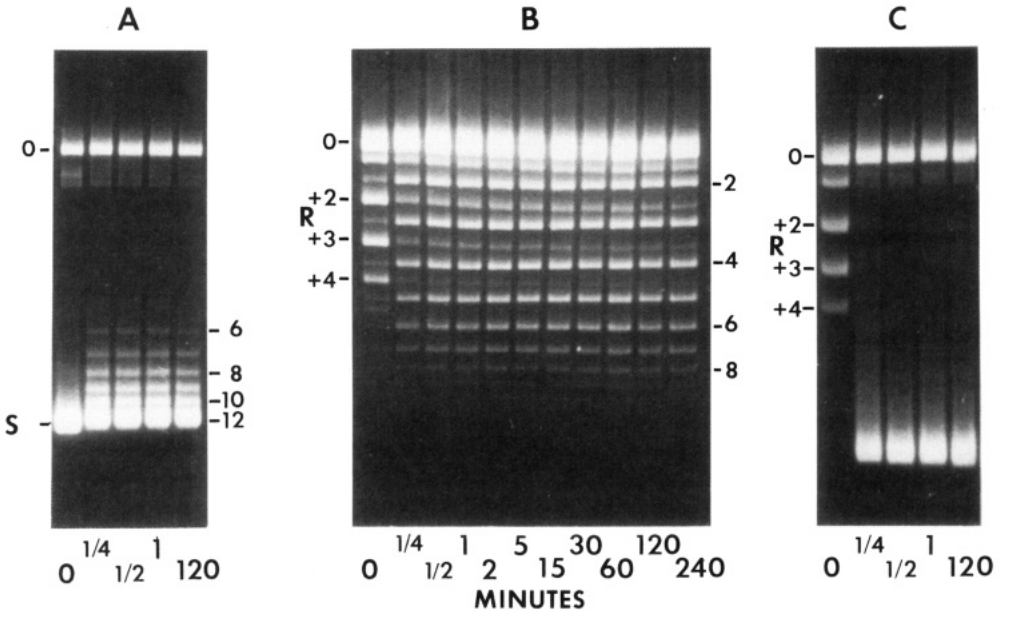

FIGURE 7: Rates of formation of stored negative stress on S–H and R–H complexes that have been cross-linked by formaldehyde. Rates of formation on cross-linked S–H complexes (A) and R–H complexes (B). (C) Cross-linked R–H complexes were treated with 5 μg/mL ethidium bromide prior to addition of topoisomerase I. As described in the legend of Figure 4, the ethidium bromide is removed from the samples of each time point prior to electrophoresis to evaluate the quantity of negative coils introduced by the nicking–closing activity of topoisomerase I. Procedures for removal of the cross-linked protein from the DNA prior to electrophoresis of the DNA are described under Materials and Methods.

1C,D). Similarly, we have utilized a reconstitution protocol which involves an exchange of core histones from mononucleosome particles to the "R" DNA in the presence of 0.9 M NaCl (Drew & Travers, 1985; Constanzo et al., 1990). Again a time- and temperature-dependent process in the formation of stored negative stress is observed (data not shown). By these criteria, the data are interpreted to indicate that the lack of nucleosome formation on the "R" DNA is independent of reconstitution protocols.

A Reversible Alteration in Nucleosome Structure Is Observed When Temperature Is Shifted from 23 to 0 °C. We have previously proposed that the inability to establish a nucleosome structure on covalently-closed, relaxed DNA is due to the requisite formation of an unrestrained positive coil when each nucleosome is formed (Pfaffle & Jackson, 1990). The rigidity of the DNA to torsional stress would be expected to play a major role in defining the amount of positive stress necessary to prevent nucleosome formation. This rigidity is expected to be temperature dependent (Horowitz & Wang, 1985), and therefore, as expected, the earliest time points (15 s) for the data of Figure 6A,B indicate that less stored negative stress is present at 0 °C than at 23 °C. Is this level of stored stress reversible? If R–H complexes are incubated at 23 °C for 60 min in the absence of topoisomerase I to allow maximum formation at the higher temperature and then the complexes reincubated at 0 °C for 10 min prior to the addition of topoisomerase I, does the number of stored negative coils revert back to the condition at 0 °C? This experiment was done and is shown in Figure 6C. The 15-s time point shows a distribution of negative coils which is similar to Figure 6A (0 °C condition) and not to Figure 6B (23 °C condition). We interpret these observations to suggest that the nucleosomes that did form at 23 °C were destabilized by the increasing rigidity of the DNA at the lower temperature. This increased rigidity amplifies the effect of the positive stress which was initially generated when the increased number of nucleosomes were formed at 23 °C. Unrestrained positive stress does appear to have a major effect on the stability of nucleosomes.

