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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

TRANSCRIPTION-COUPLED DNA SUPERCOILING IN *ESCHERICHIA COLI*:
MECHANISMS AND BIOLOGICAL FUNCTIONS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Xiaoduo Zhi

2013

To: Dean Kenneth G. Furton
College of Arts and Sciences

This dissertation, written by Xiaoduo Zhi, and entitled Transcription-coupled DNA supercoiling in Escherichia coli: Mechanisms and Biological functions, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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ABSTRACT OF THE DISSERTATION

TRANSCRIPTION-COUPLED DNA SUPERCOILING IN *ESCHERICHIA COLI*:

MECHANISMS AND BIOLOGICAL FUNCTIONS

by

Xiaoduo Zhi

Florida International University, 2013

Miami, Florida

Professor Fenfei Leng, Major Professor

Transcription by RNA polymerase can induce the formation of hypernegatively supercoiled DNA both *in vivo* and *in vitro*. This phenomenon has been explained by a “twin-supercoiled-domain” model of transcription where a positively supercoiled domain is generated ahead of the RNA polymerase and a negatively supercoiled domain behind it. In *E. coli* cells, transcription-induced topological change of chromosomal DNA is expected to actively remodel chromosomal structure and greatly influence DNA transactions such as transcription, DNA replication, and recombination.

In this study, an IPTG-inducible, two-plasmid system was established to study transcription-coupled DNA supercoiling (TCDS) in *E. coli topA* strains. By performing topology assays, biological studies, and RT-PCR experiments, TCDS in *E. coli topA* strains was found to be dependent on promoter strength. Expression of a membrane-insertion protein was not needed for strong promoters, although co-transcriptional synthesis of a polypeptide may be required. More importantly, it was demonstrated that the expression of a membrane-insertion *tet* gene was not sufficient for the production of hypernegatively supercoiled DNA. These phenomenon can be explained by the “twin-

supercoiled-domain” model of transcription where the friction force applied to *E. coli* RNA polymerase plays a critical role in the generation of hypernegatively supercoiled DNA.

Additionally, in order to explore whether TCDS is able to greatly influence a coupled DNA transaction, such as activating a divergently-coupled promoter, an *in vivo* system was set up to study TCDS and its effects on the supercoiling-sensitive *leu-500* promoter. The *leu-500* mutation is a single A-to-G point mutation in the -10 region of the promoter controlling the *leu* operon, and the AT to GC mutation is expected to increase the energy barrier for the formation of a functional transcription open complex. Using luciferase assays and RT-PCR experiments, it was demonstrated that transient TCDS, “confined” within promoter regions, is responsible for activation of the coupled transcription initiation of the *leu-500* promoter. Taken together, these results demonstrate that transcription is a major chromosomal remodeling force in *E. coli* cells.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABBREVIATIONS	FULL NAME
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
DTT	Dithiothreitol
IPTG	Isopropyl- β -D-thiogalactopyranoside
<i>E. coli</i>	<i>Escherichia coli</i>
LB	Luria broth
mRNA	Messenger ribonucleic acid
NIH	National Institutes of Health
OD	Optical density
RLU	Relative light units
RNA	Ribonucleic acid
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
SD	Shine-Dalgarnon
TCDS	Transcription-coupled DNA supercoiling

Chapter 1: Transcription-coupled DNA supercoiling in *Escherichia coli topA* mutants

1.1 Transcription-coupled DNA supercoiling (TCDS): the “twin-supercoiled-domain” model

DNA is typically negatively supercoiled in bacteria (Cozzarelli, 1990; Bates and Maxwell, 2005). Changes in the level of DNA supercoiling have been found to play important roles on DNA transactions such as replication, transposition, transcription, and recombination (Cozzarelli, 1990; Kornberg and Baker, 1992; Wang, 1996; Champoux, 2001; Bates and Maxwell, 2005; Wang, 2009). It has been demonstrated that DNA supercoiling is tightly regulated by different DNA topoisomerases *in vivo* (Wang, 1996; Champoux, 2001). There are four DNA topoisomerases in *Escherichia coli*: topoisomerase I, gyrase, topoisomerase III and topoisomerase IV (Wang, 1996; Champoux, 2001). In bacteria, the DNA supercoiling level is tightly regulated by the opposing activities of two enzymes: topoisomerase I and gyrase (Pruss and Drlica, 1989; Zechiedrich et al., 2000; Snoep et al., 2002). They act differently on negatively and positively supercoiled DNA. Topoisomerase I (Massé and Drolet, 1999; Massé and Drolet, 1999), the product of the *topA* gene, functions to relax the negative supercoils in intracellular DNA while DNA gyrase (topo II) (Gellert et al., 1976), which is composed of GyrA and GyrB subunit proteins and has a tetrameric structure, acts to convert a fraction of the transient positive supercoils to “permanent” negative supercoils at the expense of ATP hydrolysis. Inactivating either enzyme results in the production of positive supercoiled DNA or hypernegative supercoiled DNA *in vivo* (Lockshon and

Morris, 1983; Pruss, 1985; Pruss and Drlica, 1986; Wu et al., 1988; Lodge et al., 1989; Tsao et al., 1989; Lynch and Wang, 1993; Ma et al., 1994). Moreover, the linking number change caused by inactivation of either topoisomerase is directly related to the transcription of the DNA and relative orientation of the transcription units (Wu et al., 1988).

In *E. coli*, countervailing activities of topoisomerase I and gyrase are homeostatically regulated (Dinardo et al., 1982): an increase in the degree of negative supercoiling reduces the transcription of the *gyrA* and *gyrB* genes, which encodes the two subunits of gyrase, and elevates the transcription of the *topA* gene, the gene that encodes DNA topoisomerase I; a decrease in the degree of negative supercoiling has the opposite effects on the expression of these genes (Menzel and Gellert, 1983; Tse-Dinh, 1985; Tse-Dinh and Beran, 1988). It has been demonstrated that *E. coli topA* strains are not viable unless they acquire compensatory mutations such as mutations in the *gyrA* and *gyrB* that encode gyrase (Dinardo et al., 1982; Pruss et al., 1982; Raji et al., 1985; Dorman et al., 1989). The suppression of the lethal phenotype of *topA* by compensatory mutations indicates the proper level of supercoiling in *E. coli* is essential and is controlled by the diametric functions of enzymes.

Early studies have shown the effects of transcription on DNA topology in *E. coli* cells. The first case was a study by Lockshon and Morris in which a substantial fraction of the plasmid pBR322 isolated from *E. coli* cells after exposure to DNA gyrase inhibitors, such as oxolinic acid and novobiocin, became positively supercoiled (Lockshon and Morris, 1983). Effects of gyrase alone cannot explain the formation of the

positively supercoiled plasmid (Liu and Wang, 1987). In a later study by Pruss (Pruss, 1985), it was demonstrated that pBR322 isolated from *E. coli* or *Salmonella typhimurium topA* mutants lacking DNA topoisomerase I is hypernegatively supercoiled. The distribution of the DNA topoisomers is much broader than that isolated from the wild type strains. In 1986, Pruss and Drlica showed that plasmid pBR322, but not pUC9 (a smaller high copy number plasmid, which is derived from pBR322), isolated from *E. coli topA* strains, became hypernegatively supercoiled (Pruss and Drlica, 1986). On the basis of these observations, Liu and Wang formulated an elegant model, the “twin-supercoiled-domain” model of transcription, to explain these results (Liu and Wang, 1987). They hypothesized that during the transcription elongation, the transcribing complex (include the polymerase, its nascent RNA, and RNA associated proteins) becomes progressively more difficult to rotate around the DNA double helix as the size of the growing RNA transcript increases. At a critical point, it is more feasible for DNA to rotate around its own helical axis to generate a transient positively supercoiled domain ahead of the RNA polymerase and a transient negatively supercoiled domain behind it. The formation of twin domains during transcription elongation is manifested by a large decrease or increase in the linking number of an intracellular plasmid when topoisomerase I or gyrase, respectively, is inhibited. This “twin-supercoiled-domain” model indicated that the state of supercoiling inside bacterial DNA is strongly modulated by transcription, it also predicted that the accumulated supercoiled DNA can be relaxed by DNA topoisomerases or neutralized each other by diffusing along the DNA helix (Liu and Wang, 1987).

Inside a bacterium, DNA gyrase and topoisomerase I act differentially on positively and negatively supercoiled domains (Wang, 1971; Gellert et al., 1976), therefore the supercoiling state of intracellular DNA is expected to be regulated by several processes, for instance, the transcription process which produces negative and positive supercoils at equal rates, the diffusion pathways which allow the cancellation of negative and positive supercoils, and the actions of DNA topoisomerases such as DNA topoisomerase I-catalyzed relaxation for negatively supercoiled DNA as well as gyrase-catalyzed negative supercoiling (Wu et al., 1988; Wang and Lynch, 1993)

There are several studies conducted to support the “twin-supercoiled-domain” model of transcription (Wu et al., 1988; Lodge et al., 1989; Tsao et al., 1989; Dröge and Nordheim, 1991; Cook et al., 1992; Dayn et al., 1992; Rahmouni and Wells, 1992; Dunaway and Ostrander, 1993; Lynch and Wang, 1993; Ma et al., 1994; Albert et al., 1996; Leng and McMacken, 2002; Leng et al., 2004; Stupina and Wang, 2005; Samul and Leng, 2007). Liu and Wang’s model also suggested that in a dilute aqueous solution, the friction force applying to the transcription complex was too small to generate significant supercoiling of the DNA template that only contain one transcription unit (Liu and Wang, 1987). We now know that it is very viscous and crowded inside a living cell (Zimmerman and Minton, 1993; Richter et al., 2008), and TCDS should be different from the dilute solution situation. It has been demonstrated that increased viscosity was able to significantly induce the DNA supercoiling in a defined protein system (Leng et al., 2004), which supports the view that *in vivo* situation is more complicated than the dilute aqueous solution. Recently, several groups re-investigated the induced torsional stress by a

transcribing RNA polymerase, they showed that even in a dilute aqueous solution, the torsional force of RNA polymerase was sufficient to generate the twin-supercoiled-domains (Nelson, 1999; Mielke et al., 2004). For instance, Nelson demonstrated that small natural bends in the DNA helix backbone was able to increase a few thousand fold of torsional stress even in a linear unanchored DNA (Nelson, 1999), this torsional force is sufficient to generate a positively supercoiled domain in front of the RNA polymerase and a negatively supercoiled domain behind it (Nelson, 1999). Another case is a Brownian dynamic study conducted by Mielke et al. (Mielke et al., 2004). These studies clearly demonstrated that a transcribing RNA polymerase alone is able to drive the formation of positively and negatively domains in a naked plasmid DNA template.

So far, studies of TCDS *in vivo* and *in vitro* were almost solely dependent on the utilization of circular plasmid in which topology of DNA was determined after transcription (Wu et al., 1988; Leng and McMacken, 2002; Samul and Leng, 2007). It has been demonstrated that TCDS on plasmids DNA required two barriers (Liu and Wang, 1987; Lodge et al., 1989; Wang and Lynch, 1993; Leng and McMacken, 2002; Stupina and Wang, 2004). The first barrier is a friction barrier generated from preventing or retarding the transcription complex from rotating around the DNA double helix; the second barrier is a topology barrier that prevents the cancellation of the positive and negative supercoiling domains (Figure 1.1).

1.2 Transcription-coupled DNA supercoiling in *E. coli topA* strains

In 1985, Pruss reported that hypernegatively supercoiled pBR322 was isolated from *E. coli* or *S. typhimurium topA* mutants lacking DNA topoisomerase I and the

isolated pBR322 DNA is extremely heterogeneous in linking number (Pruss, 1985). In a latter study, Pruss and Drlica showed that plasmid pBR322, but not pUC9 (a smaller high copy number plasmid, which is derived from pBR322), isolated from *E. coli topA* strains, became hypernegatively supercoiled, suggesting *tet* gene was responsible for this hypernegative supercoiling in *topA* strain (Pruss and Drlica, 1986). Further studies demonstrated that anchoring the transcribing RNA polymerase to the cell membrane through a nascent membrane-bound peptide or protein was required for the generation of hypernegative supercoiling in *topA* strains (Lodge et al., 1989; Cook et al., 1992; Lynch and Wang, 1993). For instance, by analyzing the transcription induced DNA supercoiling of plasmid pBR322 and its derivatives, Lodge et al. found that transcription-driven twin supercoiled domains were generated only if the DNA template was anchored to a large cellular structure by coupled transcription, translation, and membrane insertion of a nascent protein (Lodge et al., 1989). Later, Lynch and Wang showed that hypernegative DNA supercoiling in *topA* strain required the anchorage of transcribing RNA polymerase to the cell membrane through a nascent membrane-bound peptide or protein (Wang and Lynch, 1993). Moreover, Cook et al. showed that transcription of membrane-associated gene products that are oppositely oriented, rapidly supercoiled the plasmid DNA in *topA* strains (Cook et al., 1992; Ma et al., 1994). In these studies, the membrane-bound transcriptional complex cannot rotate around the DNA double helix freely and therefore a friction barrier was produced which generates the twin-supercoiled domains on the DNA templates (Lodge et al., 1989; Wang and Lynch, 1993; Ma et al., 1994).

It should be pointed out that all these studies regarding TCDS in *E. coli topA* strains utilized a combination of *E. coli* RNA polymerase and its promoters. As

demonstrated before, plasmid pBR322 and its derivatives contain several *E. coli* RNA polymerase promoters and the length and location of RNA transcripts cannot be controlled precisely (Sutcliffe, 1979; Bujard, 1981; Tomizawa and Som, 1984). In this case, TCDS may result from simultaneously transcribed of several transcriptional units on these plasmid DNA templates. Therefore, it was difficult to determine the factors that affect TCDS in *E. coli topA* strains. Apparently, a more specific model was required to identify the parameters that influence TCDS in *E. coli*.

In 2007, we established a new system to study TCDS in *E. coli* (Samul and Leng, 2007). This new system consists of a set of plasmids (i.e., the pLUC plasmids (Leng et al., 2004)) that produced RNA transcripts of different lengths by T7 RNA polymerase (Leng et al., 2004) and a *E. coli topA* strain, *VS111(DE3)* or *DM800(DE3)*, in which a λ DE3 prophage containing a T7 RNA polymerase gene under the control of the *lacUV5* promoter has been integrated into the chromosome. Compared to *topA* strain *VS111(DE3)*, *DM800(DE3)* has a compensatory mutation in the *gyrB* gene that produces a less active DNA gyrase (Sternglanz et al., 1981; McEachern and Fisher, 1989). Using this *in vivo* system, the length and location of RNA transcripts could be precisely controlled. We found that transcription by T7 RNA polymerase significantly induced the formation of hypernegatively supercoiled DNA. We also discovered that TCDS was dependent on the lengths of RNA transcripts, as predicted by the “twin-supercoiled-domain” model of transcription. More importantly, our results showed that hypernegative supercoiled DNA induced by T7 RNA polymerase did not require the anchorage of plasmid DNA to the bacterial cytoplasmic membrane, which is contrary to previous studies (Lodge et al., 1989; Cook et al., 1992; Lynch and Wang, 1993). This discovery indicated that a

transcribing RNA polymerase along is sufficient to cause a change of local DNA superhelicity. These results can be explained by the “twin-supercoiled-domain” model of transcription (Figure 1.1). As discussed above, TCDS requires two barriers. The physical barrier comes from preventing or retarding the transcriptional complex (including the transcribing RNA polymerase and the newly synthesized RNA transcript) from rotating around the DNA double helix, which results in the generation of the twin supercoiled domains. In our study, a fast-moving T7 RNA polymerase was used to produce sufficient friction force in order to generate the twin supercoiled domains, which is the main reason why the anchorage of transcription complex to the membrane is not required in *E. coli topA* strains. The second barrier is a DNA topology barrier that prevents or retards the diffusion and merger of DNA supercoiling along the longitudinal helix axis of the DNA template. In this case, the positively supercoiled DNA and negatively supercoiled DNA produced during transcription cannot cancel each other. In addition, DNA gyrase functions to convert the positively supercoiled DNA into “permanent” negative supercoiled DNA to generate hypernegatively supercoiled DNA in *E. coli topA* strains. Initially, a big cellular structure such as cell membrane was thought to be required for the production of a topological barrier. However, as discussed above, recently, our group found that certain nucleoprotein complexes, for example, those that contain sharply bent DNA sites or unwound DNA sequences, could form such topology barrier to block the diffusion and merger of the oppositely DNA domains (Leng et al., 2011). For pLUC plasmids, each of them contain one or more DNA-binding sites for several sequence-specific DNA binding proteins, such as ArgR and IHF, which have potentials to form nucleoprotein complexes that can serve as a topological barrier. In addition, *E. coli* RNA

polymerase may be able to associate with *E. coli* promoters on these DNA plasmids to stimulate the coupled supercoiling during transcription elongation. In summary, the topological barrier does exist for TCDS on pLUC plasmids. In this case, transcription by a fast-moving T7 RNA polymerase should significantly supercoil the DNA templates through the “twin-supercoiled-domain” mechanism (Figure 1.1).

1.3 Effects of transcription-coupled DNA supercoiling on the *Salmonella typhimurium leu-500* promoter

DNA supercoiling plays a crucial role in transcription regulation, as demonstrated in several systems (Cozzarelli, 1990; Wang and Lynch, 1993). The supercoiling effects could also play an important role on promoter function *in vivo* (Chen et al., 1992). One of the best characterized examples is activation of the *S. typhimurium leu-500* promoter by TCDS. The *leu-500* promoter, isolated from leucine auxotroph of *S. typhimurium*, is a single A-to-G point mutation in the -10 region of the promoter which controls the *leu* operon (Mukai and Margolin, 1963; Dubnau and Margolin, 1972; Gemmill et al., 1984). The *supX* locus (initially named *su leu 500*) was first described by Mukai and Margolin (Mukai and Margolin, 1963). Mutation in the *supX* locus could restore the leucine prototrophy and the chromosomal location of *supX* was identified between the *tryptophan* operon and *cysteine B* locus in *S. typhimurium* (Mukai and Margolin, 1963; Dubnau and Margolin, 1972). Later, the *supX* gene was identified the same gene as *topA* which encodes for topoisomerase I (Sternglanz et al., 1981; Trucksis and Depew, 1981; Trucksis et al., 1981; Wang and Becherer, 1983; Margolin et al., 1985). The AT to GC mutation was expected to increase the energy barrier to form a functional transcription

complex and this barrier can be overcome by the mutation in topoisomerase I gene that causes the loss of topoisomerase I's activity (Trucksis et al., 1981; Richardson et al., 1984; Margolin et al., 1985; Richardson et al., 1988). Intriguingly, Lilley and Higgins showed that activation of the *leu-500* promoter was only dependent on the *topA* background and did not correlate with global supercoiling, as measured from extracted DNA templates (Lilley and Higgins, 1991). In addition, when the *leu-500* promoter was cloned into a plasmid, the *topA* background was no longer required for activation of the *leu-500* promoter (Richardson et al., 1988; Lilley and Higgins, 1991; Chen et al., 1992). These phenomena suggested an unknown regulation factor rather than the *topA* background was responsible for the activation of the *leu-500* promoter. Studies conducted by Wu's group and Lilley's group pointed out *leu-500* promoter could be activated when it was coupled to transcription of another divergently oriented promoter, suggested that transcription-driven localized supercoiling was responsible for the activation of the *leu-500* promoter (Chen et al., 1992; Chen et al., 1993; Tan et al., 1994; Mojica and Higgins, 1996; Spirito and Bossi, 1996; Chen et al., 1998). Furthermore, after analyzing the *leuO* operon and the surrounding elements in *S. typhimurium* chromosome, Wu and co-workers found that *ilvIH* promoter and the *leuO* gene were located upstream of the *leu-500* promoter and they were transcribed divergently to the *leu-500* promoter (Haughn et al., 1986; Wu et al., 1995). On the basis of these results, Wu et al. proposed a promoter relay mechanism to explain the expression of the *ilvIH-leuO-leuABCD* gene cluster that is coordinated in a sequential manner (Fang and Wu, 1998; Fang and Wu, 1998; Wu and Fang, 2003). In this model, *leuO* promoter is activated by *ilvIH* promoter, which is located within the 1.9 kb intervening sequence. Both the *leuO* promoter and expression of

the LeuO protein are required for subsequent activation of the *leu-500* promoter (Fang and Wu, 1998). The key component in this model is TCDS which generates transient changes on DNA templates. In addition, recently, Hanafi and Bossi showed the orientation of the DNA supercoiling could affect neighboring promoters: transcription-induced positively supercoiling suppressed the promoter function while transcription-driven negatively supercoiling could activate the *leu-500* promoter (Hanafi and Bossi, 2000).

