Reaction parameters of TFIIIA-induced supercoiling catalyzed by a *Xenopus laevis* cell-free extract

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ABSTRACT

In addition to its fundamental role of nucleating the formation of stable transcription complexes, the *Xenopus laevis* 5S RNA specific transcription factor, TFIIIA, promotes a variety of DNA-associated metabolic reactions. We report that TFIIIA can induce a DNA supercoiling catalyzed by the *Xenopus laevis* S-150 cell-free extract on plasmids containing a single copy of the Xenopus 5S RNA gene (somatic-type). Stimulated supercoiling occurs in the presence of high concentrations of ATP (4 mM) and at a factor to DNA ratio of 1 through a mechanism most likely involving type I topoisomerase. The highest level of stimulated supercoiling occurs when TFIIIA is incubated with DNA prior to the addition of the S-150 extract. Taken together, the experiments outlined in this report establish a reliable and seminal system in which TFIIIA-induced DNA supercoiling can be observed reproducibly.

INTRODUCTION

TFIIIA is the 5S RNA gene specific transcription factor, essential for the establishment of stable transcription complexes and the subsequent expression of the Xenopus 5S RNA genes (1). The development of cell-free transcription systems afforded the opportunity to define the molecular activity of TFIIIA *in vitro* (2,3,4). From these and other basic studies, a biochemical scenario emerged depicting the construction of the 5S RNA transcription complex. TFIIIA binds first to the intragenic region of the 5S gene forming a metastable nucleoprotein complex (1,5). This complex is stabilized by sequential binding of transcription factor(s) TFIIIC, TFIIIB (6) and finally RNA Polymerase III. Such stable nucleoprotein transcription units consisting of factors A, B and C are thought to exist within chromatin *in vivo* (7,8,9). The development of an *in vitro* chromatin assembly system (10) allowed for a direct analysis of the influence of TFIIIA in modulating 5S transcription from chromatin templates.

Kmiec and Worcel (11) demonstrated that exogenously purified TFIIIA can be excluded from the intragenic control region of the 5S gene via the steric hindrance of nucleosome deposition, consistent with the results of Gottesfeld and Bloomer (12). The assay for such an exclusion phenomena was based on a TFIIIA-induced DNA supercoiling catalyzed by the *Xenopus laevis* S-150 cell-free extract. In this assay, exogenously purified TFIIIA was prebound onto a template containing a single copy of the 5S RNA gene within a circular plasmid. A TFIIIA-depleted S-150 was then added to the nucleoprotein complex and the conversion of DNA from a relaxed to the supercoiled form was monitored. These authors found that TFIIIA promoted DNA supercoiling under conditions that were refractory to supercoiling catalyzed by the S-150 alone. After several laboratories failed to reproduce these experiments in their systems, we decided to take a fundamentally reductionist approach to developing conditions at the biochemical level in which TFIIIA induced supercoiling could be observed reliably.

The erratic results most likely arose from the experimental protocol. Among a number of possible sources of variability are the proteolysis/ inactivation of the exogenously purified TFIIIA (13,14,15); varying levels of endogenous TFIIIA (16); varying levels of endogenous ATP in the S-150 (17). In addition, several of the initial experimental designs were seriously flawed and the reaction conditions were simply not optimized. For example, DNA flexibility was demonstrated by the addition of the antibiotic novobiocin or the enzyme topoisomerase I to a preformed nucleoprotein template (18). In both cases, the underlying supercoiled DNA reverted to the relaxed conformation. As described by Felts *et al.* (19), the addition of novobiocin to such reactions can lead to the precipitation of histones or transcription factors and thus DNA relaxation may have resulted from protein dissociation. DNA flexibility promoted by the action of exogenous topoisomerase I was visualized only after the addition of a large excess of enzyme (30 units).

As information about the biochemical nature of TFIIIA accumulated, it became apparent that TFIIIA-promoted reactions had different and rather precise requirements. For example, the TFIIIA-catalyzed ATPase activity has an optimal temperature of 30°C (20) instead of 26°C (room temperature) or 37°C. It has also been reported that several biochemical properties of TFIIIA are observed only when TFIIIA is purified in a particular way. Specifically, the dissociation of 7S particles by treatment with 7M urea, and the subsequent purification of TFIIIA, leads to protein preparations that do not possess ATPase activity (20).

Although we have been able to reproduce factor-induced supercoiling using the published conditions, we have generally obtained variable results. Hence, we sought to define reaction conditions under which TFIIIA-induced supercoiling can be

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observed reliably by examining the biochemical characteristics of the Xenopus S-150 cell-free extract and purified TFIIIA. In this manuscript we report the successful development of these optimized reaction conditions.

METHODS AND MATERIALS

Preparation of [^{32}P]-labeled Plasmid DNA, TFIIIA and Xenopus S-150 Cell-free Extract

Plasmid DNA, pXbsf201 (somatic 5S RNA gene) (2,960 base pairs) (21) was linearized by restriction enzyme digestion, labeled at the EcoRI site and circularized with DNA Ligase as previously described (22). The concentration of each DNA preparation was determined spectrophotometrically and by comparison to reference DNA concentrations in agarose gels. Transcription factor TFIIIA was prepared according to the methods of Dignam *et al.* (23) and Smith *et al.* (24). We found it important to extensively dialyze the urea out of the sample (6−8 hours) prior to use, but either method yielded purified TFIIIA that could induce DNA supercoiling. TFIIIA was stored in high protein concentration (500 μg/ml) at −80°C for several months without loss of activity, however, we found it essential to use TFIIIA immediately after thawing. The Xenopus S-150 cell-free extract was prepared as described by Glikin *et al.* (10) using oocyte-positive female frogs. Most S-150 extracts have a protein concentration of between 2 and 3 mg/ml.

DNA Supercoiling Reactions

DNA supercoiling reactions contained ATP and $MgCl_2$ (at the concentrations indicated figure legends), [^{32}P]-labeled relaxed, circular pXbsf201 DNA and the S-150 cell-free extract. All reactions were carried out in siliconized tubes (0.5 ml Eppendorfs). TFIIIA was incubated with the 5S DNA template for 30 minutes (at 26°C) in the presence of ATP and $MgCl_2$ prior to the addition of the S-150 extract (zero time). TFIIIA was diluted into Germinal Vesicle Buffer (10) to the proper concentrations by serial steps of 10 and added to the reaction mixture in a 1 μl volume. The reaction time, temperature and other assay modifications are outlined in the figure legends. The reactions were terminated by the addition of Stop mix (1% SDS, 25 mM EDTA) and Proteinase K. The analysis of DNA topology was carried out by electrophoresis through 1% agarose or 1.2% agarose laden with 15 μg/ml chloroquine diphosphate followed by autoradiography. Hexokinase (10 units) was purchased from Sigma and added to the reaction in conjunction with 10 mM glucose. As judged by thin layer chromatography, all the detectable ATP is converted to ADP within 15 seconds.

