The *Bacillus subtilis* primosomal protein DnaD untwists supercoiled DNA

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ABSTRACT

The essential *Bacillus subtilis* DnaD and DnaB proteins have been implicated in the initiation of DNA replication. Recently DNA remodelling activities associated with both proteins were discovered that could provide a link between global or local nucleoid remodelling and initiation of replication. DnaD forms scaffolds and opens up supercoiled plasmids without nicking to form open circular complexes while DnaB acts as a lateral compaction protein. Here we show that DnaD-mediated opening of supercoiled plasmids is accompanied by significant untwisting of DNA. The net result is the conversion of writhe (Wr) into negative twist (Tw), thus maintaining the linking number (Lk) constant. These changes in supercoiling will reduce the considerable energy required to open up closed circular plectonemic DNA and may be significant in the priming of DNA replication. By comparison, DnaB does not affect significantly the supercoiling of plasmids. Binding of the DnaD C-terminal domain (Cd) to DNA is not sufficient to convert Wr into negative Tw, implying that the formation of scaffolds is essential for duplex untwisting. Overall our data suggest that the topological effects of the two proteins on supercoiled DNA are different; DnaD opens up, untwists and converts plectonemic DNA to a more paranemic form whereas DnaB does not affect supercoiling significantly and condenses DNA only via its lateral compaction activity. The significance of these findings in the initiation of DNA replication is discussed.

INTRODUCTION

DNA replication is the most fundamental function in all biology. It is divided in three main stages known as initiation, elongation and termination. Initiation involves remodelling of a replication origin (*ori*C) through the action of the main initiator protein DnaA and primosomal multi-protein cascades that ultimately load two replicative ring helicases, one on each strand of the DNA duplex. The helicases in turn recruit the DNA primases thus signalling the switch from initiation to elongation and the two replication forks migrate in opposite directions, one on the leading and one on the lagging strand (11, 21). Progression to the elongation stage does not guarantee completion of DNA replication as replication forks could be challenged and arrested anywhere along the DNA. In *Escherichia coli*, reconstitution of an active replication fork at arrested sites is mediated by PriA and/or PriC primosomal pathways that include the PriA, PriB, PriC and DnaT proteins (19, 25) while priming at the *oriC* is mediated by DnaA. Homologues of both DnaA and PriA are found in gram positive bacteria but another primosomal cascade involving the DnaD, DnaB and DnaI proteins is found only in some low G+C content gram positive bacteria, including *Bacillus subtilis*. While DnaI is believed to be the gram positive functional homologue of the *Escherichia coli* helicase-loader DnaC (not to be confused with the *Bacillus subtilis* DnaC helicase), both DnaD and DnaB have no homologues in gram negative bacteria. They are essential for viability and required for both DnaA and PriA-mediated initiation of DNA replication (3, 27). The molecular events that underpin the priming mechanism are unclear at present but data so far indicate that DnaD interacts with DnaA, PriA and DnaB (16, 20) while DnaI interacts with the

DnaC helicase (15, 26, 32, 36). DnaD is believed to act early in the cascade setting the stage for helicase recruitment.

We have recently discovered that DnaD and DnaB have global DNA-remodelling activities. DnaD forms scaffolds and converts supercoiled DNA to an open circular form whereas DnaB compacts laterally supercoiled and linear DNA (35, 40). The biological significance of these activities is not clear at present but we have proposed a functional model with DnaD and DnaB being the link between global or local nucleoid remodelling and the initiation of DNA replication (35, 40). Atomic force microscopy (AFM) revealed that DnaD converts all the writhe of supercoiled plasmids into twist, and suggested that this DNA remodelling might be accompanied by significant untwisting of the duplex (40). Untwisting of the DNA may compensate for the considerable force required to open up supercoiled plasmids without nicking. Once opened up the plasmid is held firmly in the perimeter of a circular protein scaffold made up of DnaD molecules.

The actual details of the molecular mechanism of the DnaD-induced topological effects are not known. In an effort to understand this mechanism we examined the effects of DnaD-binding to DNA on the activity of *Escherichia coli* topoisomerase I (topo I). Topo I relaxes negatively supercoiled DNA and changes in topology can be probed by effects on its activity. We reveal that DnaD untwists the DNA in a concentration dependent manner. Untwisting requires the formation of scaffolds by intact DnaD, since a C-terminal domain (Cd) that binds to DNA and exhibits a DNA-dependent oligomerisation activity (5) is unable to significantly untwist supercoiled plasmids. By comparison, DnaB

does not affect the topology of DNA and its effect on DNA condensation is simply the result of lateral compaction. Our combined data suggest that the two proteins have different DNA remodelling effects. The significance of our findings and the putative roles of these proteins in DNA replication are discussed.

MATERIALS AND METODS

Protein purifications

DnaD and DnaB proteins and the Cd domain of DnaD were purified as described before (5, 35, 40).

Topo I relaxation assays

pBluescript plasmid was purified from XL1Bule *Escherichia coli* cells using a plasmid midi-prep kit (Sigma). The same batch of pBluescript was used for all of the Topo I relaxation assays shown in this manuscript. pBluescript (18 nM) was incubated in a total volume of 25 µl with varying concentrations of protein(s) at 37 °C for 20 minutes to allow complex formation. Subsequently, 3 µl of 10×NEB buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT), 1 µl BSA (30 mg/ml), and 1 µl of Topo I (New England Biolabs; 4u/µl) were added and the reaction mixture (total volume 30 µl) was incubated at 37 °C for 40 minutes. All proteins in the reaction mixture were removed by digestion with excess of PK at 37 °C for 20 minutes. 5 µl of 6×DNA gel loading buffer was added and the mixture loaded onto 1% w/v agarose gel casted in TAE buffer. The gel was run at 40 V in TAE buffer, in a cold room

for 7~8 hours, stained in 1 μg/ml ethidium bromide for 40 minutes, and photographed with a digital camera.

