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Transcription-coupled Hypernegative Supercoiling of Plasmid DNA by T7 RNA Polymerase in *Escherichia coli* Topoisomerase I Deficient Strains

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Summary

Transcription by RNA polymerase can stimulate negative DNA supercoiling in *Escherichia coli topA* strains. This phenomenon has been explained by a "twin-supercoiled-domain" model of transcription in which positive DNA supercoils are generated in front of a translocating RNA polymerase and negative supercoils behind it. However, since a specific system is lacking to study the factors governing this biologically important process, the parameters regulating transcription-coupled DNA supercoiling (TCDS) in *Escherichia coli* still remain elusive. In this paper, we describe our efforts to study TCDS in *Escherichia coli* using a newly developed system. This system consists of a *topA strain*, VS111(DE3) or DM800(DE3), in which a λDE3 prophage containing a T7 RNA polymerase gene under control of the *lacUV5* promoter has been integrated into the cell chromosome, along with a set of plasmids producing RNA transcripts of various lengths by T7 RNA polymerase. Using this system, we found that transcription by T7 RNA polymerase strikingly induced formation of hypernegatively supercoiled plasmid DNA. We also discovered, for the first time, that TCDS was dependent on the length of RNA transcripts *in vivo*, precisely predicted by the "twin-supercoiled-domain" model of transcription. Furthermore, our results demonstrated that hypernegative supercoiling of plasmid DNA by T7 RNA polymerase did not require anchoring of DNA to the bacterial cytoplasmic membrane. These results indicate that a transcribing RNA polymerase alone is sufficient to cause change of local DNA superhelicity, which can have a powerful impact on the conformation and function of critical DNA sequence elements, such as promoters and DNA replication origins.

Keywords

DNA supercoiling; the "twin-supercoiled-domain" model; T7 RNA polymerase; *topA* strains; transcription

Introduction

DNA supercoiling is a fundamental property of the DNA double helix. In bacteria, DNA is typically negatively supercoiled. Free energy constrained in negative superhelical tension greatly promotes a number of essential DNA transactions, such as DNA replication, transcription, and recombination.[1–4] In *Escherichia coli*, the DNA supercoiling level is

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primarily regulated by opposing actions of DNA topoisomerase I and gyrase.[5–7] Inactivation of either enzyme results in production of positively or hypernegatively supercoiled plasmid DNA.[8,9]

A number of studies have demonstrated that transcription can induce localized DNA supercoiling.[9–12] Liu and Wang have formulated a "twin-supercoiled-domain" model of transcription to explain this phenomenon.[13] This elegant model hypothesizes that the transcribing complex becomes progressively more difficult to rotate around the DNA double helix as the size of the growing RNA transcript increases. At a critical point, energetically, it is more feasible for DNA to rotate around its own helical axis to generate a transient positively supercoiled domain in front of RNA polymerase and a transient negatively supercoiled domain behind it. The two transient supercoiled domains may be relaxed by DNA topoisomerases or neutralize each other by diffusion along the helical axis.[13]

There is substantial evidence to support the "twin-supercoiled-domain" model of transcription in *Escherichia coli*.[9,14–18] Earlier studies showed that plasmid pBR322, but not pUC9 (a smaller high copy number derivative of pBR322 without *tet*, the gene encoding the tetracycline resistance protein), isolated from *Escherichia coli topA* strains lacking DNA topoisomerase I, became hypernegatively supercoiled.[14] Further studies showed that hypernegative supercoiling of plasmids in *topA* strains requires anchoring the transcribing RNA polymerase to the membrane through a nascent hydrophobic membrane-bound peptide or protein.[15–17] Moreover, Cook *et al.* demonstrated that transcription of oppositely oriented membrane-associated gene products rapidly supercoiled the plasmid DNA templates in *topA* strains.[16, 18] In these cases, the membrane-bound transcriptional machinery cannot freely rotate around the DNA double helix and therefore provides a friction barrier to produce the twin supercoiled domains on the DNA templates.[19] It should be pointed out that all these studies regarding transcription-coupled DNA supercoiling (TCDS) in *Escherichia coli topA* strains utilized a combination of *Escherichia coli* RNA polymerase and its promoters. As demonstrated previously, pBR322 and its derivatives contain several *Escherichia coli* RNA polymerase promoters[20] where the length and location of RNA transcripts cannot be precisely controlled. TCDS may result from simultaneously transcribing several transcriptional units on these plasmid DNA templates. Therefore, it was difficult to determine the factors influencing TCDS in *topA* strains. Apparently, a more specific model system is needed to identify parameters that regulate TCDS in *Escherichia coli*.

In this article, we report our efforts to create a new system to study TCDS in *Escherichia coli*. This *in vivo* system consists of an *Escherichia coli topA* strain, VS111(DE3) or DM800 (DE3), in which a λDE3 prophage containing a T7 RNA polymerase gene under control of the *lacUV5* promoter has been integrated into the cell chromosome, and a set of supercoiling reporter plasmids that produces specific locations of RNA transcripts of various lengths, as determined by T7 promoters and the transcription terminators. Our results demonstrated that transcription by T7 RNA polymerase significantly supercoiled the plasmid DNA templates containing a T7 promoter in *Escherichia coli topA* strains. TCDS in the present system was dependent on the length of RNA transcripts and did not require anchoring of DNA to the bacterial cytoplasmic membrane.

Results

We have recently established an *in vitro* defined protein system, composed of an RNA polymerase and *Escherichia coli* DNA gyrase, which allows us to examine the factors affecting the efficiency of TCDS.[12,21] We utilized a T7 RNA polymerase that specifically initiates transcription at the T7 promoter and also multiple Rho-independent transcription terminator sequences. In such a case, it is possible to regulate precisely the regions of individual

supercoiled DNA templates that are transcribed and control the lengths of the RNA transcript that are produced. Using this system, we have shown that certain sequence-specific DNA-binding proteins potently stimulated TCDS in the minimal transcription-supercoiling (T-S) system.[12] We also demonstrated that TCDS takes place by two independent pathways *in vitro*: the R-loop and the twin-domain pathways.[21] The twin-domain pathway was much more potent and became predominant *in vitro* when certain sequence-specific DNA-binding proteins were present during transcription in which DNA supercoiling efficiency was proportional to RNA transcript length and was greatly stimulated by macromolecular crowding agents.[21] In this article, we describe an *in vivo* T-S system that is aimed at elucidating how transcription stimulates DNA supercoiling in *Escherichia coli*.

Construction of *Escherichia coli topA*(DE3) strains for study of hypernegative supercoiling of plasmids induced by transcription of T7 RNA polymerase

In order to study TCDS in *Escherichia coli*, topoisomerase I deficient (*topA*) strains, VS111 and DM800 were used. Both strains are topoisomerase I deletion strains: VS111 is a Δ*topA* strain without a compensatory mutation[22] and DM800 is a Δ*topA* strain with a compensatory mutation in the *gyr* B gene that produces a less active DNA gyrase.[23,24] Our first step was to insert a T7 RNA polymerase gene under control of *Escherichia coli lacUV5* promoter into the host strain chromosome in which expression of T7 RNA polymerase can be induced by addition of IPTG. VS111(DE3) and DM800(DE3) were made by co-infection of the host strains with three phages of the λDE3 Lysogenization Kit as described under “Materials and Methods” and verified by plating the T7 tester phage (provided by the manufacturer) that only produced plaques in the presence of IPTG (data not shown). Western blotting analyses confirmed that VS111(DE3) and DM800(DE3) were successfully constructed (Figures S1 and S4). Expression of T7 RNA polymerase was IPTG-inducible; the expression level was dependent on the IPTG concentration added into the media (Figure S1a, compare lanes 2 to 6) and also on the induction time (Figure S1b, compare lanes 2 to 6). We estimated that approximately 10 to 50 molecules of T7 RNA polymerase were produced in each cell after 5 min induction in the presence of 50 μM IPTG, based upon a concentration gradient of the purified T7 RNA polymerase in the Western blotting experiment (data not shown).