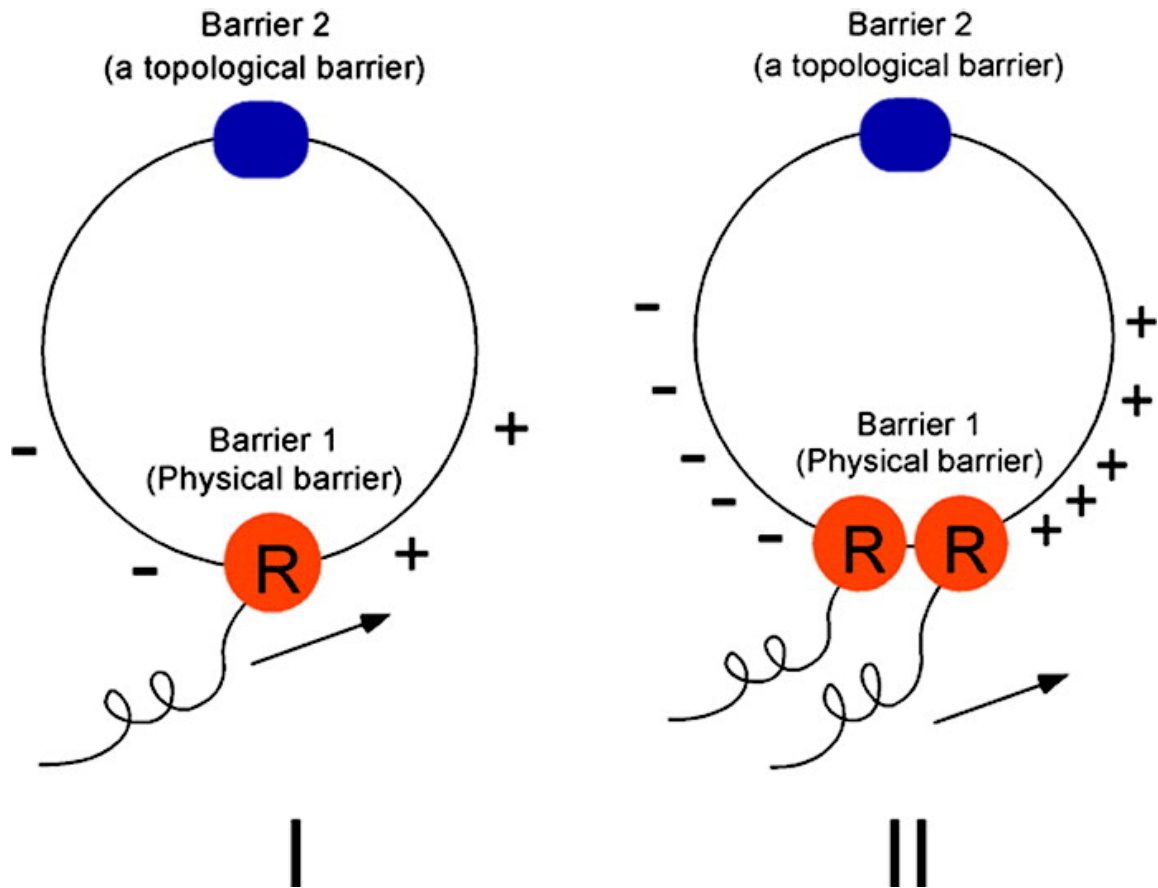


Figure 1.1 A two-barrier model to explain TCDS on circular plasmids DNA. As RNA polymerase binds to the DNA double helix and transcribed counterclockwise, positively supercoils will be generated ahead of the transcribing RNA polymerase and negatively supercoils behind it. A physical barrier (barrier 1) is formed by preventing or retarding of RNA polymerase from rotating around the DNA double helix. A topological barrier (barrier 2) may be generated from the formation of some nucleoprotein complexes. When there is more than one RNA polymerase present, greater extent of TCDS will be achieved.

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Chapter 2: Dependence of transcription-coupled DNA Supercoiling on promoter strength in *Escherichia coli* topoisomerase I deficient strains

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2.1 Abstract

Transcription by RNA polymerase can induce the formation of hypernegatively supercoiled DNA *in vitro* and *in vivo*. This phenomenon has been nicely explained by a “twin-supercoiled-domain” model of transcription where a positively supercoiled domain is generated ahead of the RNA polymerase and a negatively supercoiled domain behind it. In *Escherichia coli topA* strains, DNA gyrase selectively converts the positively supercoiled domain into negative supercoils to produce hypernegatively supercoiled DNA. In this article, in order to examine whether promoter strength affects transcription-coupled DNA supercoiling (TCDS), we developed a two-plasmid system in which a linear, non-supercoiled plasmid was used to express *lac* repressor constitutively while a circular plasmid was used to gage TCDS in *E. coli* cells. Using this two-plasmid system, we found that TCDS in *topA* strains is dependent on promoter strength. We also demonstrated that transcription-coupled hypernegative supercoiling of plasmid DNA did not need the expression of a membrane-insertion protein for strong promoters; however, it might require co-transcriptional synthesis of a polypeptide. Furthermore, we found that for weak promoters the expression of a membrane-insertion *tet* gene was not sufficient for the production of hypernegatively supercoiled DNA. Our results can be explained by the “twin-supercoiled-domain” model of transcription where the friction force applied to *E. coli* RNA polymerase plays a critical role in the generation of hypernegatively supercoiled DNA.

2.2 Introduction

DNA supercoiling plays fundamental roles in a number of essential DNA metabolic pathways, such as DNA replication, recombination, and transcription (Bates and Maxwell, 2005; Cozzarelli and Wang, 1990; Wang, 2009). In *Escherichia coli*, DNA is typically negatively supercoiled. DNA supercoiling status inside *E. coli* cells is primarily set by counter actions of two DNA topoisomerases, DNA gyrase, and topoisomerase I (Champoux, 2001; Snoep et al., 2002; Wang, 1996; Zechiedrich et al., 2000). Inactivating DNA gyrase or topoisomerase I results in the production of positively (Lockshon and Morris, 1983) or hypernegatively (Pruss, 1985) supercoiled DNA, respectively.

Since the 1980s, it has been demonstrated that transcription by RNA polymerase could introduce supercoils to plasmid DNA templates *in vitro* and *in vivo* (Leng and McMacken, 2002; Leng et al., 2004; Lockshon and Morris, 1983; Pruss, 1985; Tsao et al., 1989; Wu et al., 1988). Liu and Wang proposed a “twin-supercoiled-domain” model of transcription to explain how transcription by RNA polymerase is able to supercoil the plasmid DNA templates (Liu and Wang, 1987). This elegant model hypothesizes that a transcribing RNA polymerase becomes increasingly more difficult to rotate around the axis of the DNA double helix as the size of the growing RNA transcript increases. At a critical point, energetically, it is more feasible for the DNA molecule to rotate around its own helix axis to produce a positively supercoiled domain in front of the RNA polymerase and a negatively supercoiled domain behind it. These two transient supercoiled domains may be relaxed by DNA topoisomerases or cancel each other by

diffusion (Leng and McMacken, 2002; Mielke et al., 2004; Nelson, 1999; Tsao et al., 1989; Wu et al., 1988).

So far, there is substantial experimental evidence to support the “twin-supercoiled-domain” model of transcription (Albert et al., 1996; Cook et al., 1992; Dunaway and Ostrander, 1993; Leng and McMacken, 2002; Lodge et al., 1989; Lynch and Wang, 1993; Ma et al., 1994; Stupina and Wang, 2004; Tsao et al., 1989; Wu et al., 1988). For instance, in *E. coli* topoisomerase I-deficient (*topA*) strains, transcription by RNA polymerases is capable of driving the plasmid DNA templates to hypernegatively supercoiled status (Cook et al., 1992; Lodge et al., 1989; Pruss, 1985; Wang and Lynch, 1993). It was shown that transcription-coupled hypernegative supercoiling of plasmid DNA required co-transcriptional synthesis of a membrane-associated protein or polypeptide for plasmid pBR322 and derivatives (Cook et al., 1992; Lodge et al., 1989; Lynch and Wang, 1993; Ma et al., 1994). A possible explanation for this requirement is that co-transcriptional synthesis of a membrane-associated protein or polypeptide substantially increased the friction force against the transcribing RNA polymerase. In this scenario, a significant amount of “twin-supercoiled-domains” is generated. After the positively supercoiled domain is converted into negative supercoils by DNA gyrase, the transcribed DNA templates become hypernegatively supercoiled.

Using a similar approach, we recently demonstrated that transcription by T7 RNA polymerase strikingly stimulated DNA supercoiling; transcription-coupled DNA supercoiling (TCDS) was dependent on the length of RNA transcripts in *E. coli topA* strains *VS111(DE3)* and *DM800(DE3)* (Samul and Leng, 2007). Additionally, we found that hypernegative supercoiling of plasmid DNA by T7 RNA polymerase did not require

anchoring of DNA to the bacterial cytoplasmic membrane (Samul and Leng, 2007). We attributed these results to the fact that a much stronger T7 promoter and a much faster T7 RNA polymerase (comparing with *E. coli* RNA polymerase (Seidel and Dekker, 2007)) were used in our transcription–supercoiling (T–S) assays. In this case, the “twin-supercoiled-domains” were efficiently generated and, as a result, TCDS did not need transcriptional machinery to couple to translation and membrane-insertion. These results also suggested that promoter strength is important to TCDS in *E. coli* cells. In order to further study how promoter strength affects the efficiency of TCDS in *E. coli topA* strains, herein we developed a new two-plasmid system: the first plasmid is a linear plasmid derived from coliphage N15 (Ravin and Ravin, 1999) and was used to express *lac* repressor constitutively. In addition, the linear plasmids cannot be supercoiled (Deneke et al., 2000) and therefore will not interfere with the supercoiling assays; the second plasmid is a circular plasmid that was used to examine TCDS by *E. coli* RNA polymerase. Using this unique two-plasmid system, we found that transcription-coupled hypernegative supercoiling of plasmid DNA templates was dependent on promoter strength and did not require the expression of a membrane-insertion protein for strong promoters, which is consistent with our results for T7 RNA polymerase (Samul and Leng, 2007).

2.3 Methods

2.3.1 Materials

Ethidium bromide, kanamycin, lysozyme, and chloroquine were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Ampicillin and bovine serum albumin (BSA) were

obtained from Fisher Scientific (Fairlawn, NJ). Tetracycline was purchased from EMI Science (Gibbstown, NJ). Isopropyl- β -D-thiogalactopyranoside (IPTG) was obtained from Anatrace, Inc. (Maumee, Ohio). All restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were bought from New England Biolabs, Inc. (Beverly, MA). *Pfu* DNA polymerase was purchased from Stratagene, Inc. (La Jolla, CA). All synthetic oligonucleotides used as primers were obtained from MWG-Biotech, Inc. (Huntsville, AL). QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, RNeasy Mini Kit, and QIAquick Nucleotide Removal Kit were bought from QIAGEN, Inc. (Valencia, CA). ThermoScript RT-PCR System plus Platinum® *Taq* DNA polymerase was purchased from Invitrogen, Inc. (Carlsbad, CA). Power SYBR Green PCR Master Mix was obtained from Applied Biosystems, Inc. (Carlsbad, CA). GFP-Ab2 Mouse Monoclonal Antibody is a product of Thermo Fisher Scientific, Inc. (Fremont, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse antibody was obtained from EMD Biosciences, Inc. (Madison, WI). Supersignal West Pico Chemiluminescent Substrate was bought from Thermo Scientific, Inc. (Rockford, IL).

2.3.2 Bacterial strains and plasmids

E. coli strain *VS111* [*F*-*LAM-rph-I* Δ *topA*] as described in Stupina and Wang (2005) was obtained from the *Coli* Genetic Stock Collection/*E. coli* Genetic Resource Center (CGSC) at Yale University. *E. coli* strain *DM800* [*F*- Δ (*topAcysB*)204 *arcA13* *gyrB225*] was kindly provided by Dr. Marc Drolet at Universite de Montreal. All linear plasmids were derived from coliphage N15-based, linear plasmid pG591 (Ravin and Ravin, 1999), which was kindly provided by Dr. Nikolai V. Ravin at Centre

“Bioengineering” RAS, Russia. Plasmid pZXD4 was constructed by inserting a 33 bp synthetic DNA fragment containing a multiple cloning site into the unique BglII site of pG591. Plasmid pZXD51 (Figure 2.1A) was constructed in two steps. First, promoter P_{lacI} controlling the expression of *LacI* in pET-30a(+) was mutated to the strong promoter P_{lacI}^q using PCR-based, site-directed mutagenesis. Then the *lacI* gene including promoter P_{lacI}^q was amplified by PCR and inserted between NheI and AflIII sites of pZXD4 to generate plasmid pZXD51. *E. coli* strains carrying pZXD51 express *LacI* constitutively.

All circular plasmids constructed in this work were derived from plasmid pBR322. Plasmid pBR322se1 was constructed by converting the -35 region of promoter $P_{anti-tet}$ into an XhoI site using PCR based, site-directed mutagenesis. In this case, promoter $P_{anti-tet}$ was removed. Plasmid pBR322se2 was created after a Shine–Dalgarno sequence (5'-AAGGAGG-3') was inserted to the upstream region of the open reading frame of the *tet* gene. Plasmid pBR322se3 was made by introducing KpnI and SacI sites into the plasmid surrounding the weak promoter P_{bla} . In this scenario, promoter P_{bla} may easily be replaced by other promoters. Plasmid pZXD7 was created by removing a Dcm sequence of pBR322se3 associated with the unique MscI recognition site using PCR-based, site-directed mutagenesis. Plasmid pZXD8 was generated after a BglII site was inserted to the downstream region of the *tet* gene of plasmid pZXD7. A 37 bp synthetic deoxyoligonucleotide containing a multiple cloning site was then inserted into the unique KpnI site of pZXD8 to yield plasmid pZXD9. Plasmid pZXD11 was constructed after a 36 bp synthetic DNA fragment containing a T7 promoter was inserted into the EcoRI and XhoI sites to replace promoter P_{tet} . Plasmid pZXD12 was made after a 64 bp synthetic DNA fragment containing an inactive promoter $P_{leu-500}$ was inserted into the HindIII and

KpnI sites of pZXD11. Plasmid pZXD14 was created when an 813 bp DNA fragment of pLUC1 carrying four tandem copies of *rrnB* T1 transcription terminators (Leng and McMacken, 2002) was inserted into the unique MscI site of pZXD12. Plasmids pZXD44, 50, 49, 47, and 48 were constructed by replacing the T7 promoter with *E. coli* promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} , respectively, between the EcoRI and XhoI sites of pZXD14.

Plasmids pZXD57, 58, 56, 55, and 54, each carrying a *GFPuv* gene under the control of *E. coli* promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} , respectively, were constructed in a few steps. First, an AgeI site was introduced between the Shine–Dalgarno sequence and the start codon of the *tet* gene of plasmid pXZD48 to generate plasmid pXZD52 using PCR-based, site-directed mutagenesis. Second, the unique XhoI site in the *GFPuv* gene of plasmid pGFPuv (Stratagene, Inc., La Jolla, CA) was silently removed without changing the open reading frame of *GFPuv* gene using PCR-based, site-directed mutagenesis to yield plasmid pZXD53. Third, a 737 bp PCR product containing the *GFPuv* gene was cloned into the AgeI and BsmI sites of plasmid pZXD52 to produce plasmid pZXD54. In this case, the *tet* gene was replaced by the *GFPuv* gene under the control of *E. coli* promoter P_{lacL8} . Plasmids pZXD57, 58, 56, and 55 were constructed by replacing the *E. coli* promoter P_{lacL8} with promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , and P_{lac} between the EcoRI and XhoI sites of pZXD54, respectively.

Plasmid pZXD59 was created when a 735 bp DNA fragment containing the *GFPuv* gene in reverse orientation amplified from plasmid pZXD53 was inserted into the AgeI and BsmI sites of plasmid pZXD57. In this scenario, *E. coli* cells carrying pZXD59 are not able to express GFPuv protein after IPTG induction. Plasmids pZXD60 and 61

were produced when *lacZ* gene in the forward and reverse orientations was amplified from plasmid pYC2/CT/*lacZ* and inserted into the AgeI and BsmI sites of pZXD57. In this case, *E. coli* cells carrying pZXD60 are able to express β -galactosidase after IPTG induction. However, *E. coli* cells carrying pZXD61 are not able to express β -galactosidase after IPTG induction. Plasmid pZXD62 was constructed after a 2.3 kb PCR fragment of *lacZ* gene was inserted between the HindIII and KpnI sites of pZXD57. Plasmid pZXD63 was produced after a 1.8 kb PCR fragment of *lacZ* gene was inserted into the HindIII and KpnI sites of pZXD44. Plasmids pZXD60A and pZXD63A were constructed where the start codon (ATG) of the *lacZ* and *tet* genes were, respectively, mutated to the stop codon TAG using PCR-based, site-directed mutagenesis. In this scenario, *E. coli* cells carrying plasmids pZXD60A and 63A are not able to express β -galactosidase and tetracycline resistance protein, respectively. Please notice that plasmids pZXD60, 61, 62, 63, 60A, and 63A have the same size, i.e., 7055 bp.

2.3.3 *In vivo* T-S assays

Escherichia coli cells carrying different plasmids were grown overnight in LB containing 50 μ g/ml of ampicillin and kanamycin. The overnight culture was then diluted (1:100) in fresh LB containing 50 μ g/ml of ampicillin and kanamycin, and grown until optical density of the cells at 600 nm reached approximately 0.5. IPTG (final concentration, 1 mM) was added to the cell culture to initiate transcription by different promoters, i.e., P_{T7A1/O4}, P_{tac}, P_{lacUV5}, P_{lac}, and P_{lacL8}. Plasmid DNA was purified using QIAGEN Miniprep Kit. The topological state of each DNA preparation was analyzed by electrophoresis in a 1% agarose gel in 1 \times TAE buffer (40 mM Tris-acetate, 1 mM EDTA, and pH 7.8) containing 2.5 μ g/ml of chloroquine. After electrophoresis, agarose gels were

stained with ethidium bromide, destained, and photographed under UV light. The net intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software. The percentage of hypernegative DNA supercoils was calculated by dividing the intensity of the hypernegatively supercoiled DNA band by the total intensity of all DNA topoisomers.

2.3.4 Western blotting experiments

Western blotting experiments were used to verify the expression of GFPuv protein in *E. coli topA* strain *VS111* after 1 mM of IPTG induction. Total protein purified from *E. coli* cells was analyzed by electrophoresis in a 15% SDS-PAGE and electrophoretically transferred to a 0.45 µm nitrocellulose membrane. The membrane blot was then blocked with a solution containing 5% nonfat skim milk in TBST (50mM Tris-HCl, pH 8.0, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20) for 45 min at room temperature and incubated with the primary antibody, GFP Ab-2 mouse monoclonal antibody, diluted 1:1000 in TBST solution overnight at 4 °C. After the overnight incubation, the membrane blot was washed three times with TBST, blocked with a solution containing 5% nonfat skim milk in TBST for 15 min at room temperature, and then incubated for 1 h with an HRP-conjugated anti-mouse IgG secondary antibody (diluted 1:5000) at room temperature. The immunoreactive GFPuv protein was detected with Supersignal West Pico Chemiluminescent Substrate.

2.3.5 RNA isolation, cDNA synthesis, and polymerase chain reaction (PCR)

Total RNA was isolated from *E. coli* cells using QIAGEN RNeasy Kit as described by the manufacturer. To determine the integrity of the total RNA samples, 16S

and 23S rRNA were resolved by electrophoresis in a 1.2% agarose gel in 1× MOPS buffer containing formaldehyde (20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA, 1% formaldehyde, and pH 7.0). After electrophoresis, agarose gels were stained with ethidium bromide, destained, and photographed under UV light. cDNA were synthesized from total RNA samples using ThermoScript RT-PCR System. 2.76 µg of RNA was mixed with a sequence-specific primer (final concentration, 0.5 µM) or random hexamer primers (50 ng/µl) and four deoxynucleotide triphosphates (dNTPs; final concentration, 1 mM). The mixtures were incubated at 65 °C for 5 min and transferred to ice for another 5 min to remove secondary structures of RNA. The denatured RNA samples were then mixed with 1× cDNA synthesis buffer with a total volume of 20 µl containing 5 mM DTT, 40 units of RNaseOut, and 15 units of ThermoScript Reverse Transcriptase, and incubated at 60 °C for 1 h to synthesize cDNA. The cDNA synthesis mixtures were transferred to an 85 °C water bath for 5 min to terminate the reactions. After the synthesis step, 2 units of RNase H were added to the reaction mixtures and incubated at 37 °C for 20 min to remove the RNA templates.

PCR reactions were carried out using cDNA samples synthesized as described above. A 50 µl PCR reaction contains 1× PCR Buffer without Mg²⁺, 1.58 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each primer (Table 2.1), 0.5 µl cDNA, and 2 units of Platinum *Taq* DNA polymerase. The reactions started at 94 °C for 2 min, proceeded for 16 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and terminated at 72 °C for 10 min. Subsequently, the PCR products were analyzed by electrophoresis in a 12% polyacrylamide gel in 1× TAE buffer. After electrophoresis, polyacrylamide gels were

stained with ethidium bromide or SYBR gold, destained, and photographed under UV light.

2.3.6 Real-time PCR Assays

Real-time PCR assays were carried out using MiniOpticon Real-time PCR system (Bio-Rad, Hercules, CA). A 20 μ l reaction contains 0.5 μ l cDNA, 0.5 μ M of each primer (Table 2.1), and 10 μ l of Power SYBR Green PCR Master Mix (2 \times). The reaction started at 95 $^{\circ}$ C for 10 min and continued for 40 cycles at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. The C_q values (quantification cycle values) were calculated from the exponential phase of each PCR amplification reaction as recommended by the manufacturer.

