Preferential Binding of Histones H3 and H4 to Highly Positively Coiled DNA†

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ABSTRACT: The interaction of histones H3 and H4 with highly positively coiled DNA has been studied. To carry out this study, it was necessary to develop a protocol for the preparation of large quantities of highly positively coiled DNA. Such preparations were obtained by maximizing transcription-induced stress in cells containing PBR322 and then applying a short-term treatment with novobiocin, which inhibits gyrase. Fractionation on CsCl−EtBr gradients gave PBR322 plasmids with topological states ranging from +0.15 to −0.043 superhelical density. In competition experiments between negatively and positively coiled DNA, histones H3,H4 preferentially bound the positively coiled DNA when the superhelical density was greater than +0.10. This preference was shown on the basis of sedimentation rate on sucrose gradients, selective aggregation by H3,H4 binding, and cross-linking experiments in which the histone−DNA content was characterized on CsCl density gradients. An analysis of the DNA helical pitch in 1.1 M NaCl, a condition in which moderately positively coiled DNA preferentially binds H3,H4, indicated that the preferential binding may be due to a decrease in helical pitch that approximates 0.06 bp/turn. Consistent with this observation is the ability of histones H3,H4 to transiently hold this altered pitch in the presence of topoisomerase I. A possible explanation for this preference is based on the known observation that histones overwind the DNA helix when in a nucleosome. These data provide an explanation for the *in vivo* observation that histones H3,H4 are rarely displaced during the transcription process. These observations are discussed with regard to mechanisms for transcription through nucleosomes.

The DNA of a eukaryotic cell is packaged into semiregular repeated structures called nucleosomes. For each nucleosome, 145 bp of DNA is wrapped in a left-handed coil 1.8 times around two each of four highly basic proteins, histones H3, H4, H2A, and H2B. The binding energies of these eight proteins with DNA are extremely high, requiring NaCl concentrations greater than 1.2 M to disrupt. This stability is due to electrostatic interactions not only between the histones and DNA but also between the eight histones themselves within the core complex. Any transient release of one histone from the DNA would be prevented from complete dissociation because of the continued interaction with the other histones in the core complex. Disruption of all eight proteins as a group from the DNA at physiological ionic strength represents a very difficult task. Yet various levels of disruption are required to facilitate access to the DNA for transcription, replication, and repair. Extensive research has been performed to understand the disruption mechanisms, and these studies have been reviewed extensively (Svaren & Chalkley, 1990; Grunstein, 1990; Thomas, 1991; Kornberg & Lorch, 1991; van Holde et al., 1992; Morse, 1992; Hansen & Ausio, 1992; Felsenfeld, 1992; Adams & Workmen, 1993; Paranjape et al., 1994; Wolffe, 1994; Lewin, 1994). Of principal interest with regard to our study are the *in vivo* observations that histones H2A,H2B are extensively disrupted from the DNA during the transcription process and that histones H3,H4 are minimally disrupted (Jackson & Chalkley, 1985; Jackson, 1990). Such an observation suggests that a "divide and conquer" approach is applied to the nucleosome, in that the first step in the process is the disruption of the interaction between H2A,H2B and H3,H4, followed by a second step which is the displacement of H2A,H2B from the DNA. Transcription clearly would be facilitated by such a process, but not totally so because of the continued maintenance of H3,H4 on the transcribed DNA. Why is it so important to maintain H3,H4 on the DNA? What is the mechanism for its maintenance in the presence of a disruptive RNA polymerase? How does the polymerase cause the initial disruption of the interaction between H2A,H2B and H3,H4?

With regard to the latter question, our studies indicate that during transcription disruption occurs due to the formation of transcription-induced topological stress. The twin-domain model for induced stress was first described by Liu and Wang (1987) and has gained general acceptance on the basis of a large number of studies demonstrating its presence in both prokaryotes (Pruss & Drlica, 1986, 1989; Wu et al., 1988; Tsao et al., 1989; Cook et al., 1992; Rahmouni & Wells, 1992; Dayn et al., 1992) and eukaryotes (Brill & Sternglanz, 1988; Giaever & Wang, 1989; Ljungman & Hanawalt, 1992; Gartenberg & Wang, 1992). The model is based on the premise that RNA polymerase is restricted from rotation around the DNA helix due to the viscous drag of the RNA transcript. Since 10−15 bp of the DNA helix is maintained in an open state at the site of transcription, the DNA is forced to rotate (Gamper & Hearst, 1982). This rotation results in overwinding in advance of the polymerase (one positive coil per 10.5 bp transcribed) and similar underwinding of the helix in the wake of the polymerase (one negative coil per 10.5 bp transcribed). This positive stress, which tends to form an unrestrained right-handed supercoil, might be expected to have a disruptive influence on the left-handed supercoil of the nucleosome. Both *in vitro* (Pfaffle et al., 1990) and *in vivo* (Lee & Garrard, 1991) evidence has been obtained to support this scenario. However, to more clearly

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define the effect of topological stress on nucleosome structure, it was necessary to study nucleosome stability in the absence of RNA polymerase.

In those studies, we observed that a positively coiled DNA of +0.011 superhelical density would severely limit nucleosome formation (Jackson, 1993). Additional data indicated that the interaction between histones H2A,H2B and H3,H4 was more disrupted when the histones were associated with the moderately positively coiled DNA than with negatively coiled DNA (Jackson et al., 1994). It was also observed that histones H2A,H2B had a 10-fold preference for binding negatively coiled DNA over positively coiled DNA and that H2A,H2B would rapidly transfer between the two DNAs at physiological ionic strength (Brooks & Jackson, 1994). These data led to a model in which it was proposed that the positive stress induced by RNA polymerase causes disruption of the H2A,H2B interphase with H3,H4, which in turn allows H2A,H2B to release from the DNA and bind to the negatively coiled DNA in the wake of the polymerase. H2A,H2B would then reform a nucleosome with the H3,H4 that remained associated with the previous region of transcribed DNA. Additional reconstitution experiments with negatively coiled DNA and histones H3,H4 provided us with a potential explanation for why H3,H4 remained associated with the DNA. In those experiments, it was observed that H3,H4 would form a stable pseudonucleosome comprising an octameric complex of H3,H4 alone (Jackson et al., 1994). It was postulated that H3,H4 release would tend to promote the formation of such structures on the negatively coiled DNA in the wake of the polymerase [see Discussion in Jackson et al. (1994)]. The question concerning the mechanism for maintenance of H3,H4 on the DNA during the transcription process was unresolved. In this report, data are presented which indicate that H3,H4 bind with high affinity to DNA that is highly positively coiled. This affinity is greater than the affinity H3,H4 have for negatively coiled DNA and therefore provides a mechanism to exclude pseudonucleosome formation in the wake of the transcribing RNA polymerase.

MATERIALS AND METHODS

Procedures for Purification of Histones H3,H4 and Topoisomerase I. Histones were purified from chick erythrocytes using a modification of the procedure of Simon and Felsenfeld (1979). Purified nuclei were prepared by four washes with 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 and 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.

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 activities, 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.

Procedures for Preparation of DNAs with Varying Topological States. The two DNA plasmids used in these studies (PBR322 and pT7/T3-19, Bethesda Research Laboratories) were grown in HB101 at 37 °C in enriched LB broth (Maniatis, 1982). The covalently closed supercoiled DNAs were purified on CsCl density gradients and yielded circular DNAs of superhelical density between −0.043 and −0.061. These densities correspond to an average of −18 coils for PBR322 (4363 bp) and −13 coils for T7/T3-19 (2238 bp), as determined by analysis on chloroquin gels (data not shown).

The following describes the defined conditions for purifying highly positively coiled DNA. Strain AS19 was used in these studies as it is highly permeable to antibiotics (a gift from Leroy Liu, UMDNJ-RW Johnson Med. Sch.). These cells were transformed with PBR322 and grown in 6 L quantities (6−2.8 L Fernbach flasks) in enriched LB broth containing 10 μg/mL tetracycline. After growth to an OD_{550} of 1.3, the cells were treated with 400 μg/mL novobiocin for 30 min and rapidly harvested by acceleration to 8000 rpm (approximately 3 min). The cell pellets were homogenized into 200 mL of 25% sucrose, 50 mM Tris (pH 8.0), and 500 μg/mL novobiocin and mixed with 50 mL of 10 mg/mL lysozyme. After incubation at 4 °C for 10 min, 70 mL of 0.25 M EDTA (pH 8.0) was added to the mixture and the incubation continued for 5 min. A lysing mix (300 mL) consisting of 2% Triton X-100, 60 mM EDTA, and 50 mM Tris (pH 8.0) was added, and the lysed cells were centrifuged at 35 000 rpm for 30 min in a Type 35 rotor. The supernatant was treated with an equal volume of 30% poly(ethylene glycol) (#8000)/1.5 M NaCl, and after incubation for 30 min at 4 °C, the sample was centrifuged at 10 000 rpm for 30 min. The pellet was homogenized into 50 mL of 10 mM Tris/10 mM EDTA, and CsCl and ethidium bromide (EtBr) were added. The sample was maintained at 4 °C overnight and then centrifuged at 10 000 rpm for 10 min to remove insoluble protein. The supernatant was adjusted to 1 mg/mL EtBr concentration and the refractive index was adjusted to 1.3950. Centrifugation was at 35 000 rpm for 90 h at 11 °C in a Type 70 rotor. Fractions were collected in subdued light, extracted with butanol to remove the EtBr, and dialyzed against 10 mM Tris/10 mM EDTA (pH 8.0) overnight at 4 °C. The samples were then phenol/chloroform extracted and ethanol precipitated. The DNA was dissolved and stored in 10 mM Tris/1 mM EDTA (pH 8.0).