Formaldehyde Cross-Linking of the Reconstituted Complexes Preserves the State of Nucleosome Structure. To further demonstrate that nucleosome structure is deficient in the R–H complexes, we have attempted to freeze the state of the histone–DNA interactions by formaldehyde fixation. Both S–H and R–H complexes were prepared by method 1 as described for Figure 1. Subsequently, the samples were incubated with 1% formaldehyde for 24 h at 4 °C. After dialysis to remove the excess fixative, the samples were treated with topoisomerase I at 23 °C. The cleavage reaction was terminated by the addition of a buffer containing SDS and proteinase K, and the samples were incubated 37 °C for 2 days to reverse the protein–DNA cross-links and degrade the proteins (Jackson, 1978). The DNA was then directly electrophoresed on agarose gels as described previously. As shown in Figure 7A for the S–H complexes, topoisomerase I within 15 s has relaxed the DNA, leaving the same number of stored negative coils that are observed without fixation (compare with Figure 1A). A further incubation of up to 2 h does not alter the distribution. The fixation does not appear to have altered the rate at which topoisomerase I is capable of cleaving the fixed S–H complexes or the number of stored negative coils that are present in the complex. When the R–H complexes are similarly treated with topoisomerase I (Figure 7B), a small subset of stored negative coils is observed at the 15-s time point which does not change with an incubation of up to 4 h. This lack of change in the number of negative coils as a function of times is in stark contrast to the pattern observed with R–H complexes that have not been treated with formaldehyde (compare with Figure 1C,D). The number of negative coils that are preserved is similar in quantity to the number of negative coils present in the 15-s time point for the 0 °C incubations of Figure 1C or Figure 7A.

The implication from these data is that formaldehyde fixation has preserved the state of nucleosome structure that is present on the DNA at 0 °C. To demonstrate that this distribution is not due to limited accessibility by topoisomerase I, the fixed R–H complexes were treated with 5 μg/mL

ethidium bromide followed by incubation with topoisomerase I. In this instance, unrestrained positive stress is artificially induced into the closed circular DNA by the intercalation of this agent. If the fixation procedure has limited the accessibility of the enzyme to the DNA, the removal of these positive coils should be prevented. As shown in Figure 7C, topoisomerase I within 15 s has removed the positive coils as indicated by the presence of the negatively coiled DNA. Note again that when ethidium bromide is remove from the DNA prior to electrophoresis, the DNA will exhibit negative coils where there was previously positive coils. Therefore, the DNA within the cross-linked R–H complexes is as accessible to topoisomerase I as the un-cross-linked complexes. The pattern observed in Figure 7C is in fact the pattern that should have been observed in the absence of ethidium bromide if one assumes that a full complement of nucleosomes were present on the "R" DNA. In that instance, the original unrestrained positive stress would have been caused by the presence of the full complement of nucleosomes. If that were the case, the 15-s time point of Figure 1D should have given a pattern of negative coils that was the same as the 15-s time point for Figure 5C. This is clearly not the case.

The data of Figure 7C also address an important point with regard to the translational flux of stress through a circular histone–DNA complex. With the addition of histone to DNA, it could be argued that the flux of stress through the DNA which requires freedom of rotation might become rate-limiting in these studies. However, the 15-s time point of Figure 7C shows that positive stress is efficiently translated through these complexes as the topoisomerase I has completely relaxed the DNA.

