

DNA Spiral Supercoiling and Intramolecular Topological Interlink

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Abstract

Escherichia coli chromosome DNA was observed previously to consist of subunits of Archimedean spiral-like supercoiling. How do cells build such a DNA structure remains unknown. In the present study, atomic force microscopy (AFM) images showed that supercoiled pBR322 DNA forms a spiral structure upon intercalation with 0.5 µg/ml ethidium bromide (EB), which used to be considered as zero superhelical density. New evidence suggested that a novel topological bond, intramolecular topological interlink (ITL), promotes the DNA spiral formation. Without intercalation, the supercoiled pBR322 DNA displays plectonemical supercoil with uneven distribution of the supercoil density. Similar observation was also made when the DNA was over intercalated by EB (20 µg/ml). The results indicated that ITL functions as a brake to block the helical double strands twisting and comparted circular DNA into different superhelical density domains. When DNA was denatured in alkaline, AFM images showed that the ITL remains constant. As the denatured pBR322 DNA was cut with restriction endonuclease *Pst*I, the digested DNA kept intramolecular interlinks to converge into a centre with two free cut ends. Interlinked intermediates can be observed when natural pBR322 DNA was digested with *Hind*III and site-specific nickase *Nb. Bpu*10I. All evidence suggests that ITL is present in pBR322 DNA and causes DNA spiral supercoiling. It was found that the DNA topoisomers with different ITL number run into a ladder of bands in electrophoresis which is distinct from the DNA topoisomers produced by *E. coli* gyrase forming a smear. In a cell free system containing *E. coli* cell extract, we demonstrate that topoisomerase IV is required for the production of ITL DNA topoisomers from relaxed cccDNA substrate. Collectively, our data suggest that ITL represents a novel element of DNA topological structure. The DNA spiral supercoiling may be a universal structure present in the cell.

Keywords: DNA spiral supercoiling; Intramolecular topological interlink; Atomic force microscopy; Topoisomerase; Cell-free system; *E. coli* chromosome.

Introduction

In 1965, Vinograd et al. [1] found that DNA molecules could have three structural forms: supercoiled DNA (DNA I, or covalent closed circular DNA, i.e. cccDNA), relaxed circular DNA (DNA II, or open circular DNA, i.e. ocDNA) and linear DNA (DNA III) [1,2]. At a high pH, the supercoiled DNA (DNA I) can be converted into alkali-denatured supercoiled DNA (DNA IV, or form IV DNA), which runs the fastest in gel electrophoresis followed by DNA I, DNA III and DNA II. These forms of DNA show different behavior in the gradient density of ultracentrifugation [3-6].

An equation, $Lk=Tw+Wr$, has been used to describe the topological structure of cccDNA. The linking number, Lk , is an integer and topological property of double-strand DNA that represents the number of one strand making acrosses through the other in a planar projection. The twisting number, Tw , reflects the helical winding of the DNA strands around each other. Writhing number, Wr , is a measure of the coiling of the double stranded DNA axis [7-10]. For cccDNA, Lk is a constant which can only be changed by breaking the DNA backbone and winding or unwinding the DNA double strands. Tw can be changed by ionic strength and binding of intercalators or proteins [11]. Wr will change following the alteration of Tw . The values of Tw and Wr could be integer or fraction. The topological equation has been helpful to describe DNA three-dimensional structures [12-17].

In 1990, Boles et al. [15] proposed two different types of supercoiled DNA, plectonemically supercoiled DNA and solenoidally supercoiled DNA [15], with the former mainly present in free form of DNA writhing between two DNA strands while the later coils around a cylinder such as eukaryotic chromatin. Both forms are interchangeable and follow the rule of $Lk=Tw+Wr$. However, this can not explain DNA

spiral supercoils observed in *E. coli* chromosome structure (see Figure 1) [25].

In previous studies, four topoisomerases, topoisomerase I (Topo I), gyrase, topoisomerase III (Topo III) and topoisomerase IV (Topo IV), have been described in *E. coli* [18-21]. They have been classified into two types: those making single-stranded breaks in DNA and catalyze linking number changes in steps of one (Topo I and Topo



Figure 1: *E. coli* chromosome DNA was observed consisted of many Archimedean spiral-like supercoiling subunits [25].