Electrophoresis was on 1.2% agarose (CalBiochem, Type C) at 85 V for 14 h at 4 °C. The electrophoresis buffer was 50 mM Tris, 45 mM acetic acid, and 1.25 mM EDTA (pH 8.0). Gels were stained by protocol no. 3 as described in the legend to Figure 4B. For two-dimensional electrophoretic analysis, a DNA sample was electrophoresed in the first dimension using the same buffer conditions. The gel was then soaked for 2 h in 15 μM chloroquin, rotated 90 °C, and reelectrophoresed in the second dimension in the presence of 15 μM chloroquin.

Procedures for Analysis of the Average Helical Pitch of DNA in High NaCl Concentrations. Camptothecin (Sigma) was dissolved in DMSO by sonication at a concentration of 10 mg/mL. This solution was added to negatively supercoiled T7/T3-19 DNA (2238 bp) in 100 mM NaCl, 50 mM TEA, and 1 mM EDTA (pH 7.4) at a final concentration of 50 μg/mL camptothecin. Topoisomerase I (400 units/μg

DNA) was added and the sample incubated for 20 min at 23 °C. Aliquots were then added to an equivalent volume of a solution containing increasing NaCl sufficient to produce 0.1−1.9 M NaCl at increments of 0.2 M. Samples were incubated at 23 °C for 4 h, adjusted to 0.1% SDS, and dialyzed against 0.1% SDS, 100 mM NaCl, 50 mM TEA, and 1 mM EDTA (pH 7.4) for 3 h at 23 °C. Samples were then adjusted to 1 mg/mL proteinase K and incubated at 37 °C for 3 h. These samples were then applied to a 1.5% agarose gel. The gel electrophoretic conditions have been established to show an average of 0 supercoils (see Figure 1A, lane a) for DNA that has been relaxed at 23 °C in 100 mM NaCl, 50 mM TEA, and 1 mM EDTA (pH 7.4). If we assume 10.50 bp/helical turn for DNA at 23 °C (Depew & Wang, 1975; Pulleybank et al., 1975; Horowitz & Wang, 1984), then the number of turns in DNA of 2238 bp is 2238/10.5 = 213.1 turns. When 1.1 M NaCl is present, an average of +1.2 coils is observed (see Figure 1A). Therefore, in 1.1 M NaCl, the DNA has 213.1 + 1.2 = 214.3 turns or a helical pitch of 2238/214.3 = 10.44 bp/turn. The average pitch change from 0.1 to 1.1 M NaCl is 10.50 − 10.44 = 0.06 bp/turn. The +1.2 coil value was determined by measuring the integrated quantities for the topoisomers at 1.1 M NaCl and then applying these values to the equation, $[\sum n(x_n)]/\sum x_n$ = average supercoils, in which n is the supercoil number of the topoisomer (+ or −) and x_n is the integrated quantity of the topoisomer. The band labeled N in Figure 1 is nicked DNA, and its integrated quantity is not used in the calculations.

Reconstitution of Histone−DNA Complexes. Histones H3,H4 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). The histone and DNA concentrations were determined by using extinction coefficients for histones of 4.2 at 230 nm and for DNA of 20.0 at 260 nm (Stein, 1979).

For analysis of the topological state of the DNA in these complexes, the reconstituted histone−DNA complexes at 100 μg/mL (DNA concentration) were treated with 400 units/μg 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 and applied to a 1.2% agarose gel. DNA was visualized by ethidium bromide staining.

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 100 mM NaCl, 50 mM TEA, and 1 mM EDTA (pH 7.4). Centrifugation was at 35 000 rpm at 3 °C in a SW41 Ti rotor. The times of centrifugation varied as indicated in the figure legends. Fractions (450 μL) were collected, and 20 μL was taken from each fraction for analysis of the DNA distribution. The remainder of each fraction was treated with 5 μg of bovine serum albumin, 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% trichloroacetic acid and, after 1 h of incubation at 0 °C, centrifuged at 27000g for 10 min to collect the pellets. The pellets were sonicated into 30 μL of 10% glycerol, 300 mM Tris, 20 mM 2-mercaptoethanol, and 1% SDS (pH 8.8) (loading buffer) and applied to an SDS−PAGE gel, which consisted of a stacking gel [2.5% acrylamide, 0.13% bisacrylamide, 125 mM Tris (pH 6.8), and 0.1% SDS] and a separating gel [18% acrylamide, 0.09% bisacrylamide, 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 dye. The 20 μL that was taken for DNA analysis was treated with an equal volume of stop buffer and electrophoresed on 1.2% agarose in the presence of 15 μM chloroquin.

Density Gradient Analysis of Cross-Linked Complexes. The reconstituted histone−DNA complexes were treated with 1% formaldehyde for 24 h at 4 °C. The samples were dialyzed against 100 mM NaCl, 50 mM TEA, and 1 mM EDTA (pH 8.0) for 24 h at 4 °C. The samples were then either treated with topoisomerase I to determine the topological state of the DNA or mixed with 1.4 g of CsCl, 1.5 g of guanidine hydrochloride (GuHCl) and 100 μL of 0.25 M EDTA (pH 8.0) in a final volume of 4.0 mL. These samples were then centrifuged to equilibrium in a SW60 Ti rotor at 40K for 70 h at 4 °C. Fractions (150 μL) were collected and dialyzed in a multiwell microapparatus against 10 mM Tris/1 mM EDTA (pH 8.0) for 12 h at 4 °C. The samples were treated with stop buffer and incubated at 37 °C for 2 days. The extended length of incubation disrupts the cross-link (Jackson, 1978, 1993). An aliquot of each sample was applied to a 1.2% agarose gel.

RESULTS

At 1.1 M NaCl the Average Helical Pitch of DNA Decreases by 0.06 bp/Turn. In a previous report (Jackson et al., 1994), we observed that in the presence of 1.1 M NaCl histones H3,H4 maintained a 5-fold preference for binding to DNA that contained a +0.011 superhelical density (SD) as compared to DNA that contained a −0.045 SD. When the reconstitution of H3,H4 with the DNAs was allowed to continue to 100 mM NaCl, the preference changed to a condition in which a 2-fold preference was observed for the negatively coiled DNA. It was hypothesized that this preference for binding to the positively coiled DNA in 1.1 M NaCl was a result of a decrease in the helical pitch of the DNA caused by the high NaCl concentration. This decrease in helical pitch would be expected to be more readily facilitated in positively coiled DNA than in negatively coiled DNA. Similarly, it was argued that at 100 mM NaCl the positive stress of +0.011 was not sufficient to maintain an altered pitch, such that the preference for binding now occurred on the negatively coiled DNA. The presence of the negative coils in the −0.045 SD DNA would be expected to more stably maintain oligomeric interactions of H3,H4 in either NaCl condition. Therefore, part of this hypothesis was a statement that the binding affinity of H3,H4 for DNA with decreased helical pitch is greater than the binding affinity for DNA that promotes higher order histone−histone interactions. To test this hypothesis, it would be useful to know the helical pitch that is present in the DNA at 1.1 M NaCl.