A second concern with regard to the data of Figure 7 is whether the formaldehyde fixation causes a change in the pitch of the DNA helix which would manifest itself in an altered topology in the DNA. We have treated both "R" and "S" DNA with formaldehyde and subsequently with topoisomerase I to test for this possibility. The results appear identical to the data of Figures 3 and 4 in that both the rate of topoisomerase I cleavage and the topological state of the fixed DNA remain the same as DNA that has not been treated with the fixative (data not shown). These observations suggest that formaldehyde fixation has preserved the state of nucleosome structure within the R–H and S–H complexes.

Formaldehyde Treatment Efficiently Cross-Links the Core Histone to the DNA in both the R–H and S–H Complexes. If incomplete fixation were to occur by these procedures (particularly on the R–H complexes), the distribution of negative coils may not be a proper representation of nucleosomes that are present in the R–H complexes. To test for incomplete fixation, the cross-linked R–H and S–H complexes were applied to CsCl/GuHCl density gradients and sedimented to equilibrium. Fractions were collected, the salts and protein were removed (see Materials and Methods), and the distribution of DNA was determined by agarose gel electrophoresis. As shown in Figure 8B, when the R–H and S–H complexes were cosedimented on the same gradient, both were distributed in the same fractions of the gradient (fractions 13–17). The distribution indicates that the same quantity of histones has cross-linked to both the "S" and "R" DNA. If a lower level of cross-linking had occurred on the "R" DNA, then the distribution of the "R" DNA should have been shifted to a higher density (lower fraction number). To further demonstrate this point, a control gradient is shown in Figure 8A in which *un-cross-linked* R–H and S–H complexes are cosedimented on the same gradient. Both "R" and "S" DNAs are distributed in fractions 4–6, which reflects the loss of associated histone. If the cross-linked R–H complexes were deficient in histone content due to incomplete fixation, the distribution of "R" DNA should have been shifted to those fractions.

The capability of these gradients to detect small differences in the histone to DNA ratio is further demonstrated in the data of Figure 8C. In this instance, the formaldehyde-treated S–H complexes were sedimented to equilibrium and subsequently the CsCl and GuHCl removed from the fractions by dialysis. Before the protein was removed to analyze the DNA, each fraction was treated with topoisomerase I at 23 °C for 60 min. The protein was then removed to reveal the topological state of the DNA in the fractions. Note in Figure 8C that the number of negative coils in fraction 13 is approximately 20% less than the number of negative coils in fraction 16. The three-fraction difference is able to resolve the decreased number of nucleosomes that were present on the "S" DNA due to the decrease in quantity of histone. If the mixed R–H complexes of Figure 8B contained a complement of fixed histones which would produce the number of nucleosomes seen in the data of Figure 7B, 80% of the histones would have had to have been displaced, and the fixed R–H complexes should have distributed at fraction 8. This is clearly not observed.

To further demonstrate the efficiency of the fixation, cross-linked R–H complexes were sedimented to equilibrium on CsCl/GuHCl gradients. After the fractions were collected, the histone–DNA cross-links were reversed (see Materials and Methods), and in this instance, the distribution of the histones in the gradient was analyzed on a SDS–PAGE gel. As shown in Figure 9, all four core histones are present in equimolar quantities. If any of the histone types were inefficiently cross-linked to the DNA, those histones would have distributed to the top of the gradient (fractions 20–24). The deficiency in storage of negative stress for the fixed R–H complexes in Figure 7 cannot be a result of incomplete cross-linking of histone to DNA.

The usefulness of formaldehyde fixation in preserving existing nucleosome structure is further illustrated in the following experiment. Reconstituted R–H complexes were prepared by method 1 and subsequently treated with topoisomerase I at 23 °C. Instead of terminating the time points with addition of SDS, these time points were terminated by the addition of formaldehyde. After cross-linking for 24 h at 4 °C and removal of the excess formaldehyde by dialysis, aliquots were treated with SDS and proteinase K to analyze the topological state of the DNA. As shown in Figure 10A, formaldehyde terminated topoisomerase I activity very efficiently as exhibited by the DNA pattern which is very similar to the pattern for Figure 1D in which SDS was used. A time-dependent formation of negative stress is observed. How firmly the nucleosome structure is maintained by the formaldehyde in these time points can now be seen in the treatment of a second aliquot of the time points with topoisomerase I for 4 h at 23 °C. This length of treatment is more than sufficient to guarantee complete relaxation of the fixed complexes. When SDS and proteinase K are now added to analyze the topology of this DNA, the DNA is found to contain a similar number of negative coils to those samples which had not received the additional topoisomerase I treatment (compare Figure 10A and Figure 10B). We interpret these data to indicate that formaldehyde has frozen the state of nucleosome structure as such structures increase in number on the R–H complexes.

