Several factors were evaluated to determine their role in facilitating the presence of transcription-induced stresses in a circular DNA. Transcription was done with T7 RNA polymerase in the presence of E. coli topoisomerase I and closed circular DNA. Positive stress was observed in hypotonic conditions or when one of the polyamines, spermidine or spermine, were present. Polycations such as polylysine, polyarginine, histone H1, histones H2A-H2B, and protamine were observed to induce minimal positive stress. It is known that polyamines influence DNA structure by causing both self-association and sequence-specific structural alterations (polyamine-induced localized bending). Experimental evidence indicates that the likely cause of the positive stress is the induced bending. In order to evaluate protein-mediated bending, transcription was done on nucleosomes. A minimum of three nucleosomes on a DNA of 6055 bp was sufficient to generate very high levels of positive stress. Histones H3-H4 in the absence of H2A-H2B were responsible for this effect. Since these histones by themselves are able to maintain negative coils on DNA, it is concluded that protein-mediated bending is yet another mechanism for placing rotational restriction on DNA. The bending of DNA by either polyamines or histones is an effective mechanism for promoting transcription-induced stresses at physiological ionic strength.

Extensive topological changes occur on DNA during the transcription process. As RNA polymerase transcribes the DNA, it opens 10–15 bp of DNA and would therefore be expected to rotate 360° for every 10.5 bp that is transcribed. However, as the length of the transcript increases, the rotational freedom of the polymerase decreases, and the DNA preferentially rotates. This rotation causes an overwinding of the helix in front of the polymerase (positive stress) and an underwinding of the helix in the wake of the polymerase (negative stress). This twin supercoiled domain model (1) has received a wealth of support in the wake of the polymerase (negative stress). This twin polymerase (positive stress) and an underwinding of the helix.

Polycations such as polylysine, polyarginine, histone H1, histones H2A-H2B, and protamine were observed to induce minimal positive stress. It is known that polyamines influence DNA structure by causing both self-association and sequence-specific structural alterations (polyamine-induced localized bending). Experimental evidence indicates that the likely cause of the positive stress is the induced bending. In order to evaluate protein-mediated bending, transcription was done on nucleosomes. A minimum of three nucleosomes on a DNA of 6055 bp was sufficient to generate very high levels of positive stress. Histones H3-H4 in the absence of H2A-H2B were responsible for this effect. Since these histones by themselves are able to maintain negative coils on DNA, it is concluded that protein-mediated bending is yet another mechanism for placing rotational restriction on DNA. The bending of DNA by either polyamines or histones is an effective mechanism for promoting transcription-induced stresses at physiological ionic strength.

In contrast to the topoisomerases of the prokaryote, the eukaryotic topoisomerases (I and II) relax both positively and negatively coiled DNA equally well. A number of studies have been done in yeast with deletion mutants of topoisomerase I and ts mutants of topoisomerase II that indicate that these proteins function in a similar way to remove topological stresses that are induced by transcription (18–21). In this instance, the presence of nucleosomes does not appear to alter the formation of these induced stresses. Indeed, the induced positive stress in advance of the polymerase may alter the structural state of the nucleosome (22, 23); an alteration that may enhance transcription through them. It has also been suggested that the negative stress in the wake of the polymerase has an important role in facilitating activation of promoters, DNA replication, and recombination (reviewed in Refs. 24–27). It is therefore of interest to evaluate the parameters that facilitate the maintenance of transcription-induced stress.

The in vitro experiments that have studied the induction of transcription-induced stress have used variable conditions of ionic strength or polyamine concentrations. We have evaluated these components further in order to define the process whereby positive stress is induced in template DNA when transcription is done in the presence of E. coli topoisomerase I. We conclude that a decrease in flexibility of DNA is a critical factor that is responsible for this effect. This decreased flexibility enhances the maintenance of transcription-induced stress in two ways as follows. 1) When reduced flexibility is present, the induced topological stress has a more profound effect on the helical state of the DNA. Any negative stress that is generated causes a greater helical distortion (single-stranded character), which can be rapidly removed by E. coli topoisomerase I. This reduced flexibility is observed during transcription in hypotonic conditions and in the presence of polyamines. 2) The reduced flexibility as exhibited with the polyamines also decreases the rotational freedom of DNA. Polyamines are known to stabilize alternative DNA structures (i.e. Z DNA) and facilitate sequence-specific bending (28–32). The stabilized bending increases the effective diameter of DNA and serves a critical role in maintaining an overall negative state in the DNA. This enzyme senses helical distortion (single-stranded character) in DNA that is caused by the transcription-induced negative stress (13–15). The activity of this enzyme decreases rapidly as the DNA becomes increasingly relaxed and therefore provides a mechanism for maintenance of a basal level of negative stress. In vitro transcription studies on closed circular DNA have shown that when this enzyme is present, positive stress is produced on the template (8). Various levels of positive stress have been observed as a result of restricting rotational freedom of both polymerase and DNA (9) or by immobilizing the nascent transcript (4, 16, 17). Therefore, this topoisomerase has been shown to be an effective reagent for the quantitation of helical distortions, as observed by the accumulation of positive stress on DNA during transcription.
restricts its rotation during transcription. These two effects, which are a consequence of reduced flexibility, enhance the maintenance of helical distortions and are required for E. coli topoisomerase I to remove the negative stress. We have also evaluated whether similar helical distortions could be produced in the presence of nucleosomes. We observe that as little as three nucleosomes in a plasmid capable of holding 30 nucleosomes will facilitate the formation of very high levels of positive stress. In this instance, a protein-mediated bending of DNA has increased the effective diameter of DNA and reduced its rotational freedom. Histones H3-H4 and not histones H2A-H2B were responsible for this reduced rotational rate, which is an indication that the presence of the stored negative coil is a primary determinant for this rotational restriction.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Salmon protamine (grade X), poly-t-lysine (4–15 kDa), poly-t-arginine (5–15 kDa), poly(Asp-Glu) (7 kDa), spermine, spermidine, and protamine were purchased from Sigma, 1,19-Bis(ethylamino)-5,10,15-tirazanonadecane (BE-4-4-4-4) was a gift from Hirac Basu (University of Technology). The protein was purified by the procedure of Fujii-Brookhaven National Laboratory). The enzyme was purified by the