Analysis of DNA Topology, and Protein Binding

In the conventional supercoiling assay, deproteinized DNA samples were electrophoresed through 1% agarose gels in a Tris-glycine buffer system at 100 volts for 3 hours or 40 volts for 16 hours at 26°C followed by autoradiography. Some reactions were electrophoresed through 1.2% agarose laden with 15 μg/ml chloroquine-diphosphate. For this gel system 40 mM Tris-base, 30 mM phosphate and 1 mM EDTA (TPE buffer) containing 15 μg/ml chloroquine was used in place of Tris-glycine and the electrophoresis carried out at 40 volts for 24 hours (26°C) with buffer recirculation. The gels were then dried and autoradiographed. TFIIIA binding to DNA was analyzed by a gel retardation assay. Reaction mixtures were loaded onto 6% polyacrylamide gels (30 cm) with an acrylamide:bis acrylamide ratio of 30:0.4. The gels were pre-electrophoresed for 3 hours at 100 volts while the samples were electrophoresed through the gel at 200 volts for 2.5 hours. DNA mobility shifts were visualized by autoradiography.

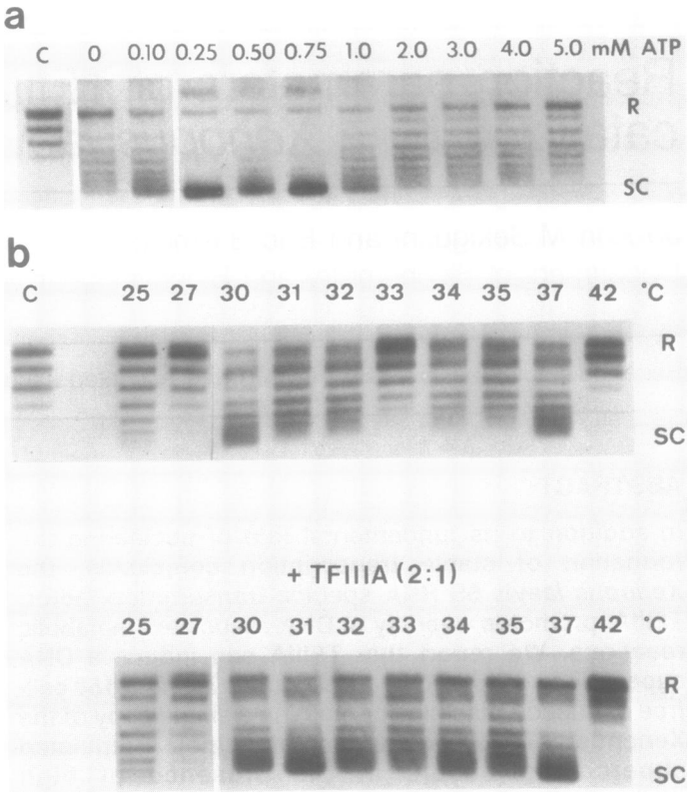

Figure 1: Reaction Parameters of TFIIIA-Induced DNA Supercoiling
a) Reaction mixtures (10 μl) containing 20 nanograms of [^{32}P]-labeled relaxed circular plasmid DNA (pXbsf201), 1 mM $MgCl_2$, 1 μl (2 μg) of the Xenopus S-150 and the indicated concentration of ATP were incubated at 26°C. After 4 hours, the reactions were terminated by addition of SDS and EDTA to a final concentration of 1% and 25mM, respectively. Proteinase K was added for a period of 2 hours at 37°C and the DNA topology visualized after electrophoresis through 1% agarose gels by autoradiography. R, relaxed circular DNA; SC, supercoiled DNA. b) Reaction mixtures (9 μl) containing 20 nanograms of [^{32}P]-labeled, relaxed circular DNA, 4 mM ATP, 1 mM $MgCl_2$ and 0.44 nanograms of TFIIIA were incubated for 30 minutes at 26°C. After this period of time, 1 μl (2 μg) of the Xenopus S-150 extract was added and the reaction continued for 4 hours at the indicated temperatures. After 4 hours, the reaction were terminated and the DNA topology analyzed as described above. The lane designated, C, represents the topology input DNA substrate. R, relaxed (covalently closed or nicked) DNA; SC, superhelical DNA.

RESULTS

It was previously reported that the *Xenopus laevis* cell-free extract (S-150) assembles properly spaced nucleosomes on circular DNA templates in an ATP-dependent fashion (10). The ATP requirement is unusual because chromatin assembly is often described as a passive, non-energy requiring metabolic process (26). Several possible roles for ATP in the S-150 catalyzed chromatin assembly have been proposed. These include the involvement of topoisomerase II in the assembly reaction or the use of ATP in the process of histone modification (10). More recently, Almouzni and Mechali (27) and Kmiec *et al.* (28) demonstrated that ATP participated in the periodic spacing of nucleosomes during chromatin assembly. Since ATP appears to be an important, yet still undefined, cofactor of the S-150's DNA supercoiling activity, our first biochemical optimization of factor-

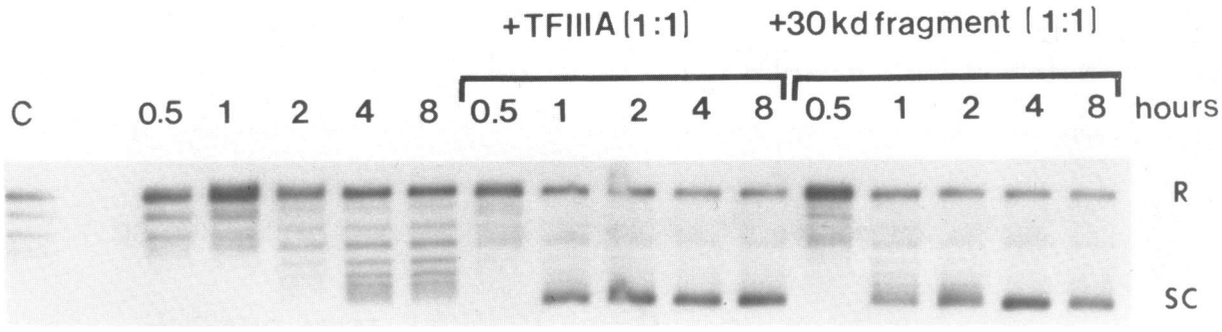

Figure 2: TFIIIA and Its 30Kd Fragment Accelerate the Rate of DNA Supercoiling Promoted by the Xenopus S-150.
Reaction mixtures (10μl) containing 20 nanograns of [^{32}P]-labeled relaxed, circular 5S plasmid DNA (pXbsf201), 1 μl (2 μg) of the Xenopus S-150, 4 mM ATP, 1 mM $MgCl_2$ and TFIIIA or the 30Kd fragment, at a protein to DNA ratio of one, were incubated at 37°C. After the indicated amounts of time the reactions were terminated as previously described and the DNA topology visualized by gel electrophoresis and autoradiography. The lane designated, C, represents the topology input DNA substrate. R, relaxed (covalently closed or nicked) DNA; SC, superhelical DNA.

induced supercoiling centered around this reaction requirement.

