Coupling DNA Supercoiling to Transcription in Defined Protein Systems†§

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Transcription of closed circular DNA templates in the presence of DNA gyrase is known to stimulate negative DNA supercoiling both in vivo and in vitro. It has proven elusive, however, to establish a general system in vitro that supports transcription-coupled DNA supercoiling (TCDS) by the “twin-domain” mechanism (Liu, L. F., and Wang, J. C. (1987) Proc. Natl. Acad. Sci. USA 84, 7024–7027) that operates in bacteria. In this report, we examine the properties of TCDS in defined protein systems that minimally contained T7 RNA polymerase and DNA gyrase. Specifically designed plasmid DNA templates permitted us to control the location and length of RNA transcripts. We demonstrate that TCDS takes place by two separate, and apparently independent, mechanistic pathways in vitro. The first supercoiling pathway, which is not likely to be significant in vivo, was found to be dependent on R-loop formation and could be suppressed by the presence of RNase H or bacterial HU protein. The second pathway for TCDS was much more potent, but became predominant in vitro only when sequence-specific DNA-bending proteins were present during transcription, and RNA transcript lengths exceeded 3 kb. This major supercoiling route was shown to be resistant to RNase H and had functional properties consistent with those predicted for the twin-domain mechanism. For example, DNA supercoiling activity was proportional to RNA transcript length and was greatly stimulated by macromolecular crowding agents. Under optimal conditions, the twin domain pathway of TCDS rapidly and efficiently generated superhelicity levels more than twice that typically found in vivo.

DNA transactions in prokaryotic cells, such as DNA replication, transcription, and recombination, are often dependent on or stimulated by negative supercoiling of the DNA template (1–3). For most of these instances, the available evidence indicates that negative superhelical tension facilitates an early step in the reaction pathway, typically, opening of the DNA double helix. The prokaryotic type II DNA topoisomerase, DNA gyrase, is the enzyme responsible for introducing negative supercoils into bacterial chromosomes and plasmids (4–6). While none of the eukaryotic DNA topoisomerases is capable of negatively supercoiling DNA, it is now apparent that some enzymes that translocate processively along DNA, such as RNA polymerase, can affect DNA superhelical tension at a local level (7–10). These localized changes in DNA supercoiling can be used to drive or regulate DNA transactions at nearby sites.

Liu and Wang (8) proposed an elegant model to explain how transcription by RNA polymerase can be used to stimulate DNA supercoiling. This model, termed the twin supercoil domain model, has received broad experimental support in studies of DNA supercoiling in living cells. The twin-domain model postulates that rotation of the RNA polymerase-RNA complex around the DNA helical axis during transcription becomes increasingly difficult as the size of the growing RNA chain increases. At a critical juncture, it becomes more feasible energetically to rotate the DNA on its axis rather than rotate the transcription complex and any associated proteins around the DNA. Further translocation of the polymerase generates transient DNA supercoils, positive supercoils in front of the polymerase and negative supercoils behind it. In bacteria, DNA gyrase acts to convert a fraction of the transient (+) supercoils to “permanent” (−) supercoils, whereas DNA topoisomerases I and IV function to relax a portion of the transient (−) supercoils (11).

Transcription-coupled DNA supercoiling (TCDS)† is also observed in vitro in minimal transcription-supercoiling (T-S) systems that contain just RNA polymerase and a DNA topoisomerase such as DNA topoisomerase I or DNA gyrase (12–14). There is substantial evidence that transient (−) supercoils can be generated on a fraction of plasmid DNA templates as a consequence of transcription in vitro (12, 13, 15). These studies concluded that the twin domain pathway of TCDS was involved, based on the functional properties of the supercoiling reactions. More recent studies, however, have raised concerns about this conclusion. For example, it has proven difficult to document that transient (+) and transient (−) supercoils are produced simultaneously during transcription as predicted by the twin domain model. Since DNA gyrase, alone among cellu-
lar topoisomerase, has the capacity to convert positive superhelical writhe to negative superhelical writhe (6), only T-S systems that contain DNA gyrase should have the capacity to convert transient DNA supercoils arising from the twin-domain mechanism to permanent negative DNA supercoils. However, studies of transcription-induced DNA supercoiling in such systems did not produce convincing evidence for the involvement of a twin-domain mechanism. Instead, Doolot and co-workers (14, 16, 17), using minimal T-S systems that contain DNA gyrase as the only topoisomerase, found that an R-loop-dependent pathway may be responsible for most of the observed supercoiling.

Recently, we reported that the addition of one or more sequence-specific DNA-bending proteins to a minimal T-S system containing DNA gyrase brought about a dramatic increase in transcription-driven DNA supercoiling (18). In the present work, we have characterized the functional properties of TCDS to help pinpoint the mechanism responsible for the stimulatory effects of DNA-bending proteins on transcription-induced DNA supercoiling. We have examined the influence of transcription length, R-loops, sequence-specific DNA-binding proteins, and other factors on TCDS in vitro. Our results suggest that DNA supercoiling is produced by two independent routes during transcription of supercoiled DNA templates in the presence of DNA gyrase. Supercoiling occurred primarily via an R-loop-dependent mechanism in a minimal enzyme system that contained just RNA polymerase and DNA gyrase. However, when this T-S system was supplemented with a specific DNA-bending protein, a twin domain type of mechanism became the predominant supercoiling route whenever long RNA transcriptions were synthesized.