**Procedures for Purification of Proteins**—Histones were purified from chicken erythrocytes as described previously (33). Briefly, histones H2A-H2B were separated from H3-H4 by elution from a hydroxylapatite column in which the chromatin had been immobilized. Elution was done with a 0.1 M NaCl step gradient between 0.6 and 2.0 M NaCl. The histones were pooled, concentrated on Amicon filters, and stored at -70 °C. Histone H1 was purified by a 0.6 M NaCl extraction of calf thymus chromatin that had been immobilized on hydroxylapatite. This extract was then treated with 5% perchloric acid in which H1 is preferentially soluble. The H1 was then extensively dialyzed against 10 mM Tris, pH 8.0, and stored at -70 °C. Topoisomerase I frequently contains H1 preparations when eluted from chromatin by NaCl. It is important to remove this activity when characterizing topological states. The perchloric acid extraction destroys this activity.

Eukaryotic topoisomerase I was isolated from MSB cells (chicken leukemic cell line) using a modification (34) of the procedure of Liu and Miller (35). One unit is defined as that quantity that achieves 100% relaxation of 0.5 μg of DNA at 30 min at 37 °C. This protein is referred to here as MSB topoisomerase I.

Prokaryotic topoisomerase I was isolated from a clone of E. coli topoisomerase I (pJW312; a gift of M. Gartenberg and J. C. Wang, Harvard). The protein was purified by the procedures for isolation as was described previously (36) except that after the final phosphocellulose column, the enzyme was further purified from DNase and RNase activity by collecting the flow-through from a Mono Q column and applying it to a Mono S column. The enzyme eluted in a 0–0.5 M KC1 gradient. We have defined 1 unit of activity as the quantity that relaxes 1 μg of DNA from -0.05 superhelical density (SD) to -0.25 SD at 37 °C for 1 min. T7 RNA polymerase was prepared from E. coli strain BL21, which contained plasmid pAR1219 (a gift of W. Studier and J. Dunn, Brookhaven National Laboratory). The enzyme was purified by the procedures of King et al. (37) except with modification as described previously (38). One unit is defined as that amount of enzyme that will incorporate 1 nmol of CTP at 37 °C for 60 min. T7 promoter 39 bp apart and a size of 2255 bp (27T7/3-19). This DNA was used as the vector for the insertion of a 3800-bp SacI fragment excised from pT207-18 (gift of P. Yau and M. Bradbury, University of California, Davis). This fragment contains 18 repeats of the 207-5 S gene of Lytechinus variegatus (40). This insertion yields a DNA of 6055 bp with two tandem promoters that directly transcribe into the insert (p2T7/3-207-18). Circular, covalently closed, negatively coiled DNA (referred to here as "S") was purified on CsCl-ethidium bromide density gradients followed by sedimentation on 5–20% sucrose gradients. For the preparation to be used as a template for transcription, the DNA was treated with MSB topoisomerase I at 0 °C in buffer conditions of 10 mM MgCl2, 10 mM triethanolamine, pH 7.4. The angle between adjacent base pairs in DNA decreases by about 0.011 rotational degree for each 1°C decrease in temperature (41–43). By including 10 mM MgCl2, the angle is reduced even further (44), so that after the topoisomerase I activity is terminated and the temperature is raised to 37 °C in isotonic conditions, the superhelical density is +0.011 SD. The 6055-bp DNA will contain an average of +6.5 coils. After treatment with the topoisomerase I, the DNA was phenol/chloroform-extracted and ethanol-precipitated prior to use. This DNA is referred to as "R." The R DNA was then used to discriminate by gel electrophoresis the template DNA that had been nicked during post-transcriptional processing of samples from template DNA that had remained covalently closed. In this way, it is possible to determine more precisely the conversion of template DNA from its original topological state (+0.011 SD) to a more positively stressed condition as a result of transcription.

**Conditions for Transcription**—The buffer conditions and quantities of T7 RNA polymerase and E. coli topoisomerase I that were used were varied as described. The remainder of the transcription conditions were kept constant and included the following: a final concentration of 50 μg/ml in template DNA and 0.8 μM each of ATP, GTP, CTP, UTP, and 5 μCi/ml [α-32P]GTP (NEN Life Science Products). The UTP was excluded in the initial mix and was added later to initiate extended transcription. The mixture was incubated for 60 min in the presence of polymerase and topoisomerase I. During this time, the promoters are saturated with the polymerase and transcription proceeds 13 bases before a UTP is required. Incubation was then continued for an additional 10 min in which the conditions were varied by including polyamines, poly-amino acids, protamine, or histones at the concentrations indicated. Synchronized transcription was then extended by adding UTP, and at specific time points the reaction was terminated by the addition of an equal volume of 0.4% SDS, 20% glycerol, 50 mM Tris, 25 mM EDTA, pH 8.0, and 0.25% bromphenol blue. Samples were directly electrophoresed for RNA analysis, or aliquots were taken for analysis of 32P incorporation by trichloracetic acid precipitation. The remainder of the transcription was treated with 20 μg/ml RNase A for 30 min followed by 200 μg/ml RNase T1 for 5 at 37 °C. DNA was also treated with 1.9% agarose gels using buffer conditions as described previously (45). DNA was visualized by staining with ethidium bromide, exposed to uv, retained a second time, extensively destained in water, photographed, and imaged on XAR-5 film for densitometric analysis. As reported previously (46), highly positively coiled DNA intercalates ethidium bromide very poorly. For accurate quantitation, the gel must be stained and destained in the dark (46). The gels were then stained a second time followed by extensive destaining in water.

**Reconstitution of Histones with NAP1**—In the transcription experiments in which histones were used, the buffer conditions were 100 mM NaCl, 40 mM Hepes, 6 mM MgCl2, 10 mM DTT (pH 7.5 or 7.9), and 0.8 mM each ATP, GTP, and CTP. The histone-NAP1 complexes were prepared in this same buffer at a final concentration of 100 μg/ml histone. After a 5-min incubation at 37 °C of the histones in this buffer, NAP1 (1:1 w/w) was added, and the incubation continued for 30 min. This solution was then directly added to the mix containing the template DNA, and the incubation continued for 10 min before transcription was initiated with the addition of UTP. This 10-min incubation is sufficient to complete the deposition of histones to the template DNA by the NAP1 (see bottom panel of Fig. 9).