2D agarose gel elctrophoresis

Topo I relaxation assays were set up, as described above. The reaction mixtures were subjected first to normal 1D agarose gel electrophoresis in 1.2 % w/v agarose gels, casted in TAE buffer, at 4 °C (cold room). The samples were loaded into a well on the top left corner of the gels, run at 40 V for 7 hours in one dimension and then soaked in TAE buffer supplemented with 4.5 μg/ml chloroquine for two hours. The gels were then turned clockwise by 90 ° and run in the second dimension at 20 V for 12 hours in TAE buffer, containing 4.5 μg/ml chloroquine. The gel was stained in 10 μg/ml ethidium bromide for 35 minutes, destained in deionised water for two hours and photographed with a digital camera.

RESULTS

DnaD untwists supercoiled plasmids in a concentration-dependent manner

Binding of DnaD to supercoiled pBR322 plasmid results in the formation of circular protein scaffolds with the plasmids held firmly around the periphery of these scaffolds (40). The contour lengths of the bound plasmids were found to be consistently longer than unbound molecules. The actual contour lengths were directly proportional to the sizes of the scaffolds, with larger scaffolds holding plasmid molecules with longer contour lengths compared to smaller scaffolds (40). The net result was the conversion of all the Wr into Tw. However, in the absence of nicking activity there will be no net

change in ΔLk, implying that the elimination of Wr must be compensated by negative twisting i.e. untwisting of the double helix. From statistical measurements of 134 pBR322 molecules bound to DnaD it has been established that this untwisting results in an average increase of the double helical turn from 10.6 to 16.1 bp (40). There is however no concrete biochemical data to reinforce these AFM observations. In an effort to provide biochemical evidence for these observations we examined the effects of DnaD binding to the activity of *E. coli* topo I. We argued that if DnaD binding to plasmids causes compensatory untwisting, treatment with topo I will relax this compensatory untwisting with a corresponding positive linking number change and subsequent removal of the bound DnaD will yield more relaxed supercoiled DNA. Because purifications of low copy number pBR322 plasmid (4,361 bp) yielded small amounts, to produce higher amounts of plasmid required for our experiments we used the high copy number pBluescript SK(-) plasmid (2,961 bp) instead.

We discovered that incubation of pBluescript with increasing concentrations of DnaD, subsequent treatment with topo I and removal of all proteins by digestion with proteinase K (PK) resulted in a stimulation of relaxation up to 16 μM DnaD, followed by apparent inhibition of relaxation at 32 μM of DnaD (Fig.1A). By comparison free pBluescript was relaxed less efficiently, as can be clearly seen from the appearance of distinct topoisomers in the absence of DnaD compared to fully relaxed plasmid in the presence of DnaD (0.1-10 μM) under identical conditions (Fig. 1B; left panel). In fact, in the presence of 10 μM DnaD four to five times less topo I was sufficient to relax the bound plasmid relative to the amount required to relax unbound plasmid (Fig. 1B; right panel). The

simplest interpretation of these data is that DnaD binding and remodelling of the DNA causes increasing compensatory untwisting as the DnaD concentration is increased. Treatment with topo I relaxes this compensatory untwisting with corresponding positive linking number changes progressively increasing with increased untwisting. Subsequent removal of the bound DnaD yields supercoiled DNA with progressively higher positive linking number changes, resulting in the appearance of more relaxed negatively supercoiled DNA. However, at high concentrations of DnaD inhibition of the topo I activity is observed because the excess bound DnaD molecules sterically prevent access to the DNA. These DnaD-induced superhelical changes were unequivocally confirmed by 2D gel electrophoresis (Fig. 1C).

Supercoiled pBluescript topoisomers were separated in the first dimension by simple 1D agarose gel electrophoresis. The most supercoiled molecules have the fastest mobility while fully relaxed molecules run slowest through the gel matrix. The gel was then soaked in 4.5 μg/ml chloroquine turned 90^{o} and topoisomers were separated in the second dimension in TAE buffer supplemented with 4.5 μg/ml chloroquine (Fig. 1C). Chloroquine relaxes negatively supercoiled DNA and can introduce positive supercoiling depending on its concentration (38). Plasmid molecules that were least negatively supercoiled will now be the most positively supercoiled and travel further toward the anode. We confirmed that incubation with increasing concentrations of DnaD (0.5, 1 and 16 μM) and subsequent treatment with topo I does indeed increase the amount of supercoiled plasmids with smaller negative linking number, compared to the supercoils with higher negative linking number in the absence of DnaD (Fig. 1C). These data verify

that topo I relaxes the compensatory untwisting induced by DnaD, resulting in an overall positive change in the Lk. At high concentrations (32 μM) DnaD inhibits the topo I relaxation activity, as manifested by the presence of more supercoils with higher negative linking number in 2D gels (Fig. 1C).