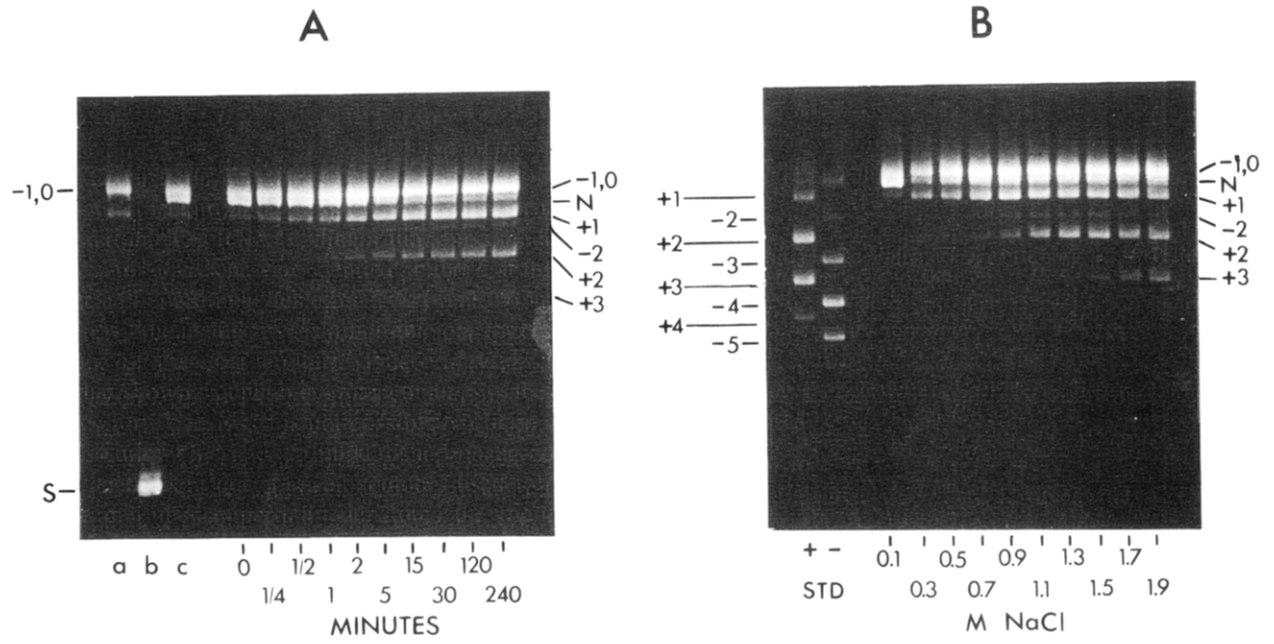

FIGURE 1: Analysis of the average helical pitch present in DNA at increasing NaCl concentrations. (A) Time course that measures the rate of conversion from the covalent topoisomerase−DNA adduct to a covalently closed DNA in 1.1 M NaCl. (B) Topological state of the DNA at increasing NaCl concentrations. For the experiment in panel A, T7/T3-19 negatively coiled DNA was incubated with topoisomerase I (400 units/μg DNA) in the absence (lane a) or presence (lane c) of 50 μg/mL camptothecin for 20 min at 23 °C. The sample containing camptothecin was then adjusted to 1.1 M NaCl by adding an equivalent volume of 2.2 M NaCl, and aliquots were taken with increasing time. Panel A also shows an experiment in which topoisomerase I (400 units/μg DNA) was incubated for 4 h at 23 °C with negatively coiled DNA in the presence of 1.1 M NaCl (lane b). For panel B, the conditions were the same as for panel A, except that the NaCl concentration was varied and a single time point of 4 h was used. In panel B, STD refers to standard DNAs that were prepared to indicate the relative mobilities of positive coils (+) and negative coils (−). The band labeled N is nicked DNA, and the band labeled S is negatively supercoiled DNA. See Materials and Methods for a description of the formulas used to calculate the helical pitch of the DNA from these data.

We have developed a protocol to measure the pitch, which is based on maintaining topoisomerase I resealing activity in these unphysiological NaCl concentrations. Camptothecin is a topoisomerase I inhibitor that stabilizes the formation of a topoisomerase I−DNA covalent adduct and does this without changing the helical pitch of the DNA (Hsiang et al., 1985). The resulting DNA therefore is in a nicked state as long as a topoisomerase I remains covalently attached to the 3′-phosphoryl group of the DNA (Figure 1, lane c). Hsiang et al. (1985) observed that at 0.5 M NaCl the covalent attachment would reverse. We have utilized this observation and found that the covalent attachment of topoisomerase I can be reversed in a wide range of NaCl concentrations in a time-dependent process. The DNA, which is originally in a nicked state and therefore free to maintain a relaxed state in the unphysiological NaCl concentrations, slowly reforms the phosphodiester bond and preserves the helical pitch. This helical pitch can be observed by measuring the topological state of the DNA as seen by agarose gel electrophoresis. A time course is shown in Figure 1A in which a camptothecin−topoisomerase I−DNA intermediate was established first, followed by the addition of NaCl to 1.1 M. Aliquots were taken with time and the topological state was analyzed. Within 30 min, the DNA was resealed as exhibited by the average of 1.2 positive coils that are observed (see Materials and Methods for a description of the formula used to obtain this value). Note that the sealing is not instantaneous upon adjustment to 1.1 M NaCl, and therefore the number of positive coils is a measure of the DNA pitch in that condition.

A further demonstration of the requirement of camptothecin for this analysis is shown in Figure 1A (lane b). In this experiment, topoisomerase I was added to negatively coiled DNA in the presence of 1.1 M NaCl. The cleavage activity of the enzyme was totally inhibited. It is, therefore, important to stabilize the initial cleavage reaction with camptothecin at 100 mM NaCl and then adjust to the higher NaCl condition. Apparently, the higher NaCl concentration displaces the camptothecin from the topoisomerase, which permits the resealing reaction to occur. This experiment has been repeated at NaCl concentrations from 0.1 to 1.9 M using a time point of 4 h. As shown in Figure 1B, there is a steady increase in the number of positive supercoils with increasing ionic strength. At 1.1 M NaCl, the condition in which histones H3,H4 form a stable interaction with the +0.011 SD DNA, an average of +1.2 coils is observed. This number of positive coils equates to a decrease in helical pitch of 0.06 bp/turn (see Materials and Methods for calculations).

Preparation of Very Highly Positively Coiled DNA. As previously indicated, H3,H4 preferentially bind to −0.045 SD DNA, as compared to +0.011 SD DNA at 100 mM NaCl. Further testing of the hypothesis could be done by establishing a topological state in the DNA, which stabilizes, at physiological ionic strength, a decreased DNA helical pitch similar to that observed at 1.1 M NaCl. Selective binding of H3,H4 to that DNA would further support the hypothesis. High levels of positive stress would be expected to decrease the helical pitch. In 1989, Tsao et al. described an experimental protocol that was used to demonstrate the twin-domain model of the transcription-induced supercoiling. Cells containing plasmids were treated with novobiocin, an inhibitor of gyrase, under conditions of active transcription. The plasmid DNAs were found to be positively coiled with a superhelical density of +0.024 SD. We have refined

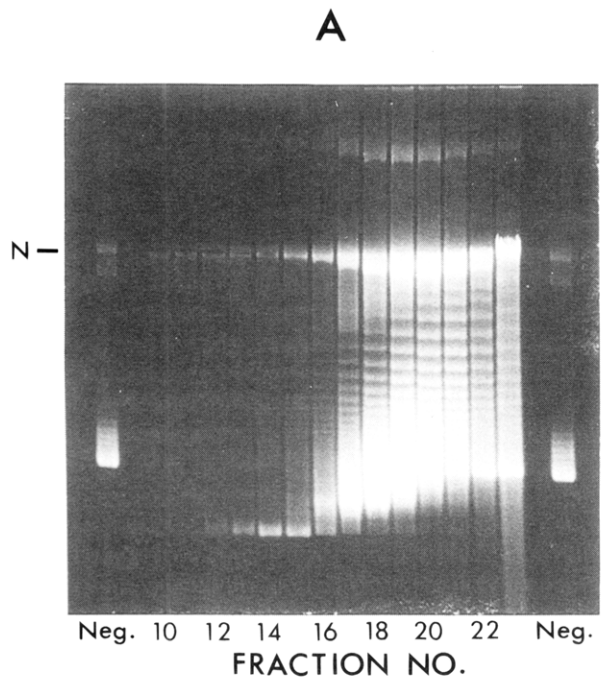

FIGURE 2: Electrophoretic analysis of PBR322 isolated from fractions of a CsCl−EtBr gradient. DNA from fractions 10−22 was electrophoresed on 1.2% agarose. Negatively coiled PBR322 (Neg) was coelectrophoresed as a standard. N indicates the location of nicked PBR322 on these gels.

their protocol and isolated very highly positively coiled DNAs, which are sufficient to test our hypothesis at physiological ionic strength (*vide infra*).

Strain AS19 cells, which have a cell wall highly permeable to antibiotics, were transformed with PBR322. The cells were grown in log culture in the presence of 10 μg/mL tetracycline and subsequently treated with 400 μg/mL novobiocin for 30 min. Cells were harvested rapidly and plasmid DNA was isolated (see Materials and Methods). The DNA was then separated into different topological states by sedimentation to equilibrium in CsCl gradients containing high concentrations of ethidium bromide (1 mg/mL). Fractions were collected, the CsCl and EtBr were removed, and the DNA was characterized on 1.2% agarose gels as shown in Figure 2. A broad spectrum of topological densities is observed from fractions 10 to 23. These plasmids are distributed at higher densities than would normally be observed for a plasmid preparation. Plasmids isolated from cells not treated with novobiocin, and therefore negatively coiled, normally distribute at fractions 23−25 (data not shown). The inability to effectively bind the EtBr suggests that these DNAs represent variations in levels of positive stress.

To establish the topological state of the DNA in these fractions, fractions 13, 16, 17, and 22 were analzyed on two-dimensional chloroquin gels. As shown in Figure 3, fraction 22 consists equally of both positively and negatively coiled DNA. As the fraction number decreases, higher and higher positively supercoiled DNAs are observed. At fraction 13 the DNA is of such high superhelical density that it runs as a single band in both dimensions. It is not possible to quantitate the superhelical density at these lower fraction numbers by gel electrophoresis alone. An alternative approach is required.

