Transcription-Driven Supercoiling of DNA: Direct Biochemical Evidence from In Vitro Studies

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Summary

The translocation of an RNA polymerase elongation complex along double helical DNA has been proposed to generate positive supercoiling waves ahead of and negative supercoiling waves behind the transcription ensemble. This twin supercoiled domain model has been tested in vitro. In the presence of prokaryotic DNA topoisomerase I, which selectively removes negative supercoils, transcription from a single promoter results in rapid and extensive positive supercoiling of the DNA template. The accumulation of positive supercoils in the DNA template requires continued movement of the elongation complex as well as sizable nascent RNA chains. These in vitro results provide direct biochemical evidence supporting the twin supercoiled domain model of transcription. Furthermore, the magnitute of DNA supercoiling (torsional) waves generated by transcription is much greater than previously expected, suggesting that transcription is one of the principal factors affecting intracellular DNA supercoiling.

Introduction

The superhelical state of intracellular DNA is an important structural determinant of DNA functions. In bacteria, two major DNA topoisomerases, DNA topoisomerase I (product of topA) and topoisomerase II (product of gyrA and gyrB), are known to regulate the state of supercoiling of intracellular DNA (reviewed in Gellert, 1981; Drlica, 1984; Wang, 1985). A proper balance of these two topoisomerases, which exhibit opposing enzymatic activities, appears important as suppression of the lethal phenotype of Escherichia coli topA mutants is often accomplished by compensatory mutations in gyrA or gyrB (DiNardo et al., 1982; Pruss et al., 1982; Raji et al., 1985). In E. coli, homeostatic regulation of topoisomerase genes at the transcription level also points to the importance of a proper balance of the cellular levels of the topoisomerases; an increase in the degree of negative supercoiling of intracellular DNA has been shown to reduce the rate of transcription of gyrase genes and to stimulate the expression of the topA gene, whereas a decrease in the superhelical tension in the DNA has the opposite effect (Menzel and Gellert, 1983; Tse-Dinh and Beran, 1988; Tse-Dinh, 1985). These results have led to the suggestion that intracellular DNA supercoiling is maintained primarily by a dynamic balance between the actions of these two opposing topoisomerases.

However, a number of observations remain unexplained by the simple concept described above: highly positively supercoiled plasmid DNA has been isolated from E. coli cells when gyrase is inactivated (Lockshon and Morris, 1983); hyper-negatively supercoiled plasmid DNA has been isolated from topA mutants, and its formation is dependent on the transcription of the tetA gene (Pruss, 1985; Pruss and Drlica, 1986); mutations in the rpoB gene, which encodes the β subunit of RNA polymerase, can suppress gyrase mutations and restore proper supercoiling (Filutowicz and Jonczk, 1983; Franco et al., 1987); mutations in the rho gene, which encodes Rho protein responsible for Rho-dependent termination of RNA transcription, affect plasmid DNA supercoiling (Fassler et al., 1986). These observations, together with results from studies in eukaryotic cells suggesting that RNA transcription may also require a swivel (Fleischmann et al., 1984; Gilmour et al., 1986; Rowe et al., 1987; Gilmour and Elgin, 1987; Stewart and Schutz, 1987; Brill et al., 1987; Zhang et al., 1988), have led to the proposal that RNA transcription is accompanied by template supercoiling (Liu and Wang, 1987). As proposed in the twin-supercoiled-domain model, translocation of the RNA polymerase complex along right-handed double helical DNA generates positive supercoiling "waves" ahead of and negative supercoiling "waves" behind the moving RNA polymerase. The local superhelical state of DNA is thus determined by a number of parameters in addition to the relative abundance of the two major topoisomerases. These parameters may include the strength of a promoter, the size of the nascent RNA, the orientation and distribution of multiple transcription units, the distribution of topoisomerase sites, and the presence of other macromolecular complexes on the same DNA (Liu and Wang, 1987; Wu et al., 1988).

The validity of the twin-supercoiled-domain model of RNA transcription has gained support from recent in vivo experiments in E. coli (Wu et al., 1988). It was demonstrated that the formation of highly positively supercoiled pBR322 DNA in E. coli cells treated with gyrase inhibitors was dependent on RNA transcription. The results were interpreted according to the twin-supercoiled-domain model of RNA transcription; the selective removal of transcription-generated negative supercoils by E. coli DNA topoisomerase I results in the accumulation of positive supercoils in the template DNA. Recent studies in yeast DNA topoisomerase mutants expressing E. coli DNA topoisomerase I provided similar results in a eukaryote (G. N. Giaever and J. C. Wang, personal communication). Our present in vitro studies, which mirror the in vivo studies, have provided direct biochemical evidence supporting the twin-supercoiled-domain model of transcription. Furthermore, our results also indicate that DNA supercoiling (torsional) waves generated by transcription are significantly greater than previously expected. RNA transcription is likely to be one of the principal factors affecting intracellular DNA supercoiling.
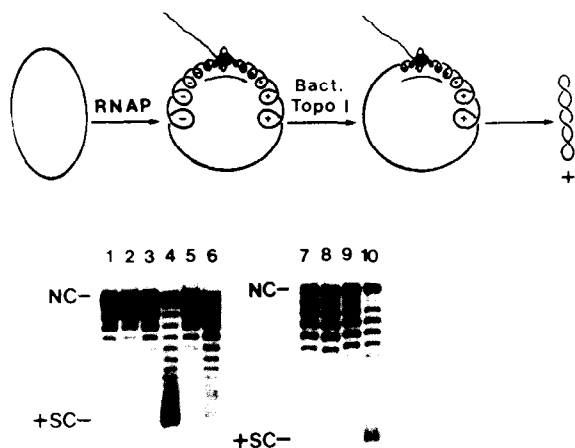
Figure 1. Testing of the Twin-Supercoiled-Domain Model of Transcription in an In Vitro Transcription System