2.4 Results and Discussion

2.4.1 Establish an IPTG-inducible, two-plasmid system to study TCDS in *E. coli topA* strains

In this study we established a two-plasmid system to examine effects of different factors on TCDS in *E. coli topA* strains *VS111* and *DM800*. The first plasmid pZXD51 (Figure 2.1A) is a linear plasmid derived from coliphage N15-based, low-copy-number plasmid pG591 (Ravin and Ravin, 1999) where a *lacI* gene was cloned under the control of the strong P_{lacI}^q promoter. In this case, *E. coli* strains carrying pZXD51 produce \sim 3000 molecules of LacI per cell constitutively (Lutz and Bujard, 1997). The second plasmid is a circular plasmid that serves as a supercoiling-reporter (Figure 2.1B). Here we constructed a series of plasmid DNA templates that contain different strengths of *E. coli* promoters (Lanzer and Bujard, 1988), i.e., $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} (Figure 2.1C), to examine effects of promoter strength on TCDS by *E. coli* RNA polymerase.

Since each promoter region contains a *lac O1* operator (Figure 2.1C), transcription initiated from these promoters is IPTG-inducible in *E. coli* cells overexpressing LacI, e.g., *E. coli* cells carrying the linear plasmid pZXD51. We also added a set of Rho-independent, *rrnB* T1 transcription terminators to each plasmid. The presence of multiple *rrnB* T1 terminators enabled us to restrict transcription to selected regions of supercoiling-reporter plasmids and to modulate the length of RNA transcripts produced. Since supercoiling-reporter plasmids have a different DNA replication origin (pMB1 origin), they can co-exist with the linear plasmid pZXD51 in *E. coli* cells. Indeed, these two types of plasmids were able to simultaneously transform *E. coli topA* strains *VS111* and *DM800*. Additionally, as mentioned above, an advantage of using a linear plasmid to express LacI is that linear plasmids cannot be supercoiled (Deneke et al., 2000) and, as a result, will not interfere with the supercoiling assays. In this case, this new system will be ideal for our *in vivo* supercoiling studies. Plasmids constructed in this study are summarized in Figure 2.1 and Table 2.2.

2.4.2 Transcription-coupled hypernegative supercoiling of plasmid DNA is dependent on promoter strength

Having established the two-plasmid system, we proceeded to examine whether TCDS is dependent on promoter strength after IPTG induction. As mentioned above, we simultaneously introduced the linear plasmid pZXD51 and a supercoiling-reporter plasmid into *E. coli topA* strains *VS111* and *DM800*. Since previous studies showed that hypernegative supercoiling of plasmid pBR322 and its derivatives is dependent on the expression of the co-transcription and translation of membrane-associated *tet* gene (Cook

et al., 1992; Lodge et al., 1989; Lynch and Wang, 1993; Ma et al., 1994; Pruss, 1985; Pruss and Drlica, 1986; Stupina and Wang, 2005), we decided to use a set of five supercoiling-reporter plasmids that carry a *tet* gene under the control of an IPTG-inducible promoter with different strengths, i.e., promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} . As expected, our RT-PCR experiments (Figure 2.2) demonstrated that the transcription level of *E. coli* strains harboring different plasmids after IPTG induction is correlated with promoter strength *in vivo* (Lanzer and Bujard, 1988). Interestingly, *E. coli* cells carrying plasmids pZXD44 and 50 were able to grow on agar plates containing 10 μ g/ml of tetracycline even in the absence of IPTG (Figure 2.7A). This resistance is most likely due to the leaky expression of the tetracycline resistance protein from the strong $P_{T7A1/O4}$ and P_{tac} promoters, since pZXD44 and 50 contain $P_{T7A1/O4}$ and P_{tac} , respectively. However, only *E. coli* cells carrying plasmids pZXD50 and 49 were able to grow on agar plates containing 10 μ g/ml of tetracycline and 1 mM of IPTG (Figure 2.7B). As demonstrated previously, overexpression of tetracycline resistance protein results in cell death (Eckert and Beck, 1989), which is likely the reason for *E. coli* cells harboring plasmid pZXD44 carrying the strong $P_{T7A1/O4}$ promoter being unable to grow on agar plates in the presence of 1 mM of IPTG. Indeed, our results showed that IPTG was able to inhibit cell growth for *E. coli* strains *VS111* and *MGI655* harboring plasmid pZXD44 (Figure 2.7C and D). Because promoters P_{lac} and P_{lacL8} are too weak, *E. coli* cells carrying plasmids pZXD47 and 48 could not produce enough tetracycline resistance protein to overcome the antimicrobial activities of tetracycline.

We next determined the topological status of the set of five supercoiling-reporter plasmids pZXD44, 50, 49, 47, and 48 in *E. coli topA* strain *VS111* harboring the linear

plasmid pZXD51. Figure 2.3 shows the results. Before IPTG induction, plasmid pZXD44, which carries a strong $P_{T7A1/O4}$ promoter, had a superhelical density, σ , of approximately -0.06 to -0.07 (Figure 2.3A and B, lane 1). In the presence of the DNA intercalator chloroquine ($2.5 \mu\text{g/ml}$), this plasmid migrated during agarose gel electrophoresis as if it contained a few negative supercoils. After IPTG induction, as expected, some topoisomers quickly became hypernegatively supercoiled (estimated $\sigma < -0.09$; hypernegatively supercoiled DNA is the fastest moving band in the gels where DNA topoisomers are no longer resolvable under our experimental conditions): the amount of hypernegatively supercoiled DNA was dependent on the IPTG concentration added to the cell culture (Figure 2.3A and 2.8) and the induction time (Figure 2.3B and 2.8). These results clearly demonstrated that the induction of expression of the membrane-insertion *tet* gene under the control of the strong $P_{T7A1/O4}$ promoter was able to drive the formation of hypernegatively supercoiled DNA, which is consistent with previous published results (Cook et al., 1992; Lynch and Wang, 1993; Pruss, 1985). Similar results were obtained for plasmids pZXD50 and 49 which carry P_{lac} and P_{lacUV5} , respectively (Figure 2.3C and 2.8). However, to our surprise, IPTG was not able to induce the production of hypernegatively supercoiled DNA for plasmids pZXD47 and 48, which harbor the weak promoters, P_{lac} and P_{lacL8} , respectively (lanes 4 and 5 of Figure 2.3C). These results demonstrated that the expression of a membrane-insertion *tet* gene is not sufficient for the production of hypernegatively supercoiled DNA. Intriguingly, our results showed that transcription-coupled hypernegative supercoiling of plasmid DNA is dependent on promoter strength: the stronger the promoter, the more hypernegatively-supercoiled DNA

produced (Figure 2.3C and D). Similar results were also obtained using *E. coli topA* strain *DM800* as the host strain (Figure 2.8G).

2.4.3 Transcription-coupled hypernegative supercoiling of plasmid DNA in *E. coli topA* strains did not require the expression of a membrane-insertion protein for strong promoters

As mentioned above, we recently found that hypernegative supercoiling of plasmid DNA by T7 RNA polymerase did not require anchoring of DNA to bacterial cytoplasmic membrane (Samul and Leng, 2007). Thus, we decided to examine whether TCDS by *E. coli* RNA polymerase in *E. coli topA* strains *VS111* and *DM800* requires anchoring of DNA to bacterial cytoplasmic membrane through co-transcriptional synthesis of polypeptides encoding membrane proteins in this new two-plasmid system. Our results showed that, for strong promoters, TCDS did not require anchoring of DNA to bacterial cytoplasmic membrane through the expression of a membrane-insertion protein.

We first constructed a set of five supercoiling-reporter plasmids, pZXD57, 58, 56, 55, and 54 that carry a cytosolic green fluorescence protein UV (*GFPuv*) gene under the control of IPTG-inducible promoters with different strengths, i.e., promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} (Figure 2.9 and Table 2.2). These plasmids were introduced to *E. coli topA* strains *VS111* and *DM800* that also harbor the linear plasmid pZXD51. As expected, after IPTG induction, *GFPuv* gene products in *E. coli* strains harboring different plasmids at transcription and expression levels were correlated with promoter strength (Figure 2.10). Interestingly and also as expected, IPTG was able to induce the

generation of hypernegatively supercoiled DNA for plasmid pZXD57 that carries a strong $P_{T7A1/O4}$ promoter (Figure 2.4). These results suggest that the expression of a cytosolic GFPuv protein was sufficient to induce the production of hypernegatively supercoiled DNA although the amount of generating hypernegatively supercoiled DNA was lower than that for plasmid pZXD44 (compare Figure 2.3A and 2.4A; also compare Figure 2.8F and 2.4C). Consistent with above described results, transcription-coupled hypernegative supercoiling of plasmid DNA for this set of five supercoiling-reporter plasmids, i.e., pZXD57, 58, 56, 55, and 54, was also dependent on promoter strength (Figure 2.4B and D). Similar results were obtained for plasmids by using *E. coli topA* strain *DM800* as the host strain (data not shown).

In order to further study transcription-coupled hypernegative supercoiling of plasmid DNA in our two-plasmid system, we next constructed six supercoiling-reporter plasmids of identical size which also carry a strong, IPTG-inducible $P_{T7A1/O4}$ promoter controlling the expression of different genes (Figure 2.11 and Table 2.2). Plasmid pZXD60 carries a *lacZ* gene under the control of $P_{T7A1/O4}$. *E. coli* cells carrying pZXD60 are able to overexpress β -galactosidase after IPTG induction. Plasmid pZXD60A is identical with pZXD60 except the start codon (ATG) of *lacZ* was mutated to the stop codon TAG (amber mutation). In this case, IPTG is not able to induce the expression of β -galactosidase for *E. coli* cells harboring pZXD60A although the transcripts from both plasmids should be almost identical. Plasmid pZXD61 contains a *lacZ* gene in the reverse orientation, which cannot direct the expression of β -galactosidase. Plasmid pZXD62 carries a *GFPuv* gene under the control of $P_{T7A1/O4}$, which is able to direct the overexpression of GFPuv protein after IPTG induction. Plasmids pZXD63 and 63A are

identical except for the start codon of *tet* gene. pZXD63 carries a *tet* gene under the control of P_{T7A1/O4} and is able to direct the overexpression of membrane insertion, tetracycline resistance protein. pZXD63A, however, has an amber mutation in the start codon of the *tet* gene (ATG to TAG). In this scenario, *E. coli* cells containing pZXD63A are not able to express tetracycline resistance protein. These six plasmids were transformed into *E. coli topA* strain *VS111* carrying the linear plasmid pZXD51 and their superhelical states were examined after IPTG induction. Figure 2.5 shows the results of these *in vivo* T-S assays. As expected, IPTG was able to induce the production of hypernegatively supercoiled DNA for plasmids pZXD60, 62, and 63 which express β -galactosidase, GFPuv protein, and tetracycline resistance protein, respectively (Figure 2.5A, B, and C; Figure 2.12A). Interestingly, our results showed that IPTG was not able to induce the generation of hypernegatively supercoiled DNA for plasmids pZXD60A, 61, and 63A although transcription added a few negative supercoils to these plasmids (Figure 2.5D, E, and F). Since plasmids pZXD60A, 61, and 63A are not able to direct the expression of a polypeptide after IPTG induction, although each plasmid carries a strong P_{T7A1/O4} promoter, these results suggest that the co-transcriptional synthesis of a protein is able to facilitate the generation of hypernegatively supercoiled DNA. Nevertheless, a close inspection of these gel images revealed that transcription added 5 to 6 negative supercoils to plasmids pZXD60A and 63A, and only 2 supercoils to pZXD61. We examined the DNA sequences of these plasmid DNA templates and found a downstream open reading frame for both pZXD60A and pZXD63A but not for pZXD61. Although both open reading frames do not contain a SD sequence, it is possible that they are still able to direct the synthesis of a polypeptide with low efficiency. These downstream open

reading frames may be the reason for the topological difference among these DNA templates. Alternatively, the different amounts of friction torque caused by various mRNA secondary structures may also result in the topological difference. Similar results were also obtained when *E. coli topA* strain *DM800* was used as the host strain (Figure 2.12B).

It has been demonstrated that the stability of *E. coli* mRNA was strongly affected by their association with ribosomes and ribosome-free mRNA was rapidly degraded *in vivo* (Deana and Belasco, 2005; Nilsson et al., 1987; Pedersen et al., 2011). Therefore, we decided to examine whether transcription-coupled hypernegative supercoiling of DNA in the new two-plasmid system is correlated with the stability of mRNA produced by these plasmids after IPTG induction. Figure 2.6 shows the results of our RT-PCR experiments. *E. coli* cells harboring plasmids pZXD60, 62 and 63, which are able to direct the overexpression of a polypeptide, i.e., β -galactosidase, GFPuv protein, and tetracycline resistance protein, respectively, produced almost the same amount of mRNA after 10 min of IPTG induction (compare lanes 1 to 3 of Figure 2.6A), suggesting that mRNA of the *lacZ*, *GFPuv*, and *tet* genes had similar stability. The introduction of an amber mutation to the start codon of *lacZ* and *tet* genes or reversing the orientation of the open reading frame of *lacZ* and *GFPuv* gene greatly reduced the stability of the RNA transcripts (Figure 2.6 and 2.13). Coincidentally, transcription was not able to drive these plasmids into hypernegatively supercoiled status (Figure 2.5D, E, and F). These results suggest that transcription-coupled hypernegative supercoiling of DNA is related to the stability of mRNA produced by these plasmids after IPTG induction.

2.5 Conclusions

In this article, we have presented strong evidence demonstrating that transcription-coupled hypernegative supercoiling of plasmid DNA in *E. coli topA* strains is dependent on promoter strength, a functional property that has not been revealed previously. Not only did we show that transcription-coupled hypernegative supercoiling of plasmids carrying a *tet* gene encoding a membrane-insertion protein required a strong promoter (Figure 2.3 and 2.8), but also we demonstrated that transcription from strong promoters, such as $P_{T7A1/O4}$ and P_{tac} , was able to induce plasmid DNA templates into hypernegatively supercoiled status for those harboring a *GFPuv* or a *lacZ* gene encoding a cytosolic protein in *E. coli topA* strains *VS111* and *DM800* (Figure 2.4, 2.5, and 2.12). Transcription from weak promoters, such as P_{lac} and P_{lacL8} , however, was not able to induce topological changes to plasmids carrying either a membrane-associated *tet* gene (Figure 2.3 and 2.8) or a cytosolic-associated gene, such as *GFPuv* or *lacZ* (Figure 2.4). Since promoter strength is correlated with transcription initiation (Brunner and Bujard, 1987; Lutz et al., 2001; McClure, 1985; Saecker et al., 2011), these results suggest that transcription initiation plays a critical role in TCDS in *E. coli* cells. However, our results also showed that transcription initiation alone was not capable of inducing plasmid DNA templates into hypernegatively supercoiled status. For instance, transcription from the strong $P_{T7A1/O4}$ promoter was not able to induce plasmids pZXD60A, 61, and 63A into hypernegatively supercoiled status although transcription added a few supercoils to these plasmids after IPTG induction (Figure 2.5D, E, and F). Nevertheless, our results are consistent with the “twin-supercoiled-domain” model of transcription (Liu and Wang, 1987) (please see below for more discussion).

In this study, we also showed that, for strong promoters, transcription-coupled hypernegative supercoiling of plasmid DNA in *E. coli topA* strains did not need the expression of a membrane-insertion protein although it required co-transcriptional synthesis of a polypeptide (Figure 2.4, 2.5, and 2.12). These results are consistent with our previously published results for T7 RNA polymerase where the strong T7 RNA polymerase efficiently drove plasmid DNA templates to hypernegatively supercoiled status even when the transcriptional machinery did not couple to translation and membrane insertion (Samul and Leng, 2007). We noticed that these results appear inconsistent with the previously published results showing that plasmid hypernegative supercoiling by *E. coli* RNA polymerase required the anchoring or insertion of the coupled transcription–translation complex into the cytoplasmic membrane (Cook et al., 1992; Lodge et al., 1989; Lynch and Wang, 1993; Ma et al., 1994; Pruss, 1985; Pruss and Drlica, 1986; Stupina and Wang, 2005). However, a careful analysis showed that both situations can be explained by the “twin-supercoiled-domain” model of transcription where the friction force (Liu and Wang, 1987) applied to *E. coli* RNA polymerase is different for promoters with different strengths. Under our experimental conditions, weak promoters with very low rates of transcription initiation might not lead to the formation of an active transcriptional ensemble including a transcribing RNA polymerase, a newly transcribed RNA, the associated ribosomes, and a newly generated polypeptide for most plasmids, which are not capable of generating sufficient friction force on *E. coli* RNA polymerase to produce the “twin-supercoiled-domains” on plasmids. In this case, TCDS is negligible. For example, recent studies showed that a synthetic weak promoter P_{lar} , a derivative of P_{lac} , only produced 4 RNAs on average in 1 h in *E. coli* cells after maximum

induction (Golding et al., 2005; Kandhavelu et al., 2011). For most times, there was no transcription initiation from P_{lar} promoter (Kandhavelu et al., 2011). Since DNA gyrase is also limited in *E. coli* cells (Taniguchi et al., 2010), the chance for weak promoters to drive the plasmid DNA templates to hypernegatively supercoiled status is low. Promoters with moderate strength, such as the P_{tet} promoter (Stuber and Bujard, 1981), may be able to generate one or two transcriptional ensembles per plasmid. Because the transcription elongation rate of *E. coli* RNA polymerase is relatively low (Golding and Cox, 2004; Uptain et al., 1997), it is possible that transcription alone may not be able to generate enough friction torque to fully prevent *E. coli* RNA polymerase from rotating against the DNA double helix and therefore cannot induce the formation of significant amounts of localized supercoiled domains. In this case, in order to generate “twin-supercoiled-domains,” the transcriptional ensemble has to anchor to the bacterial cytoplasmic membrane through co-transcriptional synthesis of polypeptide encoding membrane proteins to maximize friction resistance. This interpretation explains why TCDS for plasmid pBR322 and its derivatives carrying the P_{tet} promoter depends on the expression of a membrane-insertion tetracycline resistance protein in *E. coli topA* strains (Cook et al., 1992; Lodge et al., 1989; Lynch and Wang, 1993; Ma et al., 1994; Pruss, 1985; Pruss and Drlica, 1986; Stupina and Wang, 2005). For strong promoters, such as $P_{T7A1/O4}$ and P_{tac} , *E. coli* RNA polymerase is able to rapidly initiate transcription from them (Brunner and Bujard, 1987; Lanzer and Bujard, 1988). Therefore, each plasmid may have multiple RNA polymerases (more than two RNA polymerases) simultaneously transcribing along the DNA template. It is possible that the friction force against multiple transcribing RNA polymerases is significantly increased and sufficient to cause the formation of the “twin-

supercoiled-domains.” In this scenario, transcription-coupled hypernegative supercoiling of plasmid DNA did not need the expression of a membrane-insertion protein. Regardless, our results showed that co-transcriptional synthesis of a polypeptide is still required for the formation of hypernegative supercoiling of plasmid DNA in *E. coli topA* strains (Figure 2.5). There are two possibilities for this requirement. The first possibility is that co-transcriptional synthesis of a polypeptide significantly increases the size of a transcriptional ensemble (including a transcribing RNA polymerase, the newly synthesized RNA transcript, the associated ribosomes, and the newly synthesized polypeptides) and therefore increases the friction torque against the transcription ensemble, which prevents or retards the transcribing RNA polymerase from rotating around the DNA double helix and helps generate the “twin-supercoiled-domains.” If this explanation is correct, anchoring of plasmid DNA to bacterial cytoplasmic membrane should increase the efficiency of TCDS. Indeed, our results showed that the efficiency of TCDS is higher for plasmids expressing tetracycline resistance protein than that for plasmids expressing GFPuv protein although the sizes of both proteins are similar (compare Figure 2.3D and 2.4D). The second possibility is that ribosomes protect mRNA from degradation by ribonucleases. Because the length of RNA transcripts, which should be proportional to the friction force applied to *E. coli* RNA polymerase, plays a critical role in the production of the “twin-supercoiled-domains” (Leng et al., 2004; Liu and Wang, 1987; Samul and Leng, 2007), co-transcriptional synthesis of a polypeptide should greatly stabilize the mRNA (Deana and Belasco, 2005; Pedersen et al., 2011) and therefore increase the efficiency of TCDS in *E. coli* cells. Our results demonstrating that transcription-coupled hypernegative supercoiling of DNA is correlated with the stability

of mRNA produced by these plasmids after IPTG induction strongly support this explanation (Figure 2.6). Additionally, the facts that transcription alone added a few supercoils to plasmids pZXD60A, 61, and 63A also support this interpretation (Figure 2.5D, E, and F). Nevertheless, these two possibilities are not mutually exclusive and may contribute together to TCDS *in vivo*.

RNA polymerases are powerful motor proteins (Bai et al., 2006; Seidel and Dekker, 2007; Wang et al., 1998) which are able to rapidly move along chromosomes and remodel chromosome structures through TCDS (Albert et al., 1996; Cook et al., 1992; Dunaway and Ostrander, 1993; Leng and McMacken, 2002; Lodge et al., 1989; Lynch and Wang, 1993; Ma et al., 1994; Stupina and Wang, 2004; Tsao et al., 1989; Wu et al., 1988). The chromosomal remodeling by RNA polymerase in *E. coli* cells is directly link to the activation of transcription and DNA replication. For example, in the *ilvYC* operon of *E. coli*, the *ilvY* promoter is divergently coupled to the *ilvC* promoter (Rhee et al., 1999). Results from Hatfield Laboratory clearly demonstrated that the transcriptional activities of the *ilvY* and *ilvC* promoters are dependent on the localized superhelical density around the promoter region and can be activated by each other (Opel and Hatfield, 2001; Rhee et al., 1999). Another well-characterized example is the activation of the *Salmonella typhimurium leu-500* promoter by divergently-coupled transcription. Results from Wu's and Lilley's Laboratories showed that transcription-driven localized supercoiling rather than the global superhelical density is responsible for activation of the *leu-500* promoter (Chen et al., 1992; Tan et al., 1994). An additional example demonstrating the biological functions of TCDS stems from the studies of bacteriophage λ DNA replication initiation. Previous studied showed that bacteriophage λ DNA

replication initiation is dependent on transcription in a nearby promoter *in vivo* (Hase et al., 1989). Our recent results showed that TCDS is responsible for the activation of λ DNA replication (Leng et al., 2011). Specifically, the O-some assembled from the DNA replication initiator O protein binding to the DNA replication origin functions as a DNA topological barrier blocks, confines, and captures TCDS. In this scenario, λ DNA replication origin is unwound and DNA replication is initiated. All these examples demonstrated that TCDS plays a critical role in certain biological events.