DnaB does not cause significant changes in plasmid supercoiling

Previous observations with AFM revealed that tetrameric DnaB binds to, and laterally compacts, supercoiled plasmids forming bead-like structures with the DNA wrapped around the protein (40). An important question to answer is whether this condensing activity is accompanied by significant changes in the superhelical properties of the DNA, analogous to that observed for DnaD. We examined the effects of DnaB binding on the activity of topo I. DnaB up to 4 μM only slightly enhanced topo I relaxation (Fig. 2A lane 4 and Fig. 2B) that can be attributed to minor non-specific effects by DnaB binding rather than specific significant untwisting, as was observed for DnaD. As the concentration was raised to above 8 μM inhibition of the topo I activity was observed (Fig. 2A lanes 5-7), because of excess bound DnaB preventing access to the DNA. We verified these data with 2D gel electrophoresis (Fig. 2B).

Taken together the above data show that DnaD-dependent opening of supercoiled plasmids is accompanied by significant concentration-dependent untwisting of the duplex, whereas DnaB-mediated compaction does not change the superhelical properties of the DNA and is simply the result of lateral compaction.

The DnaD scaffold is essential for untwisting the duplex

DnaD consists of two domains with distinct activities (5). Its N-terminal domain (Nd) has a DNA-independent oligomerisation activity while its C-terminal domain (Cd) binds to DNA and exhibits a separate DNA-dependent oligomerisation activity (5). An important mechanistic detail to reveal is whether DNA-binding per sec is sufficient to untwist the duplex. To answer this question we investigated the effect of Cd binding to pBluescript on the activity of topo I (Fig. 3A). Increasing concentrations of Cd 0.5-1 μM did not affect the activity of topo I (Fig. 3A, compare lanes 3, 4 and 5) but at 8 μM a slight stimulation of topo I was apparent (Fig. 3A, compare lanes 3 and 6). This was better than the non-specific effect observed for DnaB (Fig. 2A lane 4 and Fig. 2B) but much less compared with the strong effect seen with the full length DnaD protein (Fig. 1), indicating that simple Cd binding to pBluescript causes only minor untwisting of the duplex. At 18 and 64 μM Cd there was apparent inhibition of topo I (Fig. 3A; lanes 7 and 8). Like before with excess DnaD and DnaB, this inhibition was simply a steric effect. These observations were verified by 2D gel electrophoresis (Fig. 3B). The combined data indicate that the DNA-binding and DNA-induced oligomerisation activities of Cd were not sufficient to untwist the DNA significantly. Therefore, it is not simply the DNA-binding event that untwists the duplex. The formation of the scaffold is also necessary and thus duplex untwisting is the result of the the sum of both DNA-binding and scaffold formation. Since the size of the scaffolds (40) and the extent of untwisting are both concentration dependent the implication is that as the scaffold increases in size at higher DnaD concentrations so does the untwisting of the duplex (see Discussion).

DnaB does not inhibit the DnaD-mediated untwisting activity

AFM images of DnaD and DnaB mixtures with supercoiled pBR322 revealed unique bipolar nucleoprotein complexes with the two proteins located at diametrically opposite ends of the plasmid (40). At higher molar ratios of DnaD relative to DnaB one end of the plasmid was partially opened up whereas at higher molar ratios of DnaB relative to DnaD the plasmid adopted a highly compacted rod-like conformation (40). These data indicated that the two proteins bind to DNA simultaneously and antagonize each other for the overall effects on supercoiled DNA. An important question to answer is whether the DnaB antagonistic effect against DnaD is accompanied by a concomitant inhibition of the DnaD duplex untwisting activity. To answer this question, we examined the effects of DnaD and DnaB mixtures on the topo I activity. We investigated the effect of 4 μM DnaB on the twisting activity of DnaD (Fig. 4B). At this concentration, DnaB does not inhibit topo I; (see Fig. 2). Increasing the concentration of DnaD (0.5-1 μM) in the presence of 4 μM DnaB resulted in a slight enhancement topo I relaxation, compared to the presence of equivalent concentrations of DnaD alone (Fig. 4B, lanes 3-6). At 8 μM DnaD plus 4 μM of DnaB non-specific steric inhibition of topo I was apparent (Fig. 4B, compare lanes 7 and 8). At 8 μM of DnaB non-specific steric inhibition of topo I was apparent throughout the range of 0.5-33 μM DnaD concentrations (Fig. 4A). The combined data suggest that DnaB does not inhibit the untwisting activity of DnaD and if anything it appears to slightly enhance it.

AFM revealed that both proteins bind simultaneously to the same plasmid resulting in characteristic bipolar structures with the two proteins bound at diametrically opposite

ends of a rod-like supercoiled plasmid (40). Under the conditions of our experiment the two proteins should be bound simultaneously to the plasmid and partial opening should occur (40). This is also supported by the observation that as the concentration of DnaD is increased in the presence of DnaB, eventually topo I is sterically inhibited suggesting that DnaD binds to DNA simultaneously with DnaB. These data suggest that DnaB binding does not inhibit untwisting by DnaD.