Superhelical Density of DNA in Fraction 13 Is Estimated To Be +0.150. The separation of the different topological states is based on the quantity of EtBr capable of intercalating the DNA. The more EtBr that is bound, the lower the density of the DNA. If one assumes that there is an end point in which no further positive coils can be put into DNA and that it does not matter whether this end point is achieved exclusively by EtBr intercalation or a combination of intercalation and the inherent positive stress in the plasmid, then the amount of EtBr that can intercalate should be inversely proportional to the amount of positive superhelicity that is present in the plasmid prior to EtBr addition. That such an end point does exist is strongly supported by the observation that negatively coiled DNA can be readily separated from the linear host DNA in the presence of EtBr, which is a procedure commonly used for the purification of plasmid DNA. Therefore, if we are able to determine superhelical densities on the basis of resolving topoisomers in regions of the gradient where the CsCl density is low (fractions 19−24), we should then be able to extrapolate to the higher density region (fractions 10−18) and determine the superhelical densities for the DNAs in that region. Figure 4A shows a plot of the CsCl densities for the gradient of Figure 2. The graph also shows an estimate of the number of coils present in fractions 19 and 24. These estimates are based on counting topoisomers of DNA that have been electrophoresed in the absence or presence of $MgCl_2$ (data not shown). Magnesium decreases the helical pitch of DNA, so that positively supercoiled DNA becomes less coiled and hence the topoisomers are easier to resolve. Having established the slope of the line on the basis of the CsCl densities, we extrapolate to fraction 13 and estimate that the number of positive coils at this fraction is +63. If we assume a helical pitch of 10.5 bp/turn on this DNA, the superhelical density is then +0.150 SD. As these high positive stresses are likely to decrease the DNA helical pitch, the actual superhelical value may be less than this value (White & Bauer, 1986). For this reason, we are reluctant in our subsequent experiments to define these DNAs in terms of superhelical density. Rather, we will describe these DNAs in terms of the average number of positive or negative supercoils.

A further demonstration of the high level of positive superhelical density present in these DNAs is shown in Figure 4B. In this instance, fractions 15−19 (lane a) were combined and electrophoresed in parallel with negatively coiled PBR322 (lane b). For condition 1, the gel was stained with EtBr in the presence of electrophoresis buffer, and the gel was photographed after destaining in the same buffer. For condition 2 the staining and destaining was done in water, and for condition 3 the gel was preexposed with ultraviolet light for 5 min before the restaining and destaining with EtBr. As shown in Figure 4B, the preexposure with ultraviolet light is critical to obtain quantitative staining with EtBr. Without this exposure, and particularly if the staining was done in electrophoretic buffer, the staining of the positively coiled DNA is very poor. The highly positively supercoiled band (arrow) is totally unstainable in condition 1. Even in the CsCl gradients that contain large quantities of EtBr and therefore would normally allow visualization of DNA, the DNA in fractions lower than fraction 16 cannot be seen. These highly positively coiled DNAs are quite resistant to EtBr intercalation. With regard to the quantitation of these

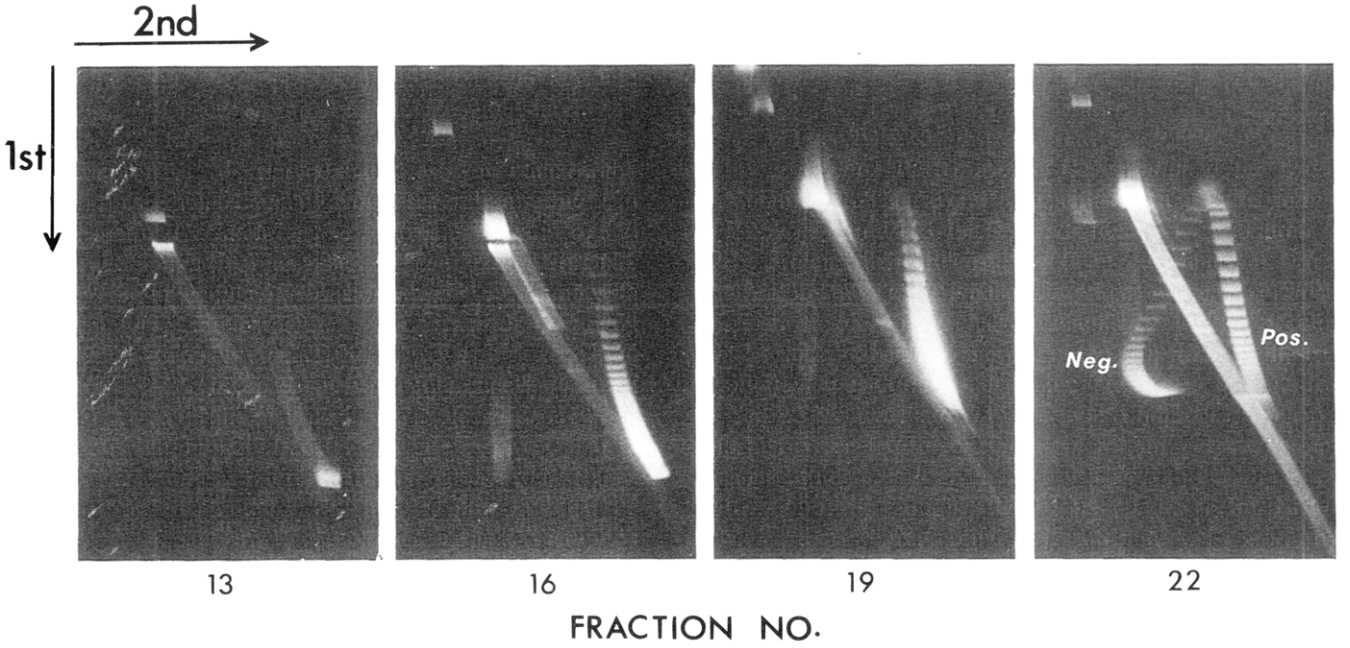

FIGURE 3: Two-dimensional gel analysis of selected fractions from the CsCl−EtBr gradient. DNA from fractions 13, 16, 19, and 22 was electrophoresed in the first dimension on 1.2% Calbiochem agarose. Subsequently the gel was equilibrated in 15 μM chloroquin and electrophoresed in the second dimension. Positive (Pos.) and negative (Neg.) coils are shown in the figure.

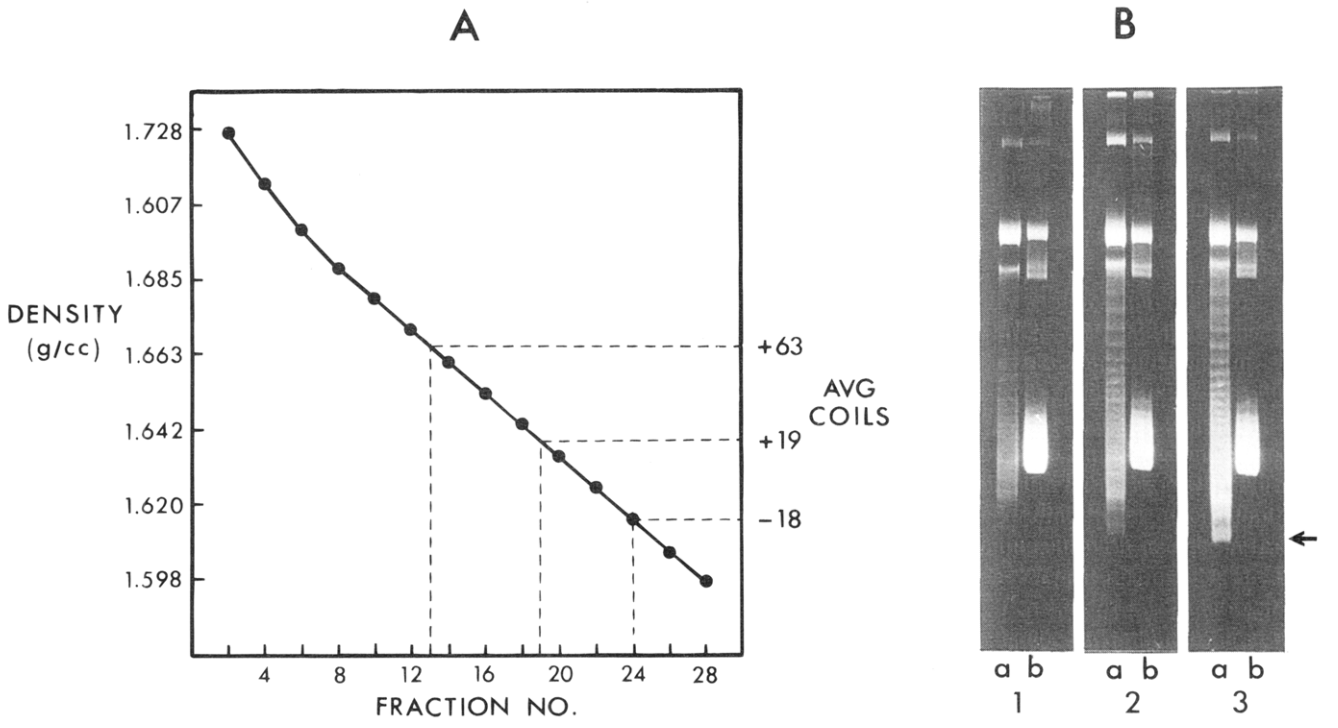

FIGURE 4: Graphical analysis to determine the number of positive coils in the fractions from the CsCl−EtBr gradient. (A) Densities of CsCl in the various fractions are plotted. These values were determined from the refractive index. Also plotted on this graph is the average number of coils (AVG COILS) present in fractions 19 and 24. With the slope of the line, these values are used to extrapolate to fraction 13 in order to determine the average number of coils present in the DNA of that fraction (+63 coils). (B) Establishing conditions for staining of positively coiled DNA with EtBr. Positively coiled DNA (lane a) from combined fractions 15−19 was electrophoresed in parallel with negatively coiled DNA (lane b). The gel strips were then exposed to various staining conditions prior to being photographed. Condition 1 is EtBr staining and destaining in electrophoresis buffer. Condition 2 is the same as condition 1 except that EtBr was used in water. Condition 3 is the same as condition 2 except that the gel was exposed to ultraviolet light for 5 min prior to restaining and destaining with EtBr. The 5 min preexposure with ultraviolet light is sufficient to nick all DNA in the gel (data not shown).

highly positively coiled DNAs on the agarose gels, all data in this report were obtained by utilizing the condition 3 protocol.