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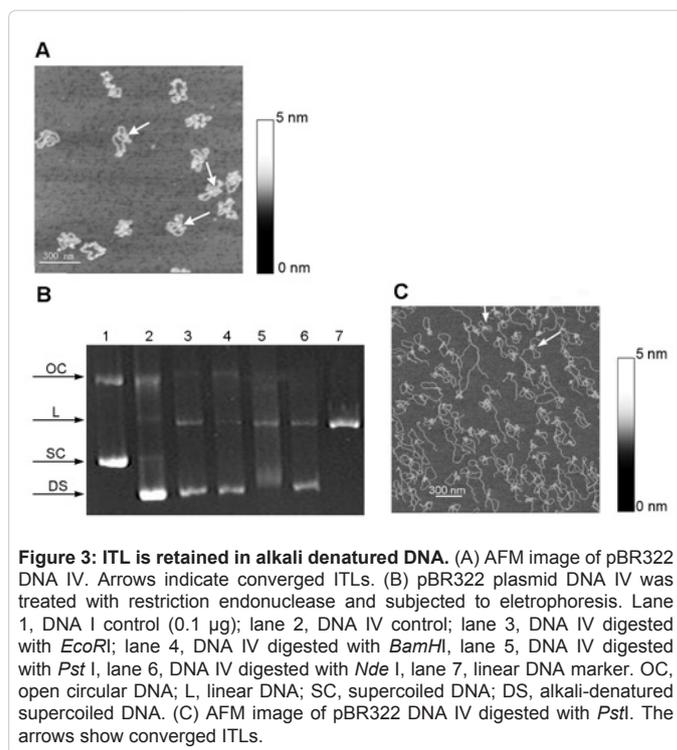
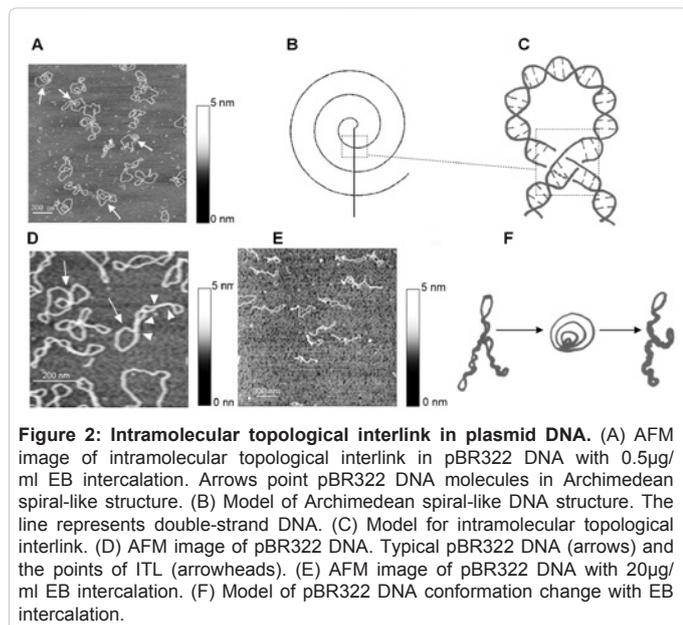
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III) and enzymes that make double-stranded breaks in DNA and catalyze linking number changes in steps of two (gyrase and Topo IV). It has been suggested that *E. coli* Topo I relaxes DNA negative supercoiling and gyrase introduces negative supercoiling into DNA. In this way, Topo I and gyrase cooperate to keep the chromosomal DNA supercoiling in an appropriate level in cells. Topo III and Topo IV have been implicated in untangling of daughter chromosomal DNA after replication. In addition, these two enzymes also participate in untying of intermediates during DNA homologous recombination. The DNA topoisomerases are involved in DNA replication, transcription, genetic recombination and chromosome separation after replication, thus are responsible for DNA topological alteration *in vivo* [22-24].

In 1974, Delius and Worcel [25] purified folded *E. coli* chromosome by density gradient centrifugation. They demonstrated that the intact chromosome consisted of many spiral supercoiling DNA structures like Archimedean spiral (Figure 1) [25]. The spiral supercoiled chromosomal DNA was converted into plectonemically supercoiled DNA at high ethidium bromide concentrations. However, the mechanism by which the spiral supercoiled structure forms inside the cells is not known.

In this study, we demonstrate the presence of a novel DNA topological bond, intramolecular topological interlink (ITL), which is created by Topo IV and readily observable in plasmid DNA isolated from *E. coli*. We have also shown that ITL promotes circular DNA to form spiral supercoiled DNA. Topo I, Topo III and Topo IV, but not gyrase, can change the number of ITL *in vitro*. ITL is the key structural element to shape DNA into a spiral form and to cause DNA running in ladder bands on agarose gel electrophoresis. Such structure does not conform to the equation of $Lk=Tw+Wr$. The ITL number of pBR322 in wild type strain of *E. coli* is about 7-8. The ITL can render cccDNA to form spiral type instead of the solenoidal type structure. Our results demonstrate that spiral supercoiling may be a normal three-dimensional structure in cellular packing of chromosomal DNA and may represent an ideal way of DNA folding and bioactivity.



Materials and Methods

Plasmid isolation

Three plasmids, pBR322(4631bp) (Fermentas, Lietuva), pUC18 (2686bp) (Fermentas, Lietuva) and pJGX15A (8176bp) [6] were used to study the DNA topological structure. Three strains, *E. coli* HB101 (*recA13, topA⁺*), *E. coli* DM800 (*topA⁺, gyrB225*) and *E. coli* SD108 (*topA⁺, gyrB225*) have been described previously [31]. Plasmid isolation and purification were performed as described previously [6].

Agarose gel electrophoresis: One-dimensional and two-dimensional electrophoresis was performed at 2V/cm on 1% agarose gel in TAE buffer, pH 7.8. The time for first dimension was 14 h-16 h and the second dimension was 6 h. Chloroquine was used at the concentration indicated in the figure legends. The electrophoresis showed in Figures 3A, 4A, 5A, 5B were performed as described previously [6]. After electrophoresis, agarose gels were stained with ethidium bromide and photographed with UVP transilluminator (UVP Inc., Upland, CA, USA).

Atomic force microscopy of DNA: AFM images of DNA were performed as described [6], except that tapping mode scanning was performed using a Nanoscope IV Multimode-AFM instrument (Digital Instruments, Santa Barbara, CA). One hundred molecules were analyzed for each sample. For EB intercalation, 1mg/ml EB solution was included in DNA samples in EB final concentration (0.5 and 20 μg/ml).

Preparation of *E. coli* HB101 cell extract

E. coli (strain HB101) was cultured in 50 ml LB medium to logarithmic phase. Cells were harvested by centrifugation at 4 and suspended in 6 ml of 25% sucrose in 0.05 M Tris-HCl, pH 8.0 and 1 mM EDTA. After addition of 5 mg lysozyme, the suspension was placed at

room temperature for 5 min. Ethylene-diamine-tetraacetate (EDTA, 0.4 ml, 0.25 M, pH 8.0) and 10% Triton X-100 (0.8 ml) were added to the cells, and the suspension was placed at room temperature for 5 min. After cell lysis, 1 M NaCl was added to the viscous solution with gentle mixing to a final concentration of 0.2 M. The lysates were stored at 4°C overnight, after which they were centrifuged at 35,000 rpm/min for 30 min in a refrigerated centrifuge. Supernatant was collected in 1 ml of aliquots and stored in a freezer at -20°C for later use.