Hypernegative supercoiling of plasmids was dependent on expression of T7 RNA polymerase

In this study we used the plasmid DNA templates pLUC3, 5, 7, and 9 as described previously (Figure S2; also see Figures 1 and S1 of reference[21]) that contain a single promoter for bacteriophage T7 RNA polymerase and multiple Rho-independent *Escherichia coli rrnB* T1 transcription terminators. As demonstrated in our previous publications,[21] the lengths and locations of RNA transcripts by T7 RNA polymerase can be controlled precisely. These plasmids were transformed into *Escherichia coli topA* strain VS111(DE3) as supercoiling reporter plasmids. Their topological status was determined after IPTG induction of expression of T7 RNA polymerase. Figure 1 shows results of such experiments for plasmids pLUC5 and pLUC9. Before IPTG induction, the plasmids had a superhelical density, σ, of approximately −0.06 to −0.07 (Figure 1a, lane 1; Figure 1b, lane 1). In the presence of the DNA intercalator chloroquine (5 μg/ml), these two plasmids migrated during agarose gel electrophoresis as if they contained a few negative supercoils. After IPTG induction, as expected, the plasmids became hypernegatively supercoiled (estimated $\sigma < -0.11$), and the amount of hypernegatively supercoiled DNA was dependent on the IPTG concentration added into the cell culture (Figure 1). Eventually, all plasmids were hypernegatively supercoiled for both DNA templates (Figure 1a, lane 6; Figure 1b, lanes 6–7). However, the amount of IPTG required to drive the plasmid DNA into hypernegatively supercoiled DNA was different for these two plasmids. For example, 25 μM of IPTG could not induce the production of hypernegatively supercoiled DNA for pLUC5 in five min (Figure 1a, lane 2); in contrast, 10 μM of IPTG was sufficient to induce

some pLUC9 into hypernegatively supercoiled DNA at an equivalent time (Figure 1b, lane 2). The IPTG-induced DNA supercoiling required the host strains to have an inducible T7 RNA polymerase gene on the chromosome and the reporter plasmids to contain a T7 promoter. Plasmids pLUC3, 5, and 9 in *Escherichia coli topA* strain VS111 that does not contain a T7 RNA polymerase gene could not be induced into hypernegatively supercoiled status by IPTG (Figure 1c, lanes 1–6). Plasmid pLUC3N that does not have a T7 promoter could not be induced into hypernegatively supercoiled in VS111(DE3) (Figure 1c, compare lane 7 to lane 8; see Figure S3 for the map). These results demonstrated that transcription by T7 RNA polymerase was required for inducing these plasmids into hypernegatively supercoiled DNA. Similar results were also obtained using *Escherichia coli topA* strain DM800(DE3) as the host strain (please see Figure S4 for details).

Hypernegative supercoiling of plasmids was dependent on the length of RNA transcripts *in vivo*

We also measured the time course of DNA supercoiling for plasmids pLUC5 and 9 in VS111 (DE3) after IPTG induction. As demonstrated previously, these two plasmids produce different lengths of RNA transcripts by T7 RNA polymerase.[21] The average size of RNA chains for pLUC5 & 9 are 162 and 3020 nucleotides, respectively.[21] As anticipated, the amount of hypernegatively supercoiled DNA was dependent on the induction time for the two plasmids: the longer the induction, the more hypernegatively supercoiled DNA was produced (Figure 2). However, the rate of producing hypernegatively supercoiled DNA is different for these two plasmids. pLUC5 that produces the shorter length of RNA transcripts had a slower kinetic rate of becoming hypernegatively supercoiled DNA (Figure 2a and 2c). It took 40 min to drive most pLUC5 DNA into hypernegatively supercoiled DNA (~ 67%, Figure 2c) after addition of 25 μM of IPTG into the cell culture (Figure 2a, lane 5). In contrast, it took about 10 min to drive most pLUC9 DNA into hypernegatively supercoiled (~ 90%, Figure 2c; Figure 2b, lane 3); all DNA became hypernegatively supercoiled after 20 min induction (Figure 2b, lanes 4 and 5; Figure 2c). These results suggest that TCDS is dependent on the length of RNA transcripts in this *in vivo* system. Similar results were obtained when 50 μM of IPTG was used to induce TCDS by T7 RNA polymerase in VS111(DE3) containing these two plasmids (data not shown).

We decided to examine more thoroughly the relationship between the RNA transcript length and TCDS in *Escherichia coli topA* strain VS111(DE3). We used the series of pLUC plasmids as described above (Figure S2; reference[21]), which produce transcripts varying from ~160 bases to ~3.1 kb in length by T7 RNA polymerase. As a first step, Northern blotting experiments were utilized to confirm that these pLUC plasmids produced various lengths of RNA transcripts in *Escherichia coli* after IPTG induction of expression of T7 RNA polymerase (Figure 3a). Two oligonucleotide probes, their sequences and targeted regions depicted in Figure S2, were used. Each probe is complementary to a unique site on the template and to the primary RNA transcripts that encompass the site. As anticipated, the *Escherichia coli rrnB* T1 transcription terminators efficiently stopped transcription initiated from the T7 promoter in *Escherichia coli* cells. Probe FL311, as designed, only hybridized with RNA isolated from *Escherichia coli* strain VS111(DE3) harboring plasmids pLUC3, 7, and 9 after IPTG induction (Figure 3a, lanes 2–4 of the upper panel). This probe did not hybridize with RNA isolated from *Escherichia coli* strain VS111(DE3) containing plasmid pLUC5 (Figure 3a, lane 1 of the upper panel). Probe FL312 only hybridized with RNA isolated from *Escherichia coli* strain VS111(DE3) containing plasmids pLUC3 and 9 after IPTG induction (Figure 3a, lanes 2 and 3 of the middle panel). It did not hybridize with RNA isolated from VS111(DE3) harboring plasmids pLUC5 and 7 (Figure 3a, lanes 1 and 4 of the middle panel). The average transcript length produced from pLUC7, 3, and 9 by T7 RNA polymerase was estimated to be about 1, 2, and 3 kb,

respectively (Figure 3a). These *in vivo* results are consistent with those obtained from *in vitro* transcription assays by T7 RNA polymerase.[21]

The superhelical status of each plasmid was examined after 5 min of IPTG induction. Figure 3b shows the results of the experiment in which 25 μM of IPTG was added into the cell culture. A small amount of hypernegatively supercoiled DNA (~7%, Figure 3c) was produced when the transcript length was ~160 nucleotides, but most DNA molecules gained only a few negative supercoils (compare lane 1 of Figure 3b with lane 1 of Figure 2a). When the average transcript length was 1 kb or longer, a large portion of the plasmid DNA was converted to a hypernegatively supercoiled state. The percentage of hypernegatively supercoiled DNA is proportional to the RNA transcript length produced during transcription by T7 RNA polymerase (Figure 3b, compare lanes 1 to 4; Figure 3c). These results clearly demonstrated that TCDS in *Escherichia coli topA* strain VS111(DE3) by T7 RNA polymerase is dependent on the length of RNA transcripts. Similar results were also obtained when 50 μM of IPTG was added into cell culture of VS111(DE3) containing these plasmids (data not shown) or when using another *Escherichia coli topA* strain DM800(DE3) as the host strain (Figure S4).