Positively Coiled DNA Preferentially Binds H3,H4 at 1.1 M NaCl. It was of interest to evaluate the binding of H3,H4 to the positively coiled DNA. The first experiment was to confirm that, in 1.1 M NaCl, the positively coiled DNA preferentially bound H3,H4, as compared to the negatively coiled DNA. The DNA from combined fractions 15−19 (an average of +49 to +19 coils) was mixed 1:1 with negatively coiled PBR322. Histones H3,H4 were then added at a ratio of 0.2:1 (wt:wt) histone:DNA in 2.0 M NaCl, and the NaCl

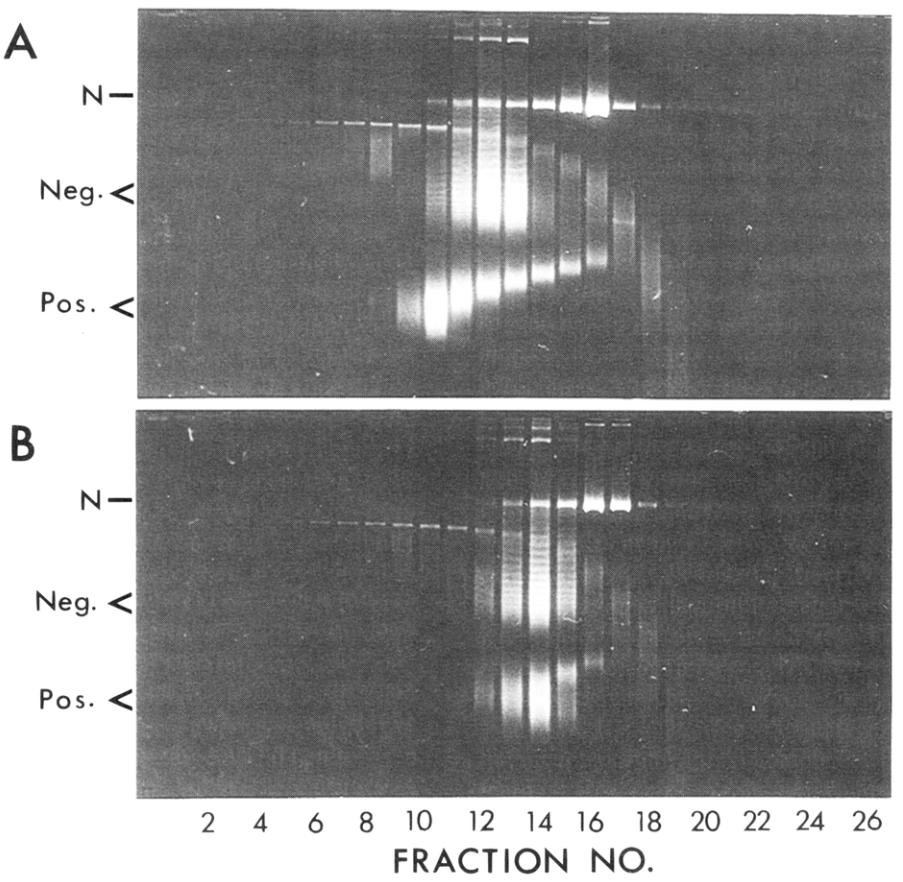

FIGURE 5: Sucrose gradient analysis in 1.1 M NaCl of H3,H4 reconstituted with both positively and negatively coiled DNA. (A) Positively coiled DNA (combined fractions 15−19) was mixed 1:1 with negatively coiled DNA (both DNAs were PBR322). Histones H3,H4 were added at a ratio of 0.2:1 histone to DNA, and after the NaCl concentration was lowered to 1.1 M, the sample was applied to a 5−20% sucrose gradient containing 1.1 M NaCl, 50 mM TEA, and 1 mM EDTA (pH 8.0). After centrifugation for 18 h at 35 000 rpm, fractions were collected and applied to a 15 μM chloroquin gel. (B) Same as panel A, except that H3,H4 were not included in the reconstitution. The locations of the positively coiled (Pos.) and negatively coiled (Neg.) DNA are indicated on the left side of the panel. N indicates the location of nicked DNA.

concentration was lowered to 1.1 M NaCl during a 3 h dialysis at 4 °C. The sample was then applied to a 5−20% sucrose solution also containing 1.1 M NaCl and centrifuged for 18 h at 35 000 rpm. Fractions were collected and analyzed on 15 μM chloroquin gels. Since both the positively and negatively coiled DNAs are PBR322, and therefore the same DNA sequence and molecular weight, chloroquin gels are necessary to determine the relative positions of the two different topologically restrained DNAs in the gradient. As shown in Figure 5A, the positively coiled DNA that migrates farthest into the gel (greatest positive superhelicity) also has a more rapid sedimentation rate than the negatively coiled DNA. This more rapid sedimentation is not due to inherent structural characteristics of positively coiled DNA, for in Figure 5B a sucrose gradient is shown in which histones H3,H4 were not present. Note that both positively and negatively coiled DNAs are located in fractions 13−15. Therefore, the more rapid sedimentation of the positively coiled DNA in Figure 5A must be due to the selective binding of H3,H4 to that DNA as compared to the negatively coiled DNA. These results confirm our earlier observations (Jackson et al., 1994) in which it was necessary for us to utilize plasmids of different molecular weights and sequences to obtain the separations necessary to measure topological preferences. These data of Figure 5 indicate that only topology defines the preference.

DNAs of Greater Than +40 Coils Are Required for Preferential Binding of H3,H4 at Physiological Ionic Strengths. With these highly positively coiled DNAs, we were in a position to further test the hypothesis that a decrease in helical pitch increases the preference for binding H3,H4 at physiological ionic strength. The protocol used was identical to that described for Figure 5, except that the reconstitution was continued by dialysis to 100 mM NaCl (see Materials and Methods). The sample was then applied to a 5−20% sucrose gradient containing 100 mM NaCl and centrifuged for 7 h at 35 000 rpm. Fractions were collected and analyzed on both SDS−PAGE gels and 15 μM chloroquin agarose gels. Figure 6A shows the distribution of H3,H4, and these proteins are found to be broadly distributed from fractions 4 to 14. From the chloroquin gel of Figure 6B, the positively coiled DNAs are also broadly distributed. Those positively coiled DNAs with the highest positive stress (greatest mobility on the gel) are the DNAs with the greatest sedimentation rate on the gradient (fractions 4−8). Their sedimentation rate is greater than that of the negatively coiled DNA (fractions 10−13). Again, this altered sedimentation rate is not due to an inherent structural characteristic of the DNA. Figure 6C shows a sucrose gradient of the same DNAs that were reconstituted in the absence of H3,H4. In this gradient, both positively and negatively coiled DNA sedimented in the same fractions (fractions 15−16). The enhanced sedimentation rate in Figure 6B of these highly