Nickase reactions

Plasmid pBR322 DNA was treated by nickase *Nb.Bpu10I* (Fermentas, Lietuva) at 20°C for various time, 1-60min. The reaction with *Nb.Bpu10I* was optimized in a 20- μ l reaction mixture consisting of 10 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 100 mM KCl, 0.1 mg/ml BSA, 1U *Nb.Bpu10I* and 0.1 μ g pBR322 DNA. Reactions were stopped by adding 2 μ l of 0.5 M EDTA.

Restriction endonuclease reactions

Plasmid pBR322 DNA was treated by restriction endonuclease *HindIII* (Fermentas, Lietuva) for various time, 0.5-30min, at 20°C or 37 °C. The reaction with *HindIII* was optimized in a 20- μ l reaction mixture consisting of 10 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 100 mM KCl, 0.1 mg/ml BSA, 1U *Hind III* and 0.1 μ g pBR322 DNA. Reactions were stopped by adding 2 μ l of 0.5 M EDTA.

Reactions with *EcoRI*, *BamHI*, *PstI*, *NdeI* were performed following the manufacturer's instructions. Twenty microliter of each reaction contains 0.1 μ g DNA. Reactions were incubated at 37 for 30 min and then stopped by adding 2 μ l of 0.5 M EDTA.

Topoisomerase reactions *in vitro*

The reactions with *E. coli* topoisomerase I (New England Biolabs, Inc.), *E. coli* gyrase (New England Biolabs, Inc.) or *E. coli* topoisomerase IV (TopoGEN, Inc. Florida) were performed following the manufacturer's instructions. Twenty microliter of each reaction contained 0.3 μ g DNA. The reaction with *E.coli* topoisomerase III, purified as described previously [34], was optimized in a 20- μ l reaction mixture consisting of 50 mM HEPES-KOH (pH 8.0), 1 mM MgCl₂, 5mM DTT, 0.1 mg/ml BSA, 1 pmol *E.coli* topoisomerase III and 0.3 μ g DNA. For the cell-free system, topoisomerase reaction mixtures were supplied with 5 μ l cell extract. Reactions were incubated at 37 for 30 min and then stopped by adding 2 μ l of 0.5 M EDTA.

Results

Spiral supercoiling is an inherent structure of pBR322 plasmid DNA

In this study, plasmids pBR322 (4631bp) [26], pUC18 (2686bp) and another ColE1 derived plasmid pJGX15A (8176bp) were used to transform *E. coli* strains. These are multiple copy number plasmids in *E. coli* and subject to modifications by DNA topoisomerases. The pBR322 DNA molecules form Archimedean spiral structures when intercalated with 0.5 μ g/ml ethidium bromide (Figure 2A). Similar to the *E. coli* chromosome DNA spiral domains shown by electron microscopy [25], the DNA spiral supercoiling of pBR322 DNA forms extending circles (Figure 2B). In the DNA spiral supercoiling, the DNA double strands are kept apart and spiral, suggesting a novel ordered DNA structure. We propose a structural element of intramolecular topological interlink (ITL) as illustrated in Figure 2C. This supposed

crossing of double strand DNA may tie DNA together at different part of double stranded molecule and form spiral shaped DNA.

The ITL causes intramolecular topological heterogeneity of pBR322 DNA

The pBR322 plasmid DNA isolated from *E. coli* showed topological heterogeneity in AFM without EB, with most DNA molecules in plectonemical supercoil (Figure 2D). It is interesting that many DNA molecules are topologically heterogeneous in different part of the molecules. For example, the AFM image of the pBR322 DNA molecule at middle right of Figure 2D clearly shows five different topological domains in a single DNA molecule. Two ends are connected to two relaxed circles of DNA, connecting two tightly plectonemical writhing DNA domains. The central domain of the DNA molecule was supercoiling with writhing number of one. The result agrees with ITL hypothesis that ITL blocked DNA double strands twisting at cross point and comparted the DNA into different topological domains.

EB intercalation does not change ITL

The natural pBR322 DNA was visualized by AFM in the presence of 20 μ g/ml EB. As the image shown in Figure 2E, most of the DNA molecules were transformed into plectonemically supercoiled DNA with intramolecular topological heterogeneity. This result supports the ITL hypothesis for the cccDNA. When a DNA molecule is in spiral, the different spirals of the DNA molecule are in different curves, thus in different supercoiling state. Hence, intramolecular topological crosses of double strands blocked DNA double strands twisting and comparted DNA molecule into topological domains with different superhelical density.

As described above, pBR322 DNA purified from bacterial cells was transformed from plectonemically supercoiling to spiral, then to another plectonemically supercoiling following EB concentration increase from 0 to 0.5 μ g/ml then to 20 μ g/ml. This course of pBR322 transformation was consistent with the behavior of cccDNA intercalation of daunomycin detected by CsCl gradient density ultracentrifugation, which showed a changing course of negative plectonemically supercoiling – relaxed cccDNA – positive plectonemically supercoiling [27]. According to the AFM images shown in Figures 2A,2D,2E, pBR322 DNA undergoes conformational change with increasing EB intercalation as illustrated in the Figure 2F.

The result demonstrated DNA topological principle that writhing number and twisting number could be changed by EB intercalation but not the linking number. When writhing number was 0 in 0.5 μ g/ml EB, pBR322 DNA becomes spiral, not to an 'open circle' as described by Waring [27]. When DNA was in plectonemically supercoil, ITL is the 'branch point' which compartes DNA into different topological domains. The evidence suggested that ITL is another topological link which differs from the classical linking number (Lk) and remains constant even after intercalation by EB.