Hypernegative supercoiling of plasmids did not require coupling transcription to translation and membrane insertion of a nascent protein

As demonstrated previously by other groups, transcription-induced hypernegative supercoiling of plasmids is dependent on anchoring DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins in *Escherichia coli topA* strains.[15–17] This conclusion came from rather rigorous analyses of the topological status of plasmid pBR322 and its derivatives in *topA* strains in which hypernegative supercoiling of these plasmids requires the cotranscription and translation of membrane-associated proteins by *Escherichia coli* RNA polymerase.[15–17] However, some complications do exist in these analyses. Plasmid pBR322 has 7 *Escherichia coli* RNA polymerase promoters (Figure 1 of reference [17]), which initiate transcription within two regions, one centering at around EcoR I site and another around the DNA replication origin.[20] Among them, three promoters, i.e., P2, P4, and P5, initiate transcription clockwise; four other promoters, i.e., P1, P3, P_P, and P_{crp}, initiate transcription counterclockwise. It is difficult to determine which promoter or promoters contribute significantly to the hypernegative supercoiling of plasmid pBR322 although P2 was assumed to contribute the most to TCDS in *Escherichia coli topA* strains.[15,17] In addition, hypernegative supercoiling of plasmid pBR322 was depending on the growth phase at which the cells were harvested: pBR322 isolated from *topA* strains in late stationary phase, e.g. overnight cell culture, did not become hypernegatively supercoiled.[9] Because of these complications, we decided to examine whether TCDS by T7 RNA polymerase in *Escherichia coli topA* strain VS111(DE3) requires anchoring DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins. Our results, however, showed that TCDS did not depend on coupling transcription to translation and membrane insertion of a nascent protein.

As described above, we used a series of pLUC plasmids to study TCDS in *Escherichia coli topA* strain VS111(DE3). These plasmids are derivatives of pUC18 in which a 614 bp fragment containing the multiple cloning sites and a *lac* promoter was removed; a 1.87 kb BamH I-Hind III fragment from pRLM4 that encodes cytosolic neomycin phosphotransferase II was inserted.[21] No membrane-associated protein or peptide was expressed by these plasmids. As shown above, these plasmids can be induced into hypernegatively supercoiled status. We also showed that TCDS in this new *in vivo* system is dependent on the length of RNA transcripts. These results demonstrated that TCDS by T7 RNA polymerase in *Escherichia coli topA* strains VS111 (DE3) did not require coupling transcription to translation and to membrane insertion of a nascent protein.

In order to further study TCDS in *Escherichia coli topA* strain VS111(DE3), two plasmids, pBGFuv and pBGRuv, have been made in which green fluorescence protein uv (*GFPuv*) gene was cloned into the unique Hind III site of pLUC1 (Figure 4). These two plasmids are identical except for the orientation of the *GFPuv* gene. pBGFuv contains the *GFPuv* gene in the correct orientation that can be induced to over-express GFPuv by IPTG (data not shown). pBGRuv contains *GFPuv* gene in the reverse orientation, which cannot direct over-expression of GFPuv after IPTG induction (no open reading frame was discovered in the reverse orientation). If TCDS requires anchoring DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins in *Escherichia coli topA* strains, these two plasmids should not become hypernegatively supercoiled after IPTG induction. Our results, however, demonstrated that both plasmids were induced into hypernegatively supercoiled status by IPTG (Figures 4 and S5). These results suggest that TCDS by T7 RNA polymerase is not dependent on coupling transcription to translation and membrane insertion of a nascent protein in *Escherichia coli topA* strain VS111(DE3). Interestingly, the supercoiling rate of pBGFuv (the correct orientation) is slightly higher than that of pBGRuv (the reverse orientation) (compare Figure 4a and 4b; Figure S5c), suggesting that coupling transcription to translation may enhance the TCDS efficiency.

We also constructed another two plasmids, pTetF and pTetR to examine whether transcription of a gene encoding a membrane-association protein affects efficiency of TCDS in our new *in vivo* system. Both plasmids have a promoterless membrane association gene, *tet* (encoding tetracycline resistance protein) downstream of the T7 promoter (Figure 5). These two plasmids are identical except for the orientation of the *tet* gene. pTetF contains a *tet* gene in the correct orientation which can be induced to express tetracycline resistance protein by IPTG. In contrast, pTetR contains a *tet* gene in the reverse orientation which cannot be induced to express tetracycline resistance protein by IPTG. We found that *Escherichia coli* strain VS111(DE3) containing pTetF grew in the presence of tetracycline and grew much faster upon addition of IPTG (data not shown). These results demonstrated that IPTG can induce expression of tetracycline resistance protein encoded by pTetF. *Escherichia coli* strain VS111(DE3) containing pTetR did not grow in the presence of tetracycline with or without IPTG, suggesting that IPTG cannot induce expression of tetracycline resistance protein for pTetR. Figure 5 shows results of the *in vivo* T-S assays by T7 RNA polymerase for pTetF and pTetR in VS111(DE3) after IPTG induction. As expected, both orientations produce hypernegatively supercoiled plasmid DNA (Figure 5a and 5b). However, the supercoiling rates of these two plasmids are similar but not identical. It takes less time for pTetR to become hypernegatively supercoiled DNA than for pTetF (Figure S6). These results further suggest that hypernegative supercoiling of plasmids does not require anchoring DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins in our *in vivo* system.

Discussion

The new *in vivo* transcription supercoiling system presented in this report has extended our previous studies on TCDS[12,21] into *Escherichia coli*. It is an IPTG-inducible system that allows precise control over the length and location of RNA transcript synthesis by T7 RNA polymerase *in vivo* (Figure 3a). Our studies of TCDS in this new system, for the first time, unambiguously demonstrated that TCDS in *Escherichia coli topA* strains is dependent on the length of RNA transcripts (Figure 3b and 3c), a functional property that has also been demonstrated in our *in vitro* transcription supercoiling system when certain sequence-specific DNA-binding proteins were present during transcription.[21] These results suggest that the two transcription supercoiling systems resemble each other in nature. Moreover, we showed that transcription by a small rapid T7 RNA polymerase potently supercoiled the DNA templates in *Escherichia coli*. The supercoiling activity did not require coupling transcription to translation

and membrane insertion of a nascent protein. These discoveries have provided new insights into TCDS *in vivo*.

Our results mostly favor the "twin-supercoiled-domain" model of transcription.[13] As demonstrated above, the hypernegatively supercoiled plasmid DNA generated in *Escherichia coli topA* strains depended on transcription by T7 RNA polymerase where expression of T7 RNA polymerase in the host strains and presence of a T7 promoter on the supercoiling reporter plasmids were required (Figure 1c). Transcription from *Escherichia coli* promoters, however, did not significantly contribute to TCDS because the plasmids did not become hypernegatively supercoiled before IPTG induction. One possible reason would be that each plasmid used in this study contains a few weak *Escherichia coli* promoters, which resulted in very low rates of transcription initiation. In this case, most plasmids in *Escherichia coli* cells might not have an active transcriptional unit to generate the formation of twin supercoiled domains on the plasmids. In addition, transcription elongation rate by *Escherichia coli* RNA polymerase is relatively low; it may not be able to induce the formation of significant amounts of localized supercoiled domains. TCDS by *Escherichia coli* RNA polymerase in this case might be negligible. In contrast, T7 RNA polymerase is one of the most rapid RNA polymerases; it is able to incorporate nucleotides into a transcript at a rate of 200–400 nucleotides per second.[25] The T7 promoter is a very strong promoter that efficiently and specifically initiates transcription by T7 RNA polymerase. Transcription elongation by T7 RNA polymerase should significantly induce production of the twin supercoiled domains in *Escherichia coli* cells. Indeed, transcription by T7 RNA polymerase greatly supercoiled all plasmids containing a T7 promoter in *Escherichia coli topA* strain VS111(DE3). Furthermore, TCDS was proportional to the length of RNA transcripts being synthesized (Figure 3), precisely predicted by the "twin-supercoiled-domain" model of transcription.[13] All evidence suggests that the twin-domain type of supercoiling mechanism is at work *in vivo*.