2.6 Acknowledgments

We would like to thank Dr. Sankar Adhya for helpful discussion and suggestions. We also thank Steven Eichelbaum for technical support and for critical reading of the manuscript before submission. We are grateful to Drs. Marc Drolet and Nikolai V. Ravin for providing us with *E. coli* strain *DM800* and linear plasmid pG591, respectively. This work was supported by grant 1SC1HD063059-01A1 from the NIH (to F.L.).

Table 2.1. DNA oligonucleotides used for primers of the RT-PCR experiments

Oligo ^a	Sequence (5'-3')	Location in the gene	Gene	PCR products
FL594F	ATTATGGCCACACCAGTGGCGC	2917-2939	<i>LacZ</i>	173bp, distal ^b
FL594R	TGACGGGCTCCAGGAGTCGTC	3069-3089	<i>LacZ</i>	173bp, distal ^b
FL654F	CACCGATCGCCCTTCCCAACAGTTG	212-236	<i>LacZ</i>	172bp, proximal ^c
FL654R	GTAGATGGGCGCATCGTAACCG	362-383	<i>LacZ</i>	172bp, proximal ^c
FL580F	GCGAGGCTGGATGGCCTTCC	990-1009	<i>tet</i>	176bp, distal ^b
FL580R	CCGTGACGATCAGCGGTCCAG	1145-1165	<i>tet</i>	176bp, distal ^b
FL657F	GGCCTCTTGCGGGATATCGTCCATTCC	184-210	<i>tet</i>	168bp, proximal ^c
FL657R	GATAGTGGCTCCAAGTAGCGAAGCG	327-351	<i>tet</i>	168bp, proximal ^c
FL590F	TCCAATTGGCGATGGCCCTGT	660-680	<i>GFPuv</i>	101bp
FL590R	GGACCATGTGGTCACGCTTTTCGT	737-760	<i>GFPuv</i>	101bp
FL586F	AGTTATCCCCCTCCATCAGG	154-135	16S rRNA	99bp
FL586R	TGCAAGTCGAACGGTAACAG	56-75	16S rRNA	99bp

^aFLXXXF and FLXXXR represent the forward and reverse primers of the PCR reactions, respectively. ^bDistal indicates the PCR products that locate in the distal region of the gene. ^cProximal indicates the PCR products locating in the proximal region of the gene.

Table 2.2. Plasmids constructed in this study

Plasmid	<i>E. coli</i> Promoter	Gene under control of the cloned promoter
pZXD44	T7A1/O4	<i>tet</i> ^a
pZXD50	tac	<i>tet</i> ^a
pZXD49	lacUV5	<i>tet</i> ^a
pZXD47	lac	<i>tet</i> ^a
pZXD48	lacL8	<i>tet</i> ^a
pZXD57	T7A1/O4	<i>GFPuv</i> ^b
pZXD58	tac	<i>GFPuv</i> ^b
pZXD56	lacUV5	<i>GFPuv</i> ^b
pZXD55	lac	<i>GFPuv</i> ^b
pZXD54	lacL8	<i>GFPuv</i> ^b
pZXD59	T7A1/O4	Reverse <i>GFPuv</i> ^b
pZXD60	T7A1/O4	<i>lacZ</i> ^c
pZXD60A	T7A1/O4	N/A ^d
pZXD61	T7A1/O4	Reverse <i>lacZ</i> ^c
pZXD62	T7A1/O4	<i>GFPuv</i> ^b
pZXD63	T7A1/O4	<i>tet</i> ^a
pZXD63A	T7A1/O4	N/A ^d

Plasmid pZXD60A and 63A have an amber mutation in the start codon of *lacZ* and *tet*, respectively. ^aThe tetracycline resistance gene of pBR322 (*tet*) encodes a 41 kD transmembrane protein TetA. ^bThe green fluorescence protein UV (*GFPuv*) gene encodes a cytosolic protein GFPuv. ^cThe *lacZ* gene encodes a cytosolic protein β -D-galactosidase. ^dN/A represents not applicable.

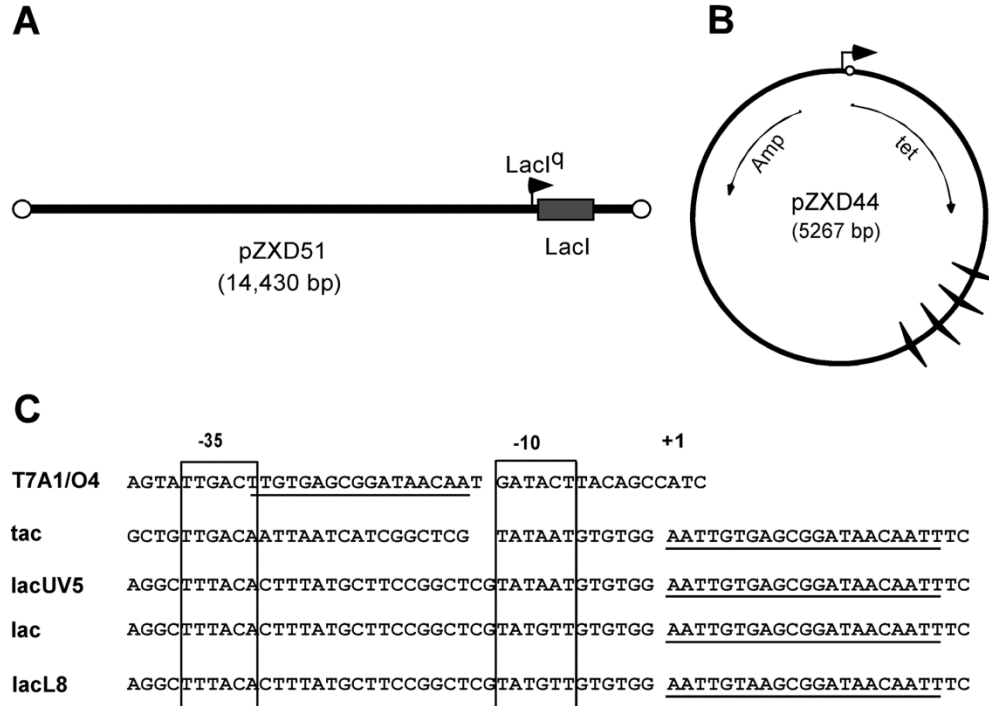


Figure 2.1 A two-plasmid system to study TCDS *in vivo*. This system contains two plasmids, a linear plasmid (A), i.e., pZXD51 and a circular, supercoiling-reporter plasmid (B), such as pZXD44. The linear plasmid is derived from linear coliphage N15-based plasmid pG591 and carries a *lacI* gene under the control of the strong P_{lacI^q} promoter. *E. coli* cells containing pZXD51 over-express *lac* repressor (LacI) constitutively, which binds to the *lac OI* operator (the open circle) on the supercoiling-reporter plasmids. The supercoiling-reporter plasmids were derived from plasmid pBR322 and constructed as detailed under Material and methods. They harbor an IPTG-inducible promoter with different strengths and a transcription unit between the promoter and a set of 4 Rho-independent *E. coli rrnB* T1 terminators (winged triangles). (C) The DNA sequence of five different *E. coli* promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} . The underlines represent the *lac OI* operators.

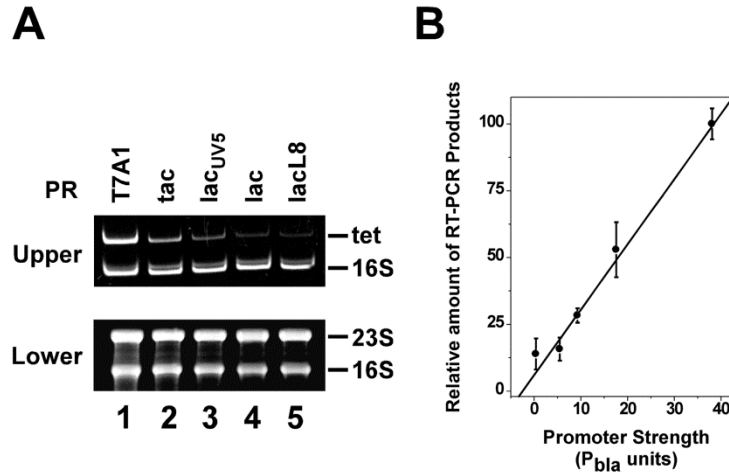


Figure 2.2 RT-PCR analysis of cDNA products of mRNA transcribed from different supercoiling-reporter plasmids pZXD44, 50, 49, 47, and 48 in *E. coli topA* strain *VS111* harboring the linear plasmid pZXD51 after 10 min of IPTG induction (1 mM). (A) RT-PCR experiments were performed as described under Materials and Methods. The lower panel is a 1.2% agarose gel containing 1% formaldehyde to show the integrity of the RNA samples used for the RT-PCR experiments. The upper panel is a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of 16S rRNA and *tet* gene cDNA synthesized from the RNA samples isolated from *E. coli* strain *VS111* carrying supercoiling-reporter plasmids pZXD44, 50, 49, 47, and 48 after 10 min of IPTG induction (lanes 1-5 respectively). Labels: PR, promoter; T7A1, the T7A1/O4 promoter; *tac*, the *tac* promoter; *lacUV5*, the *lacUV5* promoter; *lac*, the *lac* promoter; *lacL8*, the *lacL8* promoter. (B) Real-time RT-PCR analyses of the *tet* gene mRNA for *E. coli* strain *VS111* carrying different supercoiling-reporter plasmids pZXD44, 50, 49, 47, and 48 after 10 min of IPTG induction (mean±SD, three independent experiments). The relative level of RT-PCR products is proportional to the promoter strength. Promoter strength in P_{bla} units was obtained from Lanzer and Bujard (1988).

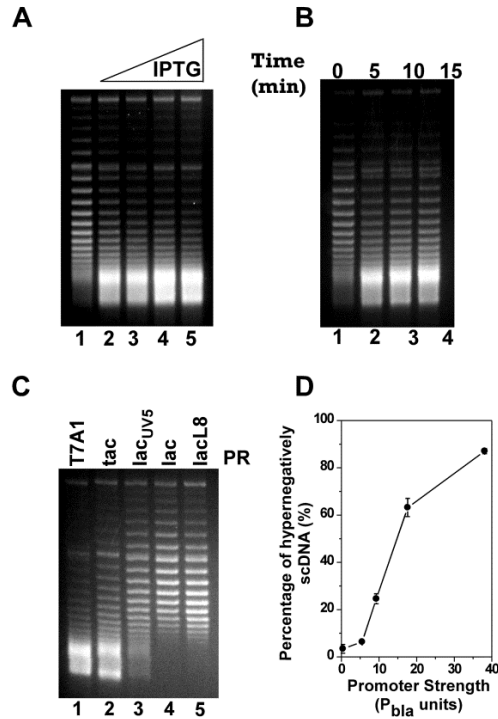


Figure 2.3 TCDS in *E. coli* strain VS111 is dependent on promoter strength. The *in vivo* T-S assays were performed as described under Materials and Methods. DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine and stained with ethidium bromide. The fastest moving band in the gels where DNA topoisomers are no longer resolvable under our experimental conditions represents the hypernegatively supercoiled DNA. (A) Dependence of TCDS on IPTG concentration for plasmid pZXD44 carrying a *tet* gene under the control of the strong $P_{T7A1/O4}$ promoter. Lane 1 contained the DNA sample isolated from *E. coli* cells prior to IPTG induction. Lanes 2–5 contained the DNA samples isolated from *E. coli* cells after 10 min of induction with 25, 50, 100, and 1000 μM IPTG, respectively. (B) Time course of the hypernegative supercoiling of plasmid pZXD44 in *E. coli* strain VS111 after 1 mM of IPTG induction. Lanes 1-4 contained, respectively, DNA samples isolated from VS111 after 0, 5, 10, and 15 min of IPTG induction. (C) Dependence of TCDS on promoter strength. Lanes 1-5 contained, respectively, DNA samples isolated from *E. coli topA* strain VS111 containing plasmids pZXD44, 50, 49, 47, and 48 after 5 min of IPTG (1 mM) induction. These plasmids carry a *tet* gene under the control of IPTG-inducible promoters with different strengths, i.e., promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} . Labels: PR, promoter; T7A1, the T7A1/O4 promoter; tac, the tac promoter; lacUV5, the lacUV5 promoter; lac, the lac promoter; lacL8, the lacL8 promoter. (D) The percentage of hypernegatively supercoiled DNA is proportional to promoter strength (the values are the average of at least three independent determinations and the standard deviations are shown). These results were calculated as described under Material and methods using the TCDS data shown in (C). Promoter strength in P_{bla} units was obtained from Lanzer and Bujard (1988).

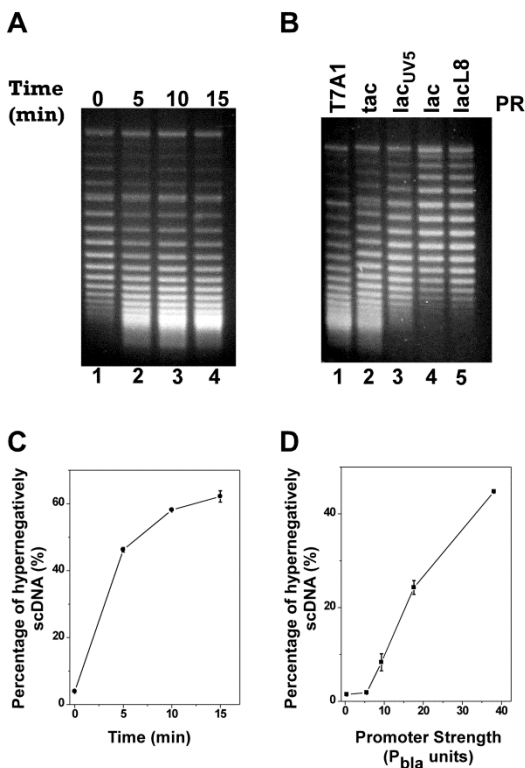


Figure 2.4 For strong promoters, TCDS in *E. coli topA* strain *VS111* did not require the expression of a membrane insertion protein. The *in vivo* T-S assays for plasmids carrying a *GFP_{UV}* gene under the control of IPTG-inducible promoters with different strengths were performed as described under Materials and Methods. DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine and stained with ethidium bromide. (A) and (C) The time course of the hypernegative supercoiling of plasmid pZXD57 carrying a *GFP_{UV}* gene under the control of the strong $P_{T7A1/O4}$ promoter in *E. coli* strain *VS111* after 1 mM of IPTG induction. Lanes 1-4 contained, respectively, DNA samples isolated from *VS111* after 0, 5, 10, and 15 min of IPTG induction. (B) and (D) Dependence of TCDS on promoter strength. Lanes 1-5 contained, respectively, DNA samples isolated from *E. coli topA* strain *VS111* containing plasmids pZXD57, 58, 56, 55, and 54, that carry a cytosolic *GFP_{uv}* gene under the control of IPTG-inducible promoters with different strengths, i.e., promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} after 5 min of IPTG (1 mM) induction. Labels: PR, promoter; T7A1, the T7A1/O4 promoter; tac, the tac promoter; lacUV5, the lacUV5 promoter; lac, the lac promoter; lacL8, the lacL8 promoter. (C) The percentage of hypernegatively supercoiled DNA for pZXD57 is a function of IPTG induction time. These results were calculated as described under Material and methods using the TCDS data shown in (A). (D) The percentage of hypernegatively supercoiled DNA is proportional to promoter strength (the values are the average of at least three independent determinations and the standard deviations are shown). These results were calculated as described under Material and methods using the TCDS data shown in (B). The promoter strength in P_{bla} units was obtained from Lanzer and Bujard (1988).

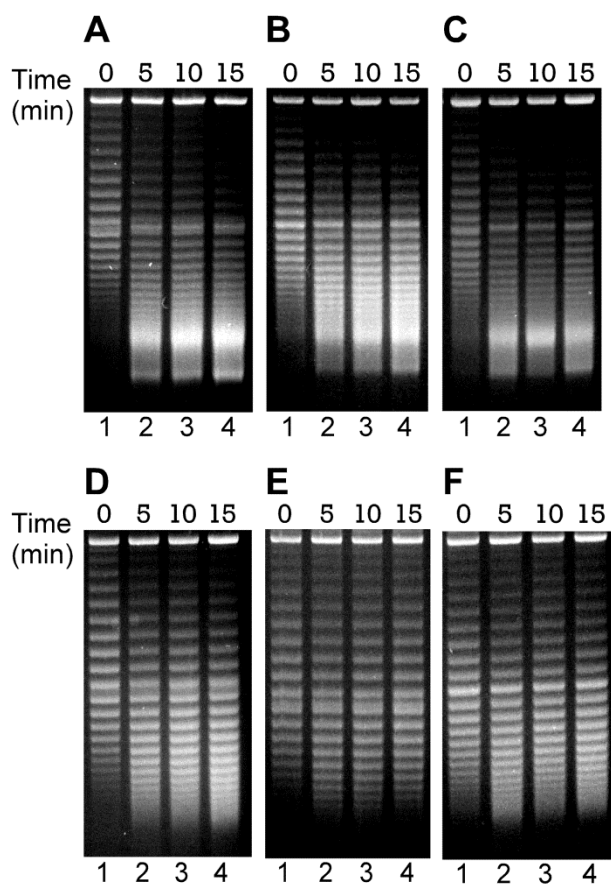


Figure 2.5 TCDS in *E. coli topA* strain *VS111* for different plasmid DNA templates with identical size carrying a strong, IPTG-inducible $P_{T7A1/O4}$ promoter. The *in vivo* T-S assays were performed as described under Materials and Methods. Transcription was induced with 1 mM of IPTG. Lane 1 contained the DNA sample before IPTG induction. Lanes 2–4 contained the plasmid DNA samples after 5, 10, and 15 min of IPTG induction, respectively. DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine and stained with ethidium bromide. Plasmids pZXD60 (A), 62 (B), and 63 (C) carry a *lacZ*, *GFPuv*, and *tet* gene under the control of the strong $P_{T7A1/O4}$ promoter, respectively. (D) Plasmid pZXD60A is identical with pZXD60 except the start codon (ATG) of *lacZ* was mutated to the stop codon TAG (amber mutation). (E) Plasmid pZXD61 contains a *lacZ* gene in the reverse orientation, which cannot direct the expression of β -galactosidase. (F) Plasmid pZXD63A is similar to pZXD63 except the start codon of the *tet* gene was mutated to the stop codon TAG (amber mutation).

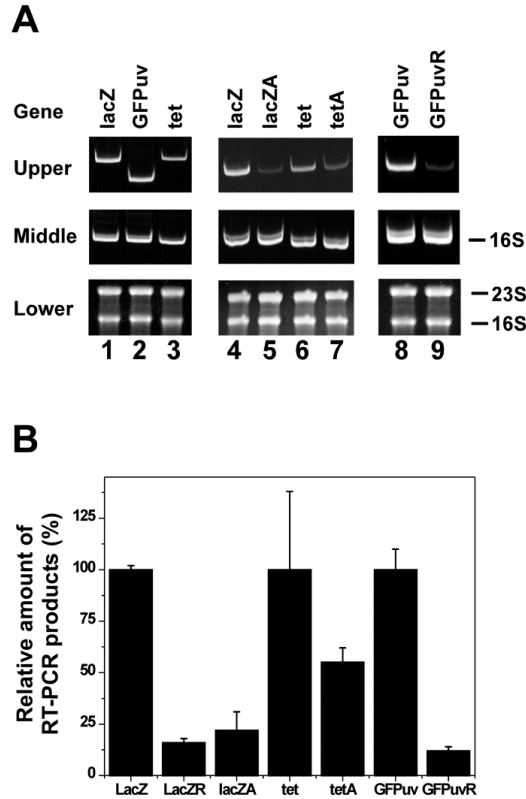


Figure 2.6 RT-PCR analyses of cDNA products of mRNA transcribed from different supercoiling-reporter plasmids pZXD60, 60A, 61, 62, 63 and 63A in *E. coli topA* strain *VS111* harboring the linear plasmid pZXD51 after 10 min of IPTG induction. (A) RT-PCR experiments were performed as described under Materials and Methods. The lower panel is a 1.2% agarose gel containing 1% formaldehyde to show the integrity of the RNA samples used for the RT-PCR experiments. The middle panel is a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of the cDNA synthesized from 16S rRNA samples isolated from *E. coli* strain *VS111* carrying different supercoiling-reporter plasmids pZXD60, 60A, 62, 63, 63A, 57, and 59 after 10 min of IPTG induction. The upper panel is also a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of the cDNA samples synthesized from the mRNA samples isolated from *E. coli* strain *VS111* carrying different supercoiling-reporter plasmids pZXD60 (lanes 1 and 4), 60A (lane 5), 62 (lane 2), 63 (lanes 3 and 6), 63A (lane 7), 57 (lane 8), and 59 (lane 9) after 10 min of IPTG induction. (B) Real-time RT-PCR analyses of the mRNA samples for *E. coli* strain *VS111* carrying different supercoiling-reporter plasmids pZXD60, 60A, 61, 62, 63, 63A, and 59 after 10 min of IPTG induction (mean±SD, three independent experiments). Labels: lacZ, the lacZ gene; lacZA, the lacZ gene with an amber mutation in the start codon; lacZR, the lacZ gene in the reverse orientation; GFPuv, the GFPuv gene; GFPuvR, the GFPuv gene in the reverse orientation; tet, the tet gene; tetA, the tet gene with an amber mutation in the start codon.