ITL is retained after alkali denaturation and *Pst I* double-strand digestion

To demonstrate ITL was inherent to cccDNA, the purified pBR322 DNA was denatured in 0.1 M NaOH, then the alkaline denatured pBR322 DNA (DNA IV) was observed under AFM. Figure 3A shows that denatured pBR322 DNA was condensed in molecular contour and retained the ITL in DNA strand crossing (shown by arrows). The result indicated that ITL does exist in DNA molecule and is character

of topological covalent link. Therefore, the ITL obeyed the topological rule, i.e. no covalent break no topological linking number change in a DNA molecule.

The purified pBR322 DNA IV was treated with restriction endonuclease *Pst* I which has a unique recognition site. Agarose gel electrophoresis showed that most DNA molecules were digested into smear bands which migrated faster than the pBR322 linear band (Figure 3B). The digested product was visualized by AFM. Figure 3C demonstrated that most of pBR322 DNA molecules were cut into linear with two free ends, but the ITLs of the cut molecules appeared to be retained and converged to a centre. The result suggested that denaturation created irregular secondary structure and blocked ITL moving between double strands, thus the cut pBR322 DNA remains in a folded state. The evidence demonstrated further that ITLs do exist between double-strand DNA.

ITL is released by single strand nicking in a time-dependant manner

The pBR322 plasmid DNA samples were digested by sequence specific single strand cutting endonuclease (nickase, only one cut point on pBR322 DNA) *Nb.Bpu10I* at 20°C for 1 min, 5 min, 10 min, 30 min and 60 min. The nickase-digested DNA samples were analyzed by agarose gel electrophoresis and the results are shown in Figure 4A. The result indicated that the nickase relaxed the DNA sample completely after 10 min (Figure 4A, lane 4). When DNA samples were treated for 1 or 5 min (Figure 4A, lane 2, 3), most DNA became intermediate which ran in the gel between the supercoiled and relaxed DNA bands. The AFM images of DNA samples treated for 10 min (Figure 4C) showed that there were less ITL than that in DNA samples treated for 1 min (Figure 4B). It suggested that single strand may migrate from interlinking point to nicking point, resulting in loss of ITL. As

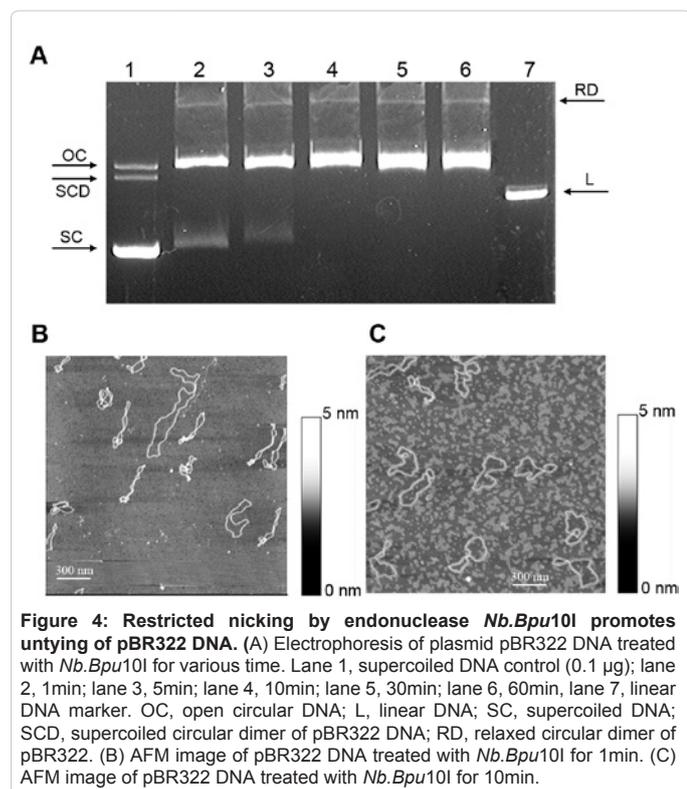
the migration may need thermic energy to break the hydrogen bonds between base pairs in double strands, therefore the multiple ITL release in the molecules may require a longer time. ITL release in molecules may be different from the nicking relaxation of plectonemical or solenoidal supercoiling DNA, because the cut of single strand would be able to release the supercoiling stress instantly by spinning the free end of the DNA *in situ* [28]. DNA with nicking in single strand complete unlinking by thermic motion, resulting in molecules stretched extensively (Figure 4C). The results demonstrated that the intramolecular interlinking points may move and nicking in single strand was able to release interlinking in a time-dependent manner. The fluorescence intensity of DNA samples increased with the progress of treatment time (Figure 4A, lane 2-6) with the maximum value at 1 h post treatment, suggesting that the relaxation by DNA nicking in single strand could increase the amount of ethidium bromide absorption by the DNA. It further suggested that the untying of the intramolecular interlinking is a slow process. Meanwhile, all the evidence suggested that ITL is not double-strand knotting because double-strand knotting could not be untied and released by single strand nicking [29].

