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Dividing a supercoiled DNA molecule into two independent topological domains

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Both prokaryotic and eukaryotic chromosomes are organized into many independent topological domains. These topological domains may be formed through constraining each DNA end from rotating by interacting with nuclear proteins; i.e., DNA-binding proteins. However, so far, evidence to support this hypothesis is still elusive. Here we developed two biochemical methods; i.e., DNA-nicking and DNA-gyrase methods to examine whether certain sequence-specific DNA-binding proteins are capable of separating a supercoiled DNA molecule into distinct topological domains. Our approach is based on the successful construction of a series of plasmid DNA templates that contain many tandem copies of one or two DNA-binding sites in two different locations. With these approaches and atomic force microscopy, we discovered that several sequence-specific DNA-binding proteins; i.e., lac repressor, gal repressor, and λ O protein, are able to divide a supercoiled DNA molecule into two independent topological domains. These topological domains are stable under our experimental conditions. Our results can be explained by a topological barrier model in which nucleoprotein complexes confine DNA supercoils to localized regions. We propose that DNA topological barriers are certain nucleoprotein complexes that contain stable toroidal supercoils assembled from DNA-looping or tightly wrapping DNA around DNA-binding proteins. The DNA topological barrier model may be a general mechanism for certain DNA-binding proteins, such as histone or histone-like proteins, to modulate topology of chromosome DNA in vivo.

The Escherichia coli chromosome is comprised of a 4.6 Mb circular, negatively supercoiled DNA molecule. A single-stranded nick or double-stranded break should release all superhelical tension and therefore relax the circular DNA molecule. However, early studies showed that multiple single-stranded nicks are required to fully relax the E. coli DNA molecule (1, 2). These studies also suggested that the E. coli chromosome consists of 40 to 100 independent topological domains in vivo (2). More recently, Postow, et al. (3) reassessed the size of the topological domains and demonstrated that the E. coli chromosome is segregated into 400 to 500 different topological domains; the sizes of the topological domains are dynamic and variable ranging from 2 to 66 kb. These studies coupled with genetic studies (4) strongly support the existence of topological barriers that divide the E. coli chromosome into different topological domains (5). One question arises from these studies: What forms topological barriers in DNA? Several models have been proposed to explain the DNA topological barriers (5, 6). For instance, because transcription by an RNA polymerase generates positive and negative supercoils (7), transcription of a gene, especially a gene producing a membrane insertion protein can induce the formation of a topological domain barrier in vivo (8). Another interesting model is that certain DNA-binding proteins especially DNA-looping proteins may constrain DNA loops to serve as topological barriers (5, 9–11). To support this model, we previously showed that certain nucleoprotein complexes, resulting from the binding of several sequence-specific DNA-binding proteins to their recognition sites, could form topological barriers that impede the diffusion and merger of independent chromosomal supercoils (12, 13). Nevertheless, conclusive evidence to support these hypothetical models is still required.

Although several attempts have been made to decipher the mechanism by which the E. coli chromosome is divided into independent topological domains (1–3), the nature of the topological barriers is still a mystery. A primary difficulty in determining the identity of the topological barriers in DNA is the lack of a simple, effective system to examine what property or properties of DNA or protein-DNA complexes can serve as topological barriers to divide a DNA molecule into different topological domains. A simple barrier might divide a small supercoiled DNA molecule into two independent topological domains. In this report, we present our efforts to establish a simple, in vitro system to examine which nucleoprotein complexes are capable of serving as topological barriers to confine free DNA supercoils within a defined region. With this unique approach, we discovered that certain sequence-specific DNA-binding proteins, such as lac repressor, gal repressor, and λ O protein, are able to act as topological barriers that prevent supercoil diffusion.