EXPERIMENTAL PROCEDURES

Proteins and Reagents—Escherichia coli RNA polymerase, phage T7 RNA polymerase, E. coli DNA gyrase and HU protein, and the phage λ O replication initiator protein were purified as previously described (18, 19). Purified E. coli RNase H was a generous gift of R. Crouch (National Institutes of Health). E. coli lac repressor (LacI) and gal repressor (GalR) were kindly provided by S. Adhya (National Institutes of Health). Replication fork triraphosphates and deoxyribonucleoside triphosphates were purchased from Amersham Biosciences. Chloroquine diphosphate, formaldehyde, agarose, polyethylene glycol 8000 (DNase- and RNase-free), and polyvinyl alcohol (30,000–70,000 molecular weight) were obtained from Sigma. All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and calf intestine alkaline phosphatase were obtained from New England Biolabs. [α-32P]UTP (3000Ci/mmol) was obtained from Amersham Biosciences. The synthetic oligonucleotides FL3 (5′-TGGAGAAAATTATACGACTCATATAGGGGGAAGCTTGGATCC-3′) and FL4 (5′-TGGAGATCCAGGATTCCTCTCCCTATTAGTGTGCTTTAATTTTC-3′) were purchased from MWG-Biotech, Inc. (High Point, NC).

Plasmid DNA Templates—All plasmids were derived from pUC18 (20), except pRLM409, which was derived from pUC8. Plasmids pRLM352, pRLM375, pRLM409, and pRLM411 were described previously (18). In the plasmid constructions outlined below, ligation of DNA fragments with non-complementary cohesive ends was accomplished following conversion of fragment termini to blunt ends by incubation with T4 DNA ligase and dNTPs. Plasmid pLUC1 was constructed by the insertion of a 43-bp DNA fragment, containing a T7 promoter (the annealing product of oligonucleotides FL3 and FL4), into the unique XhoI site of pRLM349 (18). Plasmid pLUC3 was constructed by insertion of a 1.87 kb BamHI-HindIII fragment from pRLM4 (21) that encodes neomycin phosphotransferase II (kanamycin resistance) between the BamHI and HindIII sites of pLUC1. Plasmid pLUC5 was made by insertion of the same 1.87 kb BamHI-HindIII fragment into the unique MscI site of pLUC6. Plasmid pLUC7 was constructed in two steps. Plasmid pLUC3 was digested with restriction enzyme BsaAI to produce 4293 bp and 740 bp DNA fragments. The larger DNA fragment was recircularized by self-ligation to create plasmid pLUC6. Next, the smaller (740 bp) BsaAI fragment from pLUC3 was inserted into the unique MscI site of pLUC6 to generate pLUC7. Plasmid pLUC9 was also constructed in two steps. Plasmid pLUC8 was generated by removal of a 270-bp KpnI-HindIII DNA fragment (that contained the T7 promoter and oriT) from pLUC3, followed by recircularization of the vector backbone (4763 bp). Subsequently, the 270-bp KpnI-HindIII fragment from pLUC3 was inserted into the unique AhdI site of pLUC8 to produce pLUC9 and pLUC10, which are identical plasmids except for the orientation of the KpnI-HindIII fragment. Genetic and partial restriction maps of plasmids pRLM352, pRLM375, and the pLUC plasmids are depicted in Fig. 1 and in Fig. S1 (Supplemental Materials). All plasmid chromosomes used as DNA templates in transcription reactions were isolated by an alkaline lysis method (22) and purified further by three successive bandings in CsCl equilibrium gradients in the presence of ethidium bromide.

In Vitro Transcription/Superciling (T-S) Assay—The standard in vitro T-S assay (30 μl) contained 40 mM HEPES/KOH (pH 7.6), 11 mM magnesium acetate, 100 mM potassium glutamate, 1 mM dithiothreitol, 4 mM ATP, 0.5 mM each of GTP, CTP, and UTP, 2.5 μg (3.8 nmol as nucleotide) of supercoiled plasmid DNA, E. coli DNA gyrase (80 nm), and either T7 RNA polymerase (20 nm) or E. coli RNA polymerase (33 nm). Where specified, additional proteins were added to the transcription mixtures. All components were mixed on ice and incubated at 30 °C for 10 min or for the indicated amount of time. The reactions were stopped either by addition of EDTA to 25 mM and SDS to 0.1%, followed by a phenol extraction, or by direct extraction with an equal volume of phenol. The DNA/RNA samples were precipitated with two volumes of ethanol and collected by centrifugation. Each precipitate was washed once with 70% ethanol and dissolved in 60 μl of 10 mM Tris-HCl buffer (pH 8.0). Each sample was digested with RNase A (5 μg) and RNase H (50 μg) for 30 min at 37 °C and then extracted once with an equal volume of phenol. The topological state of each DNA preparation was analyzed by electrophoresis in TAE buffer (23), pH 7.5, in a one-dimensional, 1% agarose gel that contained 5 μg/ml chloroquine. Where indicated, DNA samples were analyzed by two-dimensional agarose gel electrophoresis, in the presence of 10 μg/ml chloroquine in the first dimension and 50 μg/ml chloroquine in the second dimension. Following electrophoresis, the agarose gels were stained with ethidium bromide.