(Top) A schematic illustrating the experimental design for testing the twin-supercoiled-domain model of transcription. Transcription on a relaxed circular DNA template is expected to generate positive supercoiling waves ahead of and negative supercoiling waves behind the RNA polymerase elongation complex. In the presence of prokaryotic DNA topoisomerase I (labeled Bact. Topo I in the figure), which selectively removes negative supercoils, the template DNA is expected to become positively supercoiled.

(Bottom) In vitro transcription in the presence of prokaryotic DNA topoisomerase I. Transcription using pC15 DNA as the template was done as described in Experimental Procedures. Lane 1: control, no RNA polymerase or topoisomerase. Lane 2: 80 ng of M. luteus topoisomerase I. Lane 3: 6 U of T3 RNA polymerase. Lane 4: 80 ng of M. luteus DNA topoisomerase I and 6 U of T3 RNA polymerase. Lane 5: 6 U of T7 RNA polymerase. Lane 6: 80 ng of M. luteus DNA topoisomerase I and 6 U of T7 RNA polymerase. Lanes 7–10: each contained 6 U of T3 RNA polymerase and 80 ng of M. luteus DNA topoisomerase I. Lane 7: UTP, ATP, and CTP were omitted. Lane 8: UTP and CTP were omitted. Lane 9: UTP was omitted. Lane 10: contained all four NTPs. Incubation was done at 37°C for 30 min. The electrophoretic positions of nicked pC15 DNA and the highly supercoiled reaction product are indicated by NC and +SC, respectively.

Results

Supercoiling of the Template DNA during Transcription in the Presence of Prokaryotic DNA Topoisomerase I

According to the twin-supercoiled-domain model of transcription, translocation of the RNA polymerase elongation complex on a right-handed DNA results in the simultaneous and continuous induction of positive and negative supercoiling "waves" (Figure 1, top panel). On a circular DNA template, the positive supercoiling waves, which are generated ahead of the RNA polymerase, and the negative supercoiling waves, which are generated behind the RNA polymerase, can propagate and annihilate each other through rotational diffusion of the intervening DNA segment (Liu and Wang, 1987; Wu et al., 1988). During steady state transcription, gradients of supercoils on the template DNA are expected, and DNA segments proximal to the RNA polymerase are expected to be under the highest superhelical tension. If the negative superhelical tension on DNA behind the RNA polymerase is sufficiently high, one can detect its existence by probing with

a prokaryotic DNA topoisomerase I (labeled Bact. Topo I in Figure 1, top panel). Selective removal of negative supercoils by prokaryotic DNA topoisomerase I is expected to result in the accumulation of positive supercoils. To test the effect of transcription on the supercoiling of the template, relaxed pC15 DNA, which contains both T3 and T7 RNA polymerase promoters, was transcribed using either T3 or T7 RNA polymerase in the presence of Micrococcal luteus DNA topoisomerase I. Indeed, RNA transcription in the presence of M. luteus DNA topoisomerase I converted a subpopulation of the relaxed pC15 DNA to products with faster electrophoretic mobilities (Figure 1, lanes 4 and 6). The formation of this possibly supercoiled DNA product requires M. luteus DNA topoisomerase I (which can be substituted by E. coli DNA topoisomerase I; data not shown), RNA polymerase, and all four ribonucleoside triphosphates. Omission of a single ribonucleoside triphosphate from the otherwise complete reaction mixture was sufficient to abolish the supercoiling reaction (Figure 1, lanes 7–10), suggesting that the process of RNA elongation is essential for the observed DNA supercoiling.

The requirement for transcription in this supercoiling reaction is further supported by experiments demonstrating promoter specificity. Plasmid DNAs lacking T3 and T7 RNA polymerase promoter sequences did not produce supercoiled DNA (Figure 2, lanes 8 and 9). Other RNA polymerases, such as E. coli RNA polymerase (Figure 2, lanes 2, 7, 12, and 17) and SP6 RNA polymerase (Figure 2, lanes 5, 10, 15, and 19), could substitute for the T3 or T7 RNA polymerase provided that the template DNA contained the appropriate promoter sequence for the respective RNA polymerase. Rifampicin (1 μM) also inhibited the supercoiling reaction when E. coli RNA polymerase was used (data not shown).