DISCUSSION

DnaD is a replication initiation protein in *Bacillus subtilis* but its relative abundance in the cell, estimated at 3,000-5,000 molecules per cell (4), implies that it may also be involved in other additional functions. The discovery of its global DNA remodelling activity offered a possible link between replication and nucleoid remodelling (35, 40) but the actual mechanism of this remodelling is unclear. This remodelling activity is the sum of three separate activities residing on two distinct domains; an Nd domain with a DNA-independent oligomerisation activity and a Cd domain with DNA-binding and DNA-dependent oligomerisation activities (5). These separate activities must be coupled to each other on the same polypeptide to remodel DNA. This remodelling when applied to a supercoiled plasmid is also accompanied by an overall increase in the contour length of the plasmid DNA (40). Such an increase can only be explained by a concomitant untwisting of the helix and a statistical analysis of several DnaD-pBR322 complexes by AFM suggested an average untwisting of the duplex from 10.6 to 16.1 bp per turn (40). Our data offer direct biochemical evidence that DnaD binding to a supercoiled plasmid causes significant duplex untwisting. Binding of DnaD to DNA via its Cd and the DNA-

induced oligomerisation event that accompanies this binding (5) are not sufficient to remodel the DNA, implying scaffold formation by Nd linked on the same polypeptide is essential (5). DnaD binds efficiently to a 19-mer oligonucleotide (5) and assuming 19 bases as the binding size, compared to the size of pBluescript (2,961 bp), this represents approximately 155 binding sites per plasmid for DnaD. Therefore, at 28 and 56 molar excess of DnaD it is unlikely that the whole of the plasmid will be open up by DnaD and only at 448 molar excess it is likely that the whole of the plasmid will have been forced to open up. In fact untwisting increased progressively as the concentration of DnaD was raised and reached its maximum level at 8 μM (Fig. 1A), representing 1:448 molar excess of DnaD over the plasmid. We hypothesize that initially at low concentrations DnaD binds to few sites along the plasmid via its Cd domain and forms localized scaffolds that untwist the duplex only partially. As the concentration of DnaD increases the sizes of the local scaffolds also increase until they join to form a large circular scaffold holding the open plasmid in the periphery. We envisage that as the scaffold grows the duplex untwisting also increases progressively until the maximum possible open/paranemic plasmid conformation is achieved (Fig. 5). We estimate this to be the case at around 1:440 molar excess of DnaD over pBluescript. Untwisting of the duplex will compensate against the considerable force required to eliminate all the Wr, open up supercoiled DNA without nicking, and increase its contour length in a paranemic conformation (Fig. 5).

Local DnaD-mediated superhelical changes at the *oriC*

The untwisting activity of DnaD may also be an essential function for *oriC* remodelling to facilitate the initiation of DNA replication. Conversion of the duplex from plectonemic

to paranemic by DnaD binding and untwisting within the *oriC*, may be an essential feature for the initiation of DNA replication in *Bacillus subtilis*. With an effective zero Lk the paranemic helix within *oriC* could be easily separated, thus exposing the necessary single strands for loading of the replicative helicase. DnaD may facilitate DnaA-binding and melting of the *oriC*. There are 15 DnaA boxes in the *Bacillus subtilis oriC* and DnaA has differing activities for these sites (23). It binds to strong sites before weaker sites *in vivo* and the affinity of the binding sites plays a role in the staged assembly of the unwound *oriC* (18, 23). Local DnaD remodelling of the *oriC* may be targeted by the direct interaction with DnaA (16) and may enhance DnaA binding particularly to the weaker sites thus stimulating the formation of the unwound orisome. Indeed, the *Escherichia coli* DNA remodelling protein HU interacts with *oriC* and enhances the ability of DnaA to unwind the origin *in vitro* (14). Changes in supercoiling within *oriC* may also affect the binding affinity and/or specificity of other primosomal proteins. Indeed such changes in other cases like the *hix* site can switch the local DNA structure from an inefficient conformation for Hin interaction to an efficient one (2). A paranemic duplex may also facilitate the helicase loading in replication restart sites away from *oriC* and targeting of DnaD to restart sites may be mediated by a direct interaction with PriA (20).

DnaD is a potential modulator of global superhelical density

The precise role of DnaD *in vivo* may not be confined to the initiation of DNA replication. For example, could its DNA remodelling activity with the significant untwisting of the DNA and stimulation of topo I activity be part of the essential

homeostatic control of global superhelical density *in vivo*? Bacterial nucleoids are negatively supercoiled closed circular compacted structures separated into distinct topological domains (6, 12, 27). Modulation of the superhelical density has been shown to be an important factor in bacterial physiology affecting global transcriptional regulation, replication, recombination and response to environmental challenges (8, 34, 37, 39), and nucleoid architectural changes are apparent during growth (1, 17). Excess negative supercoiling is growth inhibitory (10) and in *Escherichia coli*, *topA* null mutants exhibit a growth defect due to increased negative supercoiling and frequently acquire compensatory mutations that inactivate DNA gyrase or over-produce topo IV resulting in a reduction of negative supercoiling (7, 9, 28, 29). It is attractive to speculate that in addition to other functions, DnaD may also act as a global regulator of superhelical density in *Bacillus subtilis*. Indeed its relative abundance in the cell (4) suggests functions additional to initiation of DNA replication. Over-production of DnaD *in vivo* may be an alternative mechanism for stimulating topo I activity and thus relieving excess negative supercoiling in *Bacillus subtilis*. A putative functional cooperation between DnaD and topo I does not imply a direct interaction between the two proteins (although this has not been excluded), as topo I activity can be modulated indirectly by DNA binding proteins in the absence of a direct interaction (31).