The Helical Pitch of DNA in the R–H and S–H Complexes Is the Same. The deficiency in nucleosome structure on the

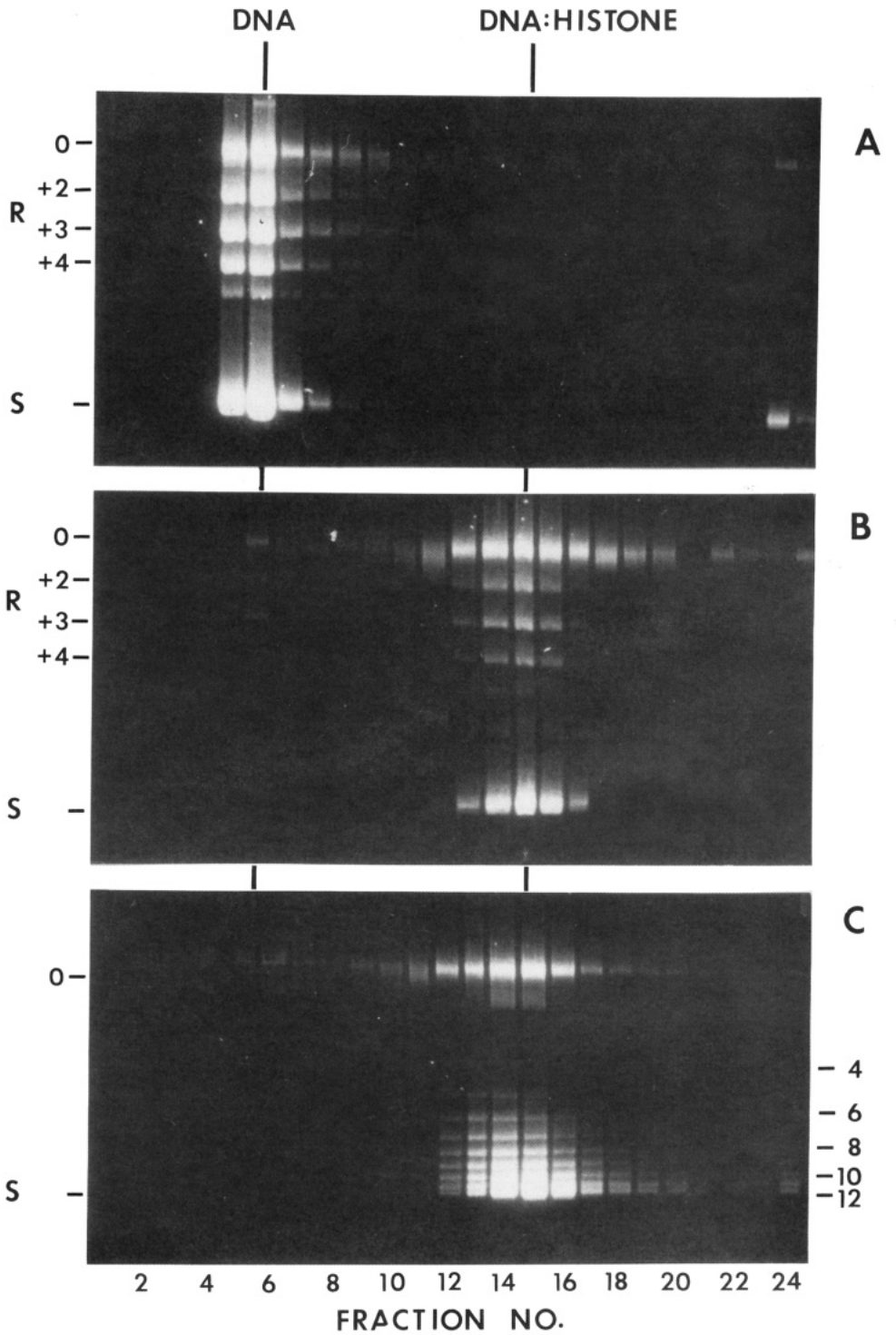

FIGURE 8: CsCl/GuHCl density gradient analysis of R–H and S–H complexes. (A) Un-cross-linked S–H and R–H complexes are combined and sedimented to equilibrium. (B) Formaldehyde cross-linked R–H and S–H complexes are combined and sedimented to equilibrium. (C) Formaldehyde cross-linked S–H complexes are sedimented to equilibrium alone. After centrifugation to equilibrium, the fractions are dialyzed to remove the salts and then either directly treated with SDS and proteinase K (panels A and B) or incubated with topoisomerase I for 60 min prior to treatment with SDS and proteinase K (panel C).

R–H complex could be in one of two forms. Either there is an altered state (decrease) in the DNA superhelix or there is an altered pitch in the DNA helix. In the first instance (model 1), the DNA helix would maintain the same standard nucleosome periodicity of 10.3 bp/turn in its interaction with the core histones. However, the H2A, H2B dimer and H3, H4 tetramer interphase would have to be disrupted such that the DNA superhelix would be similarly altered. This condition