Measurements of RNA Synthesis and the Efficiencies of Transcription Termination—Transcription reactions were performed as described above except that each reaction mixture contained [α-32P]UTP (2.5 μCi). Unless specified otherwise, reaction mixtures were incubated for 40 min at 30 °C. RNA synthesis was measured by determining the level of incorporation of labeled nucleotide into acid insoluble material, which was collected on glass fiber filters (Whatman 934-AH) and counted in a liquid scintillation spectrometer. For analyses of RNA chain lengths, the reaction mixtures were immediately extracted with an equal volume of phenol following completion of RNA synthesis. RNA transcripts were precipitated with two volumes of ethanol and dissolved in 60 μl of RNA gel loading buffer (20 mM MOPS, pH 7.0, 8 mM NaAc, 1 mM EDTA, 50% (v/v) formamide, 6.6% (v/v) formaldehyde, 1% (v/v) glycerol, 0.05% bromophen blue, and 0.05% xylene cyanol). The RNA transcripts were resolved by electrophoresis through 1.3% agarose gels prepared in MOPS buffer (20 mM MOPS, pH 7.0, 8 mM NaAc, 1 mM EDTA, and 1% formaldehyde). The gels were subsequently dried and visualized by autoradiography or quantitated using a Fuji FLA 3000 image analyzer. The fraction of the total radioactivity (applied to each gel lane) that was present in each transcript was determined. This information, taken together with the length of the RNA chain in the band, permitted calculation of the number of moles of transcript in each band. The transcription termination efficiency at a given terminator was determined from a comparison of the amount of transcript in the termination band with the total amount of all transcripts comprising longer RNA chains.

RESULTS

We recently established a defined protein system, composed of RNA polymerase and DNA gyrase, which permitted us to examine the factors that influence the coupling of DNA supercoiling to transcription (18). In our previous report, we described our discovery that addition of various sequence-specific DNA-bending proteins to the minimal T-S system brought about a dramatic increase in the rate and extent of DNA supercoiling if the DNA template contained one or more recognition sites for the binding protein. The discovery of this simple method for improving the coupling of DNA supercoiling to transcription in vitro prompted us to investigate more comprehensively the molecular mechanisms underlying this process. In this article, we describe studies of the minimal T-S system.
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that are aimed elucidating how transcription stimulates DNA supercoiling, both in the absence of specific DNA-binding proteins and in their presence.

Transcription Specificity in the T-S System—The plasmid DNA templates used in the basic T-S system (Fig. 1) typically contain a single promoter for bacteriophage T7 RNA polymerase and multiple (5–8) E. coli rrrB T1 plasmid pBR322 P4 transcription terminators. With this setup, it should be possible to regulate precisely the regions of individual supercoiled DNA templates that are transcribed, as well as control the lengths of RNA transcripts produced. The specificity of transcription termination is a pertinent issue in the T-S system, considering that phage T7 RNA polymerase must function with E. coli RNA polymerase transcription terminators. Moreover, the T-S system contains DNA gyrase and other proteins that interact with the DNA template and transcription is carried out on negatively supercoiled DNA templates, rather than on the linear DNA templates more commonly used for in vitro studies of transcription. To examine transcription specificity in vitro, we measured the lengths of RNA transcripts produced in a T-S system that contained both DNA gyrase and O protein. Our results indicated that transcription was initiated and terminated with very high specificity at the known promoter and transcription terminator DNA elements on each DNA template tested (Fig. S2, Supplemental Material). Additionally, we found that RNA transcripts greatly exceeded 9 kb in length when all of the transcription terminators on the plasmid template were oriented in the inverse (i.e. nonfunctional) direction (Fig. S2, lane 6; Supplemental Material).

Our measurements indicate the transcription by T7 RNA polymerase terminated with an efficiency of ~75–80% at each rho-independent terminator on supercoiled DNA templates in T-S reactions that contained both O protein and E. coli HU protein (Table I and Fig. S2, Supplemental Materials). These results are consistent with, or even exceed, measurements of the efficiencies of transcription termination by T7 RNA polymerase at a variety of rho-independent terminators on linear DNA templates in standard transcription reactions (24, 25). We conclude that the lengths and locations of RNA transcripts in the T-S system can be controlled precisely.