The role of DnaB

The precise function(s) of DnaB in the initiation of DNA replication is rather ambiguous. It has been suggested to act in conjunction with DnaI, effectively forming a pair of helicase loaders that load DnaC onto DNA (36) or alternatively as a membrane

attachment protein to regulate initiation of DNA replication by regulating the recruitment of DnaD from the cytoplasm to the membrane (13, 30, 33). The latter suggestion is consistent with the localization of GFP-DnaD and GFP-DnaB fusions proximal to the cell membrane (22) suggesting that the two proteins are likely to interact at some stage during primosomal assembly. A mutation in *dnaB* (DnaBS371P) suppresses the temperature sensitive phenotype caused by the *dnaD23ts* mutation and while DnaB does not interact with DnaD, the resulting DnaBS371P mutant interacts directly with DnaD (30). These data indicate that there is a genetic link between the two proteins and that DnaB can adopt a conformation (induced by the S371P mutation) that interacts directly with DnaD. Indeed, a DNA-dependent interaction between the two proteins has been detected (20) but it has not been established whether this direct interaction alters the mode of DnaD binding to DNA. Both proteins can bind simultaneously to a supercoiled plasmid resulting in opposing remodelling effects (40) and in this paper we have established that although DnaB opposes DnaD-mediated remodelling it does not inhibit the untwisting activity of DnaD. On the contrary it slightly enhances untwisting.

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FIGURES

Figure 1

DnaD stimulates the topo I activity.

A. The effect of increasing concentrations of DnaD on the activity of topo I.

pBluescript (18 nM) was incubated with increasing concentrations of DnaD (0.5, 1, 8, 16 and 32 μM; lanes 4-8, respectively) and then treated sequentially with topo I (4 units) and PK prior to electrophoresis. Controls are shown in lanes 1-3, as indicated. Relaxed, linear and negatively supercoiled plasmid is indicated by R, L and SC(-), respectively. Increased relaxation of the SC(-) plasmid is observed up to 8 μM DnaD (lanes 4-6), manifested by the increasing appearance of R. At higher concentrations of DnaD, topo I is inhibited as manifested by the appearance of supercoils with higher electrophoretic mobility in lanes 7 and 8.

B. The left panel shows that the initial stimulation of the topo I relaxation activity is evident at 1 and 10 μM DnaD (lanes 6 and 7, respectively) whereas at 0.1 and 0.01 μM DnaD there was no observable effect (lanes 4 and 5, respectively). Controls are shown in lanes 1-3, as indicated. The right panel compares the relaxation of pBluescript by increasing concentrations of topo I (4, 8 and 16 units) in the presence and absence of 10 μM DnaD, as indicated. 16 units of topo I was required to relax pBluescript with approximately the same efficiency as 4 units in the presence of 10 μM DnaD (compare lanes 4 and 7). Controls are shown in lanes 1-3, as indicated.

C. 2D gel electrophoresis verifying that DnaD untwists duplex DNA.

The gels from left to right show 2D gels of the products from the reactions in lanes 3, 4, 5, 7 and 8 of panel **A**, respectively. The positions of the R, L and SC(-) plasmids are

indicated. Topoisomers with progressively increasing negative Lk are apparent from top to bottom. Only topoisomers with Lk values from 0 to -9 are indicated for clarity.

Figure 2

DnaB does not alter the supehelical properties of DNA.

A. The effect of increasing concentrations of DnaB on the activity of topo I.

pBluescript (18 nM) was incubated with increasing concentrations of DnaB (4, 8, 16 and 26.7 μM; lanes 4-7, respectively) and then treated with topo I (4 units). The reaction was terminated and the mixture was treated with PK prior to electrophoresis. Controls are shown in lanes 1-3, as indicated. Relaxed, linear and negatively supercoiled plasmid is indicated by R, L and SC(-), respectively. Marginal non-specific stimulation is barely visible at 4 μM DnaB (lane 4) and inhibition is observed at 8, 16 and 26.7 μM DnaB (lanes 5, 6 and 7). Overall there is no significant effect on the topo I activity.

B. 2D gel electrophoresis verifying that DnaB does not untwist duplex DNA.

2D gels showing reactions in the absence (left) and presence of 4 and 8 μM DnaB (middle and right, respectively), equivalent to the products of the reactions in lanes 3, 4 and 5 from panel **A**. The positions of the R, L and SC(-) plasmids are indicated together with the position of the Lk=0 topoisomer. Topoisomers with progressively increasing negative Lk are apparent from top to bottom.

Figure 3

The Cd of DnaD cannot untwist duplex DNA.

A. The effect of increasing concentrations of Cd on the activity of topo I.

pBluescript (18 nM) was incubated with increasing concentrations of Cd (0.5, 1, 8, 16 and 64 μM; lanes 4-8, respectively) and then treated sequentially with topo I (4 units) and PK prior to electrophoresis. Controls are shown in lanes 1-3, as indicated. No effect on topo I activity was observed at 0.5 and 1 μM Cd (compare lanes 3, 4 and 5) but a slight stimulation of relaxation was observed at 16 μM Cd (compare lanes 3 and 6). At higher Cd concentrations (16 and 64 μM) inhibition of relaxation was observed.

B. 2D gel electrophoresis verifying that Cd inhibits the topo I at high concentration.

2D gels showing reactions in the absence (left) and presence of 8 and 64 μM Cd (middle and right), equivalent to the products of the reactions in lanes 3, 6 and 8 from panel **A**. The positions of the R, L and SC(-) plasmids are indicated together with the position of the Lk=0 topoisomer. Topoisomers with progressively increasing negative Lk are apparent from top to bottom.

Figure 4

DnaB inhibits untwisting by DnaD.