The Reaction Product Is Highly Positively Supercoiled DNA

To determine the supercoiled state of the DNA template being transcribed in the presence of bacterial DNA topoisomerase I, the product DNA from the complete reaction was purified and analyzed by two-dimensional gel electrophoresis. The pC15 DNA reaction product migrated in the two-dimensional gel as a streak of topoisomer spots (Figure 3, streak a to streak c). Topoisomers migrating at position c (Figure 3) have been previously shown to represent highly positively supercoiled DNA (Wu et al., 1988). Negatively supercoiled pC15 DNA was mixed with the purified reaction product and served as an internal DNA control and a mobility marker (Figure 3, streak a). The identification of the reaction product as highly positively supercoiled pC15 DNA was further confirmed by treatment with topoisomerases. The pC15 DNA reaction product was completely relaxed by treatment with HeLa DNA topoisomerase I (Figure 3C), but not with M. luteus DNA topoisomerase I (Figure 3B), while the negatively supercoiled pC15 DNA (the internal DNA control) was converted to relaxed topoisomers with either eukaryotic or prokaryotic DNA topoisomerase I treatment (Figures 3B and 3C). Since prokaryotic DNA topoisomerase I is known to selectively relax negatively supercoiled DNA, these
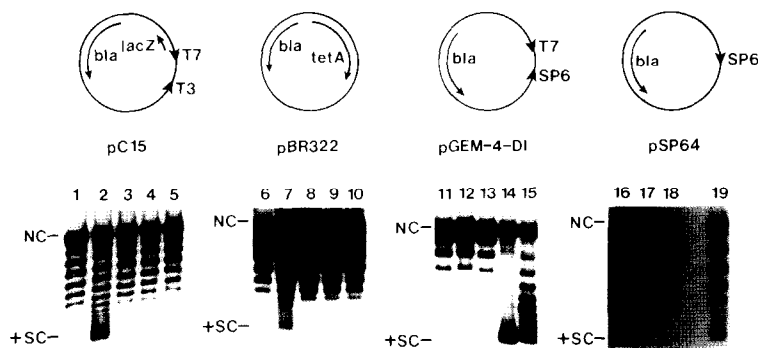
pC15   pBR322   pGEM-4-DI   pSP64

Figure 2. Supercoiling of the Template DNA by RNA Transcription Is Promoter-Specific

Transcription reactions in the presence of M. luteus DNA topoisomerase I (80 ng in each reaction) were performed as described in Experimental Procedures. A number of different RNA polymerases (1.5 U in each reaction) and plasmid DNAs containing different promoters were used in this experiment. Lanes 1, 6, 11, and 16: control DNA, no RNA polymerase. Lanes 2, 7, 12, and 17: E. coli RNA polymerase. Lanes 3, 8, 13, and 18: T3 RNA polymerase. Lanes 4, 9, and 14: T7 RNA polymerase. Lanes 5, 10, 15, and 19: SP6 RNA polymerase. Plasmid DNAs used in this series of experiments are: pC15 DNA (lanes 1–5); pBR322 (lanes 6–10); pGEM4-D1 (lanes 11–15); and pSP64 (lanes 16–19).

results strongly suggest that the pC15 reaction product is highly positively supercoiled DNA. Additionally, S1 nuclease was also used to probe the conformation of the pC15 DNA reaction product. While highly negatively supercoiled pC15 DNA (Figure 3A, the lower smear in the re-

gion labeled a; topoisomers in the lower smear have lower linking numbers than the topoisomers in the upper smear) was converted to nicked (Figure 3D, spot b) and linear forms (Figure 3D, spot f), the pC15 DNA reaction product (Figure 3D, streak c) was unaffected. Since highly negatively supercoiled DNA, but not positively supercoiled DNA, is known to be sensitive to S1 nuclease, this result again confirms the identification of the pC15 DNA reaction product as highly positively supercoiled DNA. The possibility that the reaction product might contain R loops or irreversibly denatured regions was ruled out by experiments demonstrating the resistance of the product to RNAase H treatment and renaturation at 65°C (data not shown). The specific linking difference of the product DNA migrating at position c (Figure 3) is greater than +0.03, as estimated by a modified two-dimensional gel electrophoresis procedure (Wu et al., 1988).
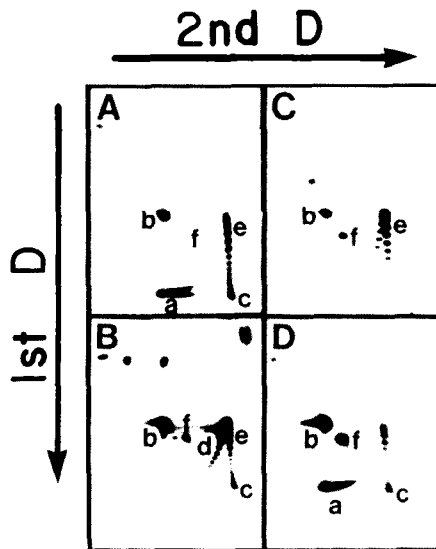