We measured the endogenous ATP level contained within six S-150 cell-free extracts by using the Luciferin-Luciferase assay system (Sigma). To our surprise, the average value was low (50 μM) with only one extract containing a concentration of 100 μM or more. It is possible that highly active ATPases are present in the S-150 and maintain the concentration of ATP at low levels. To explore the influence of ATP on DNA supercoiling catalyzed by the S-150, we fixed the $MgCl_2$ concentration at 1 mM (exogenous addition) and added increasing levels of ATP. As displayed in Figure 1a, the conversion of a [^{32}P]-labeled relaxed, circular 5S DNA template to the supercoiled form by the S-150 extract is highly dependent on the amount of exogenously added ATP. At low levels of ATP, relatively little DNA supercoiling is catalyzed by the S-150. Maximal levels of DNA supercoiling are seen when the added amount of ATP ranged from 0.25 mM to 0.75 mM, and higher levels of added ATP gradually inhibited DNA supercoiling. Our results are essentially in agreement with Knezetic and Luse (29), and indicate that ATP is an important cofactor in S-150 promoted DNA supercoiling. These observations also reveal that an optimal level of ATP exists for observing DNA supercoiling. Furthermore, in concert with our goal of developing reaction conditions for observing factor-stimulated DNA supercoiling, the endogenous DNA supercoiling activity had to be removed. Previously, excess 5S-plasmid DNA was used to titrate this activity (11) and may have contributed to some of the erratic results. The present data demonstrating inhibition at higher ATP levels affords the opportunity to use an alternate and more reliable protocol for inhibiting endogenous supercoiling activity.

The next reaction parameter examined was temperature. Using the high ATP/$MgCl_2$ ratio (4 mM/1mM), we examined DNA supercoiling at varying temperatures. The S-150 promotes DNA supercoiling over a wide range of temperatures, but with two distinct optima (Figure 1b, upper panel). Both low and high reaction temperatures (30−32°C, and 37°C) are conducive for partial DNA supercoiling. When transcription factor TFIIIA is prebound to the relaxed DNA template in the presence of 4 mM ATP and 1 mM $MgCl_2$ and at a factor to DNA ratio of 1, DNA supercoiling catalyzed by the S-150 is enhanced at all temperatures (Figure 1b, lower panel). These results confirm the fact that transcription factor, TFIIIA, enhances supercoiling of plasmids containing 5S RNA genes *in vitro*. It is important to note that the best stimulation by TFIIIA occurs at temperatures that are most conducive to the partial DNA supercoiling catalyzed by the S-150 alone (31°C and 37°C). Two-dimensional gel electrophoresis (2nd dimension included chloroquine) revealed that only negative supercoils are induced by TFIIIA (data not shown).

We carried several preliminary experiments designed to examine the chromatin structure assembled on circular templates in the absence or presence of TFIIIA. Enzymatic digestions of these nucleoprotein complexes reveal similar nucleosomal or subnucleosomal patterns (data not shown). At present, we can make no statements concerning altered nucleosome structures as a result of TFIIIA-induced supercoiling. We are currently investigating this reaction parameter in much greater detail.

At Low Factor to DNA Ratios TFIIIA Accelerates DNA Supercoiling Promoted by the S-150 Cell-Free Extract

Kmiec and Worcel (11) proposed that TFIIIA accelerated the rate of DNA supercoiling catalyzed by the S-150 extract. We repeated this experiment by preincubating TFIIIA with [^{32}P]-labeled 5S DNA, at a stoichiometry of one, in the presence of 4 mM ATP and 1 mM $MgCl_2$. The reaction was initiated by the addition of the S-150 extract. As illustrated in Figure 2, TFIIIA was found to accelerate the rate of DNA supercoiling. Highly supercoiled DNA molecules appear after only 1 hour of reaction time if the DNA template is prebound by TFIIIA prior to the addition of the S-150. Furthermore, the 30 kilodalton papain-generated fragment of TFIIIA, containing the entire DNA binding segment (24), was also effective in accelerating the rate of DNA supercoiling. The addition of histone H1 or DNA Gyrase or histone H3 in place of TFIIIA did not lead to an elevated level of supercoiling beyond that which is catalyzed by the S-150 alone.

In the previous experiments, TFIIIA-stimulated reactions were carried out at a factor to DNA ratio of one. Next, we tested the stoichiometric demands of the reaction by varying the TFIIIA to DNA ratio during the prebinding phase of the reaction. TFIIIA, the 30 kD fragment or the 7S particle (containing TFIIIA bound to 5S RNA) was prebound to [^{32}P]-labeled 5S DNA in the relaxed circular form at varying factor to DNA ratios and in the presence of 4 mM ATP and 1 mM $MgCl_2$. The S-150 was added and the reaction incubated for 1 hour at 37°C. As illustrated in Figure 3a, both TFIIIA and the 30 kD fragment were found to stimulate DNA supercoiling at a factor to DNA ratio of one, confirming previous observations. In addition, TFIIIA, but not the 30 kD fragment stimulated DNA supercoiling at a 0.5 to 1