Results

A Unique Strategy to Study DNA Topological Barriers In Vitro. In this study, we developed a unique strategy to examine whether certain sequence-specific DNA-binding proteins can block supercoil diffusion along DNA. Our first step was to construct a series of plasmids that contain one copy or several tandem copies of one or two distinct DNA-binding sites in one or two different locations (Fig. 1, Fig. S1, and Table S1). The DNA-binding sites at two locations divide the plasmid into two regions of different sizes, ~2.9 and ~1.2 kb. We also placed nicking restriction endonuclease recognition sites for Nt.BbvCI and Nb.BstI into the plasmid, such that the Nt.BbvCI site resides in the 1.2 kb region and the Nb.BstI site in the 2.9 kb region (Fig. 1, Fig. S1). For this report, we made DNA templates that contain multiple DNA-binding sites for lac repressor (LacI), gal repressor (GalR), or λ O protein (Table S1). Our next step was to examine whether these sequence-specific DNA-binding proteins can divide a supercoiled DNA molecule into two independent topological domains. For this purpose, we developed two methods: the DNA-nicking and gyrase methods (Fig. 2). In the DNA-nicking method (Fig. 24), one DNA-binding protein; e.g., LacI, will bind to the two groups of DNA-binding sites on the supercoiled DNA template. If the DNA-binding protein stably blocks supercoil diffusion, a nick generated by either Nt.BbvCI or Nb.BstI should not fully release the superhelical stress of the DNA molecule. After the single nick...
E. coli LacI Blocked Supercoil Diffusion and Divided a Supercoiled DNA Molecule into Two Independent Topological Domains. E. coli LacI, a homotetramer (14) was chosen as the first DNA-binding protein for this study. As demonstrated previously, LacI simultaneously binds to two of three potential chromosomal sites; i.e., lac O1, O2, or O3 operators to form a DNA loop (15). A DNA template, pCB115 (Fig. 1) that contains two pairs of lac O1 operators in two different locations was used in our assays. The space between the two lac O1 operators in each location is 25 bp (Table S1). Because lac O1 site is a 21 bp DNA sequence and the head-to-tail distance of the lac O1 operators is 46 bp, it is reasonable to assume that each LacI tetramer cannot simultaneously bind to the neighboring lac O1 operators of pCB115 which face in opposite directions. Instead, two LacI tetramers are able to simultaneously bind to the four lac O1 operators to form two highly stable LacI-lac O1 nucleoprotein complexes. In this case, two stable DNA loops are formed. Fig. 3 shows results of the DNA-nicking and gyrase assays, which clearly demonstrate that LacI successfully blocked supercoil diffusion and divided the DNA molecule into two independent topological domains. In the absence of LacI, Nt.BbvCI or Nb.BtsI fully relaxed the plasmid pCB115 (Fig. 3, lanes 1, 4, and 7). In the presence of LacI, however, neither Nt.BbvCI nor Nb.BtsI alone could remove all supercoils from the (−) supercoiled DNA molecule (Fig. 3, compare lane 2 to lane 1 and lane 5 to lane 4). Nt.BbvCI removed ∼7 (−) supercoils that equal to supercoils constrained in the 1.2 kb region where Nt.BbvCI recognition site is located [Fig. 1; the supercoiling density of pCB115 was determined to be ∼−0.06; i.e., it has ∼26 (−) supercoils]. Nb.BtsI removed ∼17 (−) supercoils that correspond to those constrained in the 2.9 kb region in which it nicks the plasmid. As expected, Nt.BbvCI and Nb.BtsI together removed all (−) supercoils from the DNA template (Fig. 3, lane 8). Interestingly and also as expected, the constraining of DNA supercoils in defined regions by LacI is sensitive to the presence of its inducer, isopropyl-β-D-thiogalactoside (IPTG), which lowers the affinity of LacI for its operators (Fig. 3, lanes 3 and 6). Similar results were obtained when the DNA-gyrase method was used (Fig. 3B, Fig. S2B). In this assay, we first digested the DNA template using Nt. BbvCI to yield a nicked plasmid for the following reactions. In the absence of LacI and DNA gyrase, the DNA supercoils should stay with the loop without the DNA nick (c). If the DNA-binding protein blocks DNA supercoil diffusion, after the inhibition of DNA gyrase by novobiocin, the DNA supercoils should stay with the loop without the DNA nick (d). After ligation by T4 DNA ligase and phenol extraction, a (−) supercoiled DNA template should be generated (e).
In this article, we also demonstrated that the LacI-mediated DNA looping is required for the formation of topological barriers. For this purpose, we constructed a similar plasmid DNA template, pCB152 that contains four lac O1 operators equally distributed between two different locations. Because the space between the two lac O1 operators in each location is 20 bp (Table S1) and the head-to-tail distance of the lac O1 operators is 41 bp, each LacI tetramer is able to simultaneously bind to the neighboring lac O1 operators of pCB152 due to these DNA-binding sites locating on the same side. In this case, LacI is not capable of mediating the formation of DNA loops. This arrangement of lac O1 operators should not support the formation of the topological barriers on pCB152. Indeed, our results showed that LacI did not block supercoil diffusion and divide the plasmid into two stable topological domains (Fig. S3 C and D), suggesting that DNA looping is required for the formation of LacI-mediated topological barriers. Furthermore, our results show that the protein-DNA-looping complex resulting from one LacI tetramer binding to two lac O1 operators is sufficient to form a topological barrier to block supercoil diffusion, although the topological barrier is much less stable comparing with those containing multiple LacI-lac O1 nucleoprotein complexes.