**RESULTS**

The Level of Positive Stress Observed Is Highly Dependent on the Ionic Environment in Which the Transcription Was Done—

These transcription studies were done on a closed circular DNA (6055 bp) that contains two tandemly arranged T7 promoters that are 39 bp apart. To facilitate the analysis of changes in the topological state of DNA during transcription, the DNA was predisposed to a density of +0.011 SD (see "Experimental Procedures"). As shown in Fig. 1A (lane R), the DNA averages +6.5 coils. As shown in Fig. 1B, when this DNA was transcribed by 400 units/μg DNA of T7 RNA polymerase in the buffer conditions of 10 mM Hepes, 6 mM MgCl2, 10 mM DTT, pH 7.9, and in the presence of E. coli topoisomerase I (60 units/μg DNA), the number of positive coils increased substantially as transcription proceeded. That this change is due to an increase
Transcription-induced Stress with Polyamines and Histones

![Analysis of the positive stress in DNA after transcription](image)

**Fig. 1. Analysis of the positive stress in DNA after transcription.** A, the 6055-hp DNA in its negatively coiled state (S) and the same DNA that has been changed to a superhelical density of +0.111 SD (R). This R DNA was used for the template in the subsequent transcription studies. The negatively coiled DNA served as a marker for superhelical density. It has a density of −0.05 SD and has the same relative mobility for a DNA that is made positively coiled at +0.05 SD. B, the template DNA after transcription in 10 mM Hepes, 6 mM MgCl₂, 10 mM DTT, pH 7.9, with 400 units/μg DNA of T7 RNA polymerase and in the presence of 60 units/μg DNA of E. coli topoisomerase I at 37 °C. The +RNase lane is an incubation in which transcription was done in the presence of 10 ng/μg DNA of RNase A for 10 min. C, a second dimensional electrophoretic analysis of the 10-min time point of B. The second dimension was done in 15 μM chloroquin (46). D, an agarose gel showing the size of RNA produced during transcription for the time points of B. The RNA size markers (M) are 4241 (a), 2360 (b), and 580 bases (c). E, the template DNA after transcription in the presence of 1600 units/μg DNA of T7 RNA polymerase. All other conditions are the same as in B. F, the template DNA after transcription in 100 mM NaCl, 40 mM Hepes, 6 mM MgCl₂, 10 mM DTT, and with 1600 units/μg DNA of T7 RNA polymerase and 60 units/μg DNA of E. coli topoisomerase I. The transcription rate in this buffer condition is 83 bases/s (see Fig. 1A). On the right side of F are indicated regions of positive superhelical density: region (reg.1) (±0.05), region 2 (±0.10), and region 1 (±0.15). These values are average numbers and are based on comparison with the negatively coiled (S) marker and analysis on CsCl-ethidium bromide gradients as described previously (46). The left side of A indicates the number of positive coils in the R DNA (average ±6.5). N marks the location for nicked DNA.

In positive coils can be shown in the two-dimensional analysis of Fig. 1C. This pattern of distribution for DNA topoisomers is characteristic of positive coils when the second electrophoretic condition is in the presence of chloroquin (46). Fig. 1D shows an analysis of the RNA that was produced during this transcription and indicates that the rate of transcription was 95 bases/s. That this formation of high levels of positive stress is dependent on the twin domain model of transcription-induced stress can be seen in Fig. 1B (+ RNase lane). The lack of increased positive stress in this lane is interpreted as indicating that the RNase A is destroying the transcript as transcription starts. The RNA polymerase is free to rotate on the DNA and therefore unable to induce stress.

We further characterized the conditions responsible for forming the positive stress with the following experiments. As shown in Fig. 1E, when the quantity of polymerase was increased 4-fold, the number of positive coils increased so extensively that now one major band of very highly positively coiled DNA was observed (Fig. 1, region 1). An estimate of the superhelical density can be determined by an analysis of the DNA on CsCl-ethidium bromide gradients (46). Using this approach, we estimate the level of stress to average ±0.15 SD (data not shown). These results are in contrast to when the buffer condition was adjusted to include 100 mM NaCl. As shown in Fig. 1F, there was a minimal change in the topological state of the DNA. This more physiological condition has negated the ability of E. coli topoisomerase I to remove negative coils that have been produced by transcription. Graphically shown in Fig. 2A is an analysis of the levels of positive stress that were generated as a function of variations in ionic strength. Fig. 2 also shows the quantity of RNA that was produced during transcription in order to correlate levels of transcription with the quantity of positive stress that was produced. When the NaCl concentration was raised from 0 to 120 mM, there was a 2-fold increase in transcription. Yet even with this 2-fold increase in transcription, there was a rapid and precipitous decrease in the ability to form positive coils on the template. We interpret this observation as indicating that the monovalent ions are increasing the flexibility of the DNA through neutralization of the phosphates (47, 48). The induced negative and positive coils are rapidly neutralized by translational diffusion around the circular plasmid before sufficient stress is generated to cause the helical perturbations (14, 15, 26).