Supercoiled pBR322 can be cut into linear DNA by restriction endonuclease via an ITL intermediate

To identify ITL was a structural element shaping DNA in spiral, pBR322 DNA isolated from bacterial cell was digested with restriction endonuclease *HindIII* for various time, 0, 30s, 1 min, 5 min, 10 min, 30 min at 20 (see Materials and Methods). The digested pBR322 DNA was subjected to agarose gel electrophoresis (Figure 5A). The results showed that supercoiled pBR322 DNA was digested into linear pBR322 DNA by the *HindIII* via intermediate components, i.e. DNA bands run at 'relaxed DNA' position after the first 10 minutes of digestion (Figure 5A, lane 2-5), and then the 'relaxed DNA' was transformed into linear DNA (Figure 5A, lane 6). By a time course, the intermediate seems stable enough to run into a DNA band on agarose gel. When the enzyme digestion time was extended, the relaxed intermediate was completely converted to linear pBR322 DNA. The digested pBR322 DNA product was visualized by AFM (Figure 5C). The image showed that most of the digested DNA generated two free ends but the preserved ITL kept the molecule in folded state. When the DNA strands were cut, ITL was not destroyed immediately, it was preserved until the crossing DNA strand moved to the cut site by thermic motion. When the enzyme reaction was carried at an increased temperature of 37, the cut pBR322 was untied into linear DNA within 5 min (Figure 5B), indicating that the natural pBR322 was really in ITL state and it could be released by thermal motion, again, suggesting the ITL was inherent to the natural pBR322 DNA.

ITL affects the diameter of the DNA molecules and their electrophoretic behaviour

Agarose gel electrophoresis has been used to resolve "DNA topoisomers" extracted from cells into a ladder of DNA bands [17,30]. To understand the relationship between DNA ITL and their mobility, the pBR322 DNA was extensively treated with topoisomerase I and IV, respectively. The enzyme-treated DNA samples were subjected to analysis with agarose gel electrophoresis and AFM. Figure 6C and Figure 6D showed the morphology of Topo I and Topo IV treated DNA samples. The ITL number of the treated pBR322 DNAs were significantly lower than that of the untreated DNA (Figure 6E), ranging 1-5 (Figure 6C & 6D). The evidence suggested that ITL of DNA molecule can be changed by topoisomerases, thus is a topological covalent link rather than a non-covalent link or binding by a protein.

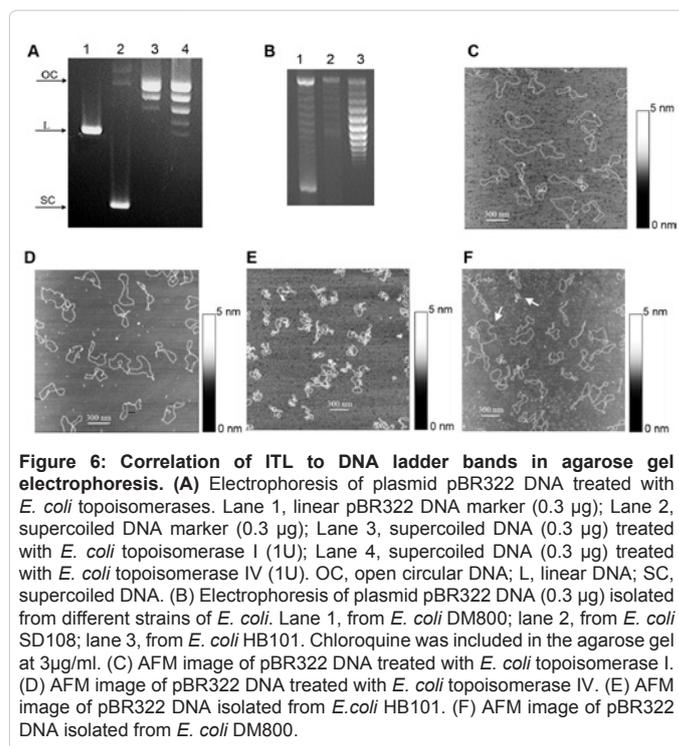


The result of electrophoresis showed that untreated DNA (Figure 6A lane 2), ran much faster than topoisomerase-treated DNAs (lane 3, 4). However, Topo I treated sample ran into a ladder with 3 DNA topoisomers, and Topo IV treated sample ran into 5 topoisomer bands. By comparing the AFM results, we found that the DNA topoisomers consisted of the ladder bands of DNA with different ITL. The evidence suggested that ITL was the main structural element responsible for the electrophoretic behavior of ladder bands in agarose gel, with DNA molecules having greater ITL number ran faster on the agarose gel than those with lower ITL number. The result also indicated that Topo I and Topo IV were unable to transform the supercoiled DNA into relaxed DNA completely.

To gain further insight, the pBR322 DNA samples were isolated from topoisomerase gene mutant *E. coli* strains. The DNA from *topA* mutant showed a long ladder of DNA bands with smear background (Figure 6B, lane 1), compared to that from a wild type *E. coli* (Figure 6B, lane 3), indicating a greater topological heterogeneity.

The *topA* deletion did not eliminate the ITL topoisomers but increase heterogeneity. In the *gyrB* mutant (*E. coli* SD108), the reduced gyrase activity in the cell lowered ITL topoisomer number and supercoiling density as showed in lane 2 of Figure 6B. The results confirmed the relationship between topoisomerases and ITL further, supporting that ITL being a topological link.

The electrophoresis results were consistent with the visual observations under AFM. In the picture of pBR322 DNA extracted

