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Type II DNA Topoisomerases: Enzymes That Can Unknot a Topologically Knotted DNA Molecule via a Reversible Double-Strand Break

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Summary

The T4 DNA topoisomerase is a recently discovered multisubunit protein that appears to have an essential role in the initiation of T4 bacteriophage DNA replication. Treatment of double-stranded circular DNA with large amounts of this topoisomerase in the absence of ATP yields new DNA species which are knotted topological isomers of the double-stranded DNA circle. These knotted DNA circles, whether covalently closed or nicked, are converted to unknotted circles by treatment with trace amounts of the T4 topoisomerase in the presence of ATP. Very similar ATP-dependent enzyme activities capable of unknotting DNA are present in extracts of Drosophila eggs, Xenopus laevis eggs and mammalian tissue culture cells. The procaryotic enzyme, DNA gyrase, is also capable of unknotting DNA. We propose that these unknotting enzymes constitute a new general class of DNA topoisomerases (type II DNA topoisomerases). These enzymes must act via mechanisms that involve the concerted cleavage and rejoining of two opposite DNA strands, such that the DNA double helix is transiently broken. The passage of a second double-stranded DNA segment through this reversible double-strand break results in a variety of DNA topoisomerization reactions, including relaxation:supercoiling; knotting:unknotting and catenation:decatenation. In support of this type of mechanism, we demonstrate that the T4 DNA topoisomerase changes the linking number of a covalently closed double-stranded circular DNA molecule only by multiples of two. We discuss the possible roles of such enzymes in a variety of biological functions, along with their probable molecular mechanisms.

Introduction

DNA toposiomerases are enzymes that catalyze the concerted breaking and rejoining of DNA backbone bonds (Wang and Liu, 1979); these reactions can interconvert the different topological isomers of circular DNA (Wang, 1971; Liu, Depew and Wang, 1976; Champoux, 1977; Kirkegaard and Wang, 1978). A variety of different DNA topoisomerases have been isolated from procaryotic and eucaryotic organisms (for review see Wang and Liu, 1979). Examples of such DNA topoisomerases are the E. coli ω protein (Eco DNA topoisomerase I) (Wang, 1971), eucaryotic nicking-closing enzymes (Champoux and Dulbecco, 1972; Baase and Wang, 1974; Keller and Wendel, 1975), procaryotic DNA gyrases (procaryotic DNA topoisomerase II) (Gellert et al., 1976a; Liu and Wang, 1978a; Peebles et al., 1979) and the *int* gene product of bacteriophage lambda (Kikuchi and Nash, 1979). It has been suggested that DNA topoisomerases participate in a variety of biological processes involving DNA metabolism. These processes include DNA replication (Wang, 1971; Champoux and Dulbecco, 1972; Gellert et al., 1976b; Sumida-Yasumoto and Hurwitz, 1977), RNA transcription (Wang, 1973; Smith, Kubo and Imamoto, 1978), genetic recombination (Champoux, 1977; Kirkegaard and Wang, 1978; Kikuchi and Nash, 1979), chromosome condensation and decondensation (Baase and Wang, 1974; Bauer et al., 1977), nucleosome assembly (Germond et al., 1979), virus encapsidation (Bauer et al., 1977) and DNA transposition (Shapiro, 1979).

A DNA topoisomerase from bacteriophage T4-infected E. coli cells has recently been isolated and characterized (Liu, Liu and Alberts, 1979; Stetler, King and Huang, 1979). The T4 DNA topoisomerase catalyzes the relaxation of superhelical DNAs (whether positively or negatively coiled) in a reaction requiring ATP hydrolysis. The purified enzyme contains multiple subunits, which are apparently coded for by T4 genes 39, 52 and 60. Genetic and biochemical studies of mutants in these genes indicate that the T4 DNA topoisomerase may be involved in the initiation of DNA replication forks (reviewed by Liu et al., 1979). Interestingly, some host reaction involving E. coli DNA gyrase can substitute partially for the T4 DNA topoisomerase function in vivo (McCarthy, 1979). For this and other reasons, we have tentatively proposed that the T4 DNA topoisomerase is an origin-specific DNA gyrase which catalyzes the ATP-dependent negative supercoiling of DNA at T4 replication origins (Liu et al., 1979).

In this communication, we first provide detailed evidence for our previous report (Liu et al., 1979) that the T4 DNA topoisomerase acts by catalyzing the concerted breaking and rejoining of both strands of the DNA double helix simultaneously. We then demonstrate that enzymes capable of completely breaking the double helix in a reversible way are widespread throughout nature, and propose that these enzymes be denoted as "type II DNA topoisomerases."