Figure 3. The Reaction Product Is Highly Positively Supercoiled DNA

pC15 DNA isolated from the in vitro transcription reaction (the same DNA sample as shown in lane 4 of Figure 1) was mixed with negatively supercoiled pC15 DNA (internal DNA control) and treated with various enzymes. Two-dimensional gel electrophoresis was done as described in Experimental Procedures. (A) No enzyme treatment. (B) Treated with 50 ng of M. luteus DNA topoisomerase I. (C) Treated with 50 ng of calf thymus DNA topoisomerase I. Both M. luteus topoisomerase I and calf thymus topoisomerase treatments were done under the standard reaction condition (Liu and Miller, 1981). (D) Treated with 0.5 U of S1 nuclease in S1 buffer (20 μl) at 37°C for 30 min. The S1 buffer contains 30 mM Na-acetate (pH 4.6), 50 mM NaCl, and 1 mM ZnCl₂. The lower case alphabets mark the electrophoretic positions of various forms of pC15 DNA. a: negatively supercoiled pC15 DNA. b: nicked pC15 DNA. c: highly positively supercoiled DNA. d: slightly negatively supercoiled DNA. e: relaxed pC15 DNA. Note that the relaxed pC15 DNA topoisomers migrated in the gel as slightly positively supercoiled DNA because of the difference in ionic environment (Depew and Wang, 1975).

The Presence of Intact Nascent RNA Is Required for Template Supercoiling

The formation of positive supercoils on the template DNA was rapid. Within 1 min, a significant proportion of pC15 DNA was converted into highly positively supercoiled DNA (Figure 4, upper panel, lane 3). The average size of the RNA transcripts was less than 6 kb within 1 min of RNA transcription (Figure 4, lower panel, lane 3). Prolonged incubation produced a progressive increase in the size of the RNA, suggesting that little chain termination occurred under these reaction conditions. These results suggest that template supercoiling during transcription is a rapid process and requires no more than 6 kb of nascent RNA chains.

According to the twin-supercoiled-domain model of transcription, unless the RNA polymerase is "anchored," the frictional drag on the nascent RNA is an important factor in the generation of supercoiling waves. To test whether intact nascent RNA is required for template supercoiling, RNAse A was added to the reaction mixture. The formation of positively supercoiled pC15 DNA was gradually inhibited with increasing concentrations of RNAase A (Figure 5A). No positively supercoiled pC15 DNA was detectable at RNAase A concentrations above 100 pg/ml (Figure 5A, lane e). That the inhibitory effect of

1 2 3 4 5 6 7 8 9 10 11 12 13

NC—

+SC—

1 2 3 4 5 6 7 8 9 10 11 12 13

7.5 —
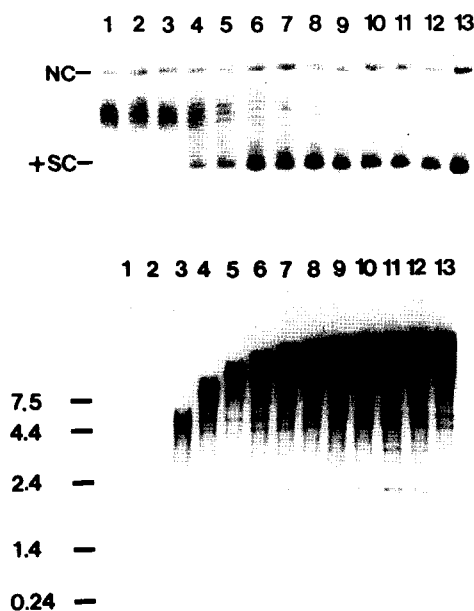4.4 —

2.4 —

1.4 —

0.24 —

Figure 4. Time Course of Positive Supercoiling of the Template DNA during Transcription

In vitro transcription reactions were performed using pC15 DNA, T3 RNA polymerase, and M. luteus DNA topoisomerase I. [α-^{32}P]GTP (2 μCi) was present in each reaction. Reactions were incubated at 37°C and terminated at various times. The change in the plasmid linking number was monitored by agarose gel electrophoresis and the result is shown in the upper panel. The size of the RNA in each sample was monitored by electrophoresis in a formaldehyde denaturing gel and the result is shown in the lower panel. The incubation times of samples shown in lanes 1–13 were 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, and 7 min, respectively. The numbers on the left of the lower panel indicate the size of the RNA in kb.

RNAse A on the supercoiling reaction is most likely due to nucleolytic degradation of RNA rather than some other nonspecific RNAase A reactions was demonstrated by the ability of RNAasIN (an RNAase A inhibitor) to reverse the inhibitory effect of RNAase A (Figure 5B) and the lack of a significant inhibitory effect of RNAase A on the rate of RNA synthesis (Figure 5C). These results suggest that a sizable nascent RNA chain is necessary to drive the supercoiling reaction.