As pointed out by Wang, Liu, and others,[13,15,19,26] TCDS on a plasmid requires two barriers (Figure 6). The first barrier comes from preventing or retarding a transcriptional machinery (including the transcribing RNA polymerase and the newly synthesized transcript) from rotating around the DNA helix, which helps generate the twin supercoiled domains.[19,26] In a dilute aqueous solution, the friction torque on a transcribing T7 RNA polymerase is too small to cause significant supercoiling.[13] However, in a highly viscous or crowded aqueous solution that more closely approximates the situation found *in vivo*, the friction torque on a transcribing T7 RNA polymerase is much larger, which should greatly supercoil the DNA templates. Indeed, we previously showed that molecular crowding agents, such as polyethylene glycol and polyvinyl alcohol, strongly stimulated TCDS in defined protein systems (Figure 8 of reference[21]) and transcripts as short as 250 nucleotides are capable of activating TCDS through the twin supercoiled domain mechanism of transcription when molecular crowding agents are present.[21] Our results presented in this paper further demonstrated that transcription by T7 RNA polymerase in *Escherichia coli topA* strains where it is very viscous and crowded significantly supercoiled plasmid DNA. These results suggest that the friction barrier applied on the transcriptional ensemble of T7 RNA polymerase *in vivo* was sufficient to produce the twin supercoiled domains. The dependence of TCDS on the length of RNA transcripts further demonstrated that the friction torque on the transcription ensemble was a determinant factor for supercoiling the plasmid DNA templates in *Escherichia coli*. As discussed above, previous studies by other groups showed that the friction barrier arising from the transcribing RNA polymerase requires anchoring or inserting the coupled transcription-translation complex into the cytoplasmic membrane,[15–17] which prevents the transcriptional machinery from rotating around the DNA double helix. Our results, however, demonstrated that anchoring or inserting the coupled transcription-translation complex into the cytoplasmic membrane is not required for TCDS by T7 RNA polymerase in *Escherichia coli topA* strains (Figures 4 and 5). This discrepancy may result from the fact that *Escherichia coli topA* strains used in studies by other

groups were those with a compensatory mutation in the gyrase gene that produces a less active DNA gyrase.[23,24] In these cases, TCDS is compromised. We tested this possibility by using *Escherichia coli topA* strain DM800(DE3) that has a less active gyrase activity and found that transcription by T7 RNA polymerase also significantly induced generation of hypernegatively supercoiled DNA (Figure S4). Obviously, a less active DNA gyrase can convert transcription-coupled supercoiling domains into permanent negative supercoils in this *in vivo* system. Another reason would be that a much faster RNA polymerase, T7 RNA polymerase was used in our studies, which efficiently generated the twin supercoiled domains even when the transcriptional machinery did not couple to translation and membrane insertion. In this scenario, DNA gyrase may work on the transient positively supercoiled domains to produce hypernegatively supercoiled plasmid DNA in *topA* strains. Nevertheless, our results showed that TCDS by T7 RNA polymerase does not require expression of membrane-association proteins in this *in vivo* system.

The second barrier is a DNA topological barrier to prevent or slow down DNA supercoiling diffusion along the longitudinal helical axis of the DNA template (Figure 6). In this case, the positively supercoiled domains cannot cancel the negatively supercoiled domains generated during transcription elongation. Therefore, DNA gyrase can convert the positively supercoiled domains into “permanent” negative supercoils to produce hypernegatively supercoiled plasmid DNA in *Escherichia coli topA* strains. Initially, a big cellular structure such as cell membrane was thought to be required for forming such a topological barrier.[13,19] However, recently, we discovered that certain nucleoprotein complexes, perhaps those that contain sharply bent DNA sites or unwound DNA sequences, can form such barriers that impede the diffusion and merger of the oppositely supercoiled DNA domains.[12] pLUC plasmids each contain one or more DNA-binding sites for several sequence-specific DNA-binding proteins, such as IHF and ArgR, which have potentials to form nucleoprotein complexes to serve as the topological barrier. In addition, *Escherichia coli* RNA polymerase, as shown previously,[12] may associate with *Escherichia coli* promoters on these DNA plasmids to promote the coupled supercoiling during transcription elongation. Nevertheless, the second barrier does exist for TCDS on pLUC plasmids. In this case, transcription by T7 RNA polymerase should significantly supercoil the DNA templates through the “twin-supercoiled-domain” mechanism (Figure 6).

An additional point needed to be discussed is the effect of the promoter strength on the coupled DNA supercoiling. As showed by Chen and Lilley[27] and also by Stupina and Wang,[26] insertion of strong promoters into *tet*-carrying plasmids greatly enhanced the efficiency of TCDS in *topA* strains. Due to the short transcripts produced in the promoter-terminator cassettes, Chen and Lilley proposed that a repeated cycle of transcription initiation and termination would introduce significant amounts of DNA unwinding into the plasmids. In *topA* strains, this DNA unwinding process may lead to accumulation of negative supercoils in the DNA templates.[27,28] T7 promoter is a very strong promoter. It is possible that this mechanism may also contribute to TCDS by T7 RNA polymerase in our new *in vivo* system.

Our results, shown in this article, suggest that a transcriptional ensemble (including the transcribing RNA polymerase and the RNA transcript) alone is sufficient to cause the change of local DNA superhelicity. This topological change of local chromosome structure can have great impact on the physiological functions of certain critical DNA sequence elements, such as the promoter regions and DNA replication origins.[29–31] Dunaway and Ostrander have previously showed that transcription by T7 RNA polymerase activated a eukaryotic promoter in *Xenopus oocytes* through transient TCDS.[29] Replication origins are often found near transcription units[31] and actively transcribed sequences are generally replicated earlier in S phase.[32] These results indicate that transient TCDS is likely to play an active role in influencing the nearby DNA transactions, such as gene expression and DNA replication.

Materials and Methods

Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG), ethidium bromide, ampicillin, kanamycim, chloroamphenical, chloroquine, and L-cysteine were obtained from Sigma-Aldrich Corporation (St. Louis, MO). All restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). *Pfu* DNA polymerase and Duralon UV Membranes were obtained from Stratagene Inc. (La Jolla, CA). All synthetic oligonucleotides used as probes or primers were purchased from MWG-Biotech, Inc. (High Point, NC). QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, RNeasy Mini Kit, and QIAquick Nucleotide Removal Kit were obtained from QIAGEN Inc. (Valencia, CA). T7 RNA Polymerase Monoclonal Antibody, HRP-conjugated anti-mouse antibody, SuperSignal Substrate, and λDE3 Lysogenization Kit were purchased from EMD Biosciences, Inc. (Madison, WI). [γ-^{32}P]ATP was obtained from Amersham Biosciences (Piscataway, NJ).

Bacterial strains and plasmids

Escherichia coli strain VS111 [F^{-} *LAM- rph-I* Δ*topA*] as described in Stupina and Wang[22] was obtained from the *Coli* Genetic Stock Collection/*Escherichia coli* Genetic Resource Center (CGSC) at Yale University. *Escherichia coli* strain DM800 [F^{-} Δ*(topA cysB)204 arcA13*, *gyrB225*] was kindly provided by Dr. Marc Drolet at Universite de Montreal. *Escherichia coli* strains VS111(DE3) and DM800(DE3) were constructed using a λDE3 Lysogenization Kit to integrate a T7 RNA polymerase gene under control of *Escherichia coli lacUV5* promoter into the host strain chromosome. A detailed procedure was provided by the manufacturer. Briefly, the host strains (VS111 and DM800) were simultaneously infected with three λ phages, a DE3 phage, a helper phage that provides the *int* function, and a selection phage incapable of integrating into the host cell. The selection phage will kill any nonlysogen λDE3 host range mutants but not the λDE3 lysogens. To test for correct integration, the selected colonies were subjected to a tester phage, which is a T7 RNA polymerase deletion mutant. In the presence of IPTG, the *Escherichia coli* cells infected with this phage form plaques indicating correct insertion of λDE3 into the host strain chromosome.