**DNA-Wrapping Proteins, such as λ O Protein and E. coli GalR also Divided a Supercoiled DNA Molecule into Two Independent Topological Domains.** Due to availability and the biological importance of λ O protein and GalR, we decided to examine whether these DNA-wrapping proteins are also able to divide a supercoiled DNA molecule into two independent topological domains. As demonstrated previously (16), λ O protein specifically binds to the four repeating sequences (iterons) of λ DNA replication origin and forms a unique nucleoprotein complex, the “O-some” to initiate DNA replication. GalR is a dimer and specifically binds to the O2 and O1 operators of E. coli galactose operon to form a loop in the presence of HIU protein (17), which inhibits transcription from two gaf promoters P1 and P2 (18). Both proteins are capable of inducing DNA wrapping upon binding to their DNA recognition sites (19). In order to test whether λ O protein is able to divide a supercoiled DNA molecule into two distinct topological domains, we made a DNA template, pCB138 containing eight λ DNA replication origins (a total of 32 λ O-binding sites) equally distributed between two locations (Fig. 1). In this scenario, the nucleoprotein complexes, which built from the DNA replication origins wrapping around λ O proteins, should divide the plasmid into two independent topological domains. Indeed, our results shown in Fig. 4 A and B, Fig. S4 C demonstrated that λ O protein was able to divide the plasmid into two distinct, stable topological domains. In the absence of λ O protein, a nick introduced by Nt.BbvCI or Nb.BstI alone could not completely remove all (-) supercoils from the DNA template (Fig. 4 A, lanes 2 and 4). These results suggested that λ O protein upon binding to its recognition sites served as DNA topological barriers to block supercoil diffusion. Consistent with our previous results (19), the binding of λ O protein to the multiple λ O-binding sites on pCB138 caused DNA wrapping and introduced about eight (++) supercoils into the DNA template (Fig. 4 A, lane 6). Nevertheless, our control experiments showed that two barriers are required to divide the DNA template into two independent topological domains (Fig. 1, Fig. S4B). Similar results were also obtained by using the DNA-gyrase assay (Fig. 4 B, lane 6).

We also cloned a few plasmids to test whether GalR is capable of dividing a supercoiled DNA molecule into two independent topological domains. Among them are pCB132 and pCB155 that carry 36 gal O2 operators equally distributed between two locations (Fig. S1, Table S1). As described under Materials and Methods, the difference between these two plasmids is the space

domains when plasmid pCB112 with four adjacent lac O1 sites was used as the DNA template (Fig. 1, Fig. S3 A and B).