The Rate of RNA Polymerase Movement Affects Template Supercoiling

The twin-supercoiled-domain model of transcription predicts that, when the frictional drags on the various macromolecular components are responsible for supercoiling of the DNA template, the two oppositely supercoiled domains are maintained by continued movement of the RNA polymerase complex. The average superhelical densities within the two supercoiled domains are proportional to the rate of RNA polymerase movement on the template DNA (Liu and Wang, 1987). Our initial experiments showed that streptolydigin (100 μM), an inhibitor that blocks transcription elongation, strongly inhibited the supercoiling reaction when E. coli RNA polymerase was used. To further test the effect of the elongation rate on template supercoiling, we controlled the rate by limiting the level of ribonucleoside triphosphates. As shown in Figure 6 (right panel), the rate of nucleotide incorporation and hence the rate of RNA chain elongation decreased rapidly after about 4 min due to rapid consumption of nucleoside triphosphates (Figure 6, right panel). The decrease in the rate of RNA chain elongation was accompanied by a parallel decrease
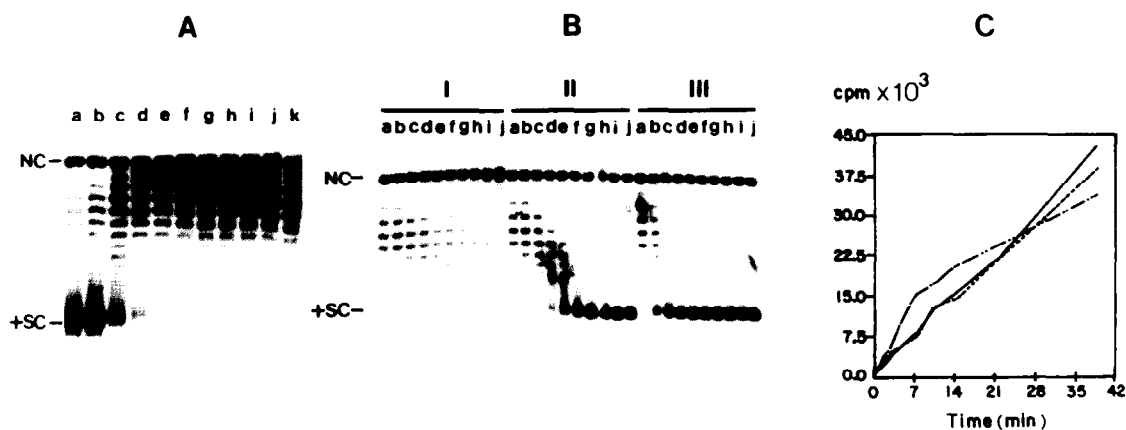
A

a b c d e f g h i j k

NC—

+SC—

B

I   II   III

a b c d e f g h i j a b c d e f g h i j a b c d e f g h i j

NC—

+SC—

C

cpm x 10^3

45.0
37.5
30.0
22.5
15.0
7.5
0.0

0   7   14   21   28   35   42

Time (min)

Figure 5. Template Supercoiling Requires Sizable Nascent RNA Chains

(A) Transcription reactions were performed using pC15 DNA, M. luteus DNA topoisomerase I, and T3 RNA polymerase. Different amounts of bovine pancreatic RNAase A were present in the reactions. Lane a: control, with 5 U of RNAasIN. Lane b: control. Lanes c–k: contained 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml of RNAase A, respectively.

(B) Transcription reactions were performed using pC15 DNA and T3 RNA polymerase as described in Experimental Procedures, except that the concentrations of rNTPs were increased 2.5-fold and the temperature of incubation was lowered to 25°C. Eighty nanograms of M. luteus DNA topoisomerase I was added to the reaction at various times after the reaction was started, and the incubation temperature was immediately shifted to 37°C for 1 min before termination of the reaction. The electrophoretic patterns of the reaction mixtures throughout the time course are displayed in I (10^{-5} mg/ml of RNAase A in each reaction), II (10^{-5} mg/ml of RNAase A in each reaction, and 10 U of RNAasIN were added to reactions after 4 min of incubation, lanes d–j), and III (no RNAase A, and 10 U of RNAasIN were added to reactions after 4 min of incubation). The incubation time (before 1 min pulse treatment with topoisomerase) of samples in lanes a–j was 0, 1, 2, 4, 5, 6, 8, 12, 20, and 36 min, respectively.

(C) Incorporation of [α-^{32}P]GTP into RNA. The solid line, the dotted line, and the dashed line represent the time courses of [α-^{32}P]GTP incorporation from samples shown in I, II, and III (B), respectively.
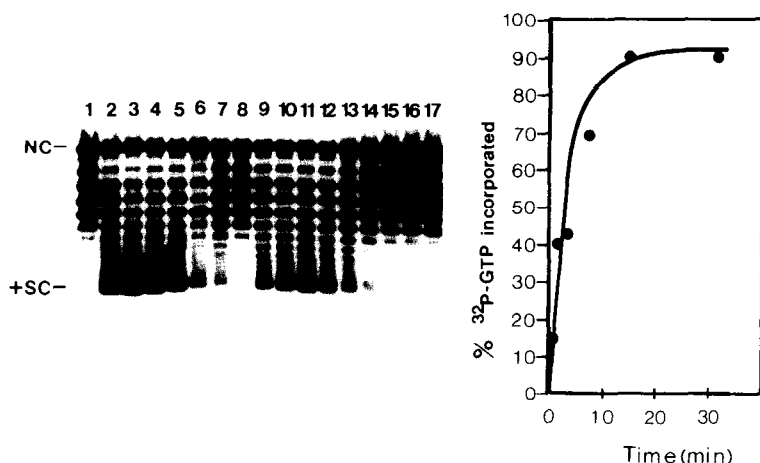
Figure 6. Template Supercoiling Is Dependent on the Continued Movement of the RNA Polymerase Elongation Complex