A. The effect of DnaB (8 μM) on the twisting activity of increasing amounts of DnaD (0.5-33 μM), as indicated. Appropriate mixtures of proteins were incubated with pBluescript (18 nM), sequentially treated with topo I and PK before electrophoresis. The positions of fully relaxed, linear and supercoiled plasmids are indicated by R, L and SC, respectively. Increasing amounts of DnaD and treatment with topo I resulted in gradual positive increases of Lk by duplex untwisting, manifested as a gradual relaxation of the negatively supercoiled plasmid (lanes 4, 6 and 8) followed by steric inhibition of topo I activity at high concentrations of DnaD (lanes 10 and 12). Under identical conditions but

in the presence of 8 μM DnaB, the untwisting activity of DnaD was totally inhibited, as manifested by the presence of mainly supercoiled plasmid throughout (lanes 5, 7, 9, 11 and 13). Controls are shown in lanes 1-3, as indicated.

B. The same experiment as the one shown in panel **A** was carried out with 4 μM DnaB at 0.5, 1 and 9.7 μM DnaD, as indicated. Increasing the concentrations of DnaD (0.5-1 μM) in the presence of 4 μM DnaB resulted in a slight enhancement of topo I relaxation the presence of equivalent concentrations of DnaD alone (lanes 3-6).

Figure 5

A schematic diagram illustrating conversion of DNA from plectonemic to paranemic by DnaD-concentration dependent untwisting. DNA remodeling by DnaD is the sum of separate DNA-independent and DNA-dependent oligomerisation activities residing on its Nd and Cd domains, respectively, plus a DNA-binding activity on the Cd domain (15). Based upon a DNA-remodelling model proposed before (15), we suggest that initial binding of DnaD to DNA via its Cd domain causes only minor untwisting of the duplex but as the scaffold forms at higher concentrations, via oligomerisation interactions mediated by the Nd domain, gradual untwisting of the duplex becomes significant and the duplex is eventually converted to a paranemic form. Only three helical turns are shown for simplicity and although untwisting is depicted to open up individual helical turns, it is likely that it will be distributed throughout all the helical turns in the circular plasmid molecule as the DnaD scaffold grows.