Transcription-coupled DNA Supercoiling in a Minimal T-S System—Having established the specificity of transcription in the T-S system, we proceeded to examine the time course of DNA supercoiling in reaction mixtures that contained simply T7 RNA polymerase and DNA gyrase. We used supercoiled DNA templates that direct synthesis of either short RNA transcripts (pRLM352) or long RNA transcripts (pRLM375). When short RNA transcripts of ~250 nucleotides in length were being produced, we observed a 5-minute lag where no significant changes in template superhelicity occurred (Fig. 2A, lane 3). By the 20 min time point, however, nearly all of the input template DNA molecules had gained at least 15 or 20 additional negative supercoils (Fig. 2A, lane 5). In contrast, an increase in the superhelicity of a portion of the pRLM375 template was noticeable in as little as 2 min of transcription when long RNA chains are being synthesized (Fig. 2B, lane 2). Despite the more rapid kinetics of supercoiling with pRLM375, however, some template molecules had essentially unchanged superhelicity after 20 min of incubation (Fig. 2B, lane 5). At the same time point, other DNA molecules in the reaction mixture were hypernegatively supercoiled, as indicated by the smear of ethidium fluorescence located below the primary supercoiled band in the chloroquine-agarose gel. This broad heterogeneity in DNA superhelicity, observed specifically when long RNA chains were being synthesized, suggests that a different molecular mechanism may account for some of the increased supercoiling of pRLM375 DNA.

Drolet et al. (14) had earlier observed that transcription-stimulated DNA supercoiling in a similar system (composed of E. coli RNA polymerase and DNA gyrase) is sensitive to RNase H. The available evidence suggested that a mechanism dependent on the formation of R-loops was responsible for the increased DNA supercoiling in their system. To determine if R-loops play a significant role in TCDs in our minimal T-S system, we examined the effects of RNase H, RNase A, and E. coli HU protein. RNase H, but not RNase A, is capable of digesting the RNA chain of R-loops. On the other hand, the histonelike HU protein, upon binding to DNA, acts to constrain endogenous DNA supercoiling (26). Since increased negative DNA superhelicity increases the stability of R-loops (27, 28), the lowering of effective DNA superhelicity by the nonspecific binding of HU to the DNA template would be expected to reduce the formation and stability of R-loops. Indeed, our analysis indicates that RNase H and HU each interfere strongly with DNA supercoiling that is coupled to transcription of pRLM352 (Fig. 3A). In contrast, the inclusion of RNase A in the T-S system to digest the product RNA chains concurrently during transcription had little to no impact on the amount of DNA supercoiling obtained. These results are consistent with a model in which DNA supercoiling during the synthesis of short RNA chains on pRLM352 is strictly dependent on the formation of R-loops. Qualitatively similar results were obtained when pRLM375, which yields long RNA chains during transcription, was the DNA template (Fig. 3B). In this case, though, the block to DNA supercoiling by RNase H was not as complete (Fig. 3B, lanes 2 and 3). This finding raises the possibility that a twin-domain supercoiling mechanism (8) might also be responsible for some portion of the DNA supercoiling when long RNA chains are being produced. To test this idea, we added RNase A to the T-S reaction mixture to prevent the formation...
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Termination of transcription by T7 RNA polymerase at E. coli rnmB T1 terminator sequences

Transcription reactions using T7 RNA polymerase were performed and analyzed as described under “Experimental Procedures” and modified as detailed in the legend to Fig. 2. Reaction mixtures were incubated at 30 °C for 40 min. RNA transcripts were resolved by electrophoresis in formaldehyde-agarose gels and quantified using a Fuji FLA 3000 image analyzer. The amount of RNA synthesis was determined from a measurement of the incorporation of [α-32P]UTP into acid-insoluble material.

### Table 1

| Plasmid template | Distance to terminatora | Overall termination efficiencyb | Ave. RNA chain sizec | RNA synthesis | No. transcripts per DNAd |
|------------------|-------------------------|---------------------------------|----------------------|---------------|--------------------------|
|                  | nt no. 1 no. 2 no. 3 no. 4 | nt nmol                        |                      |               |                          |
| pRLM352          | 200                      | 79.5 95.4 99.5 99.9            | 250                  | 50            | 410                      |
| pLUC5            | 110                      | 77.6 93.8 98.5 99.8            | 162                  | 34            | 560                      |
| pLUC7            | 1236                     | – – – 99.4                    | 1280                 | 68            | 140                      |
| pLUC3            | 1976                     | – – – 99.5                    | 2030                 | 96            | 130                      |
| pLUC9            | 2985                     | – – – 98.9                    | 3020                 | 120           | 110                      |
| pLUC10           | –                       | 0 0 0 0                       | > 9000               | 290           | 51                       |
| pRLM375          | –                       | 0 0 0 0                       | > 9000               | 290           | 40                       |

a Predicted length in nucleotides of the shortest RNA transcript produced when T7 RNA polymerase transcription is terminated at the first functional (properly oriented) rho-independent transcription terminator.
b Overall percentage of RNA transcripts terminated following encounter of T7 RNA polymerase with each successive functional transcription terminator.
c The average size of RNA chains produced was calculated from the amounts and measured (or predicted) sizes of the four smallest RNA chains (comprising 99% or more of all transcripts).
d The average number of transcripts produced per input plasmid DNA template molecule was calculated from the amount of RNA product synthesized and the average RNA transcript length. The average transcript length was assumed to be 15 kb for templates pLUC10 and pRLM375.