would represent an "opened" nucleosomal state and would eliminate the storage of one negative coil. In the second inst ce (model 2), the DNA would be wrapped around the hist e core, maintaining the proper left-handed superhelix whi would of necessity generate a negative coil. However, in o r to neutralize the presence of the negative coil, the heli itch of the DNA would need to be increased such that the overall nucleosome structure would not produce a torsionally constrained negative coil. This rotational change in pitch would need to be on average from 10.3 to 9.7 bp/turn (see the legend of Figure 11 for calculations). This altered structure could not be classified as a nucleosome as it would

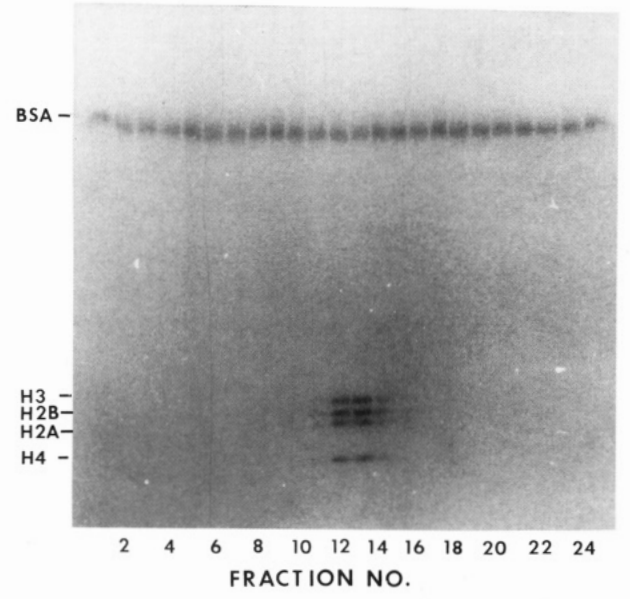

FIGURE 9: Analysis of histone distribution on a CsCl/GuHCl density gradient of the cross-linked R–H complexes. Cross-linked R–H complexes were sedimented to equilibrium, fractions were collected, and after reversal of the cross-link as described under Materials and Methods, the fractions were electrophoresed on 18% PAGE–SDS gels. BSA was added as a carrier to each fraction to ensure that in the subsequent precipitation with TCA quantitative yield of all histones is achieved.