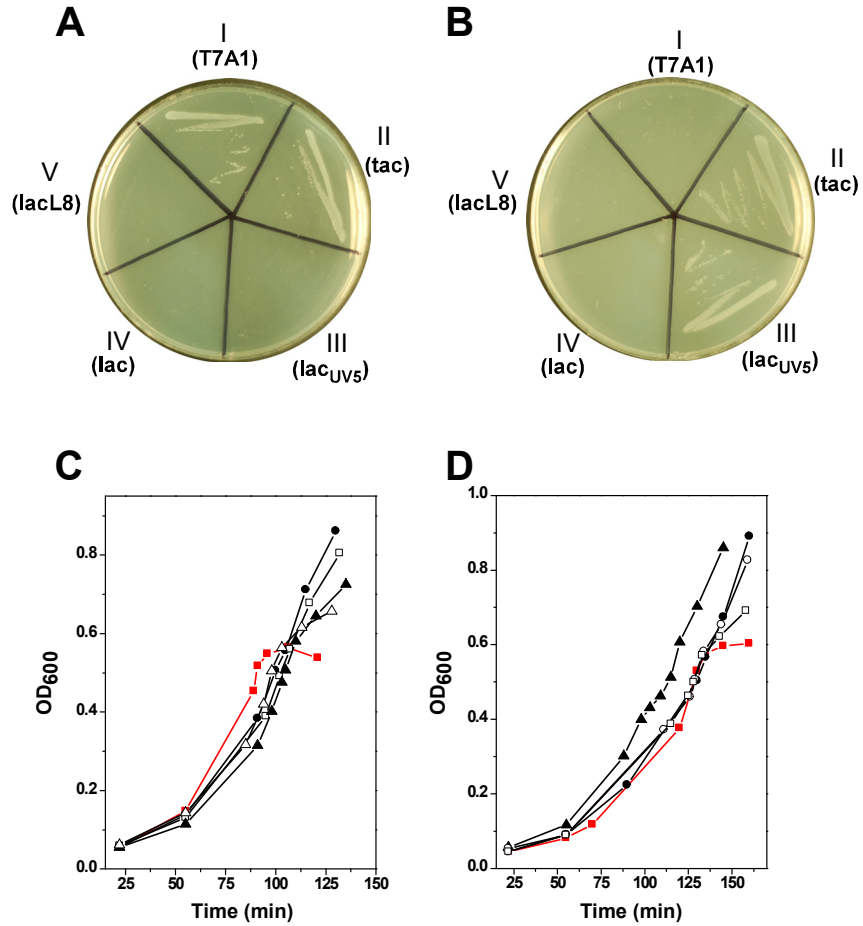


Figure 2.7 The tetracycline sensitivity assays. *E. coli topA* strain *VS111/pZXD51* containing different plasmid DNA templates was grown at 37 °C overnight on LB agar plates containing 10 µg/ml of tetracycline in the absence (A) or presence (B) of 1 mM of IPTG. These agar plates also contained 50 µg/ml of kanamycin and ampicillin. Sections I-V contained *VS111/pZXD51* carrying plasmids pZXD44, pZXD50, 49, 47, and 48, respectively. (C) and (D) are cell growth curves of *E. coli* strains *MG1655/pZXD51* and *VS111/pZXD51* carrying different plasmids in the absence of tetracycline, monitored at OD₆₀₀. 1 mM of IPTG was added to the cell cultures when OD₆₀₀ reached ~0.5. The red lines and squares represent cell growth curves for *E. coli* strains carrying plasmid pZXD44 that harbors a *tet* gene under the control of the strong P_{T7A1/O4} promoter. Other symbols represent cell growth curves for *E. coli* strains carrying plasmids pZXD50 (open triangles), 49 (solid triangles), 47 (solid circles), and 48 (open squares).

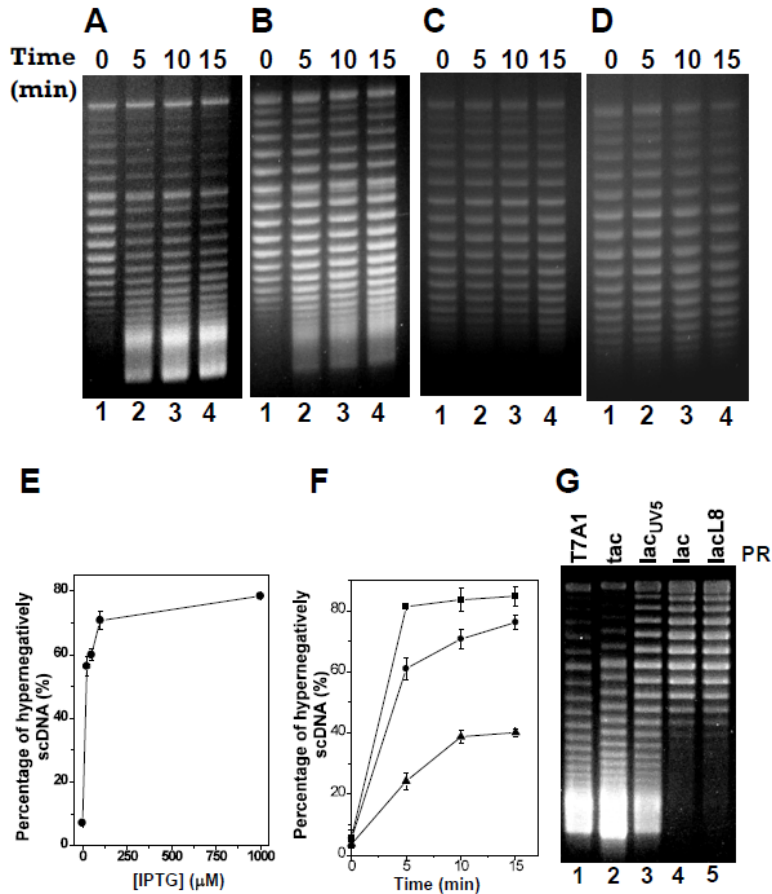


Figure 2.8 TCDS in *E. coli topA* strains is dependent on promoter strength. The *in vivo* T-S assays were performed as described under Materials and Methods. DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine and stained with ethidium bromide. (A), (B), (C), and (D) Time courses of the hypernegative supercoiling of plasmid pZXD50, 49, 47, and 48 in *E. coli* strain *VS111/pZXD51* after 1 mM of IPTG induction, respectively. Lanes 1-4 contained DNA samples isolated from *VS111/pZXD51* after 0, 5, 10 and 15 min of IPTG induction. (E) The percentage of hypernegative supercoiled DNA of plasmid pZXD44 is a function of IPTG concentration. These results were calculated as described under Materials and Methods using the TCDS data shown Figure 2.3A. (F) The percentage of hypernegatively supercoiled DNA for pZXD44 (squares), 50 (circles), and 49 (triangles) 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) and Figure 2.3B. (G) Dependence of TCDS on promoter strength. Lanes 1-5 contained, respectively, DNA samples isolated from *E. coli topA* strain *DM800/pZXD51* containing plasmids pZXD44, 50, 49, 47, and 48 after 5 min of IPTG (1 mM) induction. These plasmids carry a *tet* gene under the control of IPTG-inducible promoters with different strengths, i.e., promoters $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} . Labels: PR, promoter; T7A1, the T7A1/O4 promoter; tac, the tac promoter; lacUV5, the lacUV5 promoter; lac, the lac promoter; lacL8, the lacL8 promoter.

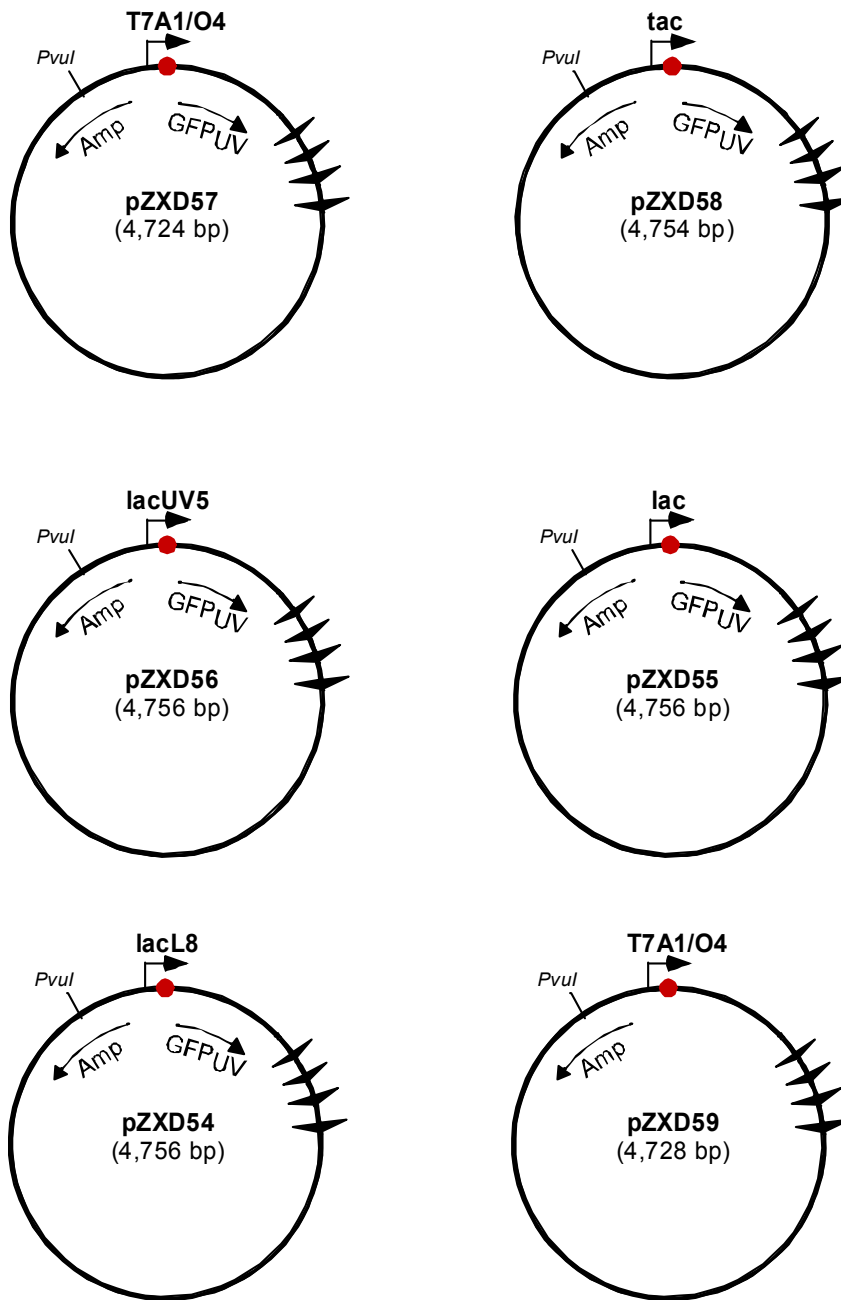


Figure 2.9 Plasmid maps of pZXD54, 55, 56, 57, 58, and 59. This series of plasmids were constructed as described under Materials and Methods. Each plasmid carries a *GFPuv* gene under the control of an IPTG-inducible promoter, i.e., $P_{T7A1/O4}$, P_{tac} , P_{lacUV5} , P_{lac} , and P_{lacL8} and a set of four copies of Rho-independent *rrnB* T1 transcription terminators (winged triangles). The arrow on each plasmid indicates the direction of transcription from the IPTG-inducible promoter. The red circle indicates the *lac OI* operator in the promoter.

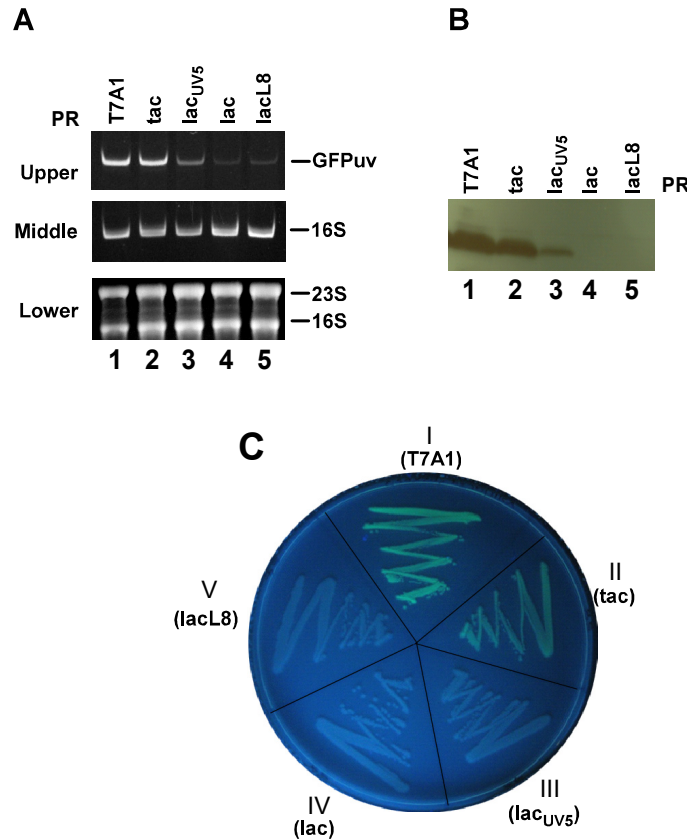


Figure 2.10 The expression level of GFPuv protein after IPTG induction in *E. coli topA* strain *VS111/pZXD51* containing different plasmids is correlated with promoter strength. (A) RT-PCR experiments were performed as described under Materials and Methods. The lower panel is a 1.2% agarose gel containing 1% formaldehyde to show the integrity of the RNA samples used for the RT-PCR experiments. The middle panel is a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of the cDNA synthesized from 16S rRNA samples isolated from *E. coli* strain *VS111/pZXD51* carrying different supercoiling-reporter plasmids. The upper panel is also a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of *GFPuv* gene cDNA synthesized from the RNA samples isolated from *E. coli* strain *VS111/pZXD51* carrying different supercoiling-reporter plasmids pZXD57 (lane 1), 58 (lane 2), 56 (lane 3), 55 (lane 4) and 54 (lane 5) after 10 min of IPTG induction. (B) Western blotting experiments were performed as described under Experimental Procedures. Lanes 1-5 contained, respectively, protein samples isolated from *E. coli VS111/pXD51* cells carrying plasmids pZXD57, 58, 56, 55, and 54 after 3 hours of 1 mM IPTG induction. (C) *E. coli topA* strain *VS111/pZXD51* containing different plasmid DNA templates was grown at 37 °C overnight on LB agar plates containing 50 µg/ml of ampicillin in the presence of 1 mM of IPTG and photographed under UV light. Sections I-V contained pZXD57, 58, 56, 55, and 54, respectively. Labels: PR, promoter; T7A1, the T7A1/O4 promoter; tac, the tac promoter; lacUV5, the lacUV5 promoter; lac, the lac promoter; lacL8, the lacL8 promoter.

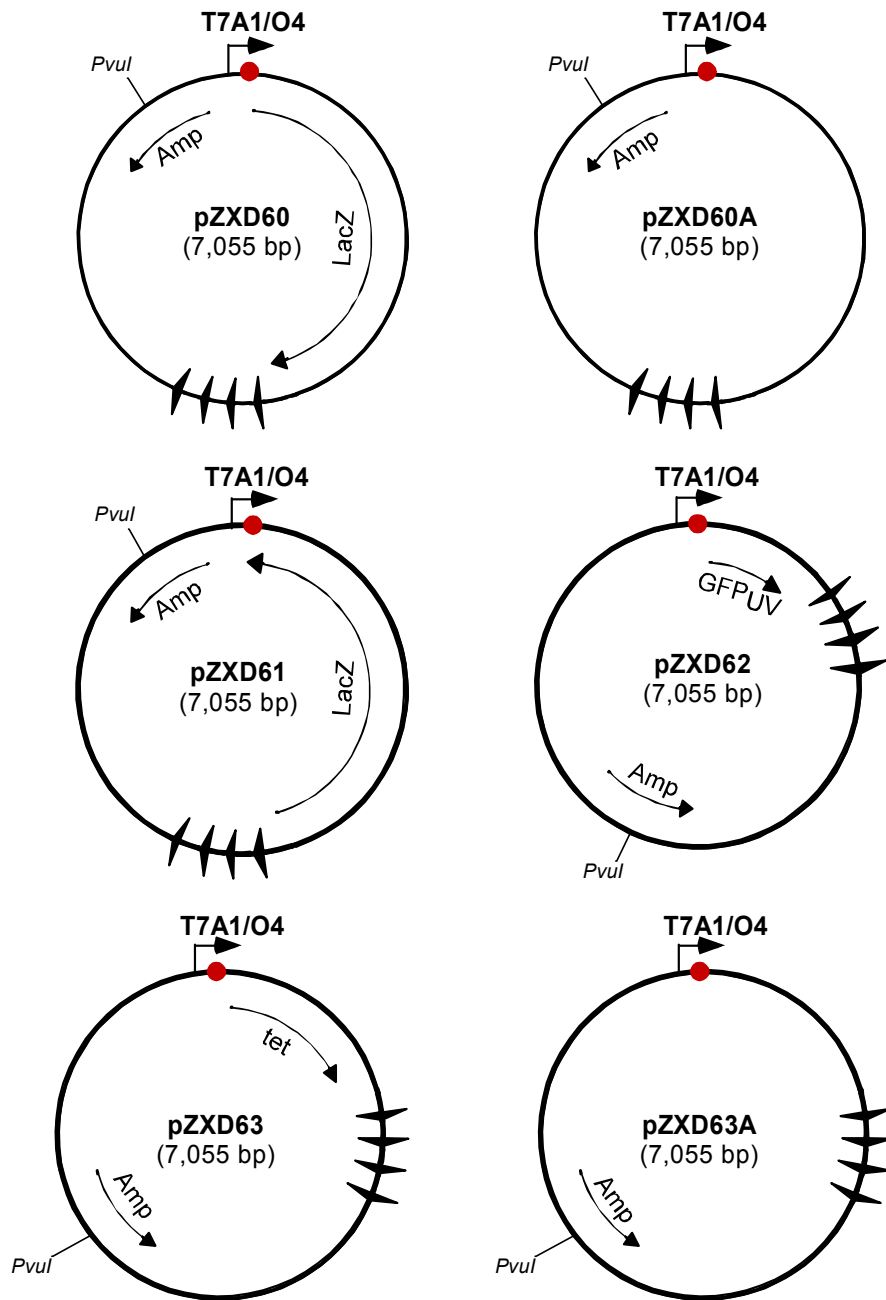


Figure 2.11 Plasmid maps of pZXD60, 60A, 61, 62, 63, and 63A. This series of plasmids of identical size were constructed as described under Materials and Methods. Each plasmid carries a strong, IPTG-inducible $P_{T7A1/O4}$ promoter and a set of four copies of Rho-independent *rrnB* T1 transcription terminators (winged triangles). The arrow on each plasmid indicates the direction of transcription from the $P_{T7A1/O4}$ promoter. The red circle indicates the *lac OI* operator in the promoter.

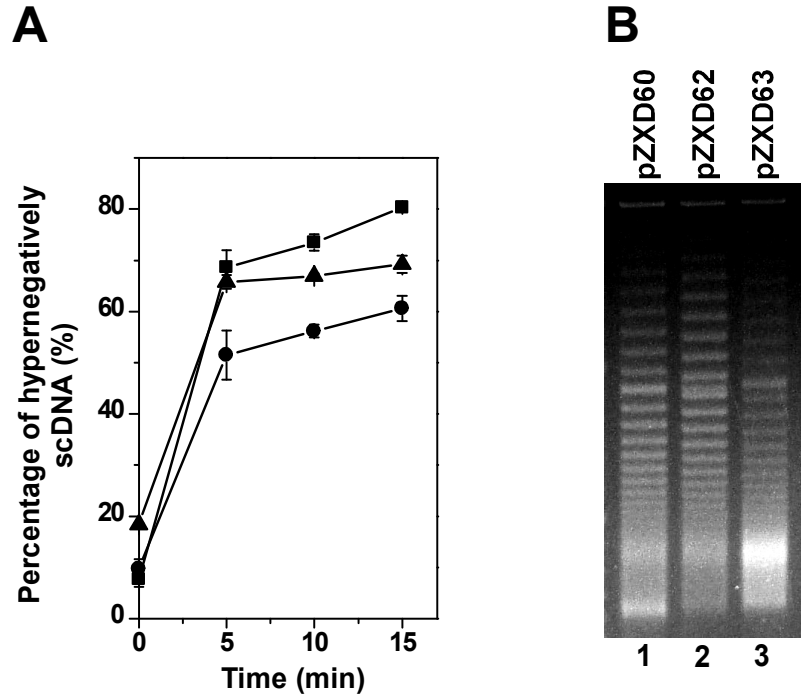


Figure 2.12 TCDS in *E. coli topA* strains *VS111/pZXD51* and *DM800/pZXD51* for different plasmid DNA templates with identical size carrying a strong, IPTG-inducible $P_{T7A1/O4}$ promoter. (A) The percentage of hypernegatively supercoiled DNA for pZXD60 (squares), 62 (circles), and 63 (triangles) is a function of IPTG induction time. These results were calculated as described under Material and methods using the TCDS data shown in Figure 2.5A, B, and C. (B) The *in vivo* T-S assays for *E. coli topA* strain *DM800/pZXD51* were performed as described under Material and methods. Transcription was induced with 1 mM of IPTG for 10 min. Lanes 1-3 contained the DNA sample for plasmids pZXD60, 62, and 63, respectively. DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 2.5 $\mu\text{g/ml}$ chloroquine and stained with ethidium bromide.