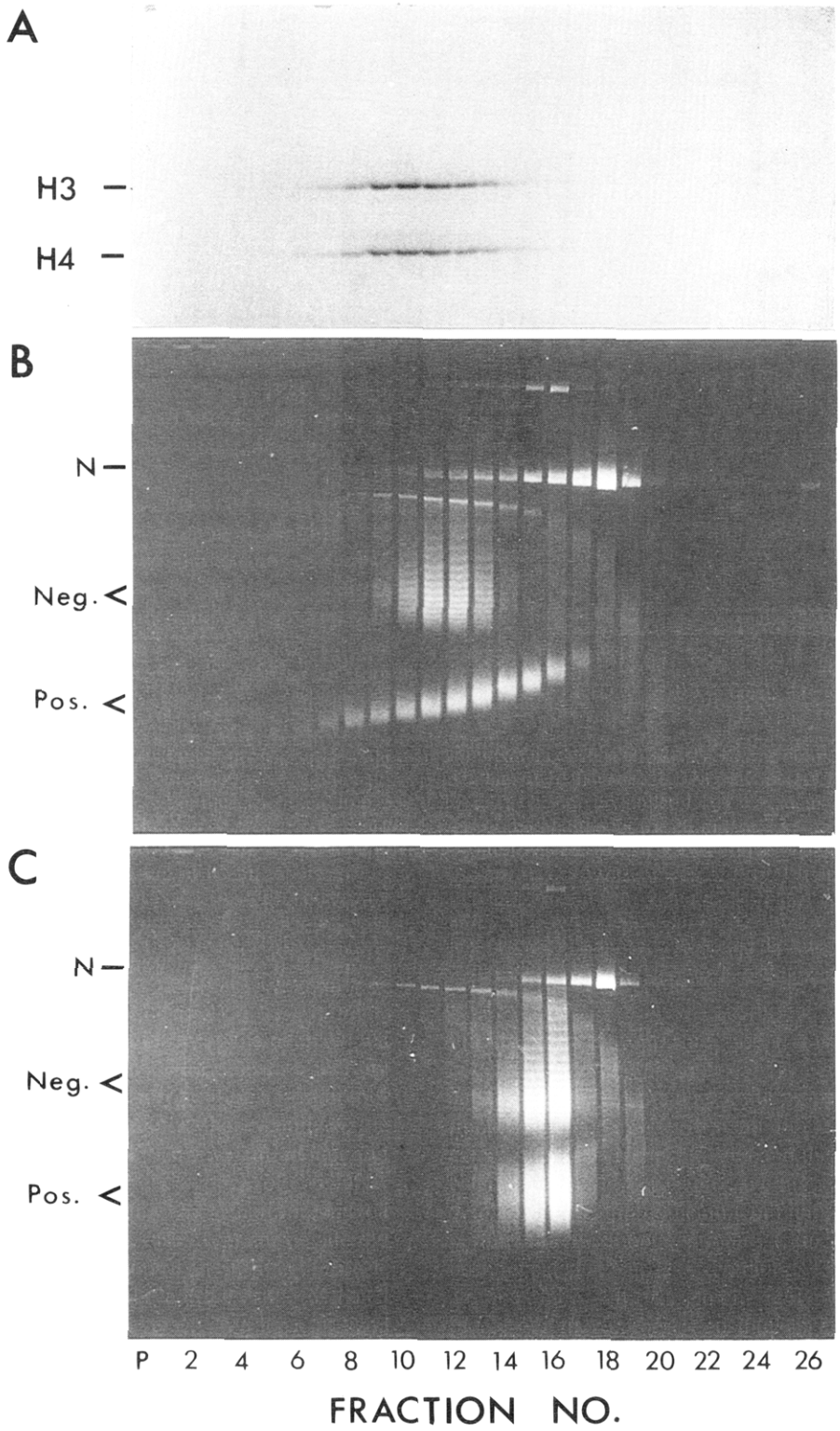

FIGURE 6: Sucrose gradient analysis in 100 mM NaCl of H3,H4 reconstituted with both positively and negatively coiled DNA. The procedures were the same as those described in the legend to Figure 5, except that during the reconstitution the NaCl concentration was lowered to 100 mM NaCl. (A) SDS−polyacrylamide gel showing the distribution of histones H3 and H4 on the sucrose gradient. (B) Corresponding chloroquin gel showing the DNA distribution for the same sucrose gradient. (C) Separate sucrose gradient in which the applied DNA was reconstituted in the absence of histones H3,H4. Centrifugation was at 35 000 rpm for 7 h. At the bottom of panel C, the P refers to the pellets from the sucrose gradients.

positively coiled DNAs must be due to the selective binding of H3,H4, a binding preference that is greater than that for negatively coiled DNA.

It should be noted that the window of superhelical densities for which this preference is observed is much narrower than that for the data of Figure 5. In fraction 11 of Figure 5A, a broad spectrum of positively coiled DNA is present. Only very highly positively coiled DNA is present in fractions 4−8 of Figure 6B. We estimate that positively coiled DNAs of at least +40 are required to maintain the binding preference of H3,H4 at physiological ionic strength. The inference from these data is that the level of positive

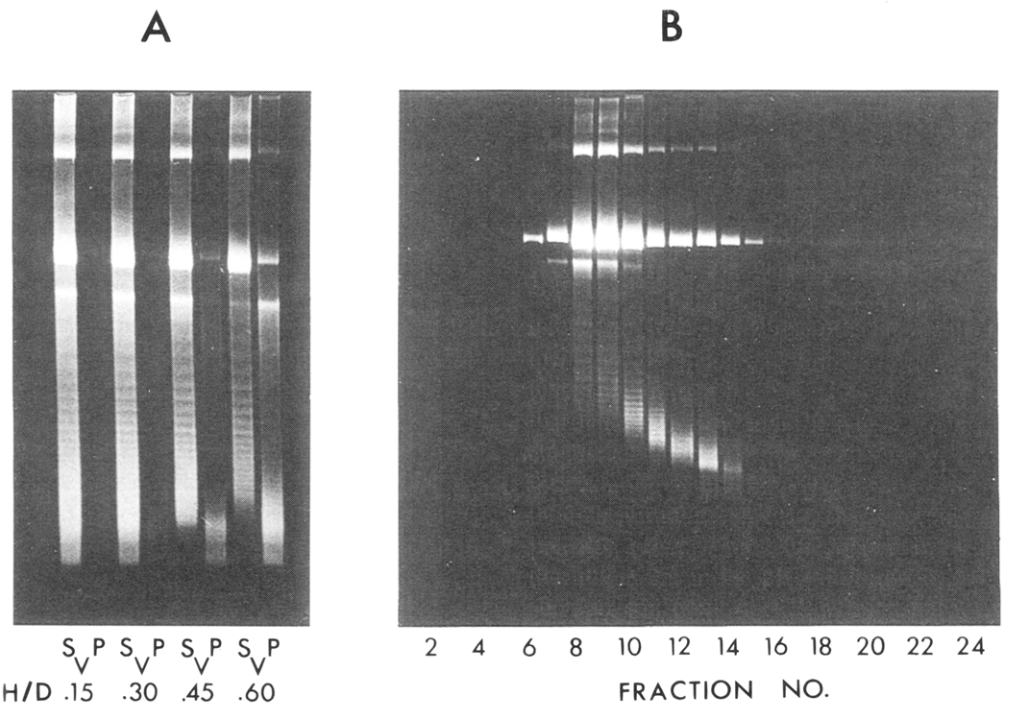

FIGURE 7: CsCl/GuHCl gradient analysis of the H3,H4 content in reconstituted complexes containing positively coiled DNA. (A) Positively coiled DNA from combined fractions 15−19 was reconstituted by stepwise dialysis with H3,H4 at histone to DNA ratios (H:D) of 0.15, 0.30, 0.45, and 0.60. The samples were then centrifuged for 2 min at 10 000 rpm in a microfuge, and the supernatants (S) and pellets (P) were analyzed on 1.2% agarose. (B) Reconstituted complex of H3,H4 with DNA at a ratio of 0.2 was cross-linked with formaldehyde and then sedimented to equilibrium on a CsCl/GuHCl gradient. Fractions were collected and the cross-links reversed (see Materials and Methods). The DNA was then analyzed on 1.2% agarose.

superhelical stress necessary to transiently decrease the DNA helical pitch by 0.06 bp/turn to maintain that preference is greater than +40 coils (approximately +0.10 SD).

In a Mixed Population of Positively Coiled DNA, Histones H3,H4 Preferentially Bind the Most Positively Coiled DNA. The data of Figure 6A have been interpreted as indicating that DNA with greater than +40 coils preferentially binds H3,H4. If this interpretation is correct, one would predict that, independent of the histone to DNA ratio, the +40 coiled DNA should bind the H3,H4 preferentially. Histones H3,H4 were added to the positively coiled DNA (combined fractions 15−19) at histone to DNA ratios that varied from 0.15 to 0.60. After reconstitution from a NaCl concentration of 2.0 M to 100 mM, the samples were centrifuged for 2 min at 10 000 rpm. The supernatants and pellets were then analyzed on the agarose gel of Figure 7A. At a histone to DNA ratio of 0.3:1, virtually all of the complexes are soluble. However, as the ratio is increased to 0.45 and then to 0.6, increasing quantities of the highly positively coiled DNA are pelleted. There are two possible explanations for this insolubility. The H3,H4 may be selectively binding this highly coiled DNA to a point at which saturation occurs and aggregation is the result. Alternatively, the H3,H4 content on the highly coiled DNA may be the same as that for the other DNAs in the sample, and the aggregation is due to the inherent insolubility of the complex when H3,H4 interact with the highly coiled DNAs. These two alternatives can be resolved by determining the histone content on the highly coiled DNAs. We previously utilized formaldehyde cross-linking and subsequent analysis on CsCl and GuHCl gradients to determine the histone content on reconstituted complexes (Jackson, 1993). These procedures were applied in this analysis. The reconstituted complexes with a histone to DNA ratio of 0.2:1 were treated with 1% formaldehyde for 24 h at 4 °C. By SDS−PAGE analysis of these cross-linked complexes, we confirmed that the condition of fixation resulted in quantitative cross-linking of H3,H4 to the positively coiled DNA (data not shown). After removal of excess formaldehyde by dialysis, the sample was centrifuged to equilibrium in the CsCl/GuHCl gradients. The formaldehyde cross-links in the isolated fractions were then reversed, and the DNA was analyzed on the agarose gel that is shown in Figure 7B. The highly positively coiled DNA is located at the lower density region of the gradient (fraction 14). DNAs with lower and lower amounts of positive coils are found in fractions of increasing CsCl density (lower fraction numbers). This difference in distribution is caused by the difference in histone content and indicates that H3,H4 preferentially bind and are cross-linked to the highly coiled DNA. The selective aggregation and precipitation observed in Figure 7A are due to oversaturation by H3,H4. These highly coiled DNAs preferentially bind H3,H4.

Histones H3,H4 Transiently Maintain Positive Stress When a Positively Coiled DNA Is Relaxed. It has been extensively reported that, when H3,H4 bind DNA, the DNA is wrapped left-handed around the H3,H4 to form negative coils on the DNA. The observation that these same proteins preferentially bind to highly positively coiled DNA is not what one would have predicted on the basis of these earlier studies. It was therefore of interest to evaluate the topological state of the DNA that is bound in intimate interaction with the H3,H4. This can be done by rapidly removing unrestrained topological stress within the DNA by treatment with topoisomerase I and then observing the remaining topological state that is maintained by the protein−DNA interactions. We have previously utilized this method to study core histone interactions with DNA (Jackson, 1993).