**Polyamines Greatly Increase the Formation of Transcriptionally Induced Positive Stress**—The polyamines, putrescine (diamine) and spermidine (triamine), are found in prokaryotic cells in millimolar concentrations (49). In eukaryotic cells, spermine (tetramine) and spermidine are found in submillimolar and millimolar concentrations, respectively (50, 51). The polyamines are thought to function at several levels of RNA and DNA processing (52). With regard to DNA, polyamines interact with both the major and minor groove in a sequence-dependent process (53–55). This selective binding can result in an increased stabilization of unique DNA structures such as Z DNA (28) as well as causing a general stiffness in the DNA by interaction with the surface phosphates (29–31). Because of this general neutralization of charge and the potential bridging of the multivalent polyamines between DNAs, higher concentrations will increase DNA-DNA affinity or self-association (56–59). As shown in Fig. 3, when transcription was done for 5 min in 100 mM NaCl and in the presence of increasing spermidine (Fig. 3A) or spermine (Fig. 3C), substantial positive stress was produced on the template. In both cases, the DNA has a superhelical density estimated to be +0.10 SD (Fig. 3C, region 2). The minimal polyamine concentrations required to obtain this superhelical density were 4 mg/ml, respectively. These data have been quantitated and are graphically shown in Fig. 4 in order to compare with the levels of transcription that produced this stress. These data indicate that the transcription rate remained relatively unchanged throughout the range in which positive stress continued to be amplified for both polyamines. The increase in positive stress must be a result of a change in the physical property of the DNA or RNA synthesized from it. This altered character could be a change in the flexibility and/or an increase in self-association (aggregation) between nucleic acid molecules. The rate at which this positive
stress is produced is shown in Fig. 3. In this experiment, the spermidine (6 mM) was added simultaneously with the initiation of transcription. High levels of positive stress were observed after 30 s, which suggests that whatever physical property is changed by the polyamines, it is changed very rapidly.

We evaluated the factors that contributed to the induced positive stress by initially characterizing the aggregative state of the RNA and DNA. Transcription was done in the presence of increasing quantities of the polyamines, during which the samples were sedimented 27,000 × g for 5 min. Both the supernatant (S) and pellet (P) were analyzed by gel electrophoresis for RNA and DNA content. As shown in Fig. 4A, when the spermidine concentration was increased, there was a proportional increase in aggregation of both RNA and DNA. At 4 mM spermidine, approximately 10% of the DNA and RNA was in the pellet. RNA was the major factor that caused this aggregation, for in the absence of transcription no DNA was in the pellet (see Fig. 7A). We can differentiate between the role of DNA flexibility and aggregation in inducing positive stress, providing we can minimize this aggregation further. As shown in Fig. 4B, transcription in the presence of 25 mg/ml BSA resulted in minimal aggregation even at high spermidine concentration.
centrations. Fig. 3B shows the positive stress that was produced in this nonaggregative condition. Minimal changes in the extent and level of positive stress are observed (Fig. 3, compare A and B), which suggests that a decrease in DNA flexibility is responsible for the effect. To assess the factors that produced positive stress with spermine, transcription was done in the presence (Fig. 4D) and absence (Fig. 4C) of poly(Asp-Glu) followed by centrifugation to remove the aggregated material. In the absence of poly(Asp-Glu), complete aggregation of both DNA and RNA was observed at 1.6 mM spermine. When it was present, approximately 15% of DNA and RNA aggregated. The poly(Asp-Glu) has substantially reduced the aggregative behavior of spermine. A comparison of panels C and D of Fig. 3 indicates that the positive stress generated in the DNA for these two conditions remains unchanged. The implication from this analysis is that the decrease in DNA flexibility is again responsible for facilitating the presence of DNA helical distortions during transcription. Does this indicate that aggregation is unable to cause transcription-induced stress? To evaluate this question, we repeated this analysis with the synthetic polyanion BE-4-4-4-4. This polyanion is extremely effective at aggregating nucleic acids at very low concentrations (60). As shown in Fig. 4E, 0.4 mM BE-4-4-4-4 was sufficient to cause complete aggregation of DNA and RNA synthesized in its presence. This is a 4-fold lower concentration than was required for spermine. If we now examine the level of positive stress generated in BE-4-4-4-4 (Fig. 3E), we observed that positive stress was generated, but at a much reduced level. We have increased the polymerase and topoisomerase content in subsequent experiments and observed that this pattern remains unchanged (data not shown). We conclude that aggregation can cause positive stress, although it is unlikely to be the major factor. The minimal effect of aggregation in promoting the formation of positive stress will be further shown by our analysis of the large polycations, polylysine and polyarginine, described below.

An additional measure for the level of aggregation caused by the polyanions is the observed catenation that occurs when circular single-stranded DNA is incubated with E. coli topoisomerase I (56, 61). As shown in Fig. 5A, M13 single-stranded DNA catenates into intramolecular knotted structures as well as intermolecular larger molecular weights when in the presence of topoisomerase and 6 mM spermidine, 6 mM MgCl₂, 10 mM Hepes, pH 8.0. At this low ionic strength, spermidine causes aggregation (Ref. 58; data not shown), and, as expected, catenates are formed. As shown in Fig. 5B, when the NaCl concentration was increased, the extent of catenation dramatically decreased such that by 80 mM NaCl, no catenation of either form was observed in the 6 mM spermidine. These observations are another indication that at the more physiological salt concentrations, spermidine minimally promotes intramolecular or intermolecular interactions of DNA.

It has been reported that the polyanions are effective at stabilizing the double-stranded character of DNA (62, 63). As a result, E. coli topoisomerase I is substantially inhibited from relaxing negatively coiled DNA when in the presence of spermidine (64, 65). This point is illustrated in Fig. 6, in which negatively coiled DNA was treated with the enzyme in the absence (A) and presence (B) of 4 mM spermidine. The presence of spermidine extensively inhibited the enzyme. These data are graphically shown in Fig. 7A, in which it is observed that only 11% of the activity remained. Higher concentrations reduced the activity even further so that at 10 mM spermidine, 4% of the activity was observed (Fig. 7A). This inhibition was not due to the aggregative state of the DNA, because, as shown in Fig. 7A, the DNA remained soluble even at 10 mM spermidine, and indeed, as shown in this figure, the eukaryotic topoisomerase I
As shown in Fig. 8A, the presence of polylysine produced minimal positive stress on the DNA. Even at concentrations in which the polylysine began to cause self-association (aggregation) of the DNA (0.65:1; data not shown) (and, as shown in Fig. 8A, significant transcription continued to occur), no increase in positive stress was observed. For polyarginine, there was a gradual increase in positive stress, but it still remained significantly less than for the polyamino acids (compare Figs. 8A and 2B). This lack of positive stress is not due to an inability of E. coli topoisomerase I to access the DNA. Control experiments have been done that show that negatively coiled DNA is readily relaxed in the presence of these poly-amino acids (data not shown). We interpret these observations as indicating that peptide-induced changes in the physical state of the DNA as exhibited by these poly-amino acids is substantially different from the polyamines.