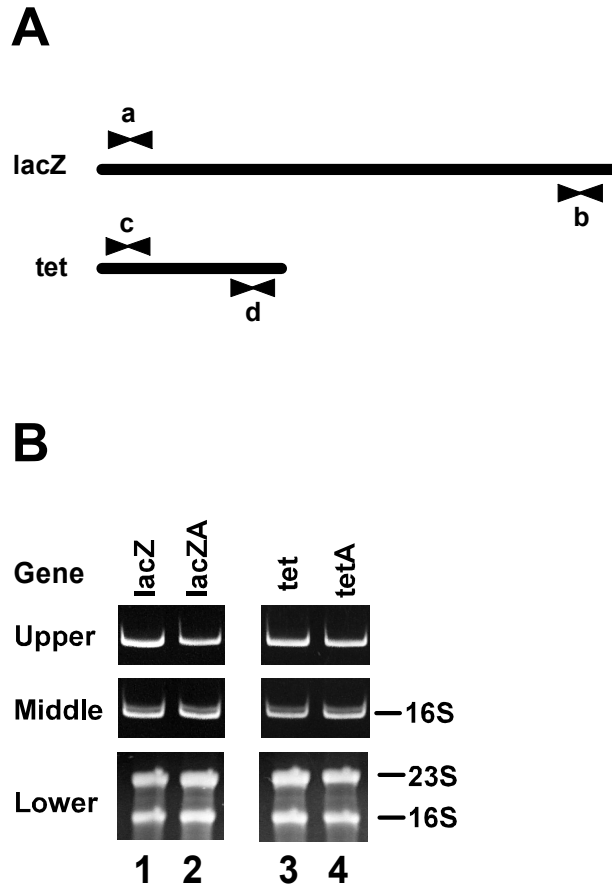


Figure 2.13 RT-PCR experiments to analyze the cDNA products synthesized from mRNA of *lacZ* and *tet* genes. (A) PCR primers to detect the proximal products (near the start codon of *lacZ* or *tet* genes; primer pairs a or c) and the distal products of cDNA (near the stop codon of *lacZ* or *tet* gene; primer pairs b and d). (B) RT-PCR experiments were performed as described under Materials and Methods. The lower panel is a 1.2% agarose gel containing 1% formaldehyde to show the integrity of the RNA samples used for the RT-PCR experiments. The middle panel is a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of the cDNA synthesized from 16S rRNA samples isolated from *E. coli* strain *VS111/pZXD51* carrying different supercoiling-reporter plasmids. The upper panel is also a 12% polyacrylamide gel in 1×TAE buffer to show the PCR products of *lacZ* and *tet* gene cDNA synthesized from the RNA samples isolated from *E. coli* strain *VS111/pZXD51* carrying different supercoiling-reporter plasmids pZXD60 (lane 1), 60A (lane 2), 63 (lane 3), and 63A (lane 4) after 10 min of IPTG induction. Primer pair a was used to detect the proximal products of *lacZ* (lanes 1 and 2). Primer pair c was used to detect the proximal products of *tet* (lanes 3 and 4). Labels: *lacZ*, the *lacZ* gene; *lacZA*, the *lacZ* gene with an amber mutation in the start codon; *tet*, the *tet* gene; *tetA*, the *tet* gene with an amber mutation in the start codon. There is no difference for the proximal PCR products between *E. coli* strains carrying pZXD60 and 60A and also between *E. coli* strains carrying pZXD63 and 63A.

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Chapter 3: Activating the *Salmonella typhimurium leu-500* promoter by transcription-coupled DNA supercoiling *in vivo*

3.1 Abstract

Transcription is able to modulate localized DNA topology both *in vitro* and *in vivo*. This phenomenon has been nicely explained by a “twin-supercoiled-domain” model of transcription in which a transcribing RNA polymerase is able to induce the formation of a transient positive supercoiled domain ahead of the RNA polymerase and a negatively supercoiled domain behind it. Transcription-induced localized, topological change of chromosome is expected to remodel chromosomal structure and greatly influence DNA transactions, such as transcription, DNA replication, and recombination. In this chapter, we developed an *in vivo* system to study TCDS and its effects on the supercoiling-sensitive *leu-500* promoter. This system consists of an *E. coli topA* strain *VS111(DE3)* and a linear plasmid DNA template in which the *leu-500* promoter is divergently coupled to the T7 promoter. Additionally, a *luciferase* gene is under the control of the *leu-500* promoter. Using the highly sensitive luciferase assay and RT-PCR experiment, we demonstrated that transient TCDS was able to activate the *leu-500* promoter. Our results are consistent with the hypothesis in which transcription is a major chromosomal remodeling force in *E. coli* cells.

3.2 Introduction

It has been well known that transcription and DNA topology are mutually linked (Fisher, 1984; Pruss and Drlica, 1989; Wang and Lynch, 1993). Effects of transcription on DNA topology have been studied for many years. In 1983, Lockshon and Morris

found plasmid pBR322 isolated from *E. coli* cells becomes positively supercoiled after exposure to DNA gyrase inhibitors (Lockshon and Morris, 1983). In a later study by Pruss (Pruss, 1985), it was demonstrated that pBR322 isolated from *E. coli* or *S. typhimurium topA* mutants lacking DNA topoisomerase I is hypernegatively supercoiled. Based on these studies, In 1987, Liu and Wang proposed the “twin-supercoiled-domain” model, to explain TCDS (Liu and Wang, 1987). They hypothesized that as the size of the RNA transcript increases, it will be more difficult for the transcribing complex to rotate around the DNA double helix. Therefore, at a critical point, it is more practical for the DNA to rotate around its own helical axis to produce a transient positively supercoiled domain ahead of the RNA polymerase and a transient negatively supercoiled domain behind it. In bacteria, DNA gyrase functions to convert a part of the transient positive supercoils to “permanent” negative supercoils, whereas DNA topoisomerase I and IV function to relax a fraction of the transient negative supercoils (Adachi et al., 1989).

DNA topology was able to affect the transcriptional efficiency of certain genes. Although both negative and positive supercoiling have been reported to inhibit transcription of some genes (Brahms et al., 1985; Wang, 1992; Gralla, 1996), it appears that negative supercoiling usually stimulates transcription. The best example came from the study of the prokaryotic *leu-500* promoter in *S. typhimurium* and *E. coli*, which is normally inactive, but it can be activated by negative supercoiling of DNA templates (Lilley and Higgins, 1991; Tan et al., 1994). The *leu-500* mutation is a single A-to-G point mutation in the -10 region of the promoter controlling the *leu* operon which results in leucine auxotrophy (Mukai and Margolin, 1963; Dubnau and Margolin, 1972). The AT

to GC mutation is expected to increase the energy barrier for the formation of a functional transcription open complex. This phenotype can be suppressed by a mutation in the topoisomerase I gene which results in a loss of topoisomerase I's activities (Trucksis et al., 1981). Intriguingly, Lilley and Higgins demonstrated that the activation of the *leu-500* promoter was only dependent on the *topA* background but did not correlate with the level of global supercoiling, as measured for extracted plasmid DNA (Lilley and Higgins, 1991). When the *leu-500* promoter alone was cloned into a plasmid, it could no longer be activated in the *topA* strains (Lilley and Higgins, 1991; Chen et al., 1992). These results suggested that an "unknown" regulating element is responsible for the activation of the *leu-500* promoter. Results from Dr. Wu's group and Dr. Lilley's group showed that transcription-driven localized supercoiling rather than the global superhelical density is responsible for activation of the *leu-500* promoter (Chen et al., 1992; Tan et al., 1994), suggesting that the "unknown" regulating element is TCDS. More recently, on the basis of a series of careful analyses, Wu and co-workers have proposed a promoter relay mechanism to explain the expression of genes in the *ilvIH-leuO-leuABCD* gene cluster which is coordinated in a sequential manner (Fang and Wu, 1998; Fang and Wu, 1998). The key component in this model is TCDS, which causes transient localized structural changes on DNA templates.

While it is clear that TCDS plays an essential role in the activation of the *leu-500* promoter, a detailed mechanism explaining how TCDS regulates gene expression is still elusive. Many studies showed that transcription has significant effects on DNA topology (Dröge, 1994). For example, transcription can induce DNA supercoiling in the presence

of *E. coli* DNA topoisomerase I or gyrase. Models such as the “twin-supercoiled-domain” model of transcription have been proposed (Liu and Wang, 1987), but they cannot completely explain all experimental results (Drolet et al., 1994; Chen and Lilley, 1999). In addition, many aspects of transcription-coupled DNA supercoiling have yet to be explored. A well-controlled system is definitely required for further studies. In addition, many DNA transactions such as the initiation of DNA replication, recombination, and transcription are coupled to transcription. In many cases, the transient topological changes of DNA templates induced by transcription are responsible for the initiation of these DNA transactions (Dröge, 1994). Thus, a study of transcription-coupled DNA supercoiling and its activation of gene expression *in vivo* are of biological significance and fundamental interest. In this chapter, we established an *in vivo* system to study transient TCDS and its effects on the supercoiling-sensitive *leu-500* promoter. Our results showed that transient TCDS was indeed able to activate the *leu-500* promoter in *E. coli* cells.

3.3 Methods

3.3.1 Materials

Ethidium bromide, Kanamycin, lysozyme and chloroquine were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Ampicillin and bovine serum albumin (BSA) were obtained from Fisher Scientific (Fairlawn, NJ). Isopropyl- β -D-thiogalactopyranoside (IPTG) was obtained from Anatrace, Inc (Maumee, Ohio). All restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were bought from New England Biolabs (Beverly, MA). *Pfu* DNA polymerase was purchased from

Stratagene, Inc. (La Jolla, CA). All synthetic oligonucleotides used as primers were obtained from MWG-Biotech, Inc. (Huntsville, AL). QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, RNeasy Mini Kit, and QIAquick Nucleotide Removal Kit were bought from QIAGEN, Inc. (Valencia, CA). ThermoScript RT-PCR System plus Platinum® *Taq* DNA polymerase was purchased from Invitrogen, Inc. (Carlsbad, CA). Power SYBR Green PCR Master Mix was obtained from Applied Biosystems, Inc. (Carlsbad, CA). Luciferase Assay System is a product of Promega Corporation (Madison, WI). SYBR® Gold Nucleic Acid Gel Stain was purchased from Life Technologies (Grand Island, NY).

3.3.2 Bacterial strains and plasmids

Escherichia coli strain *VS111* [F^- , λ^- , *rph-I*, $\Delta topA$] and *MG1655* [F^- , λ^- , *rph-I*] were obtained from the *Coli* Genetic Stock Collection/*E. coli* Genetic Resource Center (CGSC) at Yale University. The linear plasmids pZXD4, pZXD51, and circular plasmid pZXD14 were described in chapter 2 of this dissertation.

All circular plasmids constructed in this work were derived from plasmid pBR322. Plasmid pZXD64 was constructed by introducing a unique AgeI site into the upstream region of the *tet* gene of pZXD14 using PCR-based, site-directed mutagenesis. Then, the *tet* gene between the unique AgeI and BsmI sites of pZXD64 was replaced by a 3,068 bp *lacZ* gene DNA fragment of plasmid pYC2/CT/*lacZ* (Life Technologies, Grand Island, NY) to generate pZXD65. Next, four Rho-independent *E. coli rrnB* T1 terminators from plasmid pLUC1 were inserted into XbaI site of pGL3 (Promega Corporation, Wisconsin, WI) to yield pZXD67. A 2,511 bp HindIII-SpeI DNA fragment of pZXD67 carrying a

modified firefly (*Photinus pyralis*) *luciferase* gene (the codon usage was optimized for mammalian cells) and four Rho-independent *E. coli rrnB* T1 terminators was inserted between the HindIII and SpeI sites of pZXD65 to produce pZXD70. Plasmid pZXD74 was created by silently removing the EcoRI site in the downstream region of *lacZ* gene of plasmid pZXD70 without changing the open reading frame of *lacZ* gene using PCR-based, site-directed mutagenesis. Plasmid pZXD76 was generated after a XbaI site was inserted into the downstream region of the *luciferase* gene of plasmid pZXD74. Then, a 1,688 bp DNA fragment of pZE15luc carrying a firefly (*Photinus pyralis*) *luciferase* gene (the codon usage was optimized for bacterial cells) was inserted into the HindIII and XbaI sites of pZXD76 to yield pZXD77.

In this study, a few plasmids that differ in the distance and DNA composition between the T7 and *leu-500* promoters were constructed. pZXD97 was created by inserting a 72 bp synthetic deoxyoligonucleotide containing the *leu-500* promoter and a unique BamHI site into the HindIII and EcoRI sites of pZXD77. pZXD99 was produced by inserting a 53 bp synthetic deoxyoligonucleotide into the BamHI and EcoRI sites of pZXD97. pZXD94 was constructed in two steps. First, a 72 bp synthetic deoxyoligonucleotide containing the *leu-500* promoter and a unique AvrII site was inserted into the HindIII and EcoRI sites of pZXD77 to create pZXD92. Second, a 720 bp DNA fragment of pZXD57 (pZXD57 was described in chapter 2) carrying part of *GFPuv* gene was inserted into AvrII and EcoRI sites of pZXD92 to yield pZXD94. pZXD93 was created by inserting a 47 bp synthetic deoxyoligonucleotide into the AvrII and EcoRI sites of pZXD92. pZXD100 was produced by inserting a 31 bp synthetic deoxyoligonucleotide into the BamHI and EcoRI sites of pZXD97. pZXD102, pZXD104

were created by inserting one or two copies of 52 bp synthetic deoxyoligonucleotide into the unique BamHI site of pZXD99, respectively. The distance between the T7 and the *leu-500* promoters for different plasmids are listed in Table 3.2.

Plasmids pZXD82, 83, 84, 85, and 86 were constructed by replacing the T7 promoter with *E. coli* promoters P_{T7A1/O4}, P_{tac}, P_{lacUV5}, P_{lac} and P_{lacL8} between the EcoRI and XhoI sites of pZXD77, respectively.

The linear plasmid pZXD80 was constructed by inserting a 6,833bp BglII-SpeI (from pZXD77) fragment carrying a T7 promoter and the *leu-500* promoter in the divergent orientations as described above into the BglII-NheI sites of pZXD4. Similarly, the linear plasmid pZXD103 was constructed by inserting a 6,763bp BglII-SpeI (from pZXD99) fragment carrying a T7 promoter and the *leu-500* promoter in the divergent orientation into the BglII-NheI sites of pZXD4. Plasmids pZXD87, 88, 89, 90, and 91 were constructed by inserting a ~ 6.9 kb BglII-SpeI (from pZXD82, 83, 84, 85, and 86) fragment carrying a P_{T7A1/O4}, P_{tac}, P_{lacUV5}, P_{lac} and P_{lacL8} promoter and the *leu-500* promoter in the divergent orientations as described above into the BglII-NheI sites of pZXD4, respectively.

3.3.3 Luciferase Assay

Luciferase Assay was used to verify the expression of *luciferase* in various *E. coli* strains carrying different plasmid DNA templates. Briefly, *E. coli* cells carrying different plasmids were grown overnight in LB containing 50 µg/ml of ampicillin or Kanamycin. The overnight culture was then diluted (1:100) in fresh LB containing 50 µg/ml of ampicillin or Kanamycin in the presence of different concentrations of IPTG, and grown

until the optical density at 600 nm reached approximately 0.5. Next, 50 μ l of cells were mixed with 10 μ l of 1 M K_2HPO_4 (pH 7.8) and 20 mM EDTA, quickly frozen in liquid nitrogen for 3 min, and equilibrated to room temperature for 30 min to yield about 60 μ l of cell lysate. Then, the cell lysate was mixed with 300 μ l freshly prepared lysis mix containing 1 \times cell culture lysis reagent (CCLR), 1.25 mg/ml lysozyme, and 2.5 mg/ml BSA, and incubated for 10 min at room temperature. Finally, 100 μ l of Luciferase Assay Reagent (Promega Corporation, Madison, WI) was added to 20 μ l of the cell lysate and used for light measurement by using a luminometer (Biocounter, Titusville, FL) with an integration time of 10 s.

3.3.4 RNA isolation, cDNA synthesis, and polymerase chain reaction (PCR)

Total RNA were isolated from *E. coli* cells using QIAGEN RNeasy Kit as described by the manufacturer. To determine the integrity of the total RNA samples, 16S and 23S rRNA were resolved by electrophoresis in a 1.2% agarose gel in 1 \times MOPS buffer containing formaldehyde (20 mM MOPS, 8 mM sodium acetate anhydrous and 1 mM EDTA, pH 7.0, and 1% formaldehyde). After electrophoresis, agarose gels were stained with ethidium bromide, destained, and photographed under UV light. cDNA were synthesized from total RNA samples using ThermoScript RT-PCR System. 2.76 μ g of RNA was mixed with random hexamer primers (50 ng/ μ l) and four deoxynucleotide triphosphates (dNTPs; final concentration: 1 mM). The mixtures were incubated at 65 $^{\circ}$ C for 5 min and transferred on ice for another 5 min to remove secondary structures of RNA. The denatured RNA samples were then mixed with 1 \times cDNA synthesis buffer with a total volume of 20 μ l containing 5 mM DTT, 40 units of RNaseOut, and 15 units of

ThermoScript Reverse Transcriptase, and incubated at 25 °C for 10 min followed by 50 °C for 50 min to synthesize cDNA. The cDNA synthesis mixtures were transferred to an 85 °C water bath for 5 min to terminate the reactions. After the synthesis step, the reaction mixtures were mixed with 2 units of RNase H and incubated at 37 °C for 20 min to remove the RNA templates.

PCR Reactions were carried out using cDNA samples synthesized as described above. A 50 µl PCR reaction contains 1×PCR Buffer without Mg²⁺, 1 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each primer, 0.5 µl cDNA and 2 units of Platinum *Taq* DNA polymerase. The reactions started at 94 °C for 2 min, proceeded 16 cycles (for linear plasmids, used 21 cycles instead) of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, and terminated at 72 °C for 10 min. Subsequently, the PCR products were analyzed by electrophoresis in a 12% polyacrylamide gel in 1×TAE buffer. After electrophoresis, polyacrylamide gels were stained with ethidium bromide, destained, and photographed under UV light.

3.3.5 Real-time PCR Assays

Real-time PCR assays were carried out using MiniOpticon Real-time PCR system (Bio-rad, Hercules, CA). A 20 µl reaction contains 0.5 µl cDNA, 0.5 µM of each primer and 10 µl of Power SYBR Green PCR Master Mix (2X). The reaction started at 95 °C for 10 min and continued for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The C_q values (quantification cycle values) were calculated from exponential phase of each PCR amplification reaction as recommended by the manufacturer.

3.3.6 *In vivo* transcription-supercoiling (T-S) assay

E. coli cells carrying different plasmids were grown overnight in LB containing 50 µg/ml of ampicillin. The overnight culture was then diluted (1:100) in fresh LB containing 50 µg/ml of ampicillin and different concentrations of IPTG grown until optical density of cells at 600 nm reached approximately 0.5. Plasmid DNA was purified using QIAGEN Miniprep Kit. The topological state of each DNA preparation was analyzed by electrophoresis in a 1% agarose gel in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8) containing 5 µg/ml of chloroquine. After electrophoresis, agarose gels were stained with SYBR Gold and photographed under UV light. The Net Intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software. The percentage of hypernegative DNA supercoils was calculated by dividing the intensity of hypernegatively supercoiled DNA band with the total intensity of all DNA topoisomers.