(Left) Transcription reactions in the presence of pC15 DNA and T3 RNA polymerase were carried out as described in Experimental Procedures except that the incubation temperature was lowered to 25°C. The extent of template supercoiling at various times during RNA transcription was estimated by the rate of positive supercoiling measured by a pulse treatment with M. luteus DNA topoisomerase I (80 ng, 1 min at 37°C). Thirty-two minutes (lane 8) after the start of the transcription reaction, fresh ribonucleoside triphosphates were added to the same initial concentration. The incubation times of samples shown in lanes 1–17 were 0, 0.5, 1, 2, 4, 8, 16, 32, 32.5, 33, 34, 36, 40, 48, 64, 96, and 152 min, respectively.

(Right) Incorporation of ribonucleoside triphosphates was monitored using the DE81 filter paper method. More than 90% of the ribonucleoside triphosphates were consumed within 20 min of incubation. The rapid drop in the rate of RNA synthesis parallels the rapid reduction in the positive supercoiling rate as measured by topoisomerase pulse treatment (lanes 6–8).

in the rate of positive supercoiling of the pC15 DNA template (Figure 6, left panel, lanes 2–8). Thirty-two minutes after RNA transcription had started, RNA chain elongation ceased (as measured by nucleotide incorporation; Figure 6, right panel), and the rate of positive supercoiling of the pC15 DNA template, measured by 1 min pulse treatment with M. luteus DNA topoisomerase I at the end of each incubation, fell to an undetectable level (Figure 6, left panel, lane 8). Addition of fresh ribonucleoside triphosphates to the reaction at this time rapidly restored positive supercoiling of the pC15 template (Figure 6, left panel, lanes 9–17), strongly suggesting that the rate of transcription is an important determinant of the supercoiling reaction. This experiment also indicates that previous RNA transcription on the template does not lead to a perma-

nent accumulation of supercoils; continued RNA synthesis is necessary to maintain the supercoiled state of the template DNA.

Transcription-Induced Supercoils on the Template DNA Are Unconstrained

One important prediction of the twin-supercoiled-domain model of transcription is the existence of unconstrained positive and negative supercoils in the DNA template during transcription. The fact that prokaryotic DNA topoisomerase I can use the template DNA as a substrate suggests that unconstrained negative supercoils are generated. To demonstrate the existence of unconstrained positive supercoils during transcription, calf thymus DNA topoisomerase I was added to a transcription reaction, containing T3 RNA polymerase and M. luteus DNA topoisomerase I, either at the start (Figure 7, lane 2) or 15 min after the start of the transcription reaction (Figure 7, lane 1). In both cases, positive supercoils on the pC15 DNA reaction product were greatly reduced, suggesting that positive supercoils generated during transcription were also unconstrained.
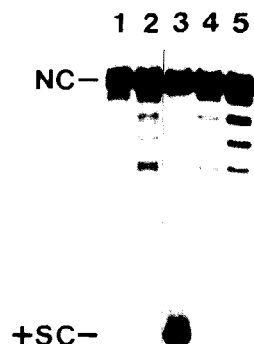
Discussion

Validity of the Twin-Supercoiled-Domain Model of Transcription

We have tested the twin-supercoiled-domain model of transcription in a purified in vitro system. Our results show that, in the presence of prokaryotic DNA topoisomerase I, movement of an RNA polymerase elongation complex on the DNA template results in rapid accumulation of unconstrained positive supercoils. The simplest interpretation is that prokaryotic DNA topoisomerase I selectively removes unconstrained negative supercoils from one of the twin-supercoiled domains (the negatively supercoiled one) generated by RNA transcription, leaving positive supercoils in the other domain. The requirement of con-

Figure 7. Supercoils Generated during RNA Transcription Are Unconstrained

Transcription reactions were performed using pC15 DNA, M. luteus DNA topoisomerase I, and T3 RNA polymerase as described in Experimental Procedures, except that the incubation time was shortened to 15 min. Calf thymus DNA topoisomerase I (30 ng) was added to the transcription reactions either at the start (lane 2) or 15 min after the start of RNA transcription (lane 1). Lane 3: no calf thymus topoisomerase I. Lanes 4 and 5: the same as lanes 2 and 3, respectively, except that T3 RNA polymerase was omitted in the reaction.

tinued RNA synthesis and sizable nascent RNA chains for the supercoiling reaction provides further support for the model.