**Extensive Positive Stress Is Generated by Transcription of DNA When Histones Are Reconstituted under Specific Conditions**—Increasing quantities of histone H1 or protamine were directly added to template DNA, and, after an incubation of 10 min, the samples were transcribed in the presence of E. coli topoisomerase I. As shown in Fig. 8B, very minimal positive stress was observed on the DNA for either protein despite the presence of substantial transcription, particularly at the lower protein-DNA ratios. E. coli topoisomerase I was unable to detect helical distortion in the DNA brought about by the transcription process. These observations are consistent with the observations concerning the poly-amino acids, in that the binding of protein to DNA does not necessarily provide conditions in which transcription-induced stresses can be preserved.

We now considered whether the presence of core histones would preserve transcription-induced stresses. Histones H2A-H2B and histones H3-H4 were reconstituted with DNA at increasingly greater ratios using the same direct addition protocol as was used for the experiments with histone H1 and protamine. Minimal positive stress was observed although substantial transcription continued to occur (Fig. 8B). However, it is known that the direct addition of core histones to DNA at physiological ionic strength severely limits nucleosome formation. Rapid binding is not conducive for proper interaction. When reconstitution is done by salt gradient dialysis (67) or by mediation with deposition factors (68), proper histone-histone and histone-DNA contacts are established, and nucleosomes will form. To evaluate the effect of nucleosomal structure in this process, core histones were pretreated with the deposition factor, NAP1, and then added to DNA in increasing quantities. The samples were split in half, and one aliquot was incubated for 10 min in the presence of MSB topoisomerase I in order to determine the number of nucleosomes that were present. The maintenance of negative coils by histones when in the presence of an eukaryotic topoisomerase is an indication that nucleosomes are present (67, 69). Each negative coil is equivalent to the presence of one nucleosome. As shown in the bottom panel of Fig. 9A, an increase in the histone-DNA ratio resulted in an expected increase in the number of negative coils on the DNA. This 10-min incubation was sufficient to complete this process, since a more prolonged 60-min incubation did not alter these levels (data not shown). The second aliquot was incubated for 10 min, during which the nucleosomes were allowed to form, except this time E. coli topoisomerase I was present. After the 10-min incubation, transcription was initiated by the addition of the UTP. Transcription was allowed to occur for 5 min, and the DNA was then analyzed as shown in the upper panel of Fig. 9A. At a histone-DNA (H:D) ratio of 0.1:1, the very highly positively coiled band (region 1) was observed. This very low ratio equates to an average of three nucleosomes on a 6055-bp
core histones were used (Fig. 9). This result is in contrast to when a ratio of 0.5:1 H:D was reached, at which point no further increase was observed. These data are graphically shown in Fig. 8A along with the level of transcription that was observed in this experiment. It is now useful to compare these data with the data of Fig. 8B, in which histones were used on the DNA in a nonnucleosomal form. There is a dramatic difference in the level of positive stress, since in the level of transcription for both situations was very similar up to the histone:DNA ratio of 0.6:1. We conclude that nucleosomes very effectively promote the presence of transcription-induced stress.

To evaluate the role of the individual core histones in producing this effect, this procedure was repeated except that only histones H3-H4 were preincubated with NAP1 prior to the addition to the DNA. As shown in Fig. 9B (lower panel), the number of negative coils increased as the ratio of H3-H4 to DNA was increased. This observation has been reported previously (46, 70) and is an indication that histones H3-H4 can hold negative coils independent of H2A-H2B. Increasing the length of incubation beyond 10 min did not appreciably change the number of negative coils that were observed and is again an indication that with these histones, deposition was complete within the 10 min (data not shown). Transcription of these reconstitutes in the presence of E. coli topoisomerase I resulted in the formation of the highly positively coiled band (Fig. 9B, upper panel). This band was first observed at the very low ratio of 0.05:1 H:D and is an indication that H3-H4 are extremely effective in maintaining transcription-induced stress. An additional point of interest regarding these data is that when a ratio of 0.5:1 H:D was reached, all of the DNA was shifted to the highly positively coiled state. This result is in contrast to when core histones were used (Fig. 9A, upper panel), in which a sizable percentage of DNA never became highly positively coiled. We interpret this difference as being due to the blockage of promoters by core histones in a subset of templates, which does not occur in the absence of H2A-H2B. It is known that nucleosomes will form on the T7 RNA polymerase promoter and repress initiation (71).

As shown in the graphical analysis of Fig. 8C, the level of positive stress that was produced in the presence of H3-H4 was 100%. In other words, this level of positive stress is equivalent to the level observed when transcription was done at low ionic strength (Fig. 2A), a condition that up to this point produced the maximum level of positive stress. The presence of H3-H4 has preserved these induced stresses in physiological conditions of ionic strength and MgCl\textsubscript{2}, a condition in which these stresses would normally not be detected by E. coli topoisomerase I.

These experiments were repeated with histones H2A-H2B in order to evaluate whether these proteins contributed to this process. In this instance, NAP1 was not used as a deposition factor, since it is ineffective in depositing H2A-H2B when H3-H4 are absent (72). Instead, H2A-H2B were deposited by direct addition to the DNA (33). The reconstitutes were then incubated with MSB topoisomerase I, and, as shown in Fig. 9C (lower panel), H2A-H2B were unable to hold negative coils. This observation has been reported previously by several investigators and is an indication that H2A-H2B in the absence of H3-H4 are unable to hold negative coils on DNA (70). The upper panel of Fig. 9 shows the quantity of positive stress that was left on the template after transcription in the presence of H2A-H2B. Even at a ratio of 1:1 H:D, the level of positive stress that was observed was very low. These data along with the level of RNA produced during transcription are graphically shown in Fig. 8C. These data illustrate the striking difference between these two sets of proteins. Histones H2A-H2B produced a 10-fold lower level of positive stress compared with H3-H4. Additional information can be obtained from this graphical analysis. For the ratios between 0.025 and 0.40 H:D, the slopes of the two lines describing the levels of positive stress that were observed for all four histones compared with H3-H4 alone are nearly identical (Fig. 8C). Similarly, if the number of stored negative coils that the four histones maintain are compared with H3-H4 alone for this same range of H:D ratios, we also observe that they are very similar (Fig. 9, A and B; compare lower panels). We therefore conclude that H2A-H2B substitute very effectively for an equimolar amount of H3-H4, providing an equimolar amount of H3-H4 is present at...
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FIG. 8. Graphical analysis of levels of transcription and positive stress that were generated in the presence of poly-amino acids (A); protamine, H1, or core histone (direct addition) (B); and H3-H4 core histone (deposited by NAP1) or H2A-H2B (C). A, percentage of production in the presence of polylysine of RNA (■) and positive stress (●) and in the presence of polyarginine of RNA (○) and positive stress (●); B, percentage of production in the presence of protamine of RNA (▲) and positive stress (●), in the presence of histone the same time. It is the storage of the left-handed supercoil by the complex of histones that provides the bending of the DNA. Histones H2A-H2B can contribute to the bending, provided that they are interacting with H3-H4 to form left-handed coils as part of the nucleosomal complex. The bending of DNA into the coil has increased the effective diameter of the DNA, which cannot be mimicked by protamine, H1, or nonnucleosomal core histones (Fig. 8B).