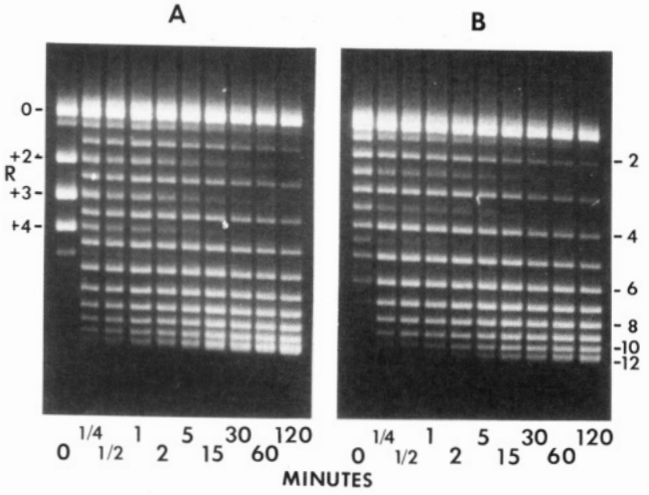

FIGURE 10: Measurement of the effectiveness of formaldehyde treatment to define the nucleosome content in R–H complexes. (A) Rates of formation are measured by terminating time points with formaldehyde rather than with SDS. The samples were cross-linked for 24 h at 4 °C followed by removal of the excess formaldehyde by dialysis. An aliquot of each time point was taken and the cross-linked protein removed prior to electrophoresis of the DNA. (B) A second aliquot of each time point containing the cross-linked complex was treated with topoisomerase I at 23 °C for 4 h after which this protein was also removed and the DNA analyzed.

not store a negative coil within its DNA superhelix. The earlier experiments we have presented would not be able to differentiate between these two altered states of nucleosome structure. The previous experiments indicated that unrestrained positive coils were not present between nucleosomes. However, if the positive coils are restrained within the pitch of the DNA (model 2), then for the DNA to return to a normal 10.3 bp/turn after topoisomerase I addition, the histone–DNA contacts would need to be disrupted. This disruption may be the rate-limiting step and account for the time- and temperature-dependent process for forming torsionally constrained negative coils on the "R" DNA. For model 1, the time-dependent process would be due to the generation of H2A, H2B, and H3, H4 interactions as the DNA is thermally twisted. These two models can be differentiated by a determination of the helical pitch in the DNA within the R–H complexes.

The treatment of nucleosomes by DNase I has been used as a highly effective procedure for establishing the pitch of the DNA helix (Noll, 1974; Lutter, 1978; Klug & Lutter, 1981; Drew & Calladine, 1987). As shown in Figure 11, S–H and R–H complexes have been treated with DNase I under three different conditions: digestion at 0 °C for 3 min (Figure 11A), digestion at 23 °C for 30 s (Figure 11B), and digestion of formaldehyde-fixed complexes at 23 °C for 30 s (Figure 11C). Each digestion was done with increasing quantities of DNase I. As shown in Figure 11B, digestion at 23 °C produces a series of DNA fragments which increase in increments of an average of 10.3 bases for both the S–H and R–H complexes. The determination of size is based on a comparison with the DNase I digest of calf thymus nuclei which is used as a standard on these gels (M). If a pitch of 9.7 bases were present, then, for example, at the 7 × 10.3 base or 72-base fragment, a fragment size of 7 × 9.7 bases or 68-base fragment should be seen. No such shift in fragment size is observed for the R–H complexes. A major limitation of digestions at 23 °C is the release of any unrestrained stress in the circular plasmid due to the initial nick by DNase I. As has been shown in Figures 1D and 7B, substantial storage of negative coils has occurred within 30 s at this temperature. To successfully detect the presence of an altered pitch, this process must be kept to a minimum. Since this same process is restricted at 0 °C, a digest with DNase I was done at that temperature, and as shown in Figure 11A, the fragment size remains the same as the control DNase I digest. As there is no indication of an altered pitch of the DNA, the data are consistent with the "opened" nucleosomal state of model 1. To further emphasize this very important point, a DNase I digest was done on the formaldehyde-fixed complexes. Fixed R–H complexes are covalently held in this altered nucleosomal state, and fixed S–H complexes are covalently held in a normal nucleosomal state. As shown in Figure 11C, both the R–H and S–H complexes maintain the same average 10.3-base periodicity. Model 1 is consistent with the data presented in this report.

DISCUSSION

Evidence has been presented which indicates that nucleosome structure is altered under conditions in which the histones are associated with circular, covalently-closed DNA which contains +3 coils. The procedures that have been used were designed to identify the presence of additional positive coils between the nucleosomes assuming normal nucleosomes are present on this DNA. Instead, we observe a time- and temperature-dependent formation of stored negative coils (i.e., nucleosome structure) as soon as topoisomerase I is added. A number of control experiments have been done which indicate that topoisomerase activity is not the rate-limiting step in this process. These results suggest that the initial +3 coils are sufficient to inhibit nucleosome formation.