All plasmids were derived from pUC18. Plasmids pLUC1, 3, 5, 7, and 9 were described previously.[12,21] Plasmid pLUC3N was constructed by deleting from pLUC3 a 963 bp Nco I DNA fragment that contains a T7 promoter. Plasmids pBGFuv and pBGRuv were constructed by inserting, into the unique Hind III site of pLUC1, a 793 bp Hind III DNA fragment containing a green fluorescence protein uv (*GFPuv*) gene that was amplified from plasmid pGFPuv (Stratagene Corporate, La Jolla, CA) using polymerase chain reaction (PCR). These two plasmids are identical expect for the orientation of the *GFPuv* gene. pBGFuv contains the correct orientation of *GFPuv* gene that can be induced to over-express GFPuv by IPTG. pBGRuv contains *GFPuv* gene in the reverse orientation, which cannot direct over-expression of GFPuv after IPTG induction. Plasmids pTetF and pTetR were also constructed by inserting, into the unique Hind III site of pLUC1, a 1229 bp Hind III DNA fragment containing a promoterless tetracycline resistance gene (*tet*) that was amplified from plasmid pBR322 using PCR. These two plasmids are identical expect for the orientation of the *tet* gene. pTetF contains the correct orientation of the *tet* gene that can be induced to over-express tetracycline resistance protein by IPTG. pTetR contains the *tet* gene in the reverse orientation, which cannot direct over-expression of tetracycline resistance protein after IPTG induction.

In vivo Transcription-Supercoiling (T-S) assay

Escherichia coli cells were grown overnight in Luria broth (LB) containing appropriate antibiotics. The overnight culture was then diluted at 1:100 ratio in fresh LB containing appropriate antibiotics and grown until optical density of cells at 600 nm reached approximately

0.5. At this point, IPTG was added directly to the culture to induce expression of T7 RNA polymerase as indicated. Plasmid DNA was extracted from cells using QIAgen Miniprep Kit. The topological status of each DNA preparation was analyzed by electrophoresis in a 1% agarose gel in 1 × TAE buffer containing 5 μg/ml chloroquine. Following electrophoresis, the agarose gels were stained with ethidium bromide, destained, and photographed under UV light. A few gels were stained with SYBR gold as needed. The intensity of the DNA topoisomers including the hypernegatively supercoiled DNA band was determined using KODAK 1D Image Analysis Software. The percentage of hypernegatively supercoiled DNA was calculated from a comparison of the intensity of the hypernegatively supercoiled band with the total intensity of all DNA topoisomers.

Western blotting experiment

Western blotting experiments were used to verify expression of T7 RNA polymerase in *Escherichia coli topA* strains VS111(DE3) and DM800(DE3) after IPTG induction. Briefly, total protein of *Escherichia coli* cells was analyzed by electrophoresis in a 10% SDS-PAGE and electrophoretically transferred to a 0.45 nm nitrocellulose membrane. After transfer, the membrane blot was blocked with a solution containing 5% non-fat skim milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature with agitation. It was then washed 3 times with TBST and incubated with the primary antibody, anti-T7 RNA polymerase monoclonal antibody, diluted 1:10,000 in a solution containing 5% BSA in TBST overnight at 4 °C with agitation. The membrane blot was washed 3 times again with TBST and incubated for 1 hour with a secondary antibody HRP-conjugated anti-mouse IgG at room temperature with agitation. The immunoreactive T7 RNA polymerase was detected with chemiluminescence SuperSignal HRP Substrate.

Northern blotting experiment

Total RNA was isolated from *Escherichia coli* cells using the Qiagen RNeasy Kit as described by the manufacturer. The RNA samples were resolved by electrophoresis through a 1.3% agarose gel that was prepared in MOPS buffer containing formaldehyde (20 mM MOPS, pH 7.0, 8 mM NaAc, 1 mM EDTA, and 1% formaldehyde). Following electrophoresis, the RNA was transferred to a Duralose nylon membrane overnight using wet capillary transfer method in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and fixed by UV cross-linking. The membrane was then prehybridized in prehybridization buffer (1M NaCl, 0.1% SDS, 500 μg/ml denatured salmon sperm DNA and 50% deionized formamide) for 1.5 hours at 42 °C and hybridized in the same prehybridization buffer containing ^{32}P-labeled nucleotide probes FL311 (5′-GACAAGACCGGCTTCCATCCGAGTAC-3′) or FL312 (5′-CTGGCAATTCCGGTTCGCTTGCTGTCC-3′) overnight at 42 °C. Following hybridization, the membrane was washed once at room temperature with 0.01 × SSC and 0.1% SDS solution and then three times at 45 °C to remove any unbound probe. Hybridization was detected by autoradiography.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TCDS
: transcription-coupled DNA supercoiling

T-S
: transcription-supercoiling

IPTG
: isopropyl-β-D-thiogalactopyranoside

GFPuv
: green fluorescence protein uv

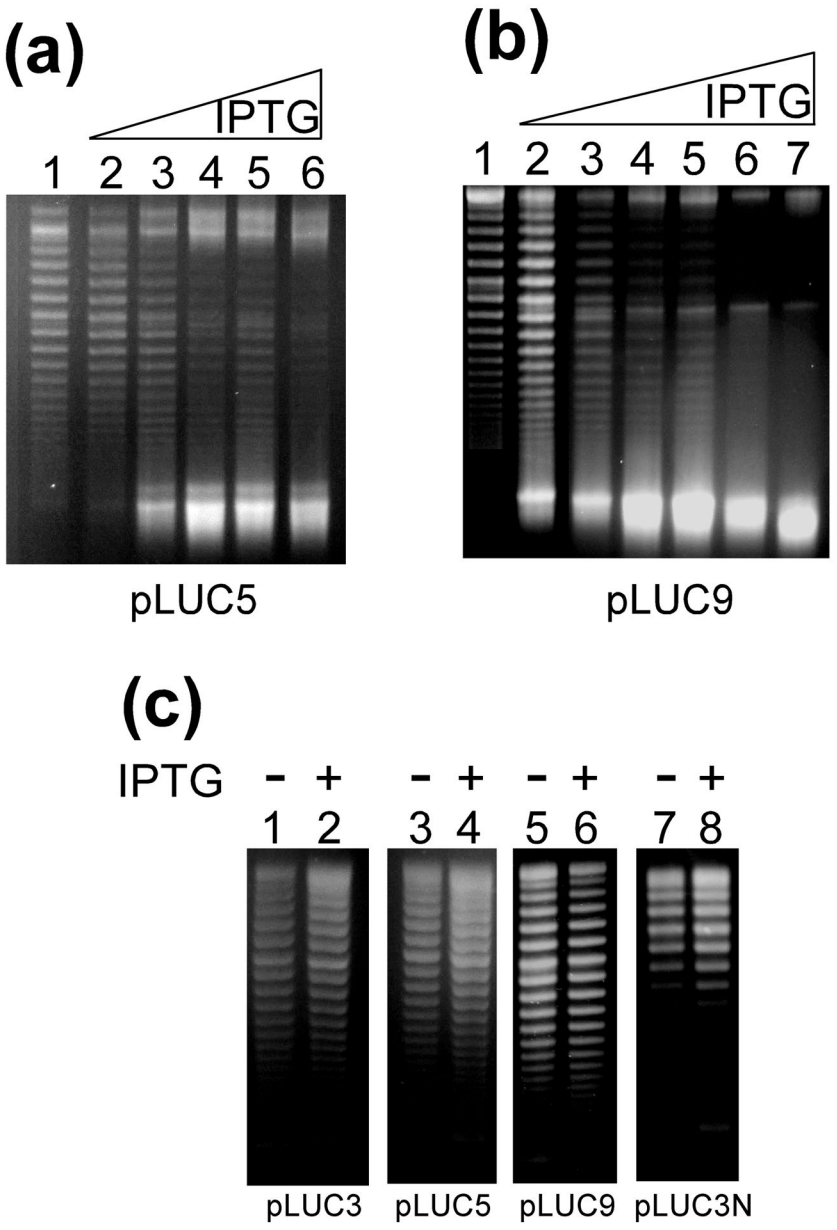

Figure 1.