We have also observed that the method of reconstitution, whether protein-mediated (NAP1) or salt-mediated (NaCl dialysis), does not alter these results. The experiments of Fig. 9 have been repeated using NaCl dialysis (67), and the same results were observed in all three cases (data not shown). In the subsequent studies of this report, we focus on the use of histones H3-H4 rather than total core histones. Histones H3-H4 do not block the T7 RNA polymerase from its promoter. We can then more effectively define increases in positive stress during transcription.

A Comparison of the Positive Stress That Is Produced with Spermidine, Spermine, and Histones H3-H4—We have observed that spermidine, spermine, and histones H3-H4 preserve transcription-induced stress at physiological ionic strength. We now compared their relative effectiveness in this process by transcribing under conditions in which both the concentration of E. coli topoisomerase I (Fig. 10, A–C) and T7 RNA polymerase (Fig. 10, D–F) were varied. The conditions that were used were 2.0 mM spermidine (Fig. 10, A and D), 0.8 mM spermine (Fig. 10, B and E), and H3-H4 at 0.5:1 H:D (Fig. 10, C and F). As shown in Fig. 10, A–C, increasing the E. coli topoisomerase I content from 3.8 to 120 units/μg DNA (lanes a–f) resulted in an increase in positive stress for all three conditions. However, any quantity of topoisomerase I greater than 15 units/μg DNA (lane c) did not succeed in significantly increasing this level of stress. Each level appears to be unique for each agent. For spermidine, the upper limit was +0.05 SD (region 3), for spermine it was +0.10 SD (region 2), and for H3-H4 it was +0.15 SD (region 1). A similar result was observed when the T7 RNA polymerase content was varied from 100 to 3200 units/μg DNA (Fig. 10, D–F). Therefore, regardless of the quantity of polymerase or topoisomerase that was used, these concentrations of spermidine, spermine, and H3-H4 preserve different levels of stress. We are observing well defined differences in rates at which the negative and positive stresses are diffusing around the circular DNA. The structural character of the DNA in these three conditions is uniquely different.

We determined the number of polymerase molecules that were actively transcribing in the experiments of Fig. 10, D–F, in order to correlate this number with the quantity of positive stress that was generated. T7 RNA polymerase (100–3200 units/μg DNA) was added to the template DNA in the presence of excess MSB topoisomerase I and transcribed for 2 min. The principle behind this analysis is that each polymerase opens the coil has increased the effective diameter of the DNA, which cannot be mimicked by protamine, H1, or nonnucleosomal core histones (Fig. 8B).

H1 of RNA (●) and positive stress (○), in the presence of core histones (by direct addition) of RNA (■) and positive stress (●). C, percentage of production in the presence of histones H3-H4 (deposited by NAP1) of RNA (●) and positive stress (○), in the presence of core histones (deposited by NAP1) of RNA (■) and positive stress (●), and in the presence of H2A-H2B (deposited by direct addition) of RNA (▲) and positive stress (●). The values of 100% for both RNA and positive stress are based on the values described in the legend to Fig. 2. The buffer condition was 100 mM NaCl, 40 mM Hapes, 6 mM MgCl2, 10 mM DTT, pH 7.9, which has a transcription rate of 83 bases/s (see Fig. 11A).
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lymerase is then displaced, the helix is restored to the double-stranded state, and a negative coil is now in the circular DNA. As shown in Fig. 10G, an average of six negative coils are present in lane d, which indicates that on average six polymerases produced the quantity of positive stress that is shown in lane d of Fig. 10, D–F. Multiple initiations are required to provide sufficient transcription-induced stress so that this upper limit of positive stress can be obtained.