The reconstituted complexes have also been treated with formaldehyde in an attempt to maintain the state of histone–DNA interactions prior to topoisomerase addition. These data also indicate that an altered nucleosomal state exists on this DNA. Formaldehyde treatment has been used extensively in studies of chromatin structure for both *in vivo* and *in vitro* experiments (Jackson, 1978; Delange et al., 1979; Martinson et al., 1979; Jackson & Chalkley, 1981a,b, 1985; Solomon &

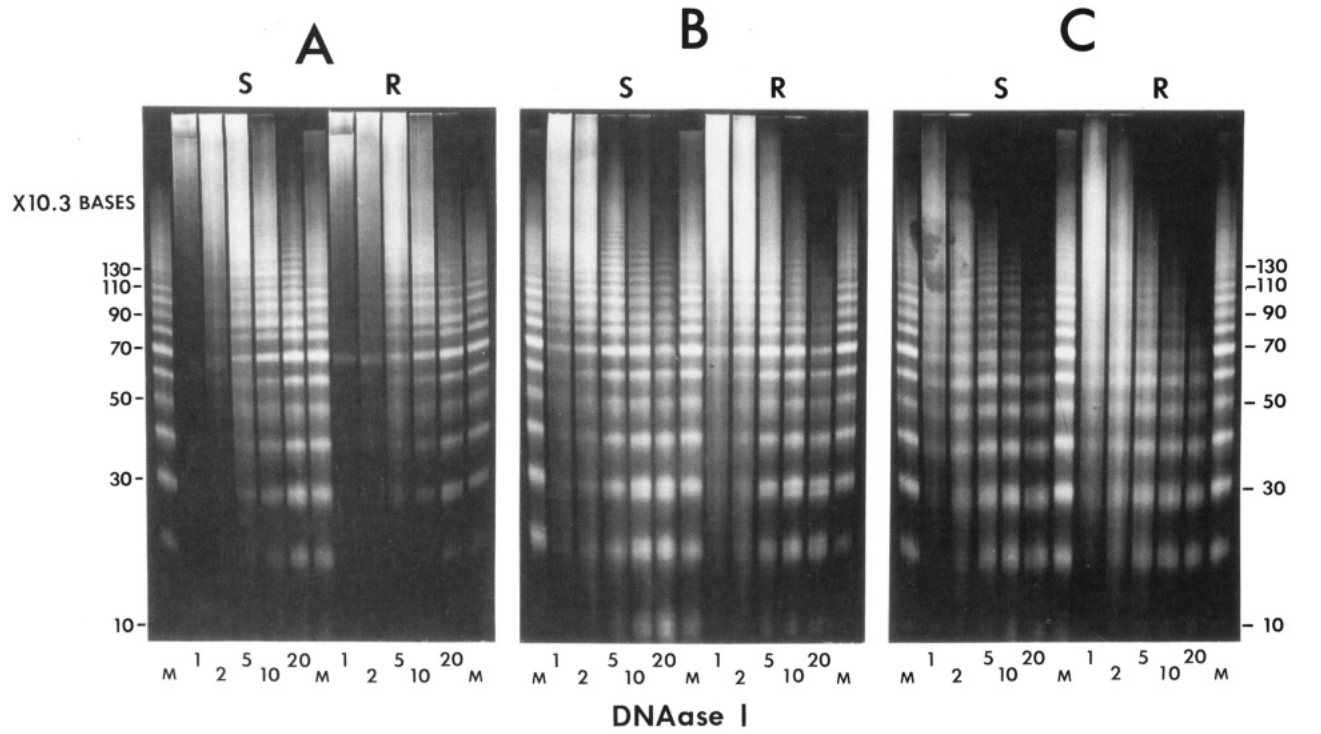

FIGURE 11: DNase I digestion and analysis of S–H and R–H complexes. (A) Digestion at 0 °C for 3 min; (B) digestion at 23 °C for 30 s; (C) digestion at 23 °C for 30 s of formaldehyde cross-linked S–H and R–H complexes. DNase I at 60 units/μL is added to 20 μg of DNA in these complexes in quantities of 1, 2, 5, 10, or 20 μL and incubated the specified times. "M" refers to marker DNA fragments which were generated by treatment of calf thymus nuclei with DNase I. The calculation for the theoretical helical pitch of 145 bp of DNA containing +1 coil is as follows: 145 bp/(10.3 bp/turn) = 14 turns in one nucleosome. Increasing it to 15 turns with the addition of the +1 coil will produce 145 bp/15 = 9.7 bp/turn. The 10.3 and 9.7 bp/turn represent an average number due to the multiple hit sites that DNase I has on the surface of the DNA (Klug & Lutter, 1981). Nevertheless, what appears to be initially a slight difference in helical pitch can be seen in a multiplied form by examining the cleavage sites toward the center of the nucleosome, i.e., seven turns of the helix. As indicated in the text, a difference of a 72-base fragment for the S–H complexes versus a 68-base fragment for the R–H complexes would be expected if model 2 were correct. A technical difficulty in these reconstitutions is the formation of nicked DNA. For these DNase I digestion studies, 20% of the DNA in the R–H complexes was nicked and therefore represents a background of normal 10.3 bp/turn periodicity due to the presence of normal nucleosomes on that DNA.

Varshavsky, 1985; Solomon et al., 1988; Ip et al., 1988; Nacheva et al., 1989; Thomas, 1989). The studies of this report have applied such treatment to an analysis of DNA topology in cross-linked histone–DNA complexes and included control experiments not previously described which further illustrate the effectiveness of formaldehyde treatment in preserving structure.