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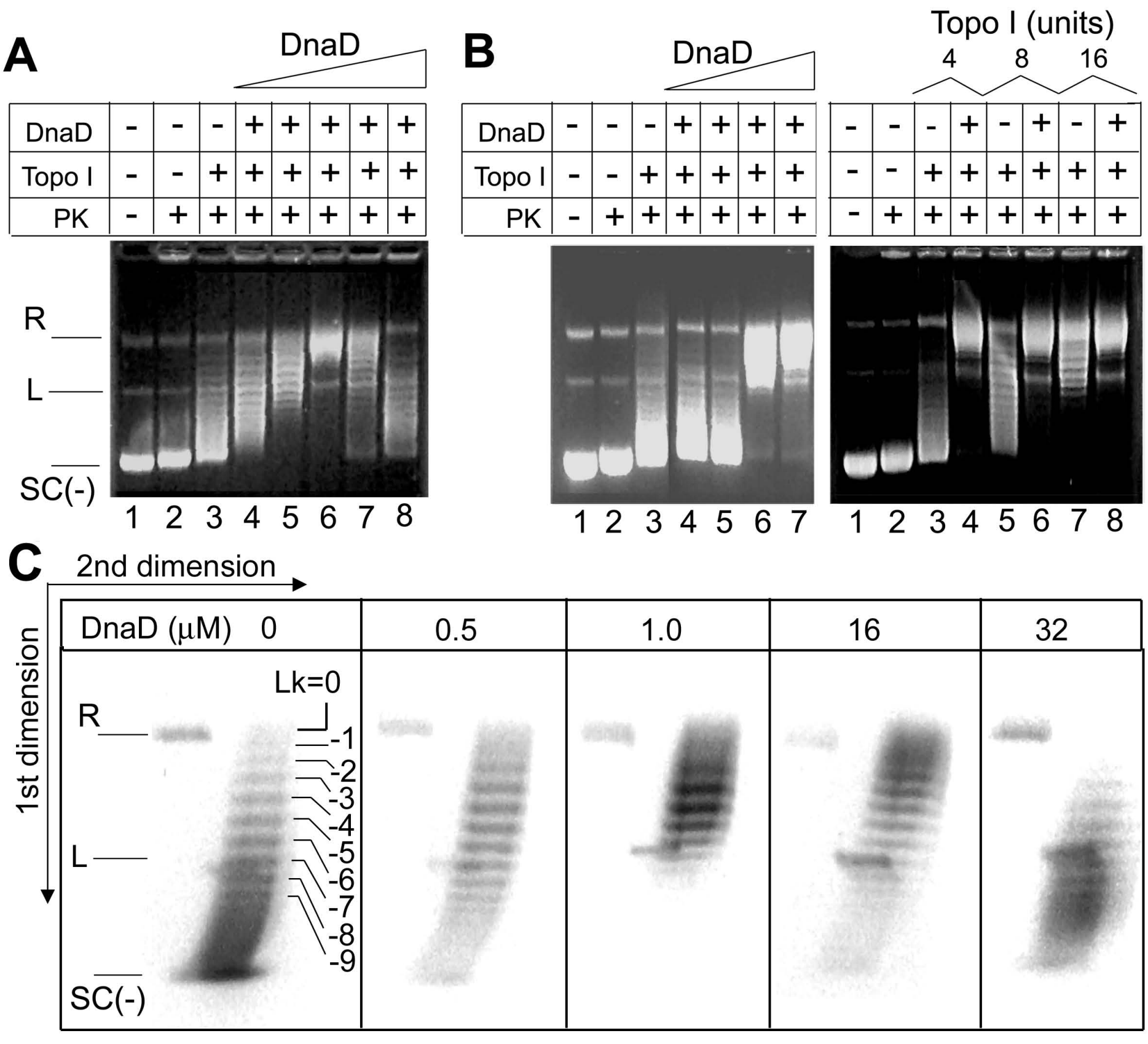
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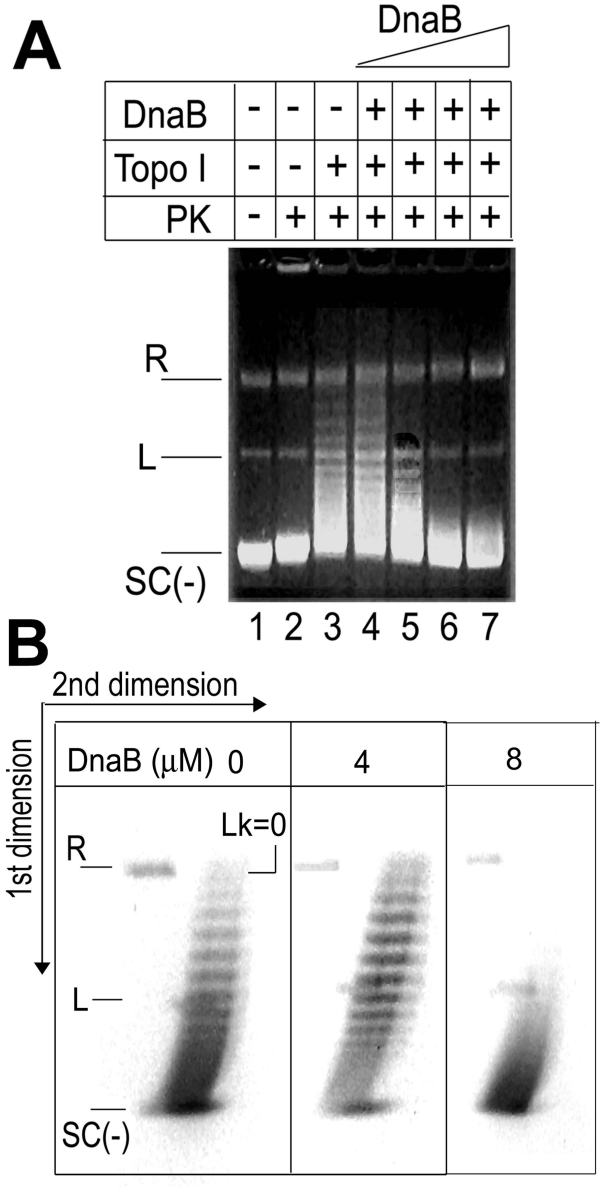
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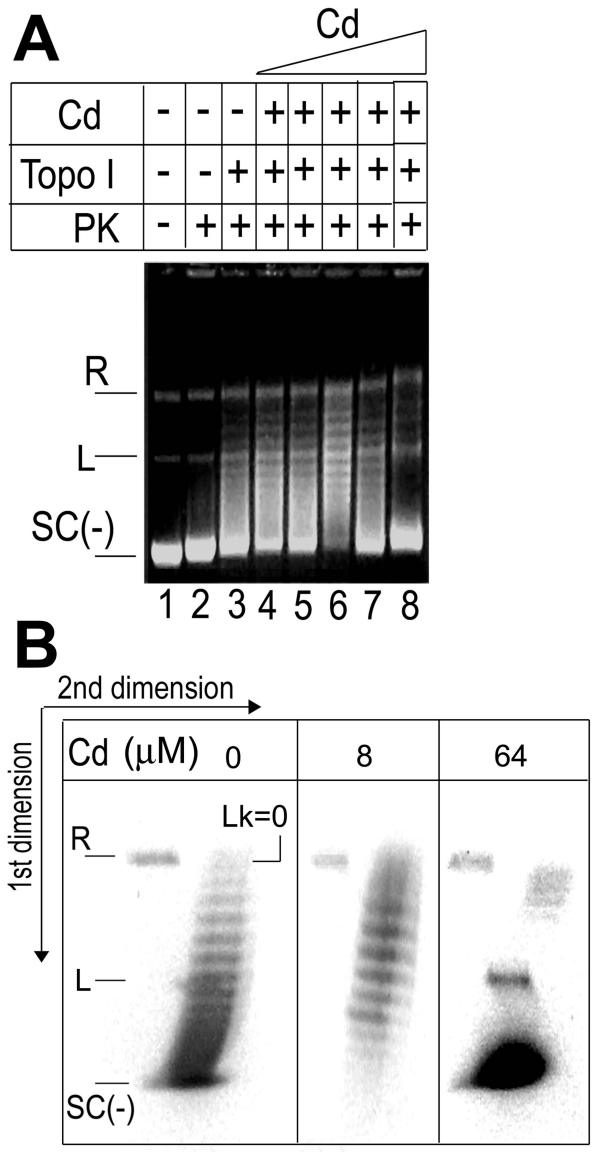
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A

DnaD (μM)