![Image](http://www.jbc.org/)

**Fig. 2.** Time course of transcription-coupled DNA supercoiling in the minimal T-S system. Transcription-supercoiling reactions using DNA templates pRLM352 (A) or pRLM375 (B), T7 RNA polymerase and E. coli DNA gyrase reactions were performed and analyzed as detailed under “Experimental Procedures.” Lanes 1–6 contained, respectively, DNA samples isolated after incubation at 30 °C for 0, 2, 5, 10, 20, and 40 min; lane 7 contained the untreated starting DNA template. DNA topoisomers were resolved by electrophoresis in 1% agarose containing 5 μg/ml chloroquine.

**Fig. 3.** RNase H and HU protein each block DNA supercoiling in the minimal T-S system. Transcription-supercoiling reactions were performed in the minimal T-S system for 10 min at 30 °C as indicated under “Experimental Procedures,” except that reaction mixtures were supplemented with RNase H, RNase A, or HU protein as specified above the gel panels. A, DNA template was pRLM352. Lane 1 contained the starting DNA template. The reaction mixtures for the DNA samples applied to lanes 2–8 contained, respectively, RNase H (25 and 50 μM), RNase A (12 and 120 μM), and HU protein (0.72 and 1.44 μM). B, DNA template was pRLM375. The untreated starting DNA template was applied to lane 6. The reaction mixtures for the DNA samples applied to lanes 2–5 contained, respectively, RNase H (55 and 157.5 μM) and RNase A (12 and 120 μM). DNA topoisomers were resolved by electrophoresis in 1% agarose containing 5 μg/ml chloroquine.

T7 RNA polymerase was permitted to synthesize long RNA transcripts on plasmid pRLM375 (Fig. 4, filled circles). However, when short RNA chains were being produced with pRLM352 as the DNA template, the rate of RNA synthesis slowed considerably after the initial 10 min incubation period (Fig. 4, open circles). Although the mass of RNA produced from pRLM375 is considerably greater than that made from pRLM352, we estimate that ~15-fold more RNA chains are made from the latter template in a 10 min incubation (based on an average transcript size of ~20 kb for pRLM375 and 0.25 kb for pRLM352 (Table 1 and Fig. S2 of Supplemental Materials).
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**Fig. 4.** Time course of RNA synthesis in the T-S system supplemented with λ O protein and *E. coli* HU protein. Transcription-supercoiling reactions (300 μl) were carried out in the presence of [α-32P]UTP as described under "Experimental Procedures," except that the reaction mixtures also contained λ O protein (100 nM, as dimer) and HU protein (0.72 μM). The RNA polymerases and transcription templates used were: open circles, T7 RNA polymerase (20 nM) and pRLM352 DNA; filled circles, T7 RNA polymerase (20 nM) and pRLM375 DNA; filled squares, *E. coli* RNA polymerase (33 nM) and pRLM409 DNA. The at the times indicated, 30-μl portions of the reaction mixtures were removed and processed to determine the level of incorporation, in the entire reaction mixture, of labeled ribonucleotide into acid-insoluble material.

**Fig. 5.** Time course of O-stimulated DNA supercoiling of plasmid pRLM375 during transcription. A, electrophoretic analysis of plasmid DNA supercoiling in one-dimensional chloroquine-agarose gels. Each T-S reaction mixture (300 μl) contained 2.5 μg of plasmid pRLM352 or pRLM375 DNA, T7 RNA polymerase (20 nM), DNA gyrase (80 nM), λ O protein (100 nM, as dimer), HU protein (0.72 μM), and RNase H (50 nM). The reaction mixtures were incubated at 30 °C for the times indicated and the plasmid DNA molecules were isolated and subjected to chloroquine-agarose gel electrophoresis as detailed under "Experimental Procedures." Lane 1 contains plasmid pRLM352 DNA that had been transcribed for 40 min; lane 2 contains pRLM375 DNA that had been transcribed for 40 min in the presence of 1.2 μM RNase A; lanes 3–8 contain plasmid pRLM375 DNA that had been transcribed for 0, 2, 5, 10, 20, and 40 min, respectively. B, electrophoretic analysis of plasmid DNA supercoiling in two-dimensional chloroquine-agarose gels. Gel panels I, II, III, and IV: plasmid DNA pRLM375 after 0, 5, 10, and 40 min of transcription as described in A above for lanes 3, 5, 6, and 8, respectively. The spots marked a, b, and c on these gels denote, respectively, the approximate migration positions of nicked circular DNA, input negatively supercoiled template DNA, and hypernegatively supercoiled supercoiled.

**Fig. 6.** Effect of RNA transcript length on λ O-stimulated transcription-coupled DNA supercoiling. A, T-S reactions were carried out with T7 RNA polymerase on a variety of pLUC plasmid DNA templates (denoted above the individual lanes) in mixtures supplemented with λ O, HU protein and RNase H as described in the legend to Fig. 5A. The plasmid DNA templates were isolated after 20 min of transcription (lanes 1–5) or 40 min of transcription (lanes 7–11) and subjected to electrophoresis in a chloroquine-agarose gel. Lane 6 contains untreated pLUC10 DNA. B, T-S reactions were carried out for 10 min as described in A above, except that the DNA template was plasmid pRLM409 and *E. coli* RNA polymerase (33 nM) polymerase replaced T7 RNA polymerase. Lane 1, the T-S reaction mixture contained no RNase A; lanes 2 and 3, the reaction mixtures contained 12 and 120 nM RNase A, respectively; lane 4, untreated pRLM409 DNA.