While our results are in qualitative agreement with the transcription model of Liu and Wang (1987), the degree of positive supercoiling generated by transcription in this in vitro system is significantly greater than predicted, considering the lack of ribosomes on the nascent RNA molecules and, in most cases, the lack of oppositely oriented transcription units on the template DNA. It is possible that the degree of supercoiling generated by RNA transcription is underestimated by Liu and Wang (1987) in their theoretical calculation. Several additional factors may contribute to the rapid and extensive positive supercoiling of the template DNA containing only a single promoter in our in vitro system. First, the number of RNA polymerase elongation complexes per template is very high, averaging ten T3 or T7 RNA polymerase molecules per template. Furthermore, transcription initiation seems highly cooperative. Only a subpopulation of the template DNA may be undergoing active transcription. The number of RNA polymerase molecules per transcribed template may therefore be significantly greater than 10. Second, the concentration of prokaryotic DNA topoisomerase I used in the in vitro reaction is significantly higher than the intracellular concentration of topoisomerase I. Our in vitro results also indicate that the fusion of positive and negative supercoils within the same DNA circle must be slow compared with the rate of topoisomerase I–catalyzed relaxation of negative supercoils. In this connection, we note that the rate of rotational diffusion of DNA, previously estimated by modeling DNA as a speedometer cable (Meselson, 1972; Liu and Wang, 1987), needs to be checked experimentally, especially for a long DNA segment.

Previous in vivo studies suggested that positive supercoiling of plasmid DNAs in cells treated with a bacterial gyrase inhibitor occurred more readily when a divergent pair of transcription units were being transcribed (Wu et al., 1988). Transcription of a single transcription unit or of multiple parallel transcription units showed much less positive supercoiling. This result may be explained by a kinetic effect due to the limiting topoisomerase I activity in cells. In E. coli DM800, a *topA* deletion strain, the transcription of a single transcription unit (e.g., *tetA*) is sufficient to induce high levels of negative supercoils (Pruss and Drlica, 1986; Wu et al., 1988). The faster rate of gyrase-catalyzed relaxation of positive supercoils than topoisomerase I–catalyzed relaxation of negative supercoils in E. coli may explain the kinetic difference between positive supercoiling upon inactivation of gyrase and negative supercoiling upon inactivation of topoisomerase I. The differential rate of relaxation of the twin-supercoiled domains during transcription may also explain why intracellular DNA is negatively supercoiled.

Effect of RNA Transcription on Local and Global Superhelical States of DNA

According to the twin-supercoiled-domain model of transcription, as long as the diffusion pathways for the twin-supercoiling waves are not blocked, only DNA segments proximal to the RNA polymerase elongation complex experience a high degree of supercoiling tension. However, intracellular DNA is often complexed with other macromolecular complexes (e.g., histone or histone-like proteins, scaffolding proteins, replication complexes, and other RNA polymerase elonation complexes) so that the dissipation of oppositely supercoiled domains by rotational diffusion pathways may be inefficient. In these cases, transcription may affect the state of supercoiling of the template DNA over a significant distance, especially when topoisomerase sites are absent in such regions. It is therefore predictable that the region upstream of a transcribed gene is under high negative superhelical tension. Such local supercoiling may be sufficient to drive structural transitions of certain DNA sequences (e.g., alternating purine pyrimidine sequences for Z-DNA formation, palindromic sequences for hairpinned DNA formation, and polypurine/polypirimidine sequences for conversion to a non-B structure), and such structural transitions may in turn affect DNA template functions (Peck et al., 1982; Peck and Wang, 1983; Lilley, 1980; Courey and Wang, 1983; Evans and Efstratiadis, 1986).

RNA transcription can also affect the overall superhelical state of intracellular DNA. For a closed circular DNA template, no net change in the linking number of the DNA accompanies the formation of twin supercoiled domains. However, because of the differential action of topoisomerases on positive and negative supercoils, the difference in their relative abundance, and the possible nonrandom distribution of topoisomerase action sites relative to the transcription unit, transcription may significantly alter the linking number of the template DNA in the presence of topoisomerases. RNA transcription can therefore affect DNA functions over a long distance within a topologically closed domain.

Until recently, the prevailing view regarding DNA supercoiling in prokaryotes is that DNA topoisomerase II (DNA gyrase) is responsible for negative supercoiling DNA in vivo (Gellert et al., 1976; reviewed in Gellert, 1981; Wang, 1985). This view is primarily based on the fact that DNA gyrase can convert relaxed DNA into negatively supercoiled DNA in a reaction coupled to ATP hydrolysis. Furthermore, a number of experiments have demonstrated the existence of negative superhelical tension on intracellular DNA in E. coli (Sinden et al., 1980; Bliska and Cozzarelli, 1987; Giaever et al., 1988). It is plausible, however, at least for DNA molecules undergoing very active transcription, that negative supercoils are generated by gyrase-catalyzed relaxation of positive supercoils. Our recent studies have shown that under a more physiological condition (120 mM KCl), gyrase catalyzes efficient relaxation of positive supercoiled DNA. No supercoiling activity can be detected under our assay conditions (Y.-P. Tsao and L. F. Liu, unpublished data). Although relaxation of positive supercoils can be mechanistically the same as negative supercoiling of DNA, our results suggest that the two reactions are mechanistically different at least under more physiological conditions. Whether DNA gyrase normally functions as a supercoiling enzyme or a relaxation enzyme in vivo requires further investigation.