The altered nucleosomal state on the "R" DNA could be in one of two forms: either an increase in the pitch of the DNA helix or a disruption of the superhelix. The DNase I digest of these complexes indicates that an average pitch of 10.3 bp/turn is maintained in both the S–H and R–H complexes. Thus, both normal nucleosomes and altered nucleosomes maintain the same DNA pitch which implies that the superhelix is altered. The unwinding of the superhelix could occur through disruption of the interphase between H2A, H2B and H3, H4 as well as a loosening of histone–DNA interactions at the entry points of the altered nucleosome. From the crystal structure of the octamer, it can be seen how the superhelix could be disrupted, resulting in a U-type structure for the DNA path (Arents et al., 1991). The H3, H4 tetramer would be seen to interact at the central axis at the bottom of the "U". The differential loosening of histone–DNA interactions at the entry point has also been observed from thermal denaturation studies and suggests that these interactions may be disrupted under stressful conditions (Simpson, 1979; Ausio et al., 1979). The higher background of DNase I cut sites in the R–H complexes near the fragment sizes of 140 bases is highly suggestive of such a process (Figure 11). The HTC histones used in this study have approximately 30% of H3 and H4 in the monoacetylated form. Acetylation has been shown to reduce histone–DNA interactions at the entry points and therefore may be a contributing factor to the loss of the stored negative stress in the "R" DNA (Norton et al., 1989, 1990).

There is considerable *in vivo* evidence to indicate that during transcription nucleosomal structure is altered, resulting in the selective release of H2A, H2B [reviewed in Jackson (1990) and van Holde et al. (1992)]. It has also been shown that *in vitro* transcription is enhanced by the partial removal of H2A, H2B (Gonzalez & Palacian, 1989) and that RNA polymerase-associated nucleosomes are depleted in those histones (Baer & Rhodes, 1983). The superhelix would need to at least partially unfold to disrupt the H2A, H2B and H3, H4 contacts to facilitate this release. We have previously observed with *in vitro* experiments that transcriptionally-induced positive stress disrupts nucleosome structure (Pfaffle et al., 1990). This same disruption by positive stress has been observed *in vivo* (Lee & Garrard, 1991). The studies of this report provide a more definitive means of evaluating the contributing factors which alter structure. Since RNA polymerase is not present to distort histone–DNA contacts, it is possible to observe the sole effect of positive stress. The altered "open" nucleosome structure is expected to be the first step. The second step may be the actual disruption of H2A, H2B interactions with DNA through intimate contact with the transcribing RNA polymerase (manuscript in preparation).

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