Dependence of TCDS on the IPTG concentration in *Escherichia coli topA* stain VS111(DE3). The *in vivo* T-S assay was performed as described under "Materials and Methods." The DNA topological status was analyzed by electrophoresis in a 1% agarose gel containing 5 μg/ml chloroquine and stained with ethidium bromide. (a) The DNA template was plasmid pLUC5 in VS111(DE3). Lane 1 contained the DNA sample isolated from *Escherichia coli* cells prior to IPTG induction. Lanes 2–6 contained the DNA samples isolated from *Escherichia coli* cells after 5 min of induction with 25, 50, 75, 100, and 1000 μM of IPTG, respectively. (b) The DNA template was plasmid pLUC9. Lane 1 contained the DNA sample isolated from *Escherichia coli* cells prior to IPTG induction. Lanes 2–7 contained the DNA samples isolated from *Escherichia coli* cells after 5 min of induction with 10, 25, 50, 75, 100, and 1000 μM of IPTG, respectively. (c) Expression of T7 RNA polymerase in host strains and presence of a T7 promoter on plasmid DNA templates are required for hypernegative supercoiling of plasmid

DNA. Lanes 1 and 2 contained plasmid pLUC3 from *Escherichia coli topA* strain VS111. Lanes 3 and 4 contained plasmid pLUC5 from *Escherichia coli topA* strain VS111. Lanes 5 and 6 contained plasmid pLUC9 from *Escherichia coli topA* strain VS111. Lanes 7 and 8 contained plasmid pLUC3N from *Escherichia coli topA* strain VS111(DE3). DNA samples were isolated from *Escherichia coli* cells before (lanes 1, 3, 5, and 7) or after 30 min of 1 mM of IPTG induction as indicated (lanes 2, 4, 6, and 8).

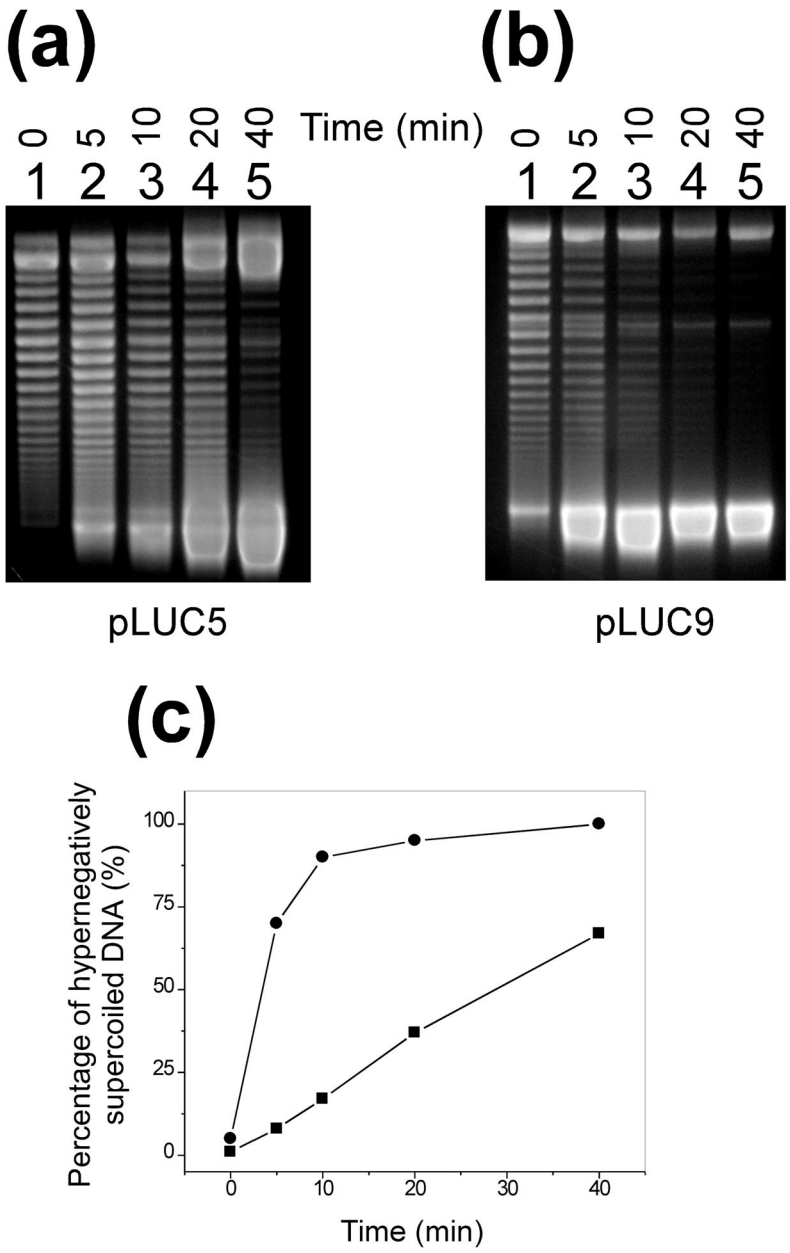

Figure 2.

Time courses of hypernegative supercoiling of plasmids in *Escherichia coli topA* strain VS111 (DE3). The *in vivo* T-S assay was performed as described under "Materials and Methods." DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 5 μg/ml chloroquine. (a) Lanes 1 to 5 contained, respectively, pLUC5 DNA samples isolated from VS111(DE3) after 0, 5, 10, 20, and 40 min of 25 μM of IPTG induction. (b) Lanes 1 to 5 contained, respectively, pLUC9 DNA samples isolated from VS111(DE3) after 0, 5, 10, 20, and 40 min of 25 μM of IPTG induction. (c) The percentage of hypernegatively supercoiled DNA for pLUC5 (squares) and pLUC9 (circles) is a function of IPTG induction time. These results were calculated as described under "Materials and Methods" using the TCDS data shown in (a) and (b).