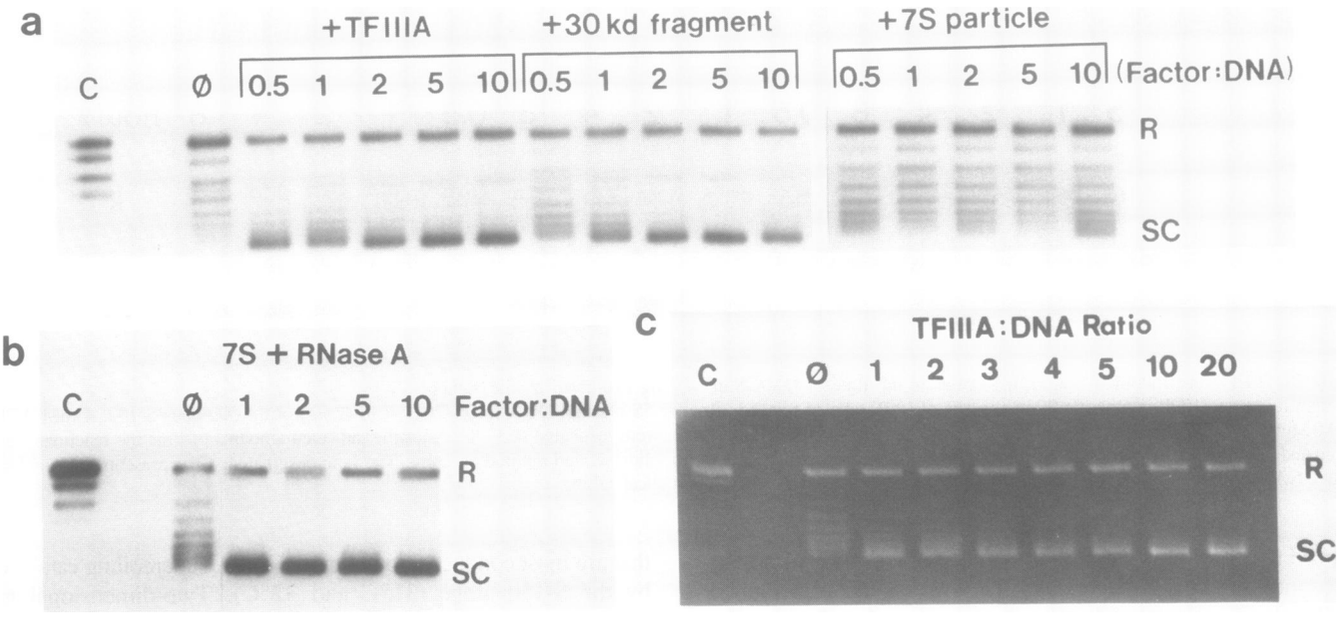

Figure 3: TFIIIA Stimulates DNA Supercoiling of [^{32}P]-Labeled and Unlabeled 5S DNA Plasmids at Low Protein-DNA Ratios
a) Reaction conditions were identical to those described in the legend of Figure 2 except that the ratio of TFIIIA to DNA was varied and the incubation time (at 37°C) was fixed at 1 hour. The appropriate amounts of TFIIIA, the 30 kD proteolytic fragment, and the 7S particle were preincubated with 20 nanograms of [^{32}P]-labeled, relaxed circular pXbsf201 at the molecular ratios of protein to DNA indicated above. DNA topology was visualized after electrophoresis through 1% agarose followed by autoradiography. R, relaxed circular DNA; SC, supercoiled DNA. b) Standard reaction conditions were previously described (Figure 2) except that in this mixture 7S particles, previously treated with RNase A for 30′ at 37°C, were added in place of purified TFIIIA at the indicated stoichiometry. The reaction mixtures were incubated for 1 hour at 37°C and processed as above (a). The lane marked Ø contained no added 7S particle. c) Reaction mixtures (10 μl) containing 100 nanograms of unlabeled relaxed circular 5S plasmid DNA (pXbsf201), 6 μl (12 μg) of the S-150 extract and TFIIIA at the indicated protein to DNA ratios were incubated at 37°C. After 1 hour the DNA topology was analyzed by electrophoresis through 1% agarose followed by staining with ethidium bromide. The lane designated, C, represents the topology input DNA substrate. R, relaxed (covalently closed or nicked) DNA; SC, superhelical DNA.

factor to DNA ratio. The original data of Kmiec and Worcel (11) indicated that a factor to DNA ratio of at least 1 was required for induced supercoiling. This difference may be attributed to the quality of the DNA; some of the labeled DNA molecules may be refractory to the supercoiling mechanism induced by TFIIIA. Alternatively, it is possible that the percentage of *fully* active TFIIIA molecules may vary from preparation to preparation. In addition, the present reaction conditions for observing factor induced supercoiling reliably now include a high level of ATP. Optimization of the reaction was not carried out previously and, as stated above, may have contributed to the erratic results. At lower factor to DNA ratios (0.25 and 0.35) no stimulated supercoiling is observed (data not shown). We conclude from this experiment that TFIIIA stimulates DNA supercoiling at low factor to DNA ratios.

The 7S particle is composed of TFIIIA plus 5S RNA. Such a nucleoprotein complex can stimulate a low level of DNA supercoiling. By treating the 7S particle with RNase A, TFIIIA is released while 5S RNA is degraded. When 7S particles are pre-treated with RNase A and the liberated protein added to a reaction mixture, a much higher level induced DNA supercoiling was observed (Figure 3b). Furthermore, the addition of 5S RNA to a reaction mixture blocks TFIIIA-induced DNA supercoiling, presumably by sequestering the factor (Sekiguchi, unpublished observations). The results of these experiments reveal two important facts: first, TFIIIA is, in all likelihood, responsible for the induced supercoiling and second, the factor must be at least partially free from RNA to be effective as a stimulant.

It could be argued that [^{32}P]-labeled circular DNA templates present an unnatural and constantly changing substrate to the enzymatic supercoiling apparatus. In fact, chloroquine agarose gel electrophoresis of the [^{32}P]-labeled DNA substrate reveals that at least 20−30% of the input circular DNA is most likely nicked. To test this possibility, we used unlabeled, covalently closed relaxed 5S plasmid DNA as the template. We elevated the amount of 5S DNA in the reaction 5 fold, while gradually raising the factor to DNA ratio during the prebinding phase of the reaction. In addition, the S-150 extract level was increased 6 fold and the reaction carried out at 37°C for 1 hour. As displayed in Figure 3c, the relaxed template (C) is only partially or weakly supercoiled by the extract (Ø). The addition of TFIIIA at molecular ratios of one or more stimulates DNA supercoiling by the S-150. Upon careful examination it is apparent that few if any additional supercoils emanate from the relaxed DNA population. Rather, the partially supercoiled molecules are more completely supercoiled as a result of the presence of TFIIIA in the reaction mixture. It is possible however, that some of the unlabeled DNA, remaining at the relaxed DNA position, is nicked since a Gaussian-Boltzman distribution is not seen in the lanes where TFIIIA has been added.