Next, we performed a time course of the DNA-nicking assay of pCB115 to examine whether the LacI-mediated topological barrier is kinetically stable. In this assay, significantly more amount of Nt.BbvCI was used, which was able to nick pCB115 within 2 min (Fig. S4 A). We then incubated the reaction mixture at 37 °C for various time before adding T4 DNA ligase to seal the DNA nick. Our results are shown in Fig. 3 C and D. At 5 min, about 91% of topoisomers were (-) supercoiled and after 120 min incubation, about 47% of topoisomers were still (-) supercoiled, indicating that the LacI-mediated DNA topological barrier was quite stable. These kinetic data were also fitted to a first-order rate equation (Fig. 3 D), producing a first-order rate constant ($k_A$) of 0.0062 min$^{-1}$ and a half-life ($t_{1/2}$) of 112 min.

**Fig. 3.** LacI divided a supercoiled DNA molecule, plasmid pCB115 into two independent topological domains. (A) The DNA-nicking assays were performed as described under Materials and Methods and Fig. 2. In addition to 0.156 nM of pCB115, as indicated at the top of the image, the reaction mixtures also contained LacI (2.5 nM), IPTG, Nt.BbvCI (4 units), and Nb.BstI (4 units). The DNA molecules (topoisomers) were isolated and subjected to agarose gel electrophoresis in the absence of chloroquine as detailed under Materials and Methods. (B) The DNA-gyrase assays were performed as described under Materials and Methods and Fig. 2. In addition to 0.156 nM of Nt.BbvCI-nicked pCB115, as specified at the top of the image, the reaction mixtures also contained LacI (2.5 nM), IPTG, E. coli DNA gyrase (5 units), and novobiofilm (3 μM). The DNA molecules (topoisomers) were isolated and subjected to agarose gel electrophoresis in the absence of chloroquine. Lanes 1 of (A) and (B) contain DNA relaxed at 37 °C (it is slightly (-) supercoiled because the gels were run at 24 °C. (C) Time course of DNA supercoiling diffusion in the presence of LacI. The DNA-nicking assays were performed as described under Materials and Methods. Each reaction mixture (320 μL) contained 0.156 nM of pCB115, 2.5 nM of LacI, and 12 units of Nt.BbvCI. The reaction mixtures were incubated at 37 °C for the time indicated. Then, a percentage of supercoiled DNA was used as the DNA template (Fig. 4 A, lanes 1, 3, and 5). In the presence of λ O protein, Nt.BbvCI or Nb.BstI alone could not completely remove all (-) supercoils from the DNA template (Fig. 4 A, lanes 2 and 4). These results suggested that λ O protein upon binding to its recognition sites served as DNA topological barriers to block supercoil diffusion. Consistent with our previous results (19), the binding of λ O protein to the multiple λ O-binding sites on pCB138 caused DNA wrapping and introduced about eight (++) supercoils into the DNA template (Fig. 4 A, lane 6). Nevertheless, our control experiments showed that two barriers are required to divide the DNA template into two independent topological domains (Fig. 1, Fig. S4B). Similar results were also obtained by using the DNA-gyrase assay (Fig. 4 B, lane 6).
separating the neighboring \( O_E \) operators: pCB155, 20 bp and pCB132, 25 bp (the head-to-tail distance of the \( O_E \) operators are 36 and 41 bp, respectively). Because the \( O_E \) operator is a 16 bp DNA sequence, it is reasonable to assume that GalR binds to the neighboring \( gal \ O_E \) operators of pCB155 on the opposite side; in contrast, GalR binds to the neighboring \( gal \ O_E \) operators of pCB132 on the same side. In this case, we were able to examine whether the operator phasing affected the ability of GalR to induce the formation of DNA topological barriers. Our results are summarized in Fig. 4 C and D. Fig. S5. These results clearly demonstrated that GalR is capable of dividing a supercoiled DNA molecule into two distinct topological domains and the operator phasing does not affect GalR as a topological barrier. Similar to \( O \) protein, the binding of GalR protein to the \( O_E \) sites on pCB155 and pCB132 also caused DNA wraping and introduced a few (−) supercoils into the DNA template, which is consistent with our previous results (19). Intriguingly, galactose did not completely abolish GalR as a topological barrier (Fig. 4 C and D, Fig. S5) although it showed some inhibitory effects on the formation of the DNA topological barriers in the DNA-nicking assay. Control experiments indicated that two barriers of GalR-OE complexes are required to divide the plasmid DNA template into two independent topological domains (Fig. S6).