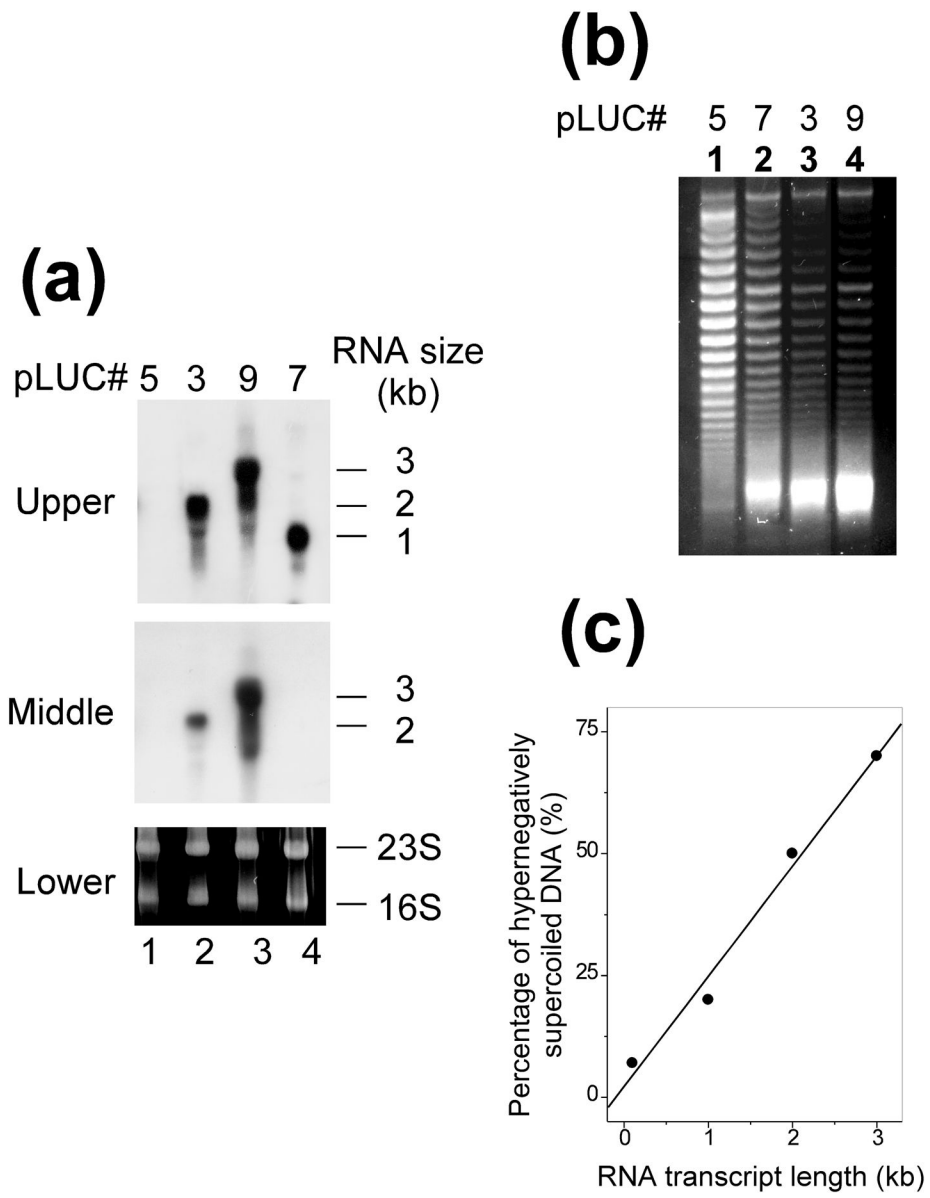

Figure 3.

TCDS was dependent on the length of RNA transcripts in *Escherichia coli topA* strain VS111 (DE3). (a) Northern blotting analyses were used to determine the length of RNA transcripts by T7 RNA polymerase after 20 min of 1 mM IPTG induction. Northern blotting experiments were performed as described under "Materials and Methods." 10 μg of RNA samples were electrophoresed through a 1.3% agarose gel that contained 1% formaldehyde and visualized by autoradiography. Lanes 1 to 4 contained, respectively, RNA samples isolated from VS111 (DE3) containing plamids pLUC5, 3, 9, and 7. The upper panel was the membrane hybridized with probe FL311. The middle panel was the membrane hybridized with probe FL312. The lower panel was the agarose gel stained by SYBR Gold stain to show the integrity of the RNA samples used for the Northern blotting experiments. (b) The *in vivo* T-S assay was performed as described under "Materials and Methods." The DNA topological status was analyzed by electrophoresis in a 1% agarose gel containing 5 μg/ml chloroquine and stained with ethidium bromide. Lanes 1 to 4 contained DNA samples isolated from *Escherichia coli topA* strain

VS111(DE3) containing plasmids pLUC5, 7, 3, and 9, respectively, after 5 min of 25 μM of IPTG induction. (c) The percentage of hypernegatively supercoiled DNA, determined as described under “Materials and Methods,” is proportional to the RNA transcript length.

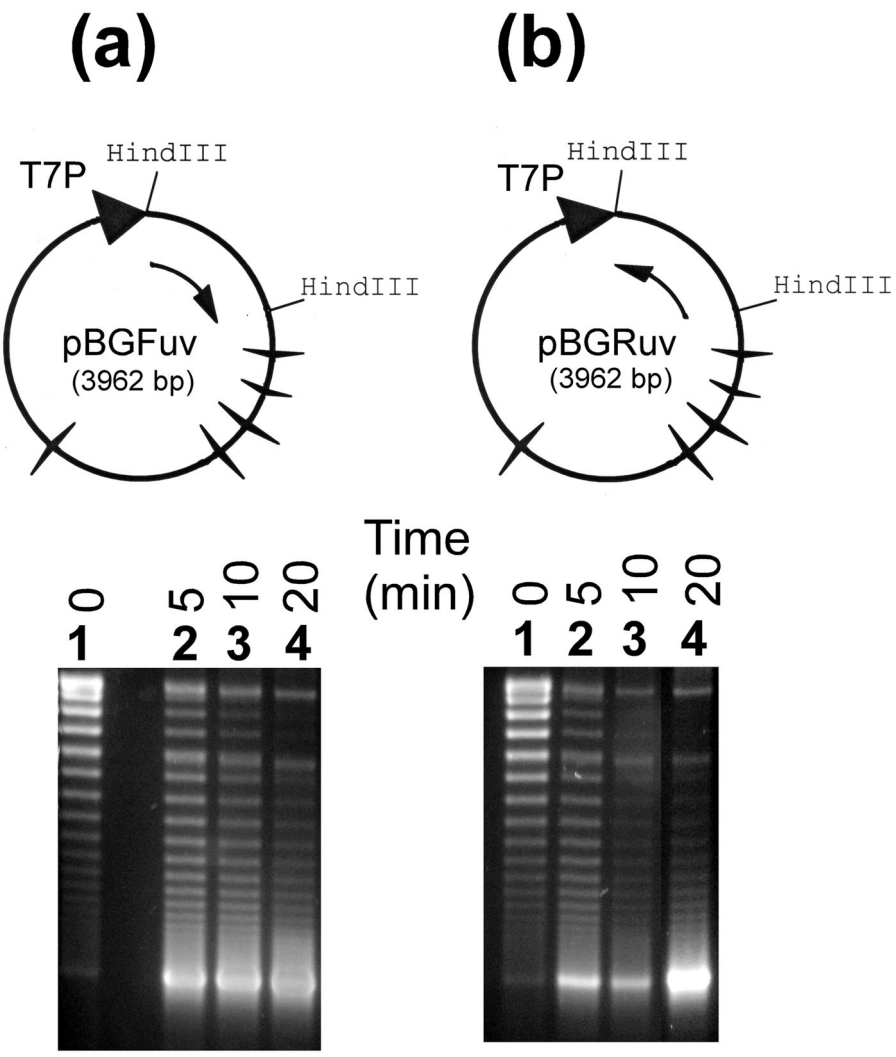

Figure 4.

Coupling transcription to translation of a nascent protein was not required for hypernegative supercoiling of plasmids by T7 RNA polymerase in *Escherichia coli topA* strain VS111(DE3). The *in vivo* T-S assay was performed as described under "Materials and Methods" for plasmids pBGFuv (a) and pBGRuv (b) in *Escherichia coli* strain VS111(DE3). Transcription was induced with 50 μM of IPTG. Lane 1 contained the DNA sample before IPTG induction. Lanes 2–4 contained the plasmid DNA samples after 5, 10, and 20 min of IPTG induction, respectively. Maps of plasmids pBGFuv and pBGRuv are shown. See the legend of Figure S2 for description of the symbols. Also see description in "Materials and Methods" for details on plasmid construction. Plasmids pBGFuv and pBGRuv each contain a *GFPuv* gene. These two plasmids are identical expect for the orientation of the *GFPuv* gene. pBGFuv contains the correct orientation of *GFPuv* gene that will be induced to over-express GFPuv with IPTG. pBGRuv contains *GFPuv* gene in the reverse orientation, which cannot direct over-expression of GFPuv after IPTG induction. The open reading frame of the *GFPuv* gene is indicated by the arrow with a small arrowhead.