A possible explanation for the mechanism of factor-induced supercoiling centers around the hypothesis that the inhibitory effect of ATP was reversed by TFIIIA's hydrolytic activity (Hazuda *et al.*, 1983). Alternatively, stimulated supercoiling could result from the chelation of $MgCl_2$ by ATP or other possible chelators. To examine this issue, we replaced the high ATP concentration in the reaction mixture with 2 mM EDTA. As illustrated in Figure 4A, TFIIIA dependent DNA supercoiling occurs efficiently in the presence of EDTA. Because the endogenous level of ATP in the S-150 is quite low (50 μM), this

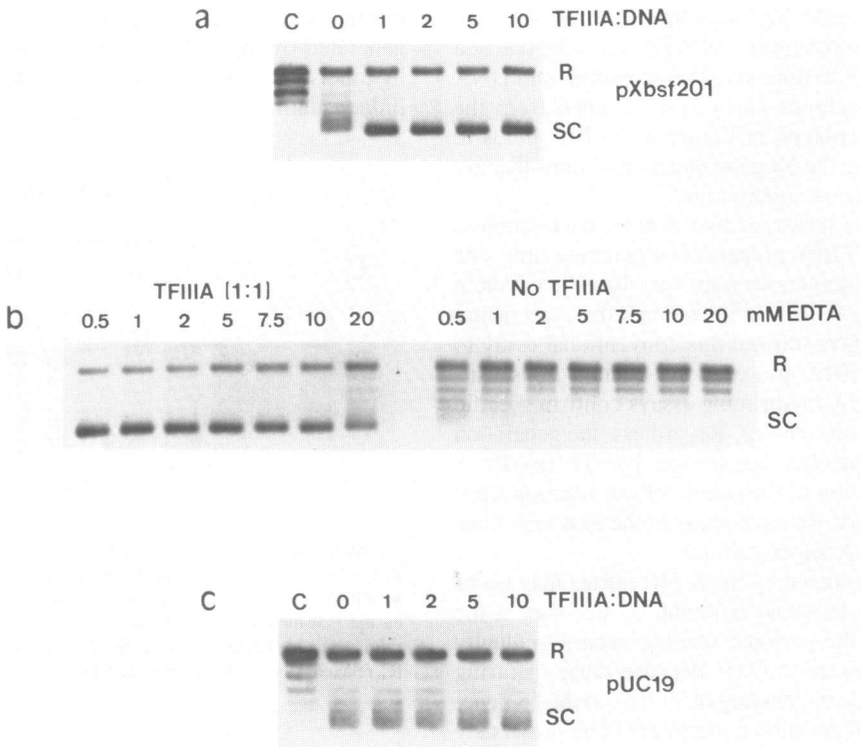

Figure 4: TFIIIA-Mediated DNA Supercoiling Occurs in the Presence of 2 mM EDTA.
(a) Reaction mixtures (10 μl) containing 20 nanograms of [^{32}P]-labeled, relaxed circular 5S plasmid DNA (pXbsf201), 1 (2 μg) of the Xenopus S-150 extract, 2 mM EDTA and the indicated amount of purified TFIIIA were incubated at 37°C. After 4 hours of incubation, the reactions were terminated by the addition of Stop Mix +and the DNA topology visualized by gel electrophoresis and autoradiography. (b) Reaction mixtures were identical to those in (a) except that the TFIIIA to DNA ratio was fixed at one and the EDTA concentration was raised gradually. (c) Reaction mixtures were identical to those described in (a) except that the [^{32}P]-labeled DNA template was pUC-18 (lacking a 5S gene). R, relaxed circular DNA; SC, supercoiled DNA; C, input labeled DNA, no addition of S-150 or TFIIIA. R, relaxed (covalently closed or nicked) DNA; SC, superhelical DNA.

data is not consistent with the former hypothesis and aligns more closely with the notion that the ATP may serve to decrease the Mg^{++} availability in the reaction mixture. At present however, no specific or mechanistic hypothesis can be put forth with unqualified confidence. In addition, we have been unable to observe ATPase activity in any of our TFIIIA preparations, although low levels of hydrolysis may not have been detected in our assay systems. TFIIIA-induced supercoiling is observed even in the presence of high levels of EDTA (up to 20 mM) (Figure 4B).

Using these conditions, we measured the influence of TFIIIA on supercoiling of plasmid molecules lacking the 5S RNA gene. As seen in Figure 4C, TFIIIA does stimulate supercoiling of plasmids that do not contain the 5S RNA gene. However, only a fraction of the molecules are supercoiled and increased levels of TFIIIA do not induce a greater amount of DNA supercoiling. This experiment was performed using [^{32}P]-labeled DNA that was greater than 90% covalently-closed (judged by chloroquine-agarose gel electrophoresis). These results are similar to the level of supercoiling induced by the 7S particle (see Figure 3B). The use of high levels of ATP in this reaction also produce the same result.

TFIIIA Binds to 5S DNA Under Reaction Conditions that Promote Induced Supercoiling

It has been well-established that TFIIIA binds to the internal control region (ICR) of the 5S RNA genes (1). To initiate the formation of a stable transcription complex, only one molecule of TFIIIA per ICR is required (5). TFIIIA binding of 5S DNA

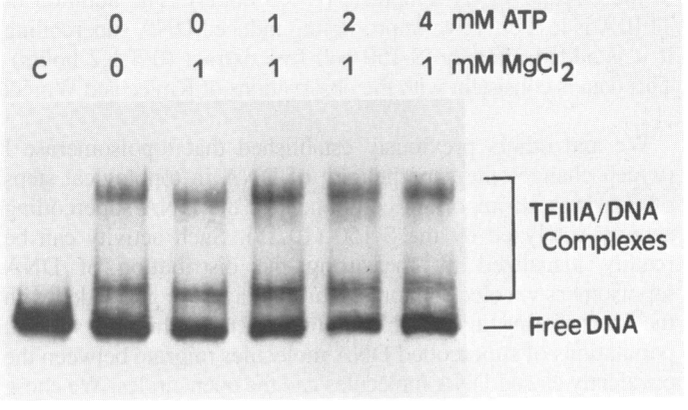

Figure 5: TFIIIA Binds 5S DNA Under Conditions That Promote Induced DNA Supercoiling
Reaction mixture (10 μl) containing 100 nanograms (5 nanograms of labeled DNA) of a 240 base pair [^{32}P]-labeled DNA fragment containing one copy of the somatic 5S RNA gene, TFIIIA (at a factor to DNA ratio of one), 1 mM $MgCl_2$, poly dI·dC and the indicated concentrations of ATP were incubated at 37°C. After 30 minutes, the samples were loaded directly onto a 6% polyacrylamide gel and electrophoresed as described in the Materials and Methods. Band shifts were visualized after autoradiography.

(somatic-type) gene under conditions that are conducive for factor induced DNA supercoiling was monitored by gel retention. A 240 base pair fragment containing one copy of the 5S RNA gene (somatic-type) was isolated as described by Razvi *et al.* (22). TFIIIA was bound to the [^{32}P]-labeled DNA fragment under

conditions that included 1 mM $MgCl_2$ in the presence of excess poly dI·dC and various concentrations of ATP. Then the reaction mixture was incubated for 30 minutes. The protein-bound DNA fragments migrated to positions easily distinguished from the unbound DNA and as displayed in Figure 5, TFIIIA binds to DNA fragments containing the 5S gene under conditions that are conducive for factor-induced supercoiling.