In this article, we made three additional plasmids, pCB160, pCB162, and pCB163 that carry multiple tandem copies of two types of DNA-binding sites in two different locations (Table S1). pCB160 contains 18 gal OE operators in one location and 16 lac O1 operators in another location; pCB162 carries 16 \( O \) protein sites in one location and another 16 lac O1 sites in a different location; and pCB163 has 16 \( O \) protein-binding sites in one location and 18 gal OE operators in another location. These plasmids were used to test whether two topological barriers deriving from unrelated DNA-binding proteins were able to divide a DNA molecule into two distinct topological domains. Our results summarized in Fig. S6 unambiguously demonstrated that two topological barriers resulting from different DNA-binding proteins are able to divide a supercoiled DNA molecule into two independent topological domains. In the absence or presence of only one DNA-binding protein; i.e., either \( O \) protein or GalR or LacI, a nick introduced by Nt.BbvCI fully relaxed the DNA templates (Fig. S6, lanes 1–3). However, in the presence of a combination of two DNA-binding proteins; i.e., \( O \) protein and GalR for pCB163, \( O \) protein and LacI for pCB162, and GalR and LacI for pCB160, Nt.BbvCI could not completely remove all (−) supercoils from these DNA templates (Fig S6, lanes 4). These results suggest that the topological barriers derived from the two unrelated DNA-binding proteins are able to confine free supercoils in a defined region and separate the supercoiled DNA molecules into two independent topological domains. As expected, galactose or IPTG had some inhibitory effects on the formation of the topological barriers (Fig. S6, lanes 5 and 6).
Discussion

In this article, we demonstrated that the binding of a DNA-binding protein to its recognition sites in two different locations on a supercoiled DNA molecule can confine free supercoils to a defined region and divide the DNA molecule into two distinct topological domains: a relaxed and a supercoiled domain. We also showed that two types of DNA-binding proteins, DNA-looping and DNA-wrapping proteins, have this functionality. For DNA-looping proteins, we used LacI, the best-characterized DNA-looping protein as a model protein for our studies. Our results show that LacI upon binding to two recognition sites on a supercoiled DNA molecule forms a unique nucleoprotein structure, such as the O-some (26). These nucleoprotein structures form a topological barrier that slow or prevent diffusion of supercoils past the nucleoprotein complex. In this scenario, it requires two such nucleoprotein complexes to divide a circular DNA molecule into two topological domains. This model also provides a reasonable explanation for a transcribing RNA polymerase to serve as a topological barrier (8, 27). First, RNA polymerases cause DNA supercoiling (12, 13). We used the "twin-supercoiled-domain" model to explain these results where nucleoprotein complexes, especially those containing stable toroidal supercoils assembled two proteins are able to form distinct, stable DNA loops under our experimental conditions. Second, our results show that a combination of two unrelated DNA-binding proteins; e.g., λ O protein and GalR also confined free supercoils to a defined region and separated the DNA molecules into two independent topological domains (Fig. S6). These results suggest that DNA wrapping, rather than looping, is the main reason for these two DNA-wrapping proteins to divide the DNA molecules into different topological domains. Nevertheless, although our AFM images showed that λ O protein and GalR are able to divide the supercoiled plasmids into distinct topological domains (Fig. S8), we cannot fully exclude the role of protein-protein interactions of λ O-DNA complexes and GalR-DNA complexes in the formation of the two distinct topological domains.