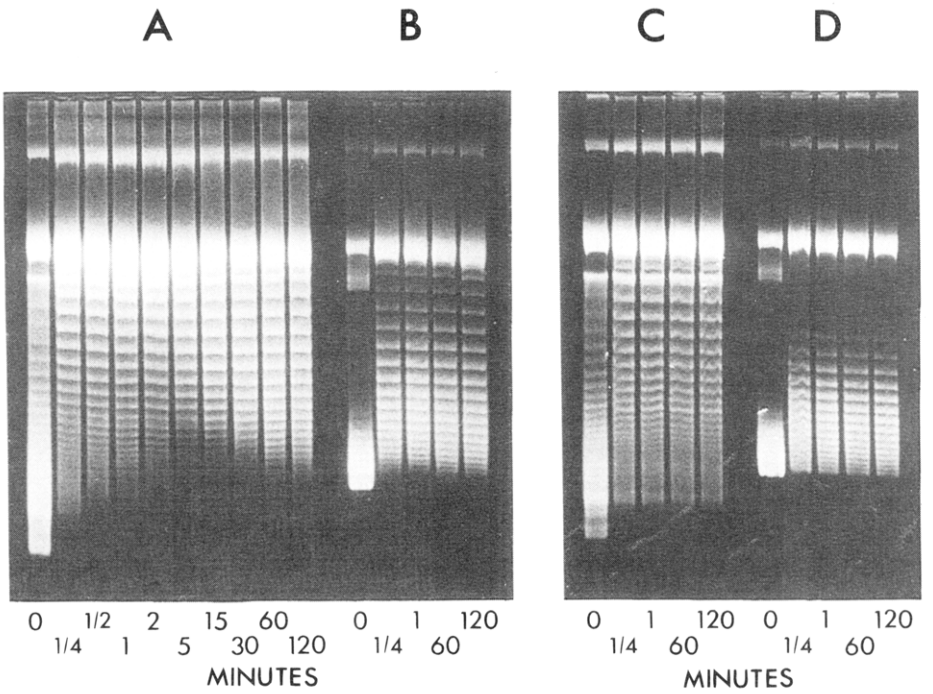

FIGURE 8: Analysis of the topology maintained by H3,H4 interaction with DNA. (A) Positively coiled DNA (combined fractions 15−19) was reconstituted at a 0.3:1 histone to DNA ratio and subsequently treated with 400 units/μg topoisomerase I at 23 °C. Aliquots were taken at specific time points and electrophoresed on 1.2% agarose gels. (B) Same as panel A except that the DNA was negatively coiled PBR322. (C) Same as panel A except that the reconstituted complex was treated with 1% formaldehyde for 24 h at 4 °C. After dialysis to remove excess reagent, the sample was treated with topoisomerase I. (D) Same as panel B except that the reconstituted complex had been cross-linked with formaldehyde as described for panel C.

Positively coiled DNA (combined fractions 15−19) and negatively coiled DNA were reconstituted separately with H3,H4 at a ratio of 0.3:1. The samples were then treated with excess topoisomerase I, and aliquots were taken with increasing time. It should be noted that eukaryotic topoisomerase I relaxes both positive and negatively coiled DNA equally well (Wang, 1985; data not shown). As shown in Figure 8B for the negatively coiled DNA, the first time point (15 s) contains a topological state that remains the same at the 120 min time point. The topological state is negative, as has been reported previously (Jackson et al., 1994), and reflects the ability of H3,H4 to store negative coils. These data also indicate that topoisomerase I activity is sufficient to remove unrestrained stress within the first 15 s. For the positively coiled DNA in Figure 8A, a similar rapid relaxation of unrestrained stress occurs within the first 15 s, followed by a gradual loss of supercoils during the next 5 min. A gradual increase in supercoils then occurs until the 60 min time point is reached. This biphasic character of the supercoil content suggests a change from positive to negative topology. To resolve these topoisomers, it is necessary to analyze the time points on two-dimensional chloroquin gels. Figure 9 shows selected time points of the two-dimensional analysis. At the 15 s time point (panel a), the DNA is still primarily in a positive state. There is an average of three negative coils on a very small percentage of plasmids. At the 1 min time point (panel b) the number of plasmids has gradually increased. By the 5 min time point (panel c), over half of the plasmids have generated a negative state. Not until the 60 min time point (panel d) have all of the plasmids made the transition from positive to negative. This very slow transition suggests either that the access of the topoisomerase I to the DNA is severely restricted and the rate-limiting step is DNA access or that the H3,H4 are intimately holding the DNA in a positive state and very slowly releasing the positive stress and converting the DNA to a negative state. These alternatives can be resolved by cross-linking the complexes with formaldehyde. Cross-linking preserves the state of the histone−DNA interactions (Jackson, 1993).

Separate aliquots of the same reconstituted complexes that were analyzed in Figure 8A,B were treated with 1% formaldehyde for 24 h at 4 °C, and after removal of excess reagent, the complexes were treated with topoisomerase I. As shown in Figure 8D for the negatively coiled DNA, the rate at which topoisomerase I removes the unrestrained stress and the number of negative coils maintained are very similar to the un-cross-linked complex (Figure 8B). The cross-linking does not alter the accessibility of the DNA to the topoisomerase. For the positively coiled DNA (Figure 8C), a similar rapid removal of unrestrained stress is observed at the 15 s time point and remains unchanged 120 min later. The topological state appears very similar to the 15 s time point of the un-cross-linked complex (Figure 8A). This similarity is more clearly demonstrated by the two-dimensional analysis of Figure 9. The topological states of the DNA at the 15 s (panel e) and 120 min time points (panel f) of the cross-linked complex are similar in both cases. The DNA is exclusively positively coiled. The superhelicity is very similar to that at the 15 s time point of the un-cross-linked complex (panel a). The data indicate that topoisomerase I access is not the rate-limiting process. The H3,H4 appear to restrain the DNA in this positive state and only reluctantly release this stress and convert the DNA to a state of negative stress. An alternative interpretation is that the histones are maintaining a decreased helical pitch in the DNA, which equates to the presence of positive coils when the histones and topoisomerase are removed prior to

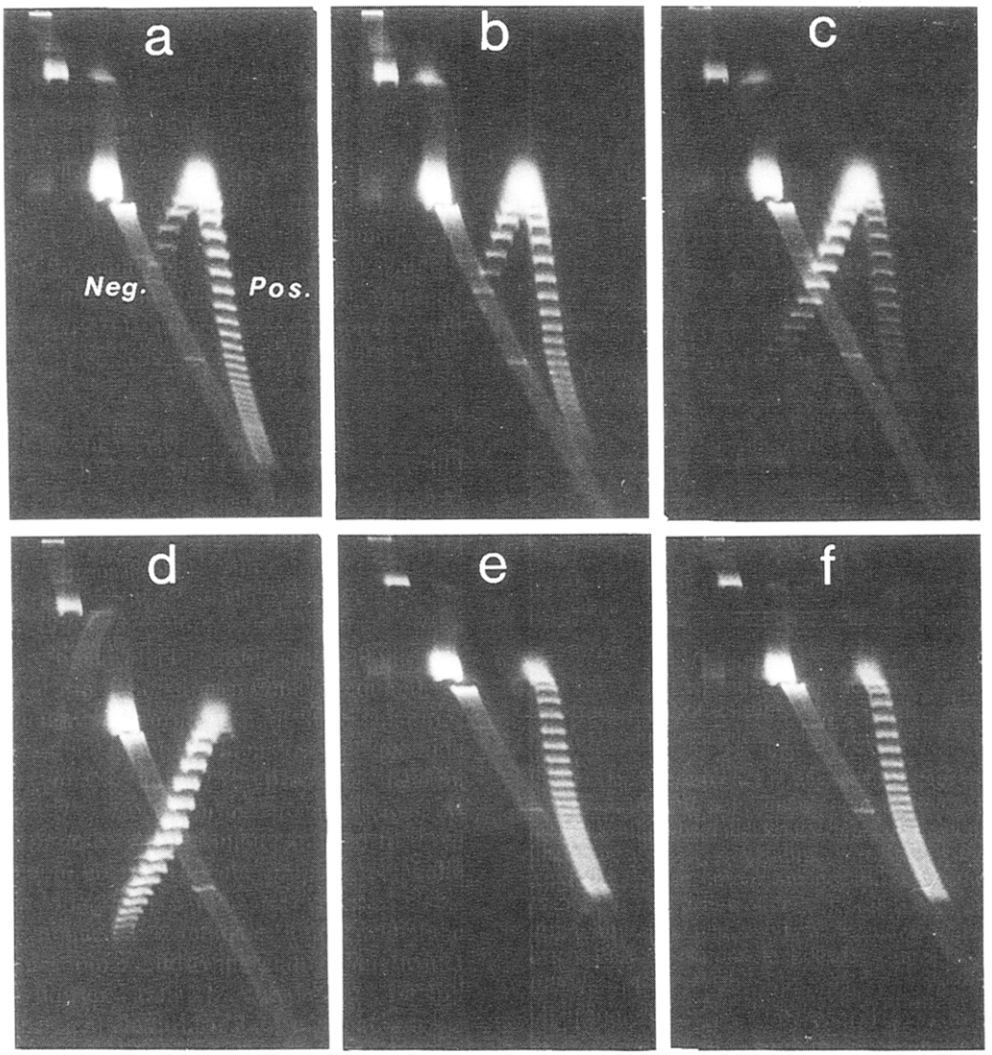

FIGURE 9: Two-dimensional analysis of selected time points from the topoisomerase I treatment of H3,H4 reconstituted complexes. Panels a−d are time points of 15 s, 1 min, 5 min, and 60 min, respectively, of the un-cross-linked reconstituted complex composed of H3,H4 and positively coiled DNA (Figure 8A). Panels e and f are of time points 15 and 120 min, respectively, of the cross-linked reconstituted complexes (Figure 8C).

gel electrophoresis. In this instance, the twist of the helix is converted into the superhelical writhe of the plasmid molecule. It is this latter interpretation that we favor and may provide a clue to the preferential binding of H3,H4 to highly positively coiled DNA.