In some cases, we have observed two distinct nucleoprotein complexes while other TFIIIA preparations generate only one complex. TFIIIA-DNA interactions may vary based on protein stability or dilution making it difficult to ascertain the exact nature of the binding (30). We have utilized this conventional assay to demonstrate that our TFIIIA preparations contain consensus activity. In addition, TFIIIA footprinting assays confirm specific binding under our reaction conditions. Regardless, the generation of one or two nucleoprotein complexes by TFIIIA-DNA interactions is not a reflection of the quality of our reaction since all TFIIIA preparations that we have made in the past year have successfully induced DNA supercoiling.

Chromatin assembly promoted by the S-150 extract may occur in cis; the circular DNA template is bound by histones at the start of the reaction and the periodic spacing occurs gradually without nucleosome dissociation (31). Because these maturing nucleosomes could preclude the binding of TFIIIA to the 5S gene, we carried out an order of addition experiment. The previously described experiments have included a prebinding phase in which purified TFIIIA is allowed to interact with the [^{32}P]-labeled circular template prior to the addition of the S-150. In this case, the experimental design was changed so that the addition of TFIIIA was ordered in relation to the start of the reaction (addition of the S-150 extract). As seen in Figure 6, TFIIIA stimulates DNA supercoiling at each measured time point. However, the highest level of induction is observed when TFIIIA is prebound to the circular DNA templates (−0.5 hours). The addition of TFIIIA is less effective in promoting induced DNA supercoiling if it is added after the S-150 cell-free extract (0.5,1,2 hours). This data is consistent with the observations of Kmiec and Worcel (11).

We and others previously established that topoisomerase I (which changes the superhelicity of DNA in topological steps of one) was an important component in the DNA supercoiling activity catalyzed by the S-150 (10,25). Such activity can be readily visualized by measuring the distribution of DNA topoisomers via electrophoresis through agarose gels laden with the intercalating agent, chloroquine (32). In this gel system, populations of supercoiled DNA molecules migrate between the covalently closed DNA molecules and the open circles. We chose conditions in which only low levels of TFIIIA-induced supercoiling are observed (the reaction temperature was 31°C; see Figure 1b). This experimental design enables us to readily follow small changes in the distribution of supercoiled molecules. Figure 7 (lane 2) displays the population of supercoiled molecules created by incubation of [^{32}P]-labeled relaxed DNA with the S-150 at 31°C for 4 hours. TFIIIA advances these populations sequentially by steps of one (Figure 7, lane 3), again demonstrating its stimulatory effect. We traced the supercoiled DNA population of the autoradiogram (lanes 2 and 3) with a laser densitometer and superimposed the graphs. The stimulation by TFIIIA and the conversion by topological steps of one are easily distinguished. From these experiments, we conclude that the stimulatory effect of TFIIIA on the supercoiling activity of the S-150 cell-free extract may occur via a topoisomerase I catalyzed mechanism. However, it is possible that the supercoils could be generated by the action of topoisomerase II and re-equilibrated by topoisomerase I. Thus, we can only firmly conclude that the linking number is not changing solely in steps of two.

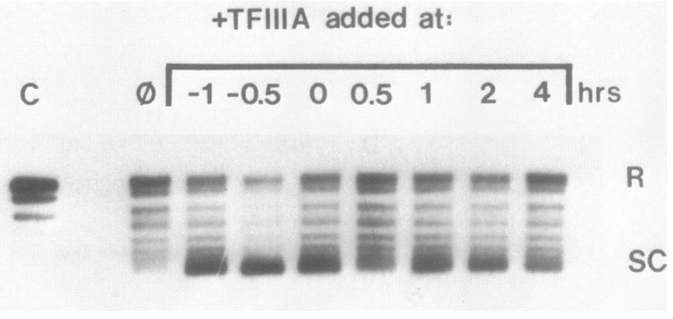

Figure 6: The Level of Induced DNA Supercoiling Can be Reduced Significantly if TFIIIA is Added After the S-150 Extract
Reaction mixtures were identical to those described in the legend to Figure 2 except that the total reaction time was 4 hours and TFIIIA- (1:1, factor-DNA ratio) was added at the times indicated. C, no S-150 added; Ø, No TFIIIA added; R, relaxed DNA; SC, supercoiled DNA.

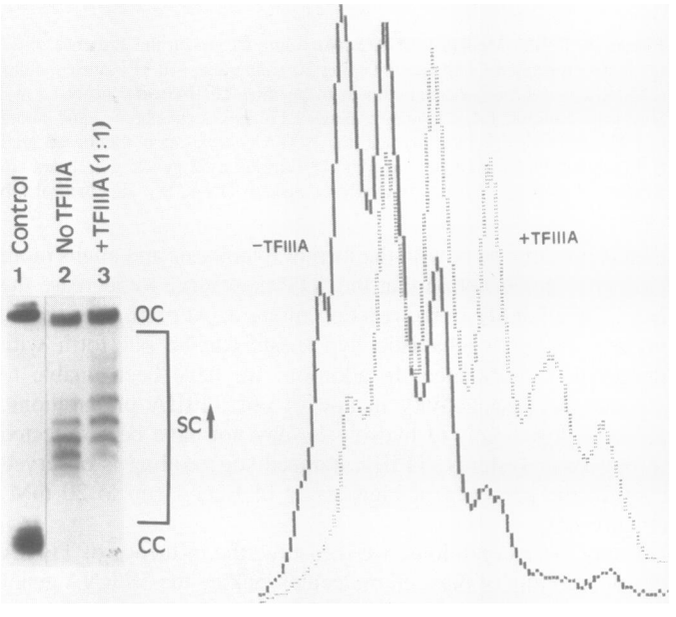

Figure 7: TFIIIA Stimulates DNA Supercoiling by a Mechanism Possibly Involving Topoisomerase I.
(left panel) [^{32}P]-labeled, relaxed circular plasmid DNA (pXbsf201, 20 nanograms) was incubated with 0.44 nanograms of TFIIIA or 1 μl of G Buffer in the presence of 4 mM ATP and 1 mM $MgCl_2$ for 30 minutes at 26°C. To each of these reaction mixtures, 1 μl (2 μg) of the Xenopus S-150 extract was added and the reaction continued at 31°C for 4 hours. The reaction mixture was terminated and deproteinized as previously described and the purified DNA was electrophoresed through 1% agarose gel containing 15 μg/ml chloroquine-diphosphate (31) followed by autoradiography. OC, open circular DNA; CC, covalently closed circular DNA; SC, supercoiled DNA. Lane 1, DNA alone; lane 2, DNA plus the S-150 extract alone; lane 3, DNA prebound by TFIIIA plus the S-150 extract. (right panel) Lanes 2 and 3 representing the population of supercoiled molecules on the autoradiogram were traced using a laser densitometer and the resulting graphs superimposed. The lane designated, C, represents the topology input DNA substrate. R, relaxed (covalently closed or nicked) DNA; SC, superhelical DNA.