0.5 1.0 9.7 16 33

DnaD	-	-	-	+	+	+	+	+	+	+	+	+
DnaB	-	-	-	-	+	-	+	-	+	-	+	-	+
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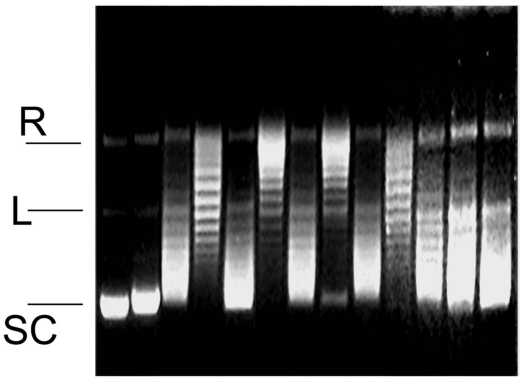


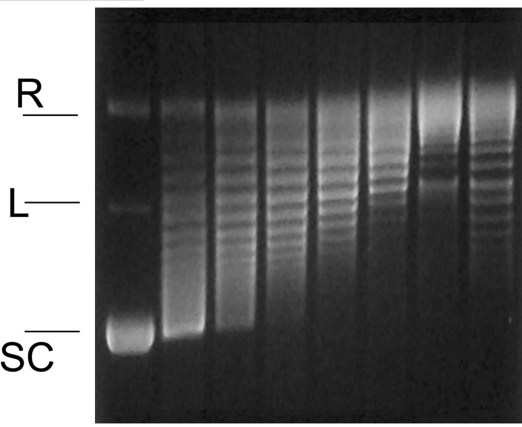
1 2 3 4 5 6 7 8 9 10 11 12 13

B

DnaD (μM)

0.5 1.0 8

DnaD	-	-	+	+	+	+	+	+
DnaB	-	-	-	+	-	+	-	+
Topo I	-	+	+	+	+	+	+	+
PK	+	+	+	+	+	+	+	+



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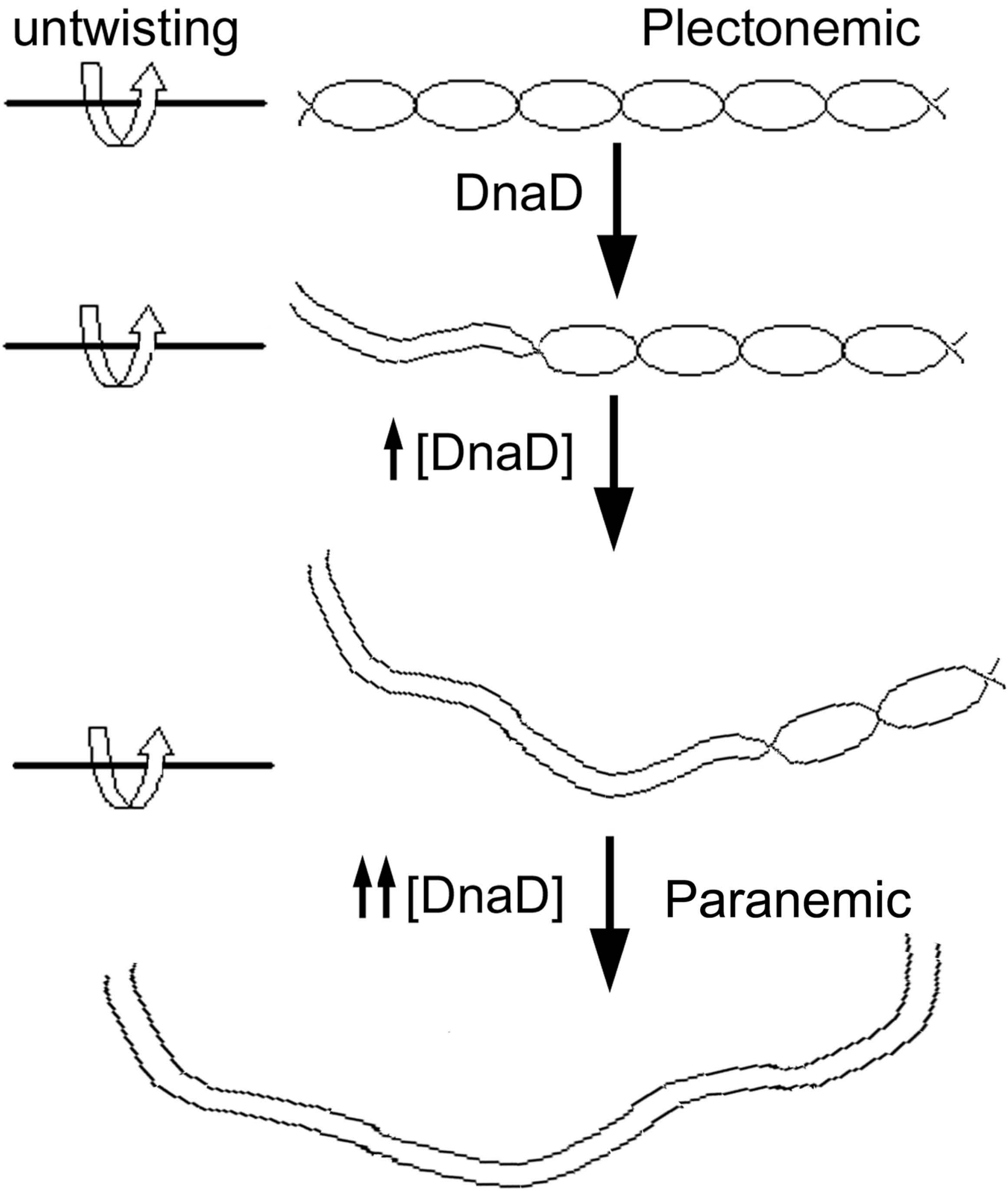
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