3.4 Results and Discussion

3.4.1 Design an *in vivo* system to examine the activation of the *S. typhimurium leu-500* promoter by transient TCDS

Previous studies showed that TCDS was able to activate the *S. typhimurium leu-500* promoter (Lilley and Higgins, 1991; Tan et al., 1994; Mojica and Higgins, 1996; Chen et al., 1998). Although it was demonstrated that transcription-driven localized supercoiling rather than the global superhelical density was responsible for the activation of the *leu-500* promoter (Mojica and Higgins, 1996), it is not clear whether transient TCDS is capable of activating the coupled *leu-500* promoter. The main reason for causing this vagueness is that previous studies almost solely relied on circular plasmids

for determining the topological change of the DNA templates (Tan et al., 1994; Mojica and Higgins, 1996; Chen et al., 1998). In this case, it is difficult to judge whether the effects come from the transient or permanent supercoiling process. In this study, we established an *in vivo* system, consisting of a circular plasmid or a linear plasmid and *E. coli topA* strain *VS111(DE3)* or wild type strain *MG1655(DE3)* to study transcriptional activation of the supercoiling sensitive *leu-500* promoter by T7 RNA polymerase. Figure 3.1 shows two plasmids that were constructed for this study. Plasmid pZXD99 is a circular plasmid derived from plasmid pBR322 (New England Biolab, Inc., Beverly, MA). It carries two divergently-coupled promoters: a unique T7 promoter for bacteriophage T7 RNA polymerase and a weak *leu-500* promoter for *E. coli* RNA polymerase. The distance between these two promoters is 77 bp (the distance was calculated between the -35 region of the *leu-500* promoter and the beginning of the T7 promoter). No other promoter-like sequences were found between these two promoters and also around these two promoters. Plasmid pZXD99 also contains a *lacZ* gene under the control of the T7 promoter and a *luciferase* gene under the control of the weak *leu-500* promoter. In this case, the expression levels initiated from these two promoters can be easily monitored (de Wet et al., 1985). Additionally, two sets of four Rho-independent *rrnB* T1 transcription terminators were used to terminate transcription initiated from these two promoters efficiently (Leng and McMacken, 2002). The presence of these two sets of transcription terminators enabled us to restrict transcription to selected regions of the circular plasmid. Plasmid pZXD103 is a linear plasmid derived from coliphage N15-based, low-copy-number plasmid pZXD4 (N V Ravin and Ravin, 1999). To construct pZXD103, the large BglIII-SpeI fragment (6,763 bp) including the *leu-500* promoter, the

T7 promoter, *luc*, *lacZ*, and two sets of four Rho-independent *rrnB* T1 transcription terminators was cloned into the multiple cloning site of pZXD4. In this case, transcriptional activation of the *leu-500* promoter can be examined in a linear plasmid background. Because linear DNA templates cannot be supercoiled (N V Ravin and Ravin, 1999; Deneke et al., 2000; Ravin, 2003), TCDS initiated from the T7 promoter is always transient. As mentioned above, we used a few *E. coli* strains for this study including a *topA* strain *VS111(DE3)* and a wild-type strain *MG1655(DE3)* (Table 3.1) in which the expression of T7 RNA polymerase is IPTG-inducible and the expression level is dependent on the IPTG concentration added to the cell culture (Samul and Leng, 2007). Using these two strains, we were able to study the activation of the *leu-500* promoter by TCDS initiated from the T7 promoter.

We also made a few plasmid DNA templates (both circular and linear plasmids) to test whether the distance and DNA sequence composition between these two promoters are critical for the activation of the *leu-500* promoter by TDCS (Table 3.2). Additionally, a series of plasmid DNA templates were constructed to examine whether TCDS initiated from an *E. coli* promoter is able to activate the divergently-coupled *leu-500* promoter and whether promoter strength is critical for the activation as well. All plasmids constructed in this study are summarized in Table 3.2.

3.4.2 Transient TCDS is able to activate the supercoiling-sensitive *leu-500* promoter

In order to examine whether TCDS by T7 RNA polymerase is able to activate the supercoiling-sensitive *leu-500* promoter, plasmid pZXD99 was transformed into *E. coli topA* strain *VS111(DE3)* and IPTG was added to the cell culture to induce the

transcription by T7 RNA polymerase. Since luciferase assays are extremely sensitive, we were measuring the firefly luciferase activities to monitor the activation of the *leu-500* promoter. As shown in Figure 3.2, in the absence of IPTG, some luciferase activities were measured, indicating that the weak *leu-500* promoter was able to direct the initiation of transcription of the *luciferase* gene. Interestingly and also as expected, the addition of IPTG to the cell culture greatly stimulated the luciferase activities. At 50 μ M of IPTG, the luciferase activity increases approximately 10-fold. We also transformed pZXD99 into a wild type strain *MG1655(DE3)* and carried out the luciferase assays. IPTG was also able to stimulate the luciferase activities in the wild type strain. However, the stimulation level was significantly reduced, suggesting that the activation of the *leu-500* promoter is greatly enhanced by a *topA* background. Additionally, we carried out a control experiment in which two compatible plasmids were transformed into *VS111(DE3)* strain. The first plasmid pZXD95 is a circular plasmid and carries a *luciferase* gene under the control of the *leu-500* promoter. This plasmid does not contain a T7 promoter. The second plasmid is a linear plasmid and carries a T7 promoter controlling the expression of *lacZ* gene. As demonstrated in Figure 3.2, IPTG could not stimulate the luciferase activities. These results suggest that transcription by T7 RNA polymerase in the same plasmid DNA template is required for the stimulation of the luciferase activities. Since the *leu-500* promoter is divergently coupled to the T7 promoter, TCDS by T7 RNA polymerase is most likely the mechanism for the activation of the supercoiling-sensitive *leu-500* promoter and the stimulation of luciferase activities. Indeed, our supercoiling assays showed that transcription by T7 RNA polymerase significantly supercoiled the

transcribed DNA template pZXD99 in the *topA* strain *VS111(DE3)* (please see below for details).

Next, we decided to examine whether TCDS by T7 RNA polymerase in a linear plasmid DNA template is able to stimulate the luciferase activities under the control of the supercoiling-sensitive *leu-500* promoter. Since linear plasmids cannot be supercoiled (N V Ravin and Ravin, 1999; Deneke et al., 2000; Ravin, 2003), TCDS driven by T7 RNA polymerase is transient. We transformed the linear plasmid pZXD103 (Figure 3.1) into *E. coli topA* strain *VS111(DE3)* and performed luciferase assays. In the absence of IPTG, we were able to detect a very small amount of luciferase activities, indicating that the weak *leu-500* promoter was capable of initiating some transcription of the *luc* gene. Nevertheless, the luciferase activities were 20 to 30 fold lower than those in *VS111(DE3)* carrying the circular plasmid pZXD99. There are two reasons for causing the lower luciferase activities. The first reason is the copy number difference between *E. coli* cells carrying these two plasmids. Plasmid pZXD99 is derived from pBR322 and each cell should contain about 15 to 20 copies of this plasmid. Plasmid pZXD103 is derived from the low copy-number, linear plasmid pG591 (N V Ravin and Ravin, 1999) and each cell only carries approximately 1 to 3 copies of the plasmid. Indeed, our unpublished results showed that *VS111(DE3)* is able to carry 10 more times of plasmid pZXD99 than plasmid pZXD103. In this case, *E. coli* cells carrying pZXD99 should produce at least 10-fold of firefly luciferase as *E. coli* cells harboring pZXD103. The second reason stems from the topology difference between these two plasmids in *E. coli* cells. Circular plasmids in *E. coli* cells are usually supercoiled with an average superhelical density of ~ -0.06 (Bauer et al., 1980; Vologodskii, 1992). Indeed, we determined the supercoiling density of

pZXD99 to be ~ -0.06 in *VS111(DE3)* in the absence of IPTG (please see below for details). As mentioned above, linear plasmids cannot be supercoiled *in vivo*. Because the *leu-500* promoter is sensitive to DNA supercoiling (Lilley and Higgins, 1991; Tan et al., 1994), the topology difference should also contribute to the difference of luciferase activities in *topA* strain *VS111(DE3)* carrying pZXD99 or pZXD103. In the next step, we added different concentrations of IPTG to *E. coli topA* strain *VS111(DE3)* harboring pZXD103 to initiate transcription from the T7 promoter. As expected, IPTG was able to significantly stimulate the luciferase activities. At 20 μM of IPTG, the luciferase activities almost increased 4-fold (Figure 3.2B). Control experiments showed that the stimulation of the luciferase activities was dependent on the transcription of the *lacZ* gene in the same plasmid (Figure 3.2B). Since the *leu-500* promoter controlling the transcription of the *luciferase* gene is divergently coupled to the T7 promoter in the linear plasmid pZXD103, the transient TCDS generated from the transcription of T7 RNA polymerase should be the mechanism for the activation of the luciferase activities. Similar results were obtained for the wild type strain *MG1655(DE3)* carrying plasmid pZXD103 (Figure 3.2B).

In this study, we also carried out RT-PCR experiments to study the stimulation of the *leu-500* promoter by TCDS generated by T7 RNA polymerase. *E. coli* strains *VS111(DE3)* and *MG1655(DE3)* harboring plasmid pZXD99 were used. As shown in Figure 3.2C and D, the addition of IPTG into both *E. coli* cell cultures significantly stimulated the transcription of the divergently-coupled *leu-500* promoter. These results clearly demonstrated that at the transcriptional level, transient TCDS driven by T7 RNA polymerase was able to activate the supercoiling-sensitive *leu-500* promoter *in vivo*.

3.4.3 The distance and DNA sequence between the divergently-coupled *leu-500* promoter and T7 promoter significantly influence the efficiency of TCDS and the activation of the *leu-500* promoter

Previous studies showed that the distance between the two divergently-coupled promoters is important for the activation of the *leu-500* promoter by TCDS (Brahms et al., 1985; Chen et al., 1993; Tan et al., 1994; Spirito and Bossi, 1996; Fang and Wu, 1998; Ravin and Lane, 1999; Ravin et al., 2000), we, therefore, decided to change the distance between the *leu-500* promoter and the T7 promoter and examine whether the distance and DNA composition between these two promoters affect the efficiency of the activation of the *leu-500* promoter by TCDS. For this purpose, we constructed two groups of plasmid DNA templates. The first group of plasmid DNA templates includes pZXD100, pZXD102, and pZXD104 which are derived from pZXD99. Synthetic DNA fragments were used to change the distance between the two promoters. In the absence of IPTG, *E. coli topA* strain *VS111(DE3)* harboring one of these plasmids had similar luciferase activities, indicating that the DNA sequence inserting between the two divergently-coupled promoters did not significantly affect the *leu-500* promoter and did not introduce new promoter activities near the *leu-500* promoter. Similar to *E. coli* strain *VS111(DE3)* harboring pZXD99, IPTG was also able to significantly activate the luciferase activities for *VS111(DE3)* carrying these three plasmids to a similar level. These results suggest that for *topA* strain *VS111(DE3)* the distance between the two divergently-coupled promoters is not very critical for the activation of the *leu-500* promoter by TCDS. The second group of plasmid DNA templates contains plasmids pZXD77, pZXD92, pZXD93, pZXD94, and pZXD97. First, DNA fragments different from those used in the first group

of DNA plasmids were inserted between the two divergently-coupled promoters. Additionally, we varied the distance between these two promoters from 36 bp to 750 bp. Surprisingly, although we did not find promoter-like sequences in the DNA fragments inserted between these two promoters, we saw a significant increase of the luciferase activities for *E. coli topA* strain *VS111(DE3)* carrying one of these plasmids in the absence of IPTG. For instance, the luciferase activity of *VS111(DE3)* carrying pZXD77 was measured to be 12,158 RLU, 4 times more than that of *VS111(DE3)* harboring pZXD99. For *VS111(DE3)* carrying pZXD93, the luciferase activity was even higher and was measured to be 68433, 24-fold as that of *VS111(DE3)* containing pZXD99. Although the mechanisms that cause the increase of the luciferase activities have not been determined yet, a possible reason is that we accidentally introduced promoter-like sequences in the region. In this case, the new promoters were able to initiate transcription of the *luciferase* gene. Another possible reason is that the DNA structure was changed when we introduced the DNA sequences into the region between the two divergently-coupled promoters. Furthermore, we may introduce some DNA-binding sequences into the region. In this case, certain transcriptional factors bind to the DNA sequence between the two promoters and stimulate transcription of the *luciferase* gene. Nevertheless, our results shown in Figure 3.3B demonstrated that IPTG was able to stimulate the luciferase activities for *E. coli* strain *VS111(DE3)* carrying these plasmid DNA templates, suggesting that TCDS by T7 RNA polymerase was capable of activating the *leu-500* promoter in all these circular plasmid templates.

In this dissertation, we also carried out luciferase assays and RT-PCR experiments for *E. coli* strains *VS111(DE3)* and *MG1655(DE3)* carrying circular plasmid pZXD77 or

linear plasmid pZXD80. Our results are shown in Figure 3.4. Similar to the results for *E. coli* strains harboring pZXD99 or pZXD103, IPTG was able to stimulate the luciferase activities at protein and RNA levels in the *topA* strain *VS111(DE3)* and the wild type strain *MG1655(DE3)*. These results suggest that transient TCDS by T7 RNA polymerase is capable of activating the *leu-500* promoter. Interestingly, the activation is dependent on IPTG concentration. The luciferase activities reached the highest point when the IPTG concentration was 20 μM in the cell culture for *E. coli* cells containing the circular plasmid pZXD77 and 10 μM for *E. coli* cells harboring the linear plasmid pZXD80. Higher concentrations of IPTG result in less luciferase activities. For wild type *E. coli* strain *MG1655(DE3)*, 100 μM of IPTG inhibited the luciferase activities. These results suggest that too much supercoiling may inhibit the *leu-500* promoter's activities. Regardless, further studies are required to rule out other possibilities.

3.4.4 The activation of the *leu-500* promoter is dependent on the promoter strength of *E. coli* RNA polymerase

So far, we only demonstrated that transient TCDS by T7 RNA polymerase was able to activate the divergently-coupled *leu-500* promoter. One question that arises from this study is whether transient TCDS by *E. coli* RNA polymerase is capable of activating the coupled *leu-500* promoter and whether promoter strength affects the activation. In order to answer this question, we constructed two sets of plasmid DNA templates: five circular and five linear plasmids that carry different strengths of IPTG-inducible *E. coli* promoters, i.e., $P_{T7A1/O4}$, P_{lac} , P_{lacUV5} , P_{lac} , and P_{lacL8} (Lanzer and Bujard, 1988); Figure 3.5; Table 3.2). we then transformed these two unique sets of DNA templates into *E. coli*

strains (*topA* and wild type) overexpressing LacI (Table 3.1) and tested the IPTG-inducible stimulation of luciferase activities. Our results are shown in Figure 3.6. As expected, IPTG was able to stimulate the luciferase activities for *E. coli* strains (both *topA* and wild type) carrying these circular and linear plasmids, suggesting that transient TCDS by *E. coli* RNA polymerase was also able to activate the *leu-500* promoter. Interestingly and also as expected, the stimulation of luciferase activities is dependent on promoter strength. For example, 50 μ M of IPTG (1mM of IPTG for *E. coli topA* strain *VS111* harboring linear plasmids pZXD87, 88, 89, 90 and 91) was able to stimulate the luciferase activities for plasmids containing $P_{T7A1/O4}$, P_{tac} , and P_{lacUV5} and however, could not stimulate luciferase activities for plasmid carrying P_{lac} and P_{lacL8} ; the stimulation level is correlated with the promoter strength for both circular and linear plasmids (Figure 3.6C and D). Since our results discussed in chapter 2 demonstrated that TCDS in *topA* strains is dependent on promoter strength, we concluded here that transient TCDS by *E. coli* RNA polymerase was able to greatly influence the nearby promoters' activities in *E. coli* cells.

3.4.5 Transcription-coupled hypernegative supercoiling of plasmid is correlated with the activation of the *leu-500* promoter in the circular plasmid DNA templates

In this dissertation, we also examined the supercoiling status of the circular plasmid pZXD77 in *E. coli topA* strain *VS111(DE3)*. In the absence of IPTG, plasmid pZXD77 (Table 3.2) isolated from *E. coli topA* strain *VS111(DE3)* has a supercoiling density of \sim -0.06. Adding IPTG to the cell culture to induce the production of T7 RNA polymerase significantly increased the supercoiling density of plasmid pZXD77 and

resulted in the generation of hypernegatively supercoiled DNA (Figure 3.7). Consistent with previously published results by our group (Samul and Leng, 2007), the production of hypernegatively supercoiled DNA was dependent on the IPTG concentration and correlated with the activation of the *leu-500* promoter in different *E. coli* strains. These results clearly suggest that TCDS induced by RNA polymerases is the mechanism responsible for the activation of the *leu-500* promoter.

3.5 Conclusions

In this chapter, we successfully established an *in vivo* system to study transient TCDS and the activation of the supercoiling-sensitive *leu-500* promoter. This system consists of an *E. coli topA* strain *VS111(DE3)* and a linear plasmid derived from linear coliphage N15. Because linear plasmid DNA templates cannot be supercoiled, TCDS initiated from T7 promoter by T7 RNA polymerase is transient. Using this unique system and the highly sensitive luciferase assays, we discovered that transient TCDS by both T7 and *E. coli* RNA polymerases was able to stimulate the supercoiling-sensitive *leu-500* promoter. These results suggest that transcription in *E. coli* cells is a major chromosomal remodeling force.

Table 3.1. *Escherichia coli* strains used in this study

Strain	Genotype	Source
<i>VS111</i>	F ⁻ λ ⁻ <i>ilvG- rfb-50 rph-I ΔtopA</i>	<i>E. coli</i> Genetic Resource Center
<i>VS111(DE3)</i>	VS111strains with λ(DE3)	<i>E. coli</i> Genetic Resource Center
<i>VS111/pZXD51</i>	VS111strain containing plasmid pZXD51	In this work
<i>VS111/pZS4Int-laci</i>	VS111strain containing plasmid pZS4Int-laci	In this work
<i>MG1655</i>	F ⁻ , λ ⁻ <i>ilvG- rfb-50 rph-1</i>	<i>E. coli</i> Genetic Resource Center
<i>MG1655(DE3)</i>	MG1655 strains with λ(DE3)	<i>E. coli</i> Genetic Resource Center
<i>MG1655/pZXD51</i>	MG1655 strain containing plasmid pZXD51	In this work
<i>MG1655/pZS4Int-laci</i>	MG1655 strain containing plasmid pZS4Int-laci	In this work

Table 3.2. Plasmids constructed in this study

Plasmid	Promoter	Distance between two promoters	Type
pZXD77	T7	147 bp	Circular
pZXD82	T7A1/O4	152 bp	Circular
pZXD83	tac	158 bp	Circular
pZXD84	lacUV5	158 bp	Circular
pZXD85	lac	158 bp	Circular
pZXD86	lacL8	158 bp	Circular
pZXD92	T7	36 bp	Circular
pZXD93	T7	77 bp	Circular
pZXD94	T7	750 bp	Circular
pZXD97	T7	36 bp	Circular
pZXD99	T7	77 bp	Circular
pZXD100	T7	55 bp	Circular
pZXD102	T7	129 bp	Circular
pZXD104	T7	181 bp	Circular
pZXD80	T7	147 bp	Linear
pZXD87	T7A1/O4	152 bp	Linear
pZXD88	tac	158 bp	Linear
pZXD89	lacUV5	158 bp	Linear
pZXD90	lac	158 bp	Linear
pZXD91	lacL8	158 bp	Linear
pZXD103	T7	77 bp	Linear

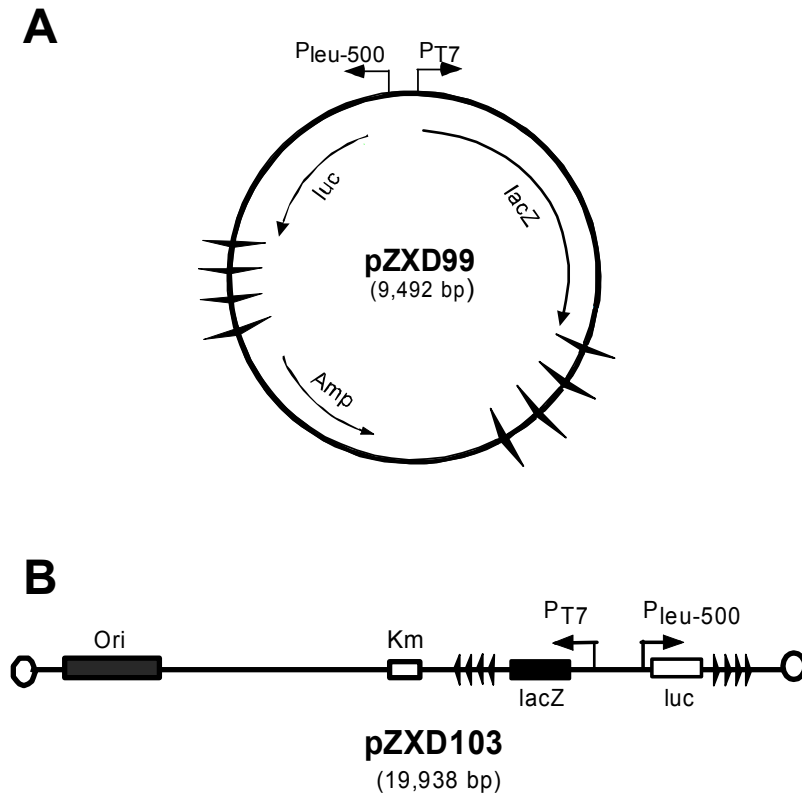


Figure 3.1 Circular plasmid pZXD99 and linear plasmid pZXD103 were constructed to study TCDS *in vivo*. (A) The circular plasmid pZXD99 was derived from pBR322. (B) The linear plasmid pZXD103 was derived from linear coliphage N15-based, low-copy number plasmid pZXD4 (pZXD4 was described in chapter 2). Plasmid DNA templates were constructed as described under Materials and Methods. Both of them contain a *lacZ* gene under the control of the T7 promoter and a *luciferase* gene under the control of the weak *leu-500* promoter. There are two sets of four Rho-independent *rrnB* T1 transcription terminators (winged triangles) on each side of the plasmids to terminate transcription initiated from two promoters. In this case, the transcription unit between the promoter and terminators could be controlled precisely.