Mechanistic Studies of TFIIIA-Induced Supercoiling

Throughout the course of this work, we have noticed the important role that ATP plays in regulating TFIIIA-induced DNA supercoiling. To more closely examine the influence of ATP in the induced supercoiling reaction, we designed an experiment in which the addition of 4 mM ATP was ordered relative to the start of the reaction. In some of the reaction mixtures, [^{32}P]-labeled plasmid DNA was prebound with TFIIIA. Next, ATP (4 mM) and $MgCl_2$ (1 mM) were added relative to the addition of the S-150. Incubation at 37°C continued for a total of 4 hours, after which the DNA topology was analyzed by agarose gel electrophoresis and autoradiography. As can be seen in the first six lanes of Figure 8 (upper panel), the inhibitory effect of ATP occurs when it is added prior to the S-150 extract. Addition of ATP after 15 minutes of reaction time does not inhibit DNA supercoiling. In sharp contrast, if TFIIIA is present with the ATP prior to the start of the reaction, no inhibition of supercoiling is observed (Figure 8, upper panel). TFIIIA somehow removes the blockage of supercoiling created by the presence of ATP. These results also demonstrate that an early phase of the supercoiling reaction promoted by the S-150 is, in all likelihood, the most sensitive to the inhibitory effect of ATP. The designation (Ø) represents a reaction mixture to which hexokinase was not added.

The results of the experiment displayed in Figure 2 were among the first to suggest that TFIIIA accelerated DNA supercoiling. In addition, we found that an incubation of at least 1 hour was required to observe TFIIIA-induced supercoiling. Based on our acquired knowledge of the role of ATP in this reaction, we wondered whether TFIIIA-dependent supercoiling was reliant upon the presence of ATP during the early phase of the reaction. To test this concept directly, we bound TFIIIA to the 5S DNA ([^{32}P]-labeled circular plasmid) in the presence of 4 mM ATP and 1 mM $MgCl_2$ for 30 minutes at 26°C. Then the S-150 was added followed by 10 units of Hexokinase and 10 mM glucose. Hexokinase converted the ATP to ADP under these conditions within 30 seconds as judged by thin layer chromatography (data not shown). Each reaction was incubated for a total of 4 hours. As shown in Figure 8 (lower panel), the removal of ATP at each point prior to one hour of incubation time had a negative influence on DNA supercoiling after 4 hours. The highest level of TFIIIA-induced supercoiling is observed when ATP is present for one hour of the reaction time. These results are consistent with those presented as Figure 2; the stimulation of DNA supercoiling by TFIIIA requires a 1 hour reaction time. In addition, the presence of ATP during this time is essential for the induction of supercoiling, perhaps suggesting that a rate-limiting, ATP-dependent step is an integral part of TFIIIA-stimulated DNA supercoiling.

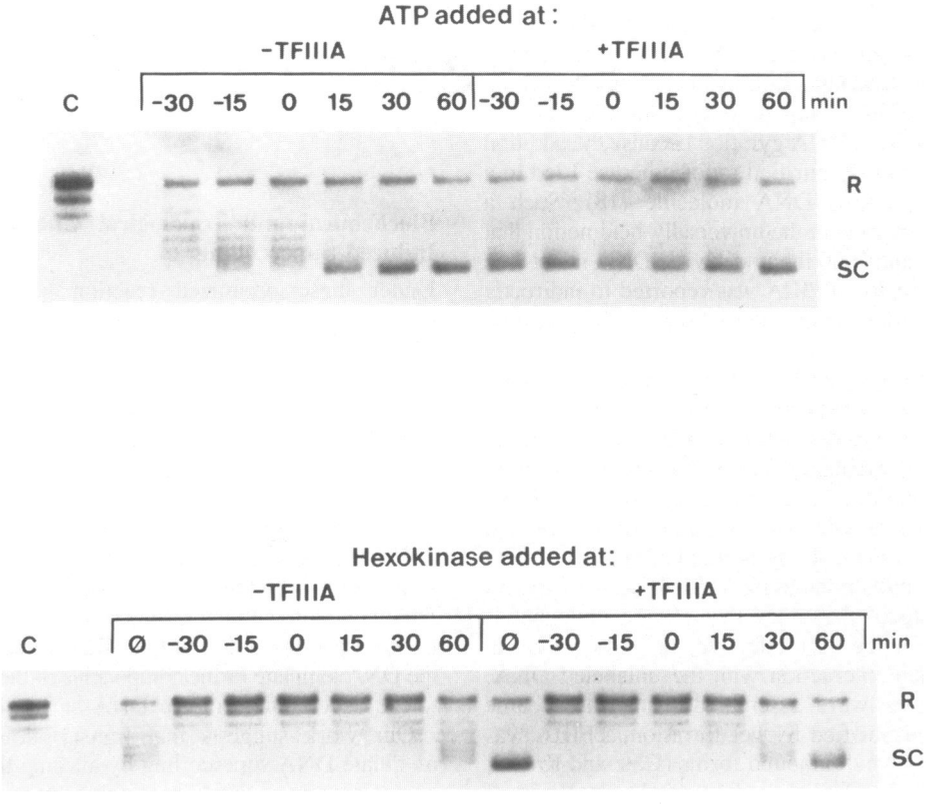

Figure 8: ATP Modulates TFIIIA Induced Supercoiling
(upper panel) Reaction mixtures (10 μl) containing 20 nanograms of [^{32}P]-labeled relaxed, circular 5S plasmid DNA (pXbsf201), 1 μl (2 μg) of the S-150 extract, 1 mM $MgCl_2$ without TFIIIA or with TFIIIA at a protein to DNA ratio of one were incubated at 37°C. At the indicated times relative to the addition of the S-150 (defined as O), 4 mM ATP was added and the reaction continued for a total of 4 hours. The reactions were terminated, processed and the DNA topology analyzed as previously described. (lower panel) Reaction mixtures were identical to those described above except that 4 mM ATP was included. TFIIIA was added to some of the reaction mixtures. Ten units of Hexokinase and 10 mM glucose were added to the reaction mixture at the indicated times and the S-150 added to all reaction mixtures at zero time. The assay was incubated at 37°C for a total of 4 hours after which the deproteinized samples were analyzed by electrophoresis through 1% agarose and autoradiography. The lane designated, C, represents the topology input DNA substrate. R, relaxed (covalently closed or nicked) DNA; SC, superhelical DNA.