Reduced Rates of Transcription at 37 °C Decrease the Formation of Positive Stress—We have observed that it is possible to decrease rates of transcription by T7 RNA polymerase at 37 °C by two approaches, adjusting the pH from 7.9 to 7.3 and/or including GDP (2 or 4 mM) during transcription. Fig. 11A shows the size of RNA that was produced at pH 7.9 (lanes a–c) and pH 7.3 (lanes d–f). When no GDP was present, the transcription rates were 83 and 47 bases/s, respectively (lanes a and c); when 2 mM GDP was present, the rates were 47 and 20 bases/s, respectively (lanes b and e); and when 4 mM GDP was present, the rates were 25 and 10 bases/s, respectively (lanes c and f). Using these procedures, we analyzed the effect of transcription rate on the ability to form positive stress. The H3-H4-NAP1 complex was reconstituted with DNA at a ratio of 0.5:1 H:D and then transcribed at four different temperatures. These temperatures were 37, 23, 13, and 4 °C. The transcription rates were 83, 27, 8.2, and 2.6 bases/s, respectively (data not shown). As shown in Fig. 12, the highly positively coiled band (region 1) was present at all four temperatures. Except for the 4 °C condition, the remaining temperatures produced equivalent quantities of this highly positively coiled DNA. The rate at which this stress was achieved was substantially slower at the lower temperatures. One possible reason for the slower rate may be a change in the activity of E. coli topoisomerase I. As noted in the data of Fig. 10, A–C, the quantity of E. coli topoisomerase I is important. We have assayed the topoisomerase at these temperatures and determined its activity relative to the activity at 37 °C. The activity was decreased 40-fold at 23 °C, 640-fold at 13 °C, and 4 × 10^4-fold at 4 °C (data not shown). These rather sizable decreases in activity are probably a major cause for the slower rate at which highly positively coiled DNA was generated at the lower temperatures. Nevertheless, the transcription rates at 23 °C (26 bases/s) and at 13 °C (8.2 bases/s) clearly produced the same high levels of stress that were observed at 37 °C (83 bases/s). It is a level substantially greater than what was observed when we produced comparable transcription rates at 37 °C in Fig. 11 (25 and 10 bases/s). The decrease in transcription rate because of the lowered temperature must have closely matched a similar reduction in thermal motion. Rates of diffusion for the transcription-induced stresses are then also reduced. A major determinant for defining the availability of negative coils that are to be neutralized by E. coli topoisomerase I is this rate of diffusion.
These data indicate that there are two potential means whereby transcription-induced stresses can be preserved within DNA: 1) changes in DNA flexibility inherent in the DNA and 2) changes in rotational flexibility because of protein interaction. The observation that low ionic strength conditions extensively increase positive stress in DNA during transcription is an indication that stiffening of DNA is a contributing factor to this process (Fig. 2A). An alternative explanation for this effect would be that the increased single-strandedness of the DNA at the lower ionic strength has increased E. coli topoisomerase I activity. We have not observed an increase in activity in this low ionic strength condition (data not shown). We have also shown that increasing the topoisomerase I activity beyond a critical level does not alter the final level of positive stress (Fig. 10). Thus, a change in DNA flexibility must be the critical factor. We surmise that the change is a lack of DNA flexibility due to the lack of counterions on the DNA surface. The decreased flexibility is predicted to increase the likelihood that a lower level of the induced stresses will induce helical distortions as these stresses diffuse around the circular DNA. The hypotonic condition, however, is nonphysiological, and the question arises as to whether a similar change in flexibility can be observed in a physiological context. Spermidine and putrescine are the major polyamines in E. coli and exist in average concentrations of 6 and 20 mM, respectively, although the effective free concentration may be less (49). Polyamines are known to decrease the flexibility of DNA (29–31), and at sufficiently high concentrations they will promote self-association of DNA (56–59). We have attempted to differentiate the relative contributions of these two effects in facilitating the formation of positive stress. We have found that when transcription is done in the presence of either BSA (Fig. 4B) or poly(Asp-Glu) (Fig. 4D), minimal aggregation of either RNA or DNA is observed. In both cases, whether with spermidine or spermine, the level of positive stress that was generated represents minimal aggregation of either RNA or DNA.

**FIG. 10.** Analysis of the positive stress in DNA that was transcribed in the presence of spermidine (A and D), spermine (B and E), and histones H3-H4 (C and F) using variable quantities of E. coli topoisomerase I (A–C) and T7 RNA polymerase (D–F). For A–C, the quantities of E. coli topoisomerase I used were 3.8 (a), 7.5 (b), 15 (c), 30 (d), 60 (e), and 120 units/μg DNA (f). The T7 RNA polymerase content was kept at 1600 units/μg DNA. For D–F, the quantities of T7 RNA polymerase used were 100 (a), 200 (b), 400 (c), 800 (d), 1600 (e), and 3200 units/μg DNA (f). The E. coli topoisomerase I content was kept at 60 units/μg DNA. Transcription was for 5 min in 2.0 mM spermidine, 0.8 mM spermine, or H3-H4 at a ratio of 0.5:1 H:D. For G, transcription was done in the presence of MSB topoisomerase I (200 units/μg DNA) for 2 min. Lanes a–f used the same quantities of T7 RNA polymerase as were used for panels D–F. Lane X is a control showing the DNA in a fully relaxed state. The numbers on the right of G are the number of negative coils (number of polymerase molecules) on the template.

**DISCUSSION**

The data presented in this section provide evidence for the existence of transcription-induced stress, which is likely to be a major factor in facilitating DNA conformational changes during transcription. The use of different polyamines and histones allows for the examination of their individual contributions to the formation of positive stress. The results demonstrate that the level of positive stress is influenced by the presence of spermidine, spermine, and histones H3-H4, with spermidine being the most effective in increasing the stress level. The use of different topoisomerases and RNA polymerases also highlights the importance of the enzyme involved in the stress formation process. The observation that increasing the topoisomerase I activity does not alter the final level of positive stress supports the hypothesis that DNA flexibility is the critical factor in stress formation. The data further suggest that changes in DNA flexibility, rather than increased enzyme activity, are responsible for the observed increases in positive stress. This conclusion is supported by the observation that even low levels of transcription can lead to significant stress formation, highlighting the importance of DNA flexibility in the transcription process.
poly(Asp-Glu) in minimizing this aggregation is probably based on their polyanionic nature. Any inter- and intranuclear bridging between nucleic acid strands that is promoted by the polycationic polyamines is competitively blocked by these agents. These observations may have relevance to the environment of a cell that is known to be in a crowded state, with macromolecular concentrations of total RNA and protein approximating 340 mg/ml (74, 75). The observation that BSA at a 10-fold lower concentration is able to modulate this aggregative behavior may be of importance in facilitating both molecular crowding and accessibility to the DNA. Therefore, the general stiffening of DNA observed in the hypotonic conditions would appear to be at least partially mimicked by the polyamines in physiological conditions.

In the data of Fig. 10 it was observed that a relatively defined level of positive stress was induced that was uniquely different for spermidine at 2 mM (+0.2 SD), spermine at 0.8 mM (+0.10), and histones H3-H4 at a H:D of 0.5 (+0.15 SD). One possibility for these differences is that they result from a variation in transcriptional activity. Transcription was increased by nearly 2-fold in the presence of polyamines (Fig. 2B). However, one would normally expect to see greater positive stress, not less, when transcription is increased. Another possibility is that the differences result from the inhibition of E. coli topoisomerase I activity when in the presence of polyamines (Fig. 7). In this instance, the reduction of activity would be expected to reduce levels of positive stress. These two variables were evaluated in the data of Fig. 10, and we observed that even when the topoisomerase I concentration was increased greater than 20-fold or the T7 RNA polymerase concentration was increased by 8-fold, no appreciable change in these defined levels of positive stress was observed. Our interpretation of this observation is that the rate-limiting step that defines these levels of stress is the rate of flux in which the positive and negative stresses are propagated around the plasmid. There must be an upper level of steady state stress that is generated that is unique to each of these three conditions.