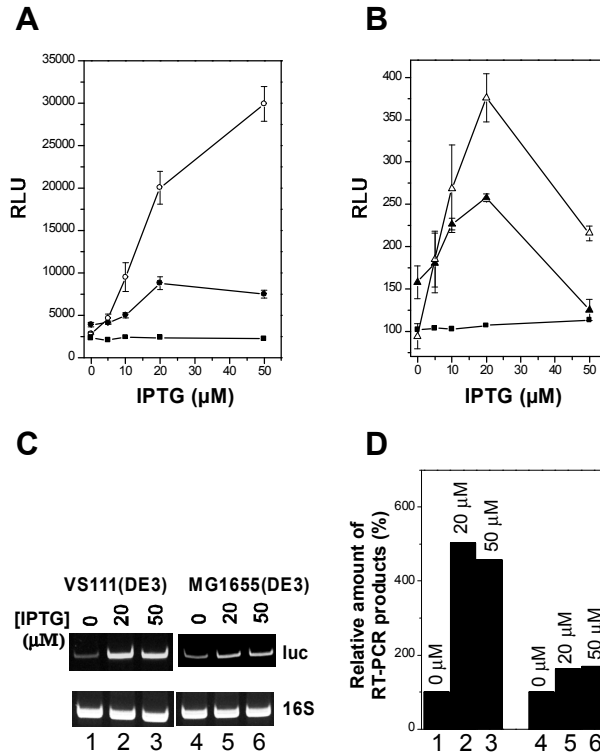


Figure 3.2 Luciferase activity was stimulated by the addition of IPTG in *E. coli topA* strain *VS111(DE3)* or wild type strain *MG1655(DE3)* containing circular plasmid pZXD99 or linear plasmid pZXD103. Luciferase assays were performed as described under Materials and Methods. Different concentrations of IPTG (ranging from 5 μM to 50 μM) were added to the cell culture to stimulate luciferase activity. Light produced was measured by a lunometer. (A) Luciferase assay for circular plasmid pZXD99 in wild type strain *MG1655(DE3)* (solid circles) or *topA* strain *VS111(DE3)* (open circle); Control experiment for *E. coli topA* strain *VS111(DE3)* harboring circular plasmid pZXD95, in which there is no T7 promoter, was shown in solid square. (B) Luciferase assay for linear plasmid pZXD103 in wild type strain *MG1655(DE3)* (solid triangles) or *topA* strain *VS111(DE3)* (open triangles); Control experiment for *E. coli topA* strain *VS111(DE3)* harboring linear plasmid pZXD91, in which there is no T7 promoter, was shown in solid square. (C) RT-PCR analysis of cDNA products of mRNA transcribed from circular plasmid pZXD99 in *E. coli topA* strain *VS111(DE3)* or wild type strain *MG1655(DE3)* to study the stimulation of luciferase activity by T7 RNA polymerase. RT-PCR experiments were performed as described under Materials and Methods. The upper and lower panels are 12% polyacrylamide gel in 1 \times TAE buffer to show the PCR products of *luciferase* gene and 16S rRNA cDNA synthesized from the RNA samples isolated from *E. coli topA* strain *VS111(DE3)* (1-3) or wild type strain *MG1655(DE3)* (4-6) without IPTG and with 20 μM , 50 μM of IPTG induction. (D) The ratios were calculated for RT-PCR analyses of PCR products pZXD99 in *E. coli topA* strain *VS111(DE3)* (1-3) or wild type strain *MG1655(DE3)* from (C).

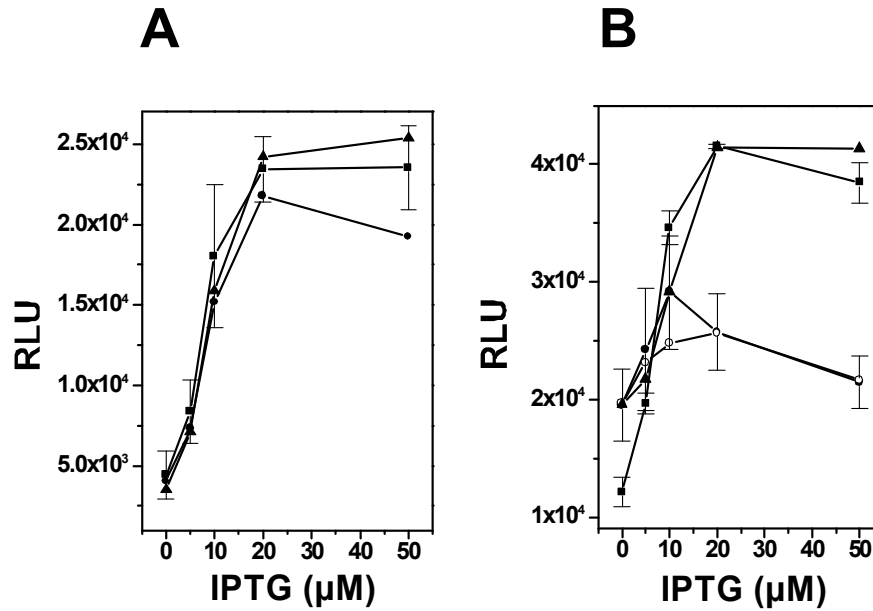


Figure 3.3 Circular plasmids that differ in the distance and DNA sequence composition between two promoters were constructed to examine the stimulation of luciferase activity by T7 RNA polymerase after the addition of IPTG in *E. coli topA* strain *VS111(DE3)*. Two groups of plasmid DNA templates that differ in the distance and DNA sequence composition between the T7 promoter and *leu-500* promoter were constructed and transformed into the *E. coli topA* strain *VS111(DE3)*. Luciferase assays were performed as described under Materials and Methods. Different concentrations of IPTG (ranging from 5 μM to 50 μM) were added to the cell culture to stimulate luciferase activity. Light produced was measured by a lunometer. (A) First group of plasmid DNA templates includes pZXD100, pZXD102, and pZXD104. The distance between two promoters are: 55 bp (solid triangles), 129 bp (solid square) and 181 bp (solid circles), respectively. (B) Second group of plasmid DNA templates includes pZXD94, pZXD77, pZXD92 and pZXD97. The distances between two promoters are: 750 bp (solid triangles), 147 bp (solid square), 36 bp (solid circles) and 36 bp (different sequence, open circles).

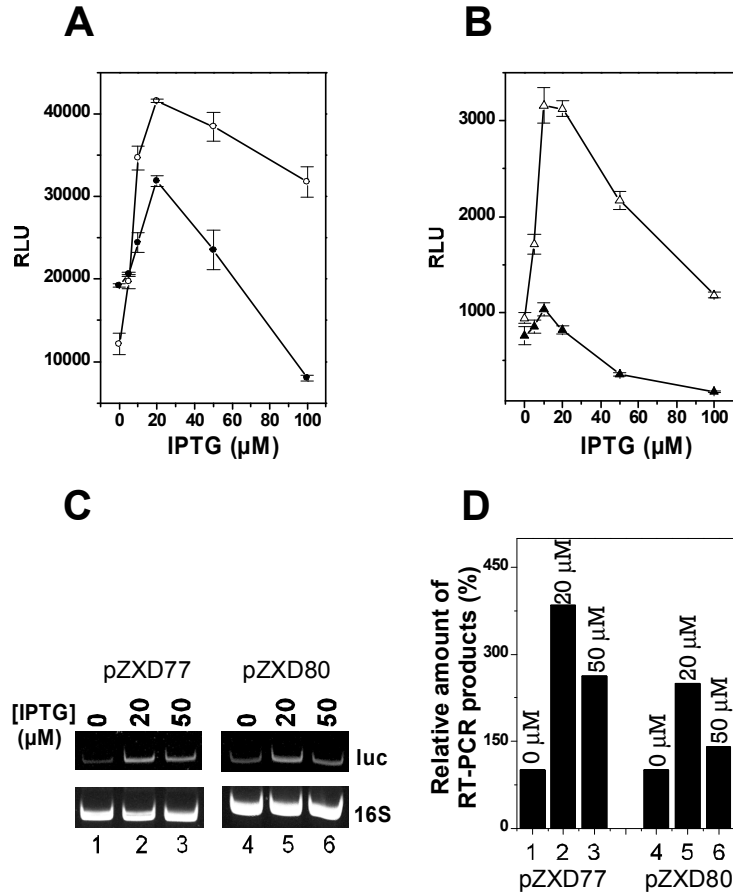


Figure 3.4 Luciferase activity was stimulated by the addition of IPTG in *E. coli topA* strain *VS111(DE3)* or wild type strain *MG1655(DE3)* containing circular plasmid pZXD77 or linear plasmid pZXD80. Luciferase assays were performed as described under Materials and Methods. Different concentrations of IPTG (ranging from 5 μ M to 100 μ M) were added to the cell culture to stimulate luciferase activity. Light produced was measured by a lunometer. (A) Luciferase assay for circular plasmid pZXD77 in wild type strain *MG1655(DE3)* (solid circles) or *topA* strain *VS111(DE3)* (open circle). (B) Luciferase assay for linear plasmid pZXD103 in wild type strain *MG1655(DE3)* (solid triangles) or *topA* strain *VS111(DE3)* (open triangles). (C) RT-PCR analysis of cDNA products of mRNA transcribed from circular plasmid pZXD77 (1-3) or linear plasmid pZXD80 (4-6) in *E. coli topA* strain *VS111(DE3)* was used to study the stimulation of luciferase activity by T7 RNA polymerase. RT-PCR experiments were performed as described under Materials and Methods. The upper and lower panels are 12% polyacrylamide gel in 1 \times TAE buffer to show the PCR products of *luciferase* gene and 16S rRNA cDNA synthesized from the RNA samples isolated from *E. coli* strain *VS111(DE3)* without IPTG and with 20 μ M, 50 μ M of IPTG induction. (D) The ratios were calculated for RT-PCR analyses of PCR products pZXD77 (1-3) or pZXD80 (4-6) in *E. coli topA* strain *VS111(DE3)* from (C).

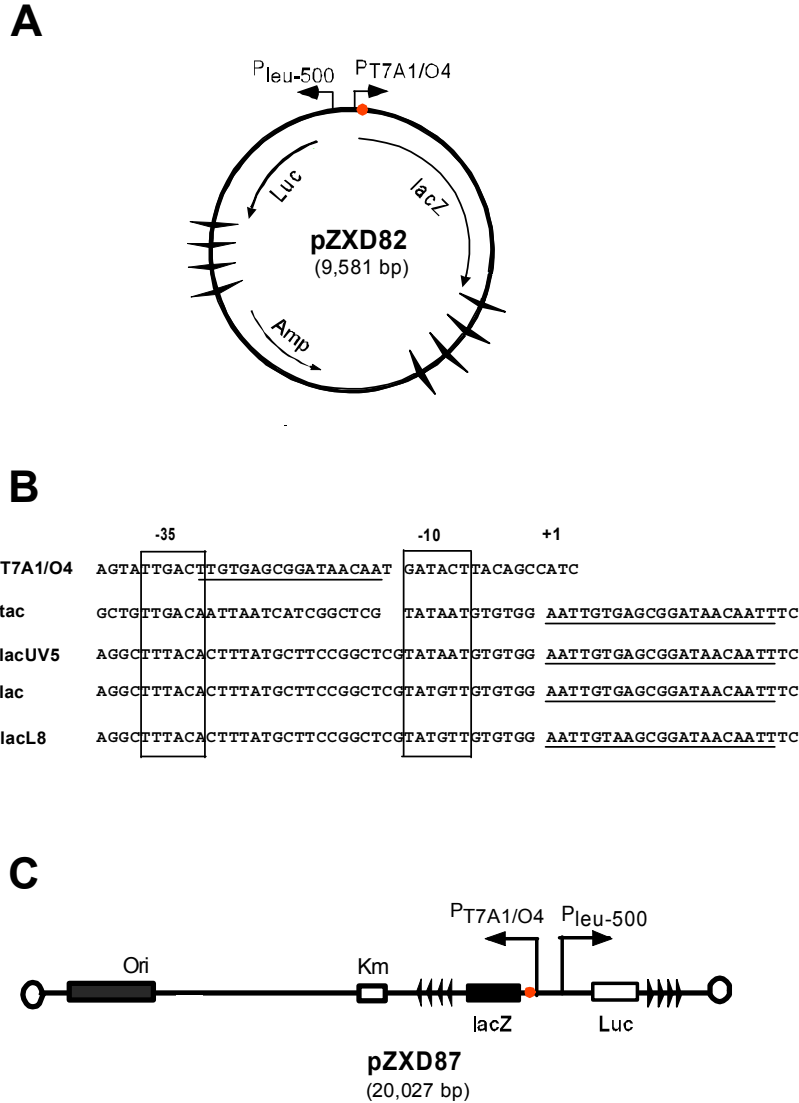


Figure 3.5 An *in vivo* system to study *E. coli* RNA polymerase induced TCDS. This system contains two plasmids, a linear plasmid pZXD51 (as described in chapter 2) or a circular plasmid pZS4Int-laci that overexpresses LacI and (A) Circular plasmid pZXD82 that carries T7A1/O4 promoter. The circular plasmids were derived from plasmid pBR322 and constructed as detailed under Materials and Methods. They harbor an IPTG-inducible promoter with different strengths and a transcription unit between the promoter and a set of four Rho-independent *E. coli rrnB* T1 terminators (winged triangles). (B) The DNA sequence of five different *E. coli* promoters P_{T7A1/O4}, P_{tac}, P_{lacUV5}, P_{lac}, and P_{lacL8}. The underlines represent the *lac OI* operators. (C) Linear plasmid pZXD87 carries T7A1/O4 promoter. The linear plasmids were derived from plasmid pZXD4 (as described in chapter 2) and constructed as detailed under Materials and Methods. They harbor an IPTG-inducible promoter with different strengths and a transcription unit between the promoter and a set of four Rho-independent *E. coli rrnB* T1 terminators (winged triangles).

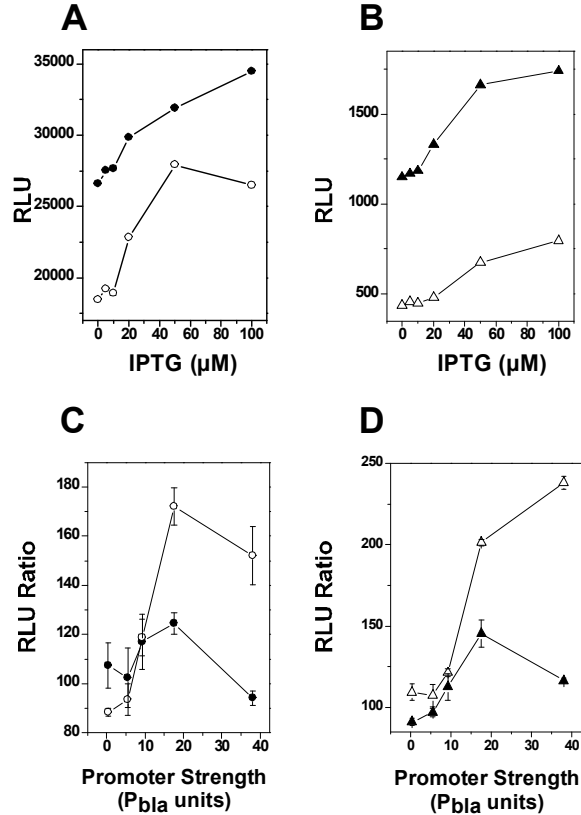


Figure 3.6 Circular plasmids and linear plasmids were transformed into *E. coli topA* strain *VS111* or wild type strain *MG1655* to examine the stimulation of the luciferase activity by *E. coli* RNA polymerase after the addition of IPTG. Luciferase assays were performed as described under Materials and Methods. Different concentrations of IPTG (ranging from 5 μM to 100 μM) were added to the cell culture to stimulate luciferase activity. Light produced was measured by a lunometer. (A) Luciferase assay for circular plasmid pZXD83 that carries tac promoter in *E. coli* wild type strain *MG1655* (solid circles) or *topA* strain *VS111* (open circle). (B) Luciferase assay for linear plasmid pZXD88 that carries tac promoter in *E. coli* wild type strain *MG1655* (solid triangles) or *topA* strain *VS111* (open triangles). (C) (D) The expression level of *luciferase* gene by *E. coli* RNA polymerase after the addition of IPTG in *E. coli topA* strain *VS111* or wild type strain *MG1655* containing different plasmids is correlated with promoter strength. Luciferase assays were performed as described under Materials and Methods. 50 μM of IPTG (for *E. coli topA* strain *VS111* harboring linear plasmids, 1 mM of IPTG was used) was added to the cell culture to stimulate luciferase activity. (C) Circular plasmids pZXD82, 83, 84, 85, and 86 in *E. coli* wild type strain *MG1655* (solid circles) or *topA* strain *VS111* (open circle). (D) Linear plasmids pZXD87, 88, 89, 90, and 91 in *E. coli* wild type strain *MG1655* (solid triangles) or *topA* strain *VS111* (open triangles). Promoter strength in P_{bla} units was obtained from Lanzer and Bujard (1988).

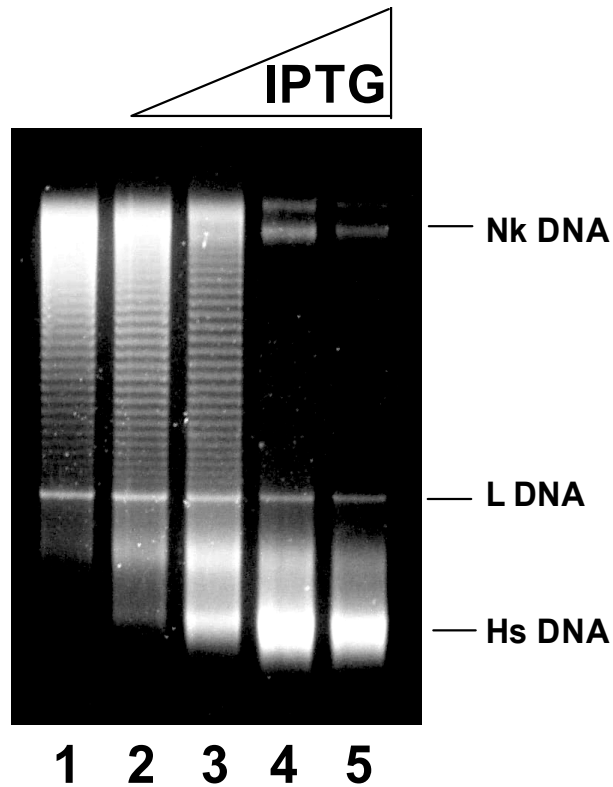


Figure 3.7 Transient TCDS in *E. coli* strain *VS111(DE3)* is responsible for the activation of *leu-500* promoter. The *in vivo* T-S assays were performed as described under Materials and Methods. DNA topoisomers were resolved by electrophoresis in a 1% agarose gel containing 5 $\mu\text{g/ml}$ chloroquine and stained with SYBR Gold. TCDS is dependent on IPTG concentration for circular plasmid pZXD77 in *E. coli topA* strain *VS111(DE3)*. Lane 1 contained the DNA sample isolated from *E. coli* cells prior to IPTG induction. Lanes 2–5 contained the DNA samples isolated from *E. coli* cells with 5 μM , 10 μM , 20 μM , and 50 μM IPTG induction, respectively. The percentage of hypernegatively supercoiled DNA is proportional to the IPTG concentration.

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Chapter 4: Conclusions and Future Directions

4.1 Conclusions

The primary objectives of this dissertation are: 1) to demonstrate that transcription is a major chromosomal remodeling force in *E. coli* cells; 2) to explore whether TCDS is able to greatly influence coupled DNA transactions, e.g., to activate a divergently-coupled *leu-500* promoter *in vivo*. To accomplish the objectives, two series of studies in *E. coli* wild-type and *topA* strains were performed.

In chapter 2, using a newly established two-plasmid system, we examined the effect of promoter strength on TCDS. Our results suggest that TCDS in *topA* strains is dependent on promoter strength. More importantly, we demonstrated that transcription-coupled hypernegative supercoiling of plasmid DNA did not require the expression of a membrane-insertion protein for strong promoters, although it might require co-transcriptional synthesis of a polypeptide. In addition, we found that the expression of a membrane-insertion *tet* gene was not sufficient for the production of hypernegatively supercoiled DNA. Our results can be explained by the “twin-supercoiled-domain” model of transcription, which suggested that friction force applied to *E. coli* RNA polymerase plays a critical role in the generation of hypernegatively supercoiled DNA.

In chapter 3, we developed an *in vivo* system to study how TCDS activates a divergently-coupled, supercoiling-sensitive *leu-500* promoter in *E. coli* cells. Our results demonstrated that transient TCDS induced by either T7 or *E. coli* RNA polymerase was able to activate the *leu-500* promoter. These observations suggested that TCDS may be a general mechanism for activating transcription *in vivo*.

Taken together, the results presented in this dissertation demonstrated that transcription is a major chromosomal remodeling force in *E. coli* cells and is able to activate a divergently coupled, supercoiling-sensitive *leu-500* promoter.

4.2 Future Directions

Future research on this topic may focus on these three areas:

1) Examining factors that regulate the activating of the *leu-500* promoter by transient TCDS in *E. coli* cells. Since we established a plasmid-based system to study the activation of the *leu-500* promoter, it should be relatively straightforward to test what factors affect the activation of the *leu-500* promoter by TCDS. These factors include the distance between the two divergently-coupled promoters, the length of RNA transcripts, the expression of a membrane-associated protein, and the presence of a topological barrier between the promoters.

2) Identifying new supercoiling-sensitive promoters in *E. coli* cells. My dissertation also provided a new method to screen and identify new supercoiling-sensitive promoters in the future.

3) Establishing a defined protein system to study the activation of the *leu-500* promoter *in vitro*.

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