DISCUSSION

The Xenopus S-150 cell-free extract catalyzes DNA supercoiling, through the assembly of chromatin on circular DNA templates in an ATP-Mg^{++} dependent fashion (10). It was previously reported that transcription factor TFIIIA stimulated DNA supercoiling promoted by the S-150 extract (11,18). However, the induced supercoiling reaction was found to be nonreproducible in several laboratories (13,14,16). The simple goal of the experiments outlined in this report was to examine biochemically TFIIIA induced supercoiling and optimize the reaction conditions so that this phenomena could be reliably observed. We define several critical and previously overlooked reaction parameters that are essential for the reproduction of TFIIIA-induced DNA supercoiling. It is our belief that factor-induced DNA supercoiling may be an important and perhaps general mechanism of gene control in eukaryotes.

Previously Defined Characteristics

In the seminal studies of TFIIIA induced supercoiling, several features of the reaction were reported. First, TFIIIA prebound to a Xenopus 5S DNA template accelerated supercoiling promoted by the S-150 extract (11). Second, a TFIIIA to DNA ratio of approximately one was required in order to observe factor-induced supercoiling, although in many cases this ratio was significantly below this value (11). Third, TFIIIA had to be present prior to the addition of the S-150 extract, presumably due to a time dependent steric hindrance by nucleosome formation (11). Fourth, the type of DNA supercoiling induced by TFIIIA was previously referred to as DNA gyration because the addition of novobiocin or exogenously purified topoisomerase I led to a relaxation of the supercoiled DNA molecule (18). Such a phenomena was inconsistent with the universally-held notion that chromatin structure is static and the underlying DNA is not free to rotate or unwind. Finally, TFIIIA was reported to indirectly stimulate 5S gene expression via the induced supercoiling reaction (11).

We successfully repeated each of these TFIIIA-promoted reactions, but continued to experience serious inconsistencies using the previously published reaction conditions. In addition to the possible sources of variability described in the Introduction, two experimental design flaws have now been recognized. First, the Xenopus extract has an endogenous supply of TFIIIA and this level was depleted previously by prebinding the S-150 with unlabeled circular plasmids containing a single copy of the 5S RNA gene. This protocol presumes that all the endogenous TFIIIA molecules are rendered ineffective for participation in future reactions by their interaction with the unlabeled DNA. When minichromosomes were assembled using these same reaction conditions and purified by gel filtration, TFIIIA was found in the DNA-bound and unbound forms (Cole and Kmiec, unpublished observations). To rely solely on the depletion of endogenous levels of TFIIIA by DNA prebinding in order to measure stoichiometry is a serious experimental error. Second, some of the endogenous TFIIIA is most likely complexed with 5S RNA to form the 7S particle (33) which as shown in Figure 3b is not capable of inducing DNA supercoiling. Since the S-150 extracts may vary in their endogenous content of 7S particles and free TFIIIA, it is difficult to analyze induced DNA supercoiling using previous protocols.

Reaction Conditions for Observing TFIIIA-Induced Supercoiling

As mentioned above, we sought a new reaction protocol that would lead to reproducibility with regard to factor induced supercoiling. We have now prepared ten S-150 extracts and purified TFIIIA six times. In each case, induced DNA supercoiling promoted by purified TFIIIA and its proteolytic fragments has been observed. Two key parameters had to be defined: the level of exogenously added ATP and the reaction temperature.

Based on previous studies from our laboratory (28) and others (29,34), the importance of ATP levels in controlling the level of endogenous DNA supercoiling has been established. Hence, as we examined assembly catalyzed by the S-150 extract (Figure 1), it became clear that DNA supercoiling resulting from nucleosome formation is sensitive to the level of exogenously added ATP. As the ATP to Mg^{++} ratio increases, the level of DNA supercoiling decreases. Since the DNA supercoiling activity of every S-150 extract tested was inhibited at high levels of ATP, we chose to block the endogenous supercoiling by ATP rather than by DNA prebinding. It is important to note that the maximal level of DNA supercoiled by each S-150 without the addition of TFIIIA should be determined first even under these new reaction conditions (Figure 2c). Our results indicate that exogenously added TFIIIA induces DNA supercoiling throughout a wide range of temperatures. Under these conditions, it appears that the population of weakly supercoiled molecules, generated by the action of the S-150 alone, are supercoiled further when TFIIIA is included in the reaction mixture. Hence, it is possible that the presence of TFIIIA simply improves the supercoiling efficiency of the endogenous machinery.

Biochemical and Topological Characteristics of TFIIIA-Induced Supercoiling

Under these optimized reaction conditions, many of the previously published reaction characteristics have held true. First, TFIIIA accelerates DNA supercoiling promoted by the S-150 extract. The mechanism by which TFIIIA induces supercoiling is operational between 30 minutes and 60 minutes of reaction time (Figures 2 and 7b). Second, a TFIIIA (or 30 kD fragment) to DNA ratio of approximately one is required for the stimulatory effect. However, a ratio of 0.5 to 1 (TFIIIA to DNA) is effective in promoting some induced DNA supercoiling (see Figure 3a). Third, the highest level of supercoiling is achieved when TFIIIA is incubated with the 5S DNA prior to the addition of the S-150. In conjunction with the results of the DNA binding experiments, these observations suggest that TFIIIA interacts productively with the DNA template or the components of the reaction mixture prior to the development of the DNA supercoiling event.

Our work suggests that transcription factor TFIIIA may modulate DNA supercoiling by altering the normal behavior of a topoisomerase, leading to the appearance of extra negative supercoils. Since the binding of TFIIIA to 5S DNA is influenced by varying ATP levels, it is possible that DNA binding alone is not sufficient to induce the supercoiling. Although we do not know the identity of all of the components of the supercoiling reaction, we have successfully isolated a group of proteins that respond to the addition of TFIIIA and catalyze factor induced DNA supercoiling (Sekiguchi *et al.*, submitted). Among the most likely protein components are topoisomerase I and histones H3

and H4; no evidence for the participation of TFIIIB or TFIIIC in the supercoiling reaction has been gathered. Furthermore, the addition of α-amanitin to the reaction mixture does not inhibit TFIIIA-induced supercoiling, thus the phenomena cannot be explained by an increase in transcriptional activity (35). At present we cannot define precisely the mechanism by which TFIIIA induces DNA supercoiling promoted by the S-150, but now the reaction is at least reproducible.

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