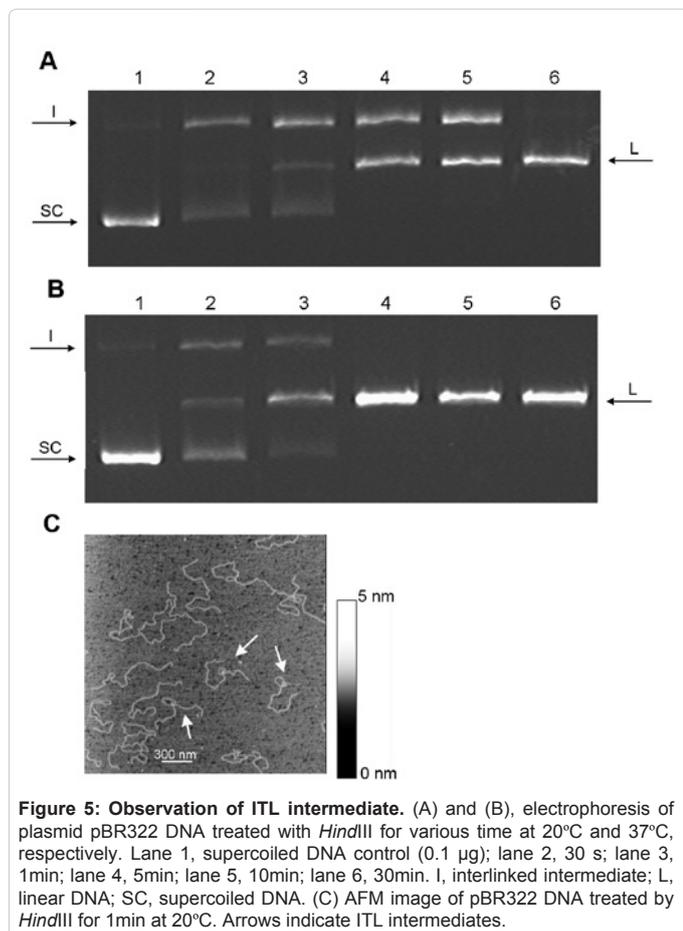


from *topA* mutant (*E. coli* DM800) (Figure 6F), the arrows showed great difference in compaction of two molecules. One of them was tight in topological interlinking and the other was untied. Quite a few of DNA molecules had a plectonemical supercoiled tail. This result indicated that these tailed DNAs may contribute to smear background as seen in lane 1 of the Figure 6B.

To understand why the decreasing ITL number of DNA molecule causes slower mobility in electrophoresis, the diameters and ITL numbers of pBR322 DNA (Figure 6E, Figures 7D) and Topo I-treated pBR322 DNA (Figure 6C) were measured from the AFM images. The results showed that the mean diameter of Topo I-treated DNA was 483 ± 50 nm and the number of ITL was 1-3 (Figure 6A). In contrast, the mean diameter of untreated DNA was 290 ± 40 nm and the average number of ITL was 7-8 (Figure 7A). This result suggested that the increase of ITL caused the decrease of molecular diameter of pBR322 DNA, indicating that the ITL shaped the DNA into spiral supercoiling and caused diameters of molecules to decrease. Thus, DNA molecules with greater ITL move faster on agarose gel with smaller contour and less resistance. The mobility of pBR322 DNA was strictly proportional to ITL number in the molecules as an integer. This provided a mechanism by which DNA isolated from cells moved in a ladder of bands according to intramolecular topological interlinking.

The AFM image of the larger plasmid pJGX15A (8176bp) DNA showed that the number of ITL was 8-11, and the mean ratio of diameter to perimeter was 0.19 ± 0.02 (Figure 7B, C). For a smaller sized plasmid pUC18 (2686bp) DNA (Figure 7B, E), the number of ITL was 1-4, and the mean ratio of diameter to perimeter was 0.25 ± 0.03 . This suggested that the restraint of DNA strand in small plasmid may disturb the ability of DNA topoisomerase to cause interlinking.

In the AFM image of pBR322 DNA IV (Figure 3C), the DNA had a proximal circular contour, but its diameter was smaller, and the ratio of



diameter to perimeter was 0.15 ± 0.02 . The results were consistent with the fact that DNA IV migrates faster than DNA I in gel electrophoresis.

DNA topoisomerases correlates to the ITL number

To determine the relationship of DNA spiral supercoiling and topoisomerases, samples of pBR322 DNA were catalyzed by four *E. coli* topoisomerases at optimum conditions. These samples were subjected to two-dimensional agarose gel electrophoresis with chloroquine, a DNA intercalator which causes gentler DNA twisting than EB (Figure 8A). The results showed that *E. coli* gyrase with the presence of ATP could convert ITL topoisomer bands into ambiguous bands even into smear, with increased moving velocity (increasing the level of negative supercoiling) (Figure 8A, panel 4). Both Topo I and Topo IV made the number of ITL to decrease, so that the supercoiling level of DNA samples were converted into positive supercoiling at the first dimensional gel with 3 $\mu\text{g}/\text{ml}$ chloroquine (Figure 6A, panel 3 and panel 6). After DNA sample was digested by Topo III, the ITL of DNA sample did not change much, except for some blurred bands.

The pBR322 cccDNA relaxed by Topo I was used as the substrates of gyrase to determine the function of gyrase for cccDNA. The samples were then subjected to two-dimensional agarose gel electrophoresis with chloroquine (Figure 8B). The result showed that gyrase catalyzed relaxed cccDNA into a series of supercoils. These supercoils mobilized as ring smear based on various superhelical densities. The catalyzed product did not form ladder bands but ran faster than untreated in gel. It suggested that gyrase introduced negative superhelical turns into relaxed cccDNA without generating new ITL. T_w and W_r in the equation $Lk = T_w + W_r$ are not integers, which can be changed following the fluctuation of environmental conditions, such as ionic strength and ion types in the solution. Alternatively, ΔLk generated by the catalysis of gyrase was partitioned on T_w and W_r . Thus, the structure of negative supercoiled DNA produced by gyrase did not possess quantum property, and the pure supercoiled DNA with different superhelical density ran in a long smear instead of ladder bands in gel. The results indicated that topological linking (Lk in equation $Lk = T_w + W_r$) generated by gyrase is different from ITL.