DISCUSSION

In 1.1 M NaCl, the observation was made that the DNA helical pitch decreased by 0.06 bp/turn (Figure 1). At this same salt condition we had previously reported that histones H3,H4 bound to a +0.011 SD DNA with a 5-fold preference over a −0.045 SD DNA (Jackson et al., 1994). When the NaCl concentration was decreased to 100 mM, this preference was lost and the H3,H4 preference changed to a 2-fold preference for the negatively coiled DNA. Since at this lower NaCl concentration the DNA helical pitch no longer would be altered, it was suggested that the decrease in helical pitch was a critical factor in creating the binding preference of H3,H4 in the high salt. Any condition that would facilitate a similar change in helical pitch at physiological ionic strength might be expected to enhance H3,H4 binding at that condition. High levels of positive superhelicity potentially could be that condition, and therefore such DNAs were reconstituted with H3,H4 in the presence of negatively coiled DNA. DNAs of greater than +40 coils/plasmid (approximately +0.10 SD) were found to preferentially bind H3,H4 at physiological ionic strength. This conclusion was based on the increased sedimentation values of these DNAs on sucrose gradients when H3,H4 were present (Figure 6), the greater histone content on these DNAs as observed by their selective aggregation (Figure 7A), and their lower density on CsCl/GuHCl gradients (Figure 7B). The inference from these observations is that H3,H4 binding is stabilized by the presence of a decreased helical pitch in the DNA. That an altered helical pitch can exist in positively coiled DNA has been shown by the studies of McClellan and Lilley (1991). In their protocol, a covalently closed, relaxed plasmid containing an A:T tract was treated with actinomycin D. Actinomycin D is an intercalator that will cause the formation of positive stress in a covalently closed plasmid. Since this intercalator has a GpC specificity, these investigators were able to directly examine the effect of positive stress on the A:T tract. This tract was found to be highly sensitive to osmium tetraoxide and was interpreted as indicating an alteration in the helical pitch. The highly positively coiled DNA in our study may undergo similar structural changes.

An explanation for the preferential binding of H3,H4 may be found in the known binding characteristics of H3,H4

within the nucleosome. The 1.8 turns of the DNA superhelix would be expected to maintain 1.8 negative coils. However, the actual number of negative coils observed approximates 1 coil per nucleosome. This linking number paradox has been interpreted to indicate that the histones substantially overwind the DNA helix (decrease the helical pitch) such that, when the histones are removed, only one negative coil remains (Travers & Klug, 1990). Numerous investigators have observed this altered helical pitch with various DNA protection protocols (Klug & Lutter, 1981; Drew & Travers, 1985; Satchell et al., 1986; Hayes et al., 1990). This overwinding of the helix would be expected to require substantial binding energy, energy that in part could be provided by the highly positively coiled DNA. For histones H3,H4, a decrease in helical pitch of 0.06 bp/turn may be sufficient to provide this enhancement and highly stabilize the interaction. The selective binding to the decreased helical pitch is further supported by the data of Figures 8 and 9. In the presence of large excesses of topoisomerase I, the H3,H4 transiently maintain the positive coils. This transient maintenance is not due to limiting topoisomerase I, since the cross-linked complexes maintain a similar level of positive stress after topoisomerase I treatment (Figures 8 and 9). Our interpretation is that H3,H4 transiently maintain the decreased helical pitch, which is translated into positive coils once the histones are removed by the addition of SDS. It could be argued that this transient maintenance is due to the binding of denatured H3,H4, which were never meant to bind positively coiled DNA. We do not believe this to be the case for two reasons. The first reason is that the binding shows topological specificity. Denatured H3,H4 presumably would bind independent of the topological state. Also, in the sucrose gradient of Figure 6 it can be seen that the H3,H4 are specifically bound to either the positively coiled or negatively coiled DNA. They do not bind DNA that is in a nicked state (fraction 16). Such specificity would not be expected of denatured histones. The second reason is based on the data of Figures 8 and 9, which indicate that, with increasing length of incubation with topoisomerase I, the H3,H4 store negative coils on the DNA. This activity would not be expected of denatured histones. We interpret the subsequent formation of negative coils as a process in which higher order H3,H4 interactions are formed, which then preserve the left-handed coil. The extent to which this decreased helical pitch is preserved by H3,H4 when the negative coils are formed is undefined by this analysis. It is expected that the pitch is similar to the normal pitch in a nucleosome.

In vivo studies on histone mobility during replication and transcription have indicated that H3,H4 mobility is minimal (Jackson & Chalkley, 1985; Jackson, 1990). Once deposited at the replication fork, the H3,H4 remain associated with DNA even when that DNA is transcribed. Histones H2A,H2B are quite mobile, and this mobility is dependent on ongoing transcription. A potential explanation for this variation in mobility may be due to differences in histone preferences for DNA topology. We have previously shown, from *in vitro* experiments (Brooks & Jackson, 1994), that histones H2A,H2B are highly mobile at physiological ionic strength and have a 10-fold preference for binding negatively coiled DNA over moderately positively coiled DNA (+0.011 SD). With *in vitro* transcription experiments, we have reported that histones within the nucleosome are unable to store the negative coil due to the presence of transcription-induced positive stress (Pfaffle et al., 1990). Those same experiments also indicated that the transcription-induced negative stress in the wake of the polymerase greatly facilitated the reformation of the nucleosome. By using these observations and additional data indicating that a nucleosome is in a more open conformation when present on a moderately positively coiled DNA (Jackson, 1993; Jackson et al., 1994), a model was proposed to describe transcription through a nucleosome (Brooks & Jackson, 1994). Key to the model was an understanding of the mechanism for maintenance of the H3,H4 interaction with DNA when assaulted by the highly disruptive RNA polymerase. The binding preference of H3,H4 to positively coiled DNA may be an important component of the mechanism. Would one expect to observe a superhelical density of greater than +0.10 SD in the transcription process? Since an integral part of the method for preparation of these highly coiled DNAs involves transcription-induced stress, the answer to the question must be in the affirmative. A superhelical density of +0.10 SD indicates that, on average, there are +2 coils present per 200 bp of DNA. Theoretically, the transcription of only 20 bases of DNA would be sufficient to generate that superhelical density (Cook et al., 1992). With an actively processing RNA polymerase, stresses substantially greater than this might be expected and are indeed observed in fraction 13 of the CsCl−EtBr gradient. The DNA in that fraction has an estimated density of +0.150 SD. On this basis, the increased binding affinity of H3,H4 for highly positively coiled DNA may provide an explanation for the *in vivo* observation that H3,H4 are relatively resistant to release from the DNA during the transcription process.

Other investigators have developed procedures for generating positively coiled DNA. Two procedures that have been the most successful involve the treatment of DNA with either reverse gyrase (Nadal et al., 1988) or *Escherichia coli* gyrase in the absence of ATP (Clark & Felsenfeld, 1994). In both instances, the maximum density obtainable is +0.03 SD. This density is not sufficient to observe the preferential binding of H3,H4 for positively coiled DNA at physiological ionic strength. It therefore was necessary to develop an alternative approach to prepare more highly positively coiled DNA. Our procedure was developed on the basis of initial observations by Lockshon and Morris (1983) and later refined by Wu et al. (1988). These latter investigators provided evidence for the twin-domain model of transcription-induced supercoiling based on the inhibition of gyrase with novobiocin. With these conditions an estimated superhelical density of +0.024 SD was observed. By maximizing the novobiocin and tetracycline concentrations, we have isolated highly positively coiled DNAs that range in superhelical density from an estimated +0.150 to −0.045 (fractions 13−22 of Figure 2). Bowater et al. (1994) used a similar protocol to produce highly negatively coiled DNA in order to study promoter activation by negative stress. In their protocol, transcription of the tet gene was done in a top A mutant, which is therefore unable to remove transcription-induced negative stress. They observed a broad range of negative topoisomers, which was interpreted as indicating that not all plasmids within a cell are actively transcribed. This is also our interpretation for the broad range of positive topoisomers observed in Figure 2. This range of topoisomers has been very useful, however, because these topoisomers

can be separated on CsCl−EtBr gradients. It is possible to select particular ranges for binding studies with histones H3,H4, and in this way, it was observed that positive superhelicity approximating +0.10 was necessary for the prefential binding of H3,H4. Subsequent studies will evaluate the role of H2A,H2B in this binding and whether there is an upper limit to the positive stress that is required to maintain this H3,H4 binding characteristic.

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