We surmise that the decrease in flexibility of DNA is the critical factor by which the polyamines facilitate stress-induced helical distortion. It has been reported that polyamines facilitate the bending of DNA in a sequence-specific manner (31, 32). This bending, which may be minimal at any particular DNA sequence, would be expected to be globally significant in the larger context of a circular DNA of multiple sequences. The increase in effective diameter would be expected to increase the viscous drag of the DNA and result in a prolonged maintenance of transcription-induced stress (48). It is our interpretation that the major factor that causes the variations in the different levels of positive stress that were observed for spermidine at 2 mM and spermine at 0.8 mM in Fig. 10 is how effectively the viscous drag (bent state) was maintained in those two conditions. Histones H3-H4 are particularly effective in bending DNA and maintaining it in that state, hence very effective in promoting this viscous drag. Thus, the level of induced positive stress with H3-H4 is the highest of the three conditions. The common theme is the bending of DNA whether by polyamines or by histones.

General stiffness, however, without the bending of DNA does not appear to be effective in the maintenance of transcription-induced stress. Minimal positive stress was obtained with polylysine, polyarginine, histone H1, protamines, or the core histones when directly added to DNA (Fig. 8). Even at higher quantities of these agents, at which point self-association of the DNA was occurring, positive stress was minimally formed. We conclude that neither the stiffness of the DNA as promoted by these agents or the self-association into aggregative complexes is sufficient to cause the rotational restriction necessary to observe high levels of induced stress. This observation was a surprise, since we had expected that such polyations would mimic the hypotonic conditions and therefore enhance the presence of helical distortions. It may be that such helical distortions are enhanced but that these particular agents mask them by their interaction with DNA. The direct interaction by the polyamines within the major and minor grooves of the DNA must produce unique structural characteristics in DNA (stiffness and bending) that cannot be mimicked by these other polycations.

Based on counterion theory, increasing the ionic environment by either mono or divalent ions would be expected to increase the flexibility of the DNA rather than decrease it (76). This increased flexibility was observed when we raised the NaCl concentration and subsequently produced a precipitous drop in levels of positive stress (Fig. 2A). The polyvalent polyamines do not appear in an additive way to increase this flexibility. Rather, the reverse is observed, which suggests that these ions maintain a more persistent interaction with DNA, rather than as a highly mobile ion shield (48). We have also studied the divalent ions Mg2+ and Ca2+ and have observed that at elevated concentrations (20 mM) a similar decrease in flexibility is observed based on the level of positive stress that is produced during transcription (11). This persistence of interaction by these polyions is expected to be responsible for the structural changes that decrease DNA flexibility. The maintenance of a consistent stiffness in the DNA may be of importance in not only facilitating stress-induced distortions of the DNA helix, which are critically important in accessing and replicating DNA (24–27), but also in ensuring that the flexibility of the DNA is not so great that it “winds itself into a ball” before the transcription-induced stresses are able to produce the helical distortions. E. coli topoisomerase I cannot relax DNA without the single-stranded character that is produced by the helical distortions. In the prokaryotic cell, it must do this in the presence of polyamines that repress helical distortions except under the conditions for which they are generated by transcription (Fig. 6).

When the core histones were deposited onto the DNA using NAP1 so that proper nucleosomes could form, substantial positive stress was observed (Fig. 9A). Even at the low ratio of 0.1:1 H:D, the region 1 band representing +0.15 SD was observed. This ratio equates to an average of three nucleosomes on a circular DNA (6055 bp), which is capable of holding 30 nucleosomes. This number of nucleosomes is not sufficient to cause an overall structural change in DNA of this size as was observed for the polyamines. It is also not likely that nucleosome-nucleosome interactions between plasmids or within plasmids can occur with this number of nucleosomes. It should be noted that the protein-induced aggregation that was observed in the higher H:D ratios of the nonnucleosomal DNA-histone complexes (Fig. 8B) produced very minimal positive stress. It is therefore very likely that the rotational restriction represents a property of the nucleosome itself. We surmise that the binding of DNA into the left-handed coil is a critical factor in this rotational restriction. Histones H3-H4 in the absence of H2A-H2B were able to form left-handed coils in DNA (Fig. 9B, lower panel) and were very effective in the maintenance of induced topological stress as seen by the presence of very high levels of positive stress (Fig. 9B, upper panel). Histones H2A-H2B are unable to form left-handed coils independent of H3-H4 and were unable to maintain induced topological stress (Fig. 9C). They were able to substitute for an equimolar amount of

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2 H. F. Peng and V. Jackson, unpublished observations.
H3-H4 as part of the nucleosome (Fig. 8C), which again emphasizes the importance of the left-handed supercoil. It is unknown in these conditions whether histone displacement occurs during transcription. We have observed that when transcription was done in the presence of MSB topoisomerase I, a displacement frequency of 1 in 4 nucleosomes was observed (77). Whether this same frequency is maintained in the presence of a high level of positive stress remains to be determined.

We have observed that the rates at which induced stresses diffuse around the circular plasmid define the level of positive stress. Reducing the transcription rate from 83 to 10 bases/s at 37 °C resulted in a substantial reduction in positive stress (Fig. 11). Insufficient transcription-induced stress was generated relative to the rate of translational diffusion. Reducing the temperature to 13 °C, which has a transcription rate of 8.2 bases/s, substantially decreased this translational diffusion so that very high levels of positive stress were now formed (Fig. 12). An implication from these observations is that independent of whatever temperature an organism lives at, the interrelationship between transcription rate and translational diffusion ensures that these transcription-induced stresses are actively present to facilitate changes in the helical state of the DNA.

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