Roles of DNA Topoisomerases in Transcription

Our present results support the conclusion from previous in vivo studies of Wu et al. (1988) that both DNA topoisomerase I and II (DNA gyrase) are involved in the elongation step of transcription. It was suggested that DNA topoisomerase I removes transcription-induced negative supercoils while DNA gyrase removes transcription-induced positive supercoils. The involvement of topoisomerases in the elongation step of transcription does not necessarily imply that DNA topoisomerases determine the proper rate of movement of the RNA polymerase elongation complex. In the absence of topoisomerases, the rate of chain elongation may not be significantly affected, especially for short transcripts. It is plausible, however, that in the absence of the topoisomerases, transcription may cause regions of DNA to be excessively supercoiled and this may have deleterious effects on certain DNA functions, including the transcriptional process itself. According to this view, DNA topoisomerases and the distribution of their sites of action on DNA are important for modulating the supercoiling waves generated by transcription.

Experimental Procedures

Enzymes and Chemicals

Calf thymus DNA topoisomerase I was purified by a slight modification of the published procedure (Liu and Miller, 1981). M. luteus and E. coli DNA topoisomerase I were prepared by published procedures (Depew et al., 1978; Kung and Wang, 1977). The in vitro transcription kit was purchased from Stratagene. RNAasIN (placenta RNAase inhibitor) was purchased from Promega. Proteinase K and RNAase A were purchased from Sigma. Streptolydigin was a generous gift from Dr. George Stadt (Lederle Co.). S1 nuclease was purchased from Pharmacia. [α-^{32}P]GTP (800 Ci/mmol) was purchased from NEN.

Plasmid DNAs

pC15 DNA was constructed by cloning a 1.8 kb human topoisomerase II cDNA fragment (ZII-1.8 clone from a HepG2 cDNA library) into the EcoRI site (between the T3 and T7 promoters) of the Bluescript SK(+) vector (Tsai-Pflugfelder et al., 1988). pGEM4-D1 DNA was constructed by cloning a 0.7 kb human topoisomerase I cDNA fragment (D1 clone from a HepG2 library) into the EcoRI site (between the T7 and SP6 promoters of the pGEM4 vector). pSP64 DNA contains an SP6 promoter.

In Vitro Transcription Reactions

Each reaction (10 µl) contained 40 mM Tris (pH 8.0), 8 mM MgCl$_2$, 2 mM spermidine, 50 mM NaCl, 0.3 mM each of ATP, CTP, UTP, and GTP, 25 mM DTT, 10 U of RNAasIN, 6 U (70 ng) of RNA polymerase, and 0.2 µg of plasmid DNA. All plasmid DNAs were relaxed to completion with calf thymus DNA topoisomerase I and purified by phenol extraction and ethanol precipitation prior to the transcription reaction. Unless otherwise indicated, incubations were done at 37°C. [α-^{32}P]GTP was added when the size of the RNA transcript or the amount of nucleoside triphosphate incorporation was determined. To monitor the superhelical state of DNA templates, reactions were terminated by adding 0.1% SDS, 10 mM EDTA, and 1 mg/ml of RNAase A (final concentrations). After a 1 hr incubation at 37°C, proteinase K was added to 100 µg/ml and the incubation was continued for 30 min. DNA samples were then analyzed by one-dimensional agarose gel electrophoresis (0.7% agarose in TBE buffer). For two-dimensional gel electrophoresis, pC15 DNA samples were purified further by phenol extraction and ethanol precipitation. The purified pC15 DNA samples were mixed with negatively supercoiled pC15 DNA as an internal control, treated with various enzymes, and then analyzed by two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis, in situ hybridization, and autoradiography were done as previously described (Wu at al., 1988).

Sizing of RNA

For determining the sizes of RNA transcripts, reactions were stopped by adding 0.1% SDS, 10 mM EDTA, and 25 µg/ml of proteinase K (final concentrations) and incubated at 37°C for 15 min. Following ethanol precipitation, the precipitated radio-labeled RNA samples were resuspended in 30 µl of RNA loading buffer (50% deionized formamide, 6.6% formaldehyde, 1% glycerol, 0.05% bromophenol blue, and 0.05% xylene cyanol in 1x MOPS buffer; Maniatis et al., 1982). Following heating at 68°C for 10 min, RNA samples were loaded onto a 1% agarose gel (in 1x MOPS buffer) containing 1% formaldehyde and 1 µg/ml of ethidium bromide.

Determination of Ribonucleoside Triphosphate Incorporation

To determine ribonucleoside triphosphate incorporation, reactions containing radiolabeled [α-^{32}P]GTP were stopped by adding 0.1% SDS and 10 mM EDTA (final concentrations). Aliquots of each sample were spotted on DE81 filter paper. The washing procedure was done as described in Maniatis et al. (1982).

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