The amount of RNA produced from pRLM352 in 40 min is about equivalent in mass to the amount of RNA synthesized from the pRLM375 DNA template in 5 min (Fig. 4). We estimate that 20–40-fold more RNA transcripts were initiated on pRLM352 DNA in 40 min than on pRLM375 in 5 min. Since the superhelical density of pRLM352 DNA is unchanged during this incubation, we conclude that neither the total mass of RNA synthesized nor the number of RNA chains has any significant impact on the level of TCDS in the T-S system. It seemed plausible that the highly preferential supercoiling of pRLM375 in the *in vitro* system was due to the capacity of this plasmid to produce very long RNA transcripts. To test this idea, we examined if concurrent cleavage of RNA transcripts by RNase A during RNA chain synthesis on pRLM375 DNA affected the final level of DNA supercoiling obtained. Indeed, supplementation of the T-S system with RNase A effectively eliminated transcription-coupled supercoiling of pRLM375 DNA (Fig. 5A, lane 2). This result supports the hypothesis that RNA transcript length determines the effectiveness of TCDS in *in vitro* when λ O or another specific DNA-bending protein is present.

**Effect of Transcription Length on Transcription-coupled DNA Supercoiling.**—We decided to examine more thoroughly the relationship between RNA transcript length and DNA supercoiling in a T-S system that contained λ O. We used the series of pLUC plasmids depicted in Fig. 1 and Fig. S1 (Supplemental Materials) as DNA templates, which yield transcripts varying from ~160 bases to over 9 kb in length. The superhelical state of each plasmid DNA template was examined after 20 min and 40 min of transcription with T7 RNA polymerase (Fig. 6A). Negligible DNA supercoiling was obtained when the transcript length averaged about 160 nucleotides (Fig. 6A, lanes 1 and 7; pLUC 5). Small amounts of hypernegatively supercoiled DNA were produced when the transcript lengths were 1.35 or 2.1 kb, but most template DNA molecules gained only 4 or 5 negative supercoils (spot c, Fig. 5B, panel II). If it is considered that hypernegatively supercoiled DNA (--- SC DNA) does not stain well with ethidium bromide, it is evident that the majority of the pRLM375 template DNA is converted to a hypernegatively supercoiled form following 10 min of transcription in the T-S system (Fig. 5B, panel III).
supercoils (Fig. 6A, lanes 2, 3, 8, and 9). However, when the average transcript length was 3.1 kb, the majority of the template DNA was converted to a hypernegatively supercoiled state after 40 min of transcription (Fig. 6A, lane 10). Finally, when transcript lengths averaged over 9 kb, essentially all of the input template DNA was converted to (−−) SC DNA by the completion of the incubation (Fig. 6A, lane 11).

To determine if the length of the RNA product also played a role in TCDS by E. coli RNA polymerase, we examined the effect of RNase A on the supercoiling of plasmid pRLM409 (18) in the T-S system. This plasmid has a single transcription terminator element, but this terminator is not functional because it is oriented opposite to the direction of transcription. Consequently, E. coli RNA polymerase produces very long transcripts that average over 9 kb on pRLM409 DNA. Thus, it is not surprising that, in the absence of added RNase A, almost all of the input pRLM409 template was converted to a hypernegatively supercoiled form after just 10 min of transcription in vitro (Fig. 6B, lane 1). Concurrent digestion of the RNA product with RNase A during transcription, which we have found shortens the effective transcript chain length to less than 100 bases, completely prevented conversion of pRLM409 to a hypernegatively supercoiled form (Fig. 6B, lanes 2 and 3). We conclude that RNA transcript chain length is a critical aspect of the phenomenon of O-protein-stimulated TCDS that takes place during transcription of covalently closed circular DNA templates by either E. coli or T7 RNA polymerase.

In a similar fashion, we used RNase A as a probe to investigate if TCDS, engendered by either E. coli gal repressor (GalR) or lac repressor (LacI) (18), is also strongly dependent on RNA transcript length. In the presence of GalR, plasmid pRLM419 was converted efficiently to a hypernegatively supercoiled form in the T-S system (Fig. 7A, lanes 3–5). However, if RNase A was included in the reaction mixture, production of (−−) SC DNA was totally blocked (Fig. 7A, lanes 7–9). Likewise, addition of RNase A to the T-S system was also found to block supercoiling of pRLM420 DNA stimulated by the presence of LacI (Fig. 7B). We infer that long RNA transcripts must be produced to observe significant transcription-coupled DNA supercoiling in the presence of either GalR or LacI.