As shown above, Topo I, Topo III and Topo IV can all decrease ITL of cccDNA *in vitro*. Interestingly, Topo IV can also promote the

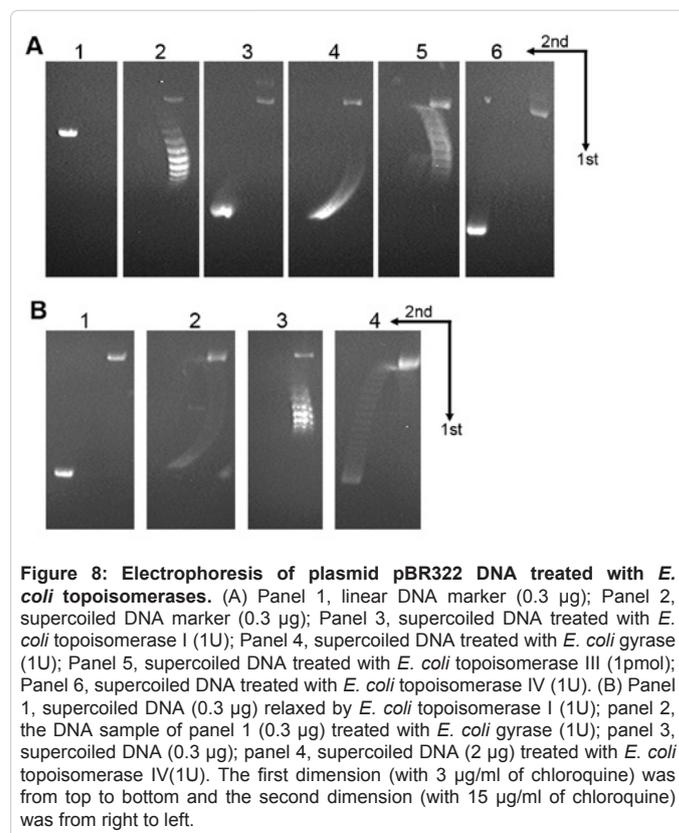


Figure 8: Electrophoresis of plasmid pBR322 DNA treated with *E. coli* topoisomerases. (A) Panel 1, linear DNA marker (0.3 μg); Panel 2, supercoiled DNA marker (0.3 μg); Panel 3, supercoiled DNA treated with *E. coli* topoisomerase I (1U); Panel 4, supercoiled DNA treated with *E. coli* gyrase (1U); Panel 5, supercoiled DNA treated with *E. coli* topoisomerase III (1pmol); Panel 6, supercoiled DNA treated with *E. coli* topoisomerase IV (1U). (B) Panel 1, supercoiled DNA (0.3 μg) relaxed by *E. coli* topoisomerase I (1U); panel 2, the DNA sample of panel 1 (0.3 μg) treated with *E. coli* gyrase (1U); panel 3, supercoiled DNA (0.3 μg); panel 4, supercoiled DNA (2 μg) treated with *E. coli* topoisomerase IV(1U). The first dimension (with 3 $\mu\text{g}/\text{ml}$ of chloroquine) was from top to bottom and the second dimension (with 15 $\mu\text{g}/\text{ml}$ of chloroquine) was from right to left.

generation of more bands in gel with chloroquine (Figure 8B, panel 4), suggesting that Topo IV can both release the ITL and increase the number of ITL. Therefore, the Topo IV is the most likely enzyme that promotes DNA to generate ITL *in vivo*. The above result (Figure 8B, panel 4) also showed that Topo IV can reduce the negative superhelical density of the DNA, possibly through consumption of energy stored in the negatively supercoiled substrate as Topo IV introduces ITL into the DNA.

Topo IV builds DNA spiral supercoiling in cooperation with gyrase in cell-free system

The *E. coli* HB101 (*recA13, topA⁺*) cell extract of logarithmic phase was prepared and used in a cell-free system to determine the biological functions of the topoisomerases. The samples of Topo I relaxed pBR322 DNA were used as substrate. Different *E. coli* topoisomerases were then added and incubated at 37 for 30 min. The DNA samples were subjected to agarose gel electrophoresis with chloroquine (Figure 9A). It is surprising that, in the presence of cell extract, all the four DNA topoisomerases played different roles. Topo I catalyzed most relaxed cccDNA into cccDNA dimers of hemicatene (lane 3, Figure 9A). The hemicatene dimers mobilized to the position of dimer supercoiled DNA [32,33]. *In vitro*, the hemicatene dimers produced by Topo I catalyzing appeared to have serial topological link points and form a tetrastrand complex of DNA (Figure 9B). The relaxed cccDNA was converted into DNA smear with high negative superhelical density when gyrase was included in the system (Figure 9A, lane 3), and the effect of Topo III on relaxed cccDNA was not apparent (Figure 9A, lane 4). The relaxed cccDNA was converted to ladder bands in gel when Topo IV was added in the cell-free system (Figure 9A, lane 5). The results