We favor models depicted in Fig. 6 to explain our results. Model (I) is for DNA-looping proteins that are able to bring two or more groups of the DNA-binding sites together to fold into a topologically constrained nucleoprotein complex. This nucleoprotein complex serves as a DNA topological barrier or divider to block supercoil diffusion. This model represents the most likely way for LacI to divide a supercoiled DNA molecule into two independent topological domains. Our AFM images strongly support this interpretation (Fig. 5, Fig. S7). Model (II) is for DNA-wrapping proteins, such as λ O protein and GalR. Specific DNA sequences wrap around these DNA-wrapping proteins to form a unique nucleoprotein structure, such as the O-some (26). These nucleoprotein structures form a topological barrier that slow or prevent diffusion of supercoils past the nucleoprotein complex. In this scenario, it requires two such nucleoprotein complexes to divide a circular DNA molecule into two topological domains. This model also provides a reasonable explanation for a transcribing RNA polymerase to serve as a topological barrier (8, 27). First, RNA polymerases cause DNA supercoiling (12, 13). We used the "twin-supercoiled-domain" model to explain these results where nucleoprotein complexes, especially those containing stable toroidal supercoils assembled two proteins are able to form distinct, stable DNA loops under our experimental conditions. Second, our results show that a combination of two unrelated DNA-binding proteins; e.g., λ O protein and GalR also confined free supercoils to a defined region and separated the DNA molecules into two independent topological domains (Fig. S6). These results suggest that DNA wrapping, rather than looping, is the main reason for these two DNA-wrapping proteins to divide the DNA molecules into different topological domains. Nevertheless, although our AFM images showed that λ O protein and GalR are able to divide the supercoiled plasmids into distinct topological domains (Fig. S8), we cannot fully exclude the role of protein-protein interactions of λ O-DNA complexes and GalR-DNA complexes in the formation of the two distinct topological domains.

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from DNA looping or tightly wrapping DNA around these DNA-binding proteins, can form topological barriers that impede the diffusion and merger of independent chromosomal supercoil domains (12). Our results in this report demonstrated that these nucleoprotein complexes are indeed able to form topological barriers to block supercoil diffusion (Figs. 3, 4, 5, Figs. S5 and S7). In addition, our results can be used to explain transcription activation of bacterial phase λ, a hallmark of λ DNA replication control in vivo (29). Our recent results showed that transcription-coupled DNA supercoiling is responsible for the activation of λ DNA replication. Specifically, the O-some (26) assembled from wrapping DNA around O protein in the replication origin blocks, confines, and captures transcription-coupled DNA supercoiling, which causes structural changes in λ DNA replication origin (30). In this case, the DNA replication origin is unwind and DNA supercoils are stabilized. This mechanism can also be used to explain transcriptional dependence of E. coli DNA replication initiation (31). In this scenario, DnaA, the E. coli replication initiator coupled with HU protein, binds to specific DnaA boxes in the oriC replication origin to form a nucleoprotein complex to unwind the AT-rich DNA sequence in the origin. Transcription-coupled DNA supercoiling should greatly stimulate this process. 

Our topological barrier model also provides unique insights into how the E. coli chromosome is divided into many different topological domains. As demonstrated previously (32, 33), the E. coli chromosomal DNA is associated with several abundant histone-like proteins, such as HU, H-NS, FIS, and IHF and folded into a compact nucleoid structure (1, 34). These histone-like DNA-binding proteins also wrap and loop DNA, which constrain DNA supercoils in vitro and in vivo (35–38). It is possible that the nucleoprotein complexes generated from these histone-like proteins serve as general DNA topological barriers to modulate localized DNA supercoiling. Indeed, our recent results showed that HU and H-NS are able to confine supercoils to a defined region. Nevertheless, the topological barriers stemming from nonspecific binding of HU or H-NS to DNA were less stable, which is consistent with the dynamic nature of E. coli chromosome topological domains (3, 39). Furthermore, nucleosomes may use the same mechanism to modulate DNA topology of eukaryotic chromosome (40).

Materials and Methods

Details of the preparation of purified proteins and the construction of plasmid DNA templates are described in SI Materials and Methods. The DNA-nicking and DNA-gyrase methods are summarized in Fig. 2. Details of the procedures are also described in SI Materials and Methods. For AFM, the LacI-DNA samples were prepared according to the DNA-nicking method. After the supercoiled DNA templates were digested by Nt.BbvCI, the LacI-DNA complexes were deposited on freshly cleaved mica and visualized with a NanoScope MultiMode AFM microscope. Details of the AFM procedure are described in SI Materials and Methods.

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