**Hydrophilic Polymers Stimulate Transcription-coupled DNA Supercoiling**—To this point, our findings in T-S systems that contain a sequence-specific DNA-binding protein are fully consistent with the involvement of a twin supercoil domain mechanism (8) in TCDS. As predicted by the Liu and Wang model, DNA supercoiling during transcription was found to be dependent on the length of the product RNA chains. The twin supercoil domain model also predicts that DNA supercoiling during transcription should be enhanced by factors that act to impede the free rotation of nascent RNA chains around the DNA template. We therefore investigated if the presence of a hydrophilic polymer, such as polyethylene glycol or polyvinyl alcohol, stimulated TCDS. At concentrations sufficient to reduce the effective solvent volume and produce macromolecular crowding, such polymers should hinder free rotation of nascent RNA transcripts. We found that supercoiling of pRLM375 DNA was greatly enhanced in a minimal T-S reaction mixture when either polyethylene glycol or polyvinyl alcohol was added to T-S reaction mixtures (Fig. 8A, lanes 3–5). Further studies will be needed to ascertain whether the stimulation of TCDS provided by these polymers arises from macromolecular crowding, from increased viscosity of the T-S reaction mixture, or from a combination of both effects.

Our results to this point suggest that TCDS in the minimal T-S system occurs by two intrinsic, yet apparently independent, pathways, one involving an R-loop mechanism and a second involving a twin supercoil domain mechanism. In the experiment depicted in Fig. 8A, we did not determine which supercoiling pathway was stimulated when hydrophilic polymers were present. To investigate this further, we examined the effect of added PVA on TCDS using conditions where the R-loop pathway was expected to be strongly suppressed. This was accomplished by the inclusion of O protein and RNase H in the T-S system (Fig. 8B). Few, if any, supercoil turns were introduced into the pRLM375 DNA template after transcription for 10 min in the minimal T-S system containing O protein and RNase H (Fig. 8B, lane 1). However, when 4% PVA was included in the reaction mixture, the majority of the template DNA was converted to a hypernegatively supercoiled form dur-
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The in vitro transcription supercoiling system used in this report permitted precise control over the length and location of RNA transcript synthesis on supercoiled DNA templates. Our studies of TCDS in this system indicate that two separate and independent pathways are used for coupling DNA supercoiling to transcription. One pathway, which is predominant in a minimal system that contains only RNA polymerase and DNA gyrase, is seemingly dependent on the formation of R-loops during transcription. In contrast, the second and more potent pathway appears to rely on the twin supercoil domain mechanism proposed by Liu and Wang (8). The twin supercoil domain pathway predominate in vitro and, as shown here, when DNA-binding and -bending proteins are present in vitro (18). The sensitivity of the two individual pathways to specific ribonucleases provided us with a means to ascertain the functional properties of each supercoiling pathway.

TCDS in a minimal system that contained only RNA polymerase and DNA gyrase appeared to be totally dependent on the formation of R-loops when the RNA transcript length was short (i.e., < -250 bases long). Supercoiling remained largely dependent on R-loop formation even when RNA transcript length exceeded 9 kb (Fig. 3B). The involvement of R-loops under these conditions is supported by the high sensitivity of TCDS to the presence of either RNase H or Hu protein (Fig. 3, A and B). RNase H action removes R-loops directly, whereas Hu likely acts indirectly to preclude R-loop formation or lessen R-loop stability. Binding of Hu to plasmid DNA constrains negative DNA supercoils and therefore diminishes the effective superhelical density of the complex. Reduced negative superhelicity, in turn, would be expected not only to slow the formation of R-loops, but also to destabilize existing R-loops as well. Further support for an R-loop-dependent supercoiling mechanism during synthesis of short RNA transcripts comes from the insensitivity of TCDS to RNase A (Fig. 3A), an enzyme that blocks TCDS by the twin-domain pathway (Figs. 5–7). Interestingly, DNA supercoiling by the R-loop-dependent pathway is not directly proportional to transcript length (Figs. 2 and 3), to the mass of RNA product (Figs. 2 and 4), or to the number of initiations made by RNA polymerase (Fig. 2, Table I). This latter finding is inconsistent with an earlier proposition that the number of transcription initiations establishes how much DNA supercoiling is produced via R-looping events (29).

Our results suggest instead that the number of elongation complexes present during steady-state transcription may determine the potency of DNA supercoiling via the R-loop-dependent pathway. This interpretation is consistent with a mechanistic model proposed for R-loop-dependent DNA supercoiling in vivo by E. coli RNA polymerase (14). We note, however, that the R-loop-dependent pathway is unlikely to be a significant source of DNA supercoiling in vivo, because of the presence of RNase H in cells, as well as Hu protein and other histone-like proteins that constrain DNA supercoiling. Moreover, DNA topoisomerase I suppresses TCDS by the R-loop-dependent pathway in vivo (30, 31).