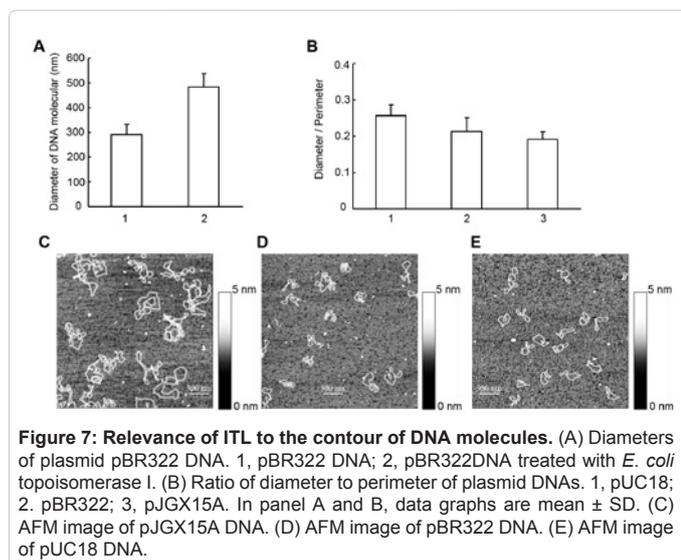
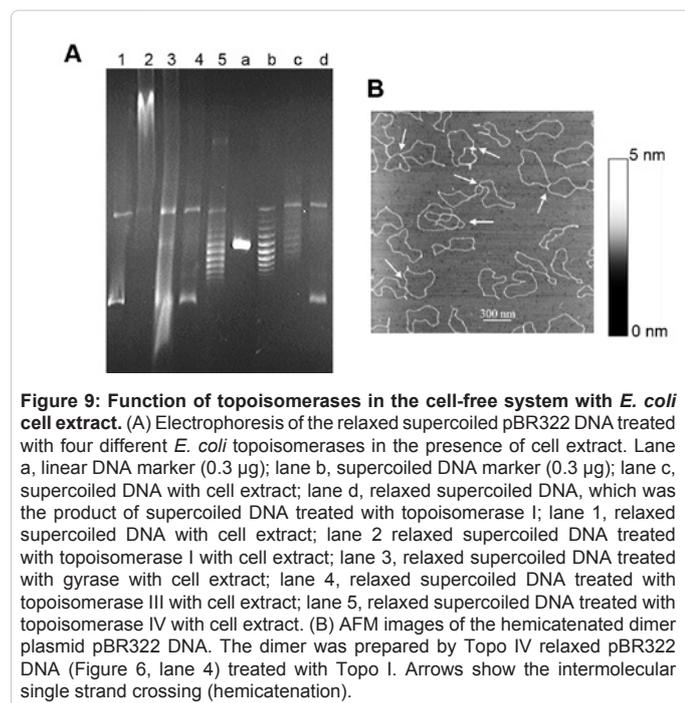


Figure 7: Relevance of ITL to the contour of DNA molecules. (A) Diameters of plasmid pBR322 DNA. 1, pBR322 DNA; 2, pBR322DNA treated with *E. coli* topoisomerase I. (B) Ratio of diameter to perimeter of plasmid DNAs. 1, pUC18; 2, pBR322; 3, pJGX15A. In panel A and B, data graphs are mean \pm SD. (C) AFM image of pJGX15A DNA. (D) AFM image of pBR322 DNA. (E) AFM image of pUC18 DNA.



suggested that the four topoisomerases have different functions in the presence of other relative protein factors in cell extract. Topo IV was the only topoisomerase that could promote relaxed cccDNA to generate ITL topoisomers, suggesting that Topo IV may be the enzyme catalyzing ITL formation *in vivo*. When supercoiled pBR322 DNA was incubated with cell extract for 30 min at 37, the product turned into blurred ladder bands in gel (Figure 9A, lane c). Incubation of the Topo I relaxed DNA with cell extract converted the DNA to a smear (Figure 9A, lane 1). The results suggested that there was endogenous gyrase activity in cell extract. The activity of endogenous gyrase did not impede the relaxed cccDNA to form ladder DNA bands in gel suggesting that gyrase activity may actually help Topo IV to introduce ITL and the Archimedean spiral supercoil formation.

Discussion

In the present study, ITL is shown to be a novel topological structural element, which supports cccDNA forming Archimedean spiral-like supercoiling. The Archimedean spiral-like supercoiling of DNA may be the normal structure for DNA packing and folding in the cell, as it is similar to the substructure of *E. coli* chromosome [25]. The DNA spiral supercoiling is superior to plectonemical supercoiling and solenoidal supercoiling for accessibility and better packing ratio.

In the present study, we show that pBR322 DNA isolated from cells can be transformed from plectonemical negative supercoiling to spiral supercoiling, then to plectonemical positive supercoiling following intercalation with increasing EB, i.e. from 0 to 0.5 μ g/ml, then to 20 μ g/ml. The result meant that plectonemical supercoiled DNA can be changed into spiral supercoiling when DNA molecule contains ITL. The natural plasmid DNA usually shows intramolecular topological heterogeneity due to the spiral supercoiling DNA having different superhelix density in different spirals. It is possible that the counterions and the protein binding, in place of EB intercalation, may cause DNA spiral *in vivo*.

Plasmid DNA isolated from *E. coli* cells consists of ITL topoisomers depending on the ITL number, forming ladder bands in gel. ITL was not a parameter in the classical DNA topological equation: $Lk = Tw + Wr$. The number of ITL is inversely proportional to the diameter of cccDNA molecules, which means that the more ITLs in a DNA molecule, the faster the DNA runs in agarose gel.

Gyrase does not contribute to ITL formation, but it could introduce negative superhelical turns into cccDNA. ΔLk generated by *E. coli* gyrase is partitioned by Tw and Wr ; therefore, negative supercoiled DNA catalyzed by gyrase forms long arc smear rather than ladder bands in gel.

We found that Topo IV could promote cccDNA to change the number of ITL of DNA *in vitro*. In the presence of cell extract, Topo IV could promote relaxed cccDNA to form ITL topoisomers displaying ladder bands in gel. The results further suggest that Topo IV, in combination with gyrase, may construct spiral supercoiling structure of chromosomes *in vivo*. It means that the DNA spiral supercoiling is built on two topological elements, supercoiling and ITL.

Topo I and Topo III belong to Type I topoisomerases, which break one DNA strand and pass the other strand through the transient break. They seem to be the optimal enzymes to catalyze ITL formation, but the results in present study suggest that they do not contribute to ITL production. Instead, our study shows that Topo IV contributes to ITL formation. In previous studies, Topo IV was classified as type II topoisomerase which breaks both strands simultaneously and passes double-stranded DNA through the break. Further studies are warranted to determine the catalytic mechanisms and functions of Topo IV and other protein factors cooperating with it *in vivo*.

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