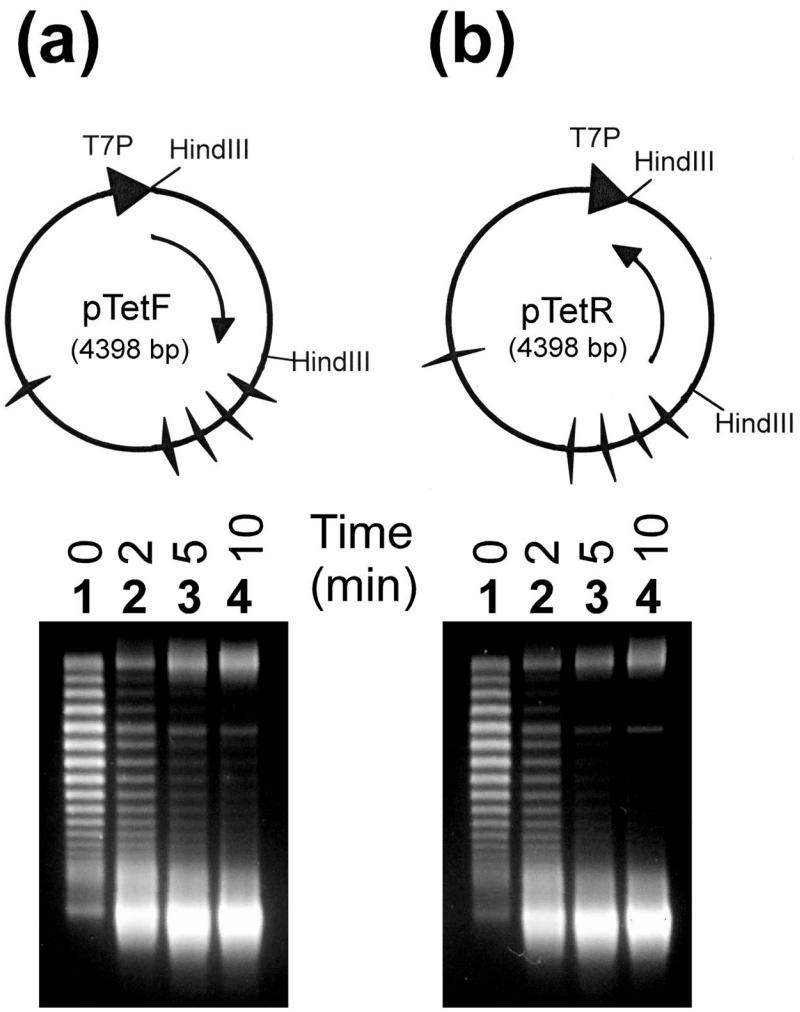

Figure 5.

Coupling transcription to translation and membrane insertion of a nascent protein was not required for hypernegative supercoiling of plasmids by T7 RNA polymerase in *Escherichia coli topA* strain VS111(DE3). The *in vivo* T-S assay was performed as described under "Materials and Methods" for plasmids pTetF (a) and pTetR (b) in *Escherichia coli* strain VS111 (DE3). Transcription was induced with 50 μM of IPTG. Lane 1 contained the DNA sample before IPTG induction. Lanes 2–4 contained the plasmid DNA samples after 2, 5, and 10 min of IPTG induction, respectively. Maps of plasmids pTetF and pTetR are shown. See the legend of Figure S2 for description of the symbols. Also see description in *Materials and Methods* for details on plasmid construction. Plasmids pTetF and pTetR each contains a tet gene. These two plasmids are identical expect for the orientation of the *tet* gene. pTetF contains the correct orientation of the *tet* gene that can be induced to over-express tetracycline resistance protein by IPTG. pTetR contains the *tet* gene in the reverse orientation, which cannot direct over-expression of tetracycline resistance protein after IPTG induction. The open reading frame of the *tet* gene is indicated by the arrow with a small arrowhead.

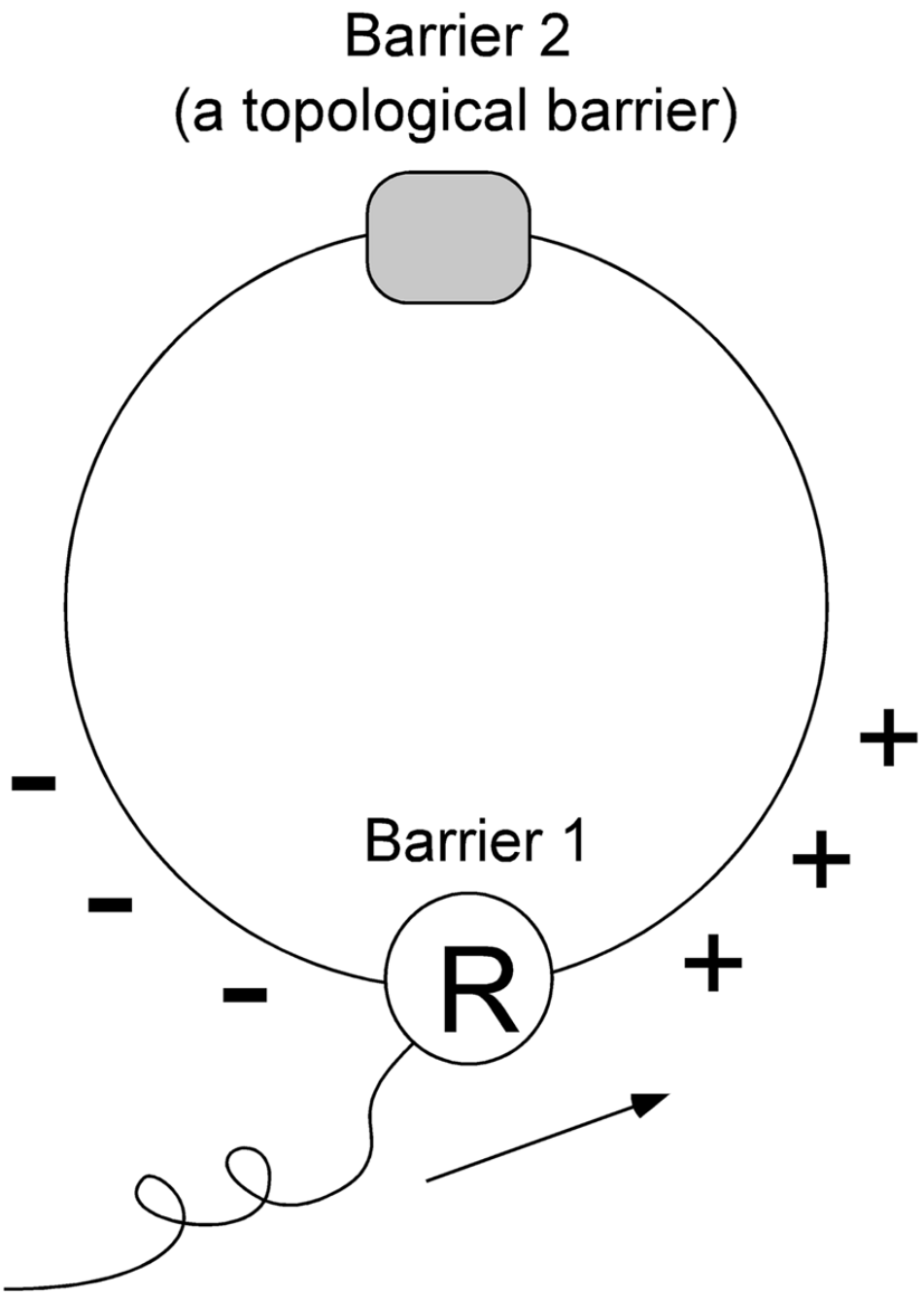

Figure 6.

A two-barrier model to explain TCDS on circular plasmids DNA derived from the "twin-supercoiled-domain" model of transcription. RNA polymerase transcribes counterclockwise to generate a positively supercoiled domain in front of the transcribing RNA polymerase and a negatively domain behind it. A friction barrier (barrier 1) is formed by preventing or retarding of RNA polymerase from rotating around the DNA double helix. A DNA topological diffusion barrier (barrier 2) may be generated from the formation of some nucleoprotein complexes, especially those that contain sharply bent DNA sites or unwound DNA sequences, which impede the diffusion and merger of the twin supercoiled domains.