The properties of TCDS in vivo were markedly changed when the minimal T-S system was supplemented with a sequence-specific DNA-binding and DNA-bending protein that interacts with recognition sites on the transcription template. Not only was DNA supercoiling strongly stimulated, as reported previously (18), but the supercoiling characteristics were also no longer consistent with the involvement of an R-loop mechanism. In the presence of a DNA-binding protein, DNA supercoiling was resistant to agents, such as RNase H or Hu protein, which abrogate the R-loop pathway (Fig. 5–8). We found clear indications that a twin domain type of supercoiling mechanism was at work when DNA-binding/bending proteins are bound to the transcription template. For example, the rate and level of DNA supercoiling in the supplemented T-S system was found to be proportional to the length of the RNA transcript being synthesized (Fig. 6). This finding is consistent with a central tenet of the twin domain model, which holds that longer RNA chains will be more difficult to rotate around the DNA axis and hence more likely to cause transient supercoiling of the DNA template in front of and behind the translocating RNA polymerase (8). Our finding that polyvinyl alcohol and polyethylene glycol stimulated TCDS significantly (Fig. 8) also argues for the involvement of the twin-supercoil-domain model in DNA supercoiling. These polymers greatly increase macromolecular crowding, as well as solution viscosity, and presumably impede free rotation of the transcription complex around the template axis.

Supercoiling by the twin domain pathway can be exceptionally potent in the presence of both λ O protein and a macromolecular crowding agent, more than 25 negative supercoils could be introduced into pRLM375 DNA after just 2 min of transcription (Fig. 8B). The superhelical density of most of the input DNA template increased from -0.06 to at least -0.13 in this short time span. Nonetheless, the efficiency of TCDS is remarkably low, even under optimal conditions. Based on the amount of RNA product made in 2 min and on the fact that two negative DNA supercoils can potentially be produced by DNA gyrase for every 10.5 base pairs transcribed (under conditions where ongoing transcription forces rotation of the DNA helix), we calculate a supercoiling efficiency of just 0.3%. Of course, supercoiling efficiencies might be higher under some circumstances in vivo, where other mechanisms for restricting rotation of the transcription complex around the DNA axis may be available. RNA transcripts of 3 kb or longer are needed in vitro under dilute aqueous conditions to activate TCDS effectively by the twin domain pathway (Fig. 6), even when a DNA-bending protein is present that partially hinders the merger of oppositely supercoiled domains by diffusion (18). It is notable, however, that transcripts as short as 250 nucleotides are capable of activating TCDS by the twin domain pathway when macromolecular crowding agents are also present (data not shown). These latter conditions should more closely approximate the situation found in vivo. We conclude that TCDS via the twin-

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domain mechanism, despite its inherent inefficiency, can result in rapid and striking changes in local DNA superhelicity. In vivo, a sharp increase in the negative superhelicity of the template DNA brought about by TCDS will eventually be counteracted by the compensatory actions of DNA topoisomerase I and DNA topoisomerase IV, enzymes that are each capable of removing excess negative DNA supercoils (11). Nevertheless, the discovery that supercoils generated by transcription are apparently relaxed more slowly by topoisomerases than are plectonemic supercoils present in the template DNA (32) indicates that regions of locally dense supercoiling arising from TCDS may persist for significant periods of time.

The specific molecular events responsible for TCDS remain obscure. For the twin domain pathway, any mechanistic description must account for the conspicuous bimodal distribution of template superhelical densities present at early stages of the in vitro reaction. At early times, only a minor subset of the input negatively supercoiled DNA template was converted into a hypernegatively supercoiled form (Fig. 5B, panel II). Sufficient RNA polymerase was present in the T-S reaction mixtures (−12−15 RNA polymerase molecules per plasmid DNA) to ensure that each template was being actively transcribed. Accordingly, transcript lengths presumably did not vary markedly between individual DNA template molecules. We infer, therefore, that no sharp threshold transcript length exists that provokes automatic formation of transient DNA supercoils as RNA polymerase translocation continues (at least under dilute aqueous conditions). Instead, some unknown event must occur on a subset of the DNA template molecules that produce long RNA chains. This precipitating event, which conceivably involves the entanglement of the growing RNA chain with other transcripts or the template, likely prevents free rotation of RNA polymerase and its RNA product around the DNA axis during ongoing transcription. Transient (+) DNA supercoils would be efficiently generated by active RNA polymerase translocation on “entangled” templates. Ultimately, negative DNA supercoils result when the transient (+) supercoils are acted upon by DNA gyrase before they diffuse and dissipate (e.g. through merger with transient (−) supercoils).

Historically, it has proven difficult to detect TCDS by the twin domain mechanism in vitro when the T-S system contains only RNA polymerase and DNA gyrase. Our results suggest that multiple factors contribute to this situation. Small plasmid DNA are generally used as the templates in these studies, which facilitates the rapid merger and annihilation of transient (+) and (−) supercoils. Second, the presence of DNA gyrase stimulates TCDS by the R-loop-dependent pathway, thereby obscuring a much more modest DNA supercoiling reaction by the twin domain pathway. Third, nearly all previous in vitro studies of TCDS used dilute aqueous reaction conditions that disfavor supercoiling by the twin domain mechanism. Finally, a DNA-bending protein must be bound to the template DNA in order to observe robust supercoiling by the twin-domain pathway under typical reaction conditions (18).