Results

Formation of a New DNA Species by T4 DNA Topoisomerase Treatment

The T4 DNA topoisomerase has been shown to require ATP in order to relax superhelical DNA circles (Liu et al., 1979; Stetler et al., 1979). In the absence of ATP, a very low level of relaxation activity can be detected

when an equal weight of the T4 DNA topoisomerase is incubated with superhelical DNAs. The DNA molecules produced in this case, however, include some new DNA species quite different from the expected partially relaxed, covalently closed DNA product. For example, in Figure 1, the time course of such a reaction with pBR322 DNA in the absence of ATP has been monitored by electrophoresis of the DNA products through an agarose gel; unexpectedly, some of the covalently closed (form I) DNA substrate in lane A is converted initially into DNA molecules that migrate slightly faster than the form I DNA in the agarose gel (lanes B, C and D). This means that these DNA molecules must be either smaller or more compact than the original supercoiled DNA circle used as substrate. Although this unusually fast-migrating DNA can subsequently be converted into a group of slower moving DNA bands by the relaxation of supercoils (lane E and F; see below), even these contain minor species that migrate at a rate different from any of the DNA bands present in a partially relaxed pBR322 DNA sample run in parallel as a control (lane G).

The electrophoretic mobilities of the fast-migrating new DNA species generated by the T4 DNA topoisomerase treatment (Figure 1, lanes B, C and D; more

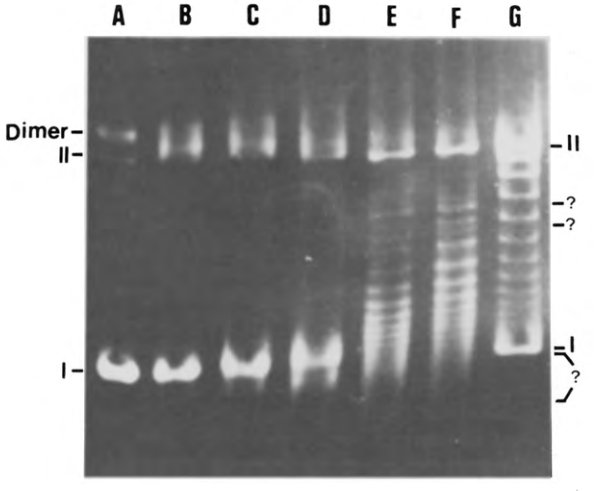

Figure 1. Electrophoretic Analysis of the Formation of New DNA Species by Treatment of Superhelical DNA Circles with T4 DNA Topoisomerase in the Absence of ATP

A reaction mixture (175 μl) containing 50 mM Tris–HCl (pH 7.4), 45 mM KCl, 7.5 mM $MgCl_2$, 0.4 mM dithiothreitol, 0.5 mM Na_3EDTA, 25 μg/ml human serum albumin, 16 μg/ml superhelical (form I) pBR322 DNA (a 4362 bp, covalently closed, double-stranded DNA circle) and 22 μg/ml T4 DNA topoisomerase was incubated at 30°C. Aliquots of 20 μl were withdrawn at various times and each was stopped by addition of 5 μl of 5% sodium dodecylsulfate (SDS), 50% glycerol plus 1 mg/ml bromophenol blue, followed by electrophoresis through a 0.8% agarose gel in MgTBE buffer as described previously (Liu et al., 1979). (A) Zero time; (B) 50 sec; (C) 1.5 min; (D) 5 min; (E) 15 min; (F) 30 min; (G) partially relaxed normal form I pBR322 DNA control. After staining with ethidium bromide, DNA was visualized by photographing the wet gel fluorescence under ultraviolet illumination (Liu et al., 1979). Note that this particular gel did not run evenly; the form I DNA migrated faster on the left than on the right.

clearly visible in Figure 8A, lane B) are unaffected by phenol extraction, 1% SDS treatment, or a combined 30 min proteinase K (100 μg/ml) and 1% SDS treatment at 60°C (data not shown). Moreover, analytical ultracentrifugation revealed no difference between the buoyant densities of the topoisomerase product DNAs and the original DNA substrate (±0.001 g cm^{-3}). It therefore seemed highly improbable that residual protein binding was involved in creating any of the new fast-migrating DNA species shown in Figure 1. In addition, the unusual migration rates observed for these new DNA species are unchanged when the DNA is annealed at temperatures just below the Tm. Thus the apparent conformational change that has occurred must involve more than just hydrogen bonding rearrangements.

Several other possibilities could explain the peculiar mobilities of the new DNA species:

—the superhelical DNA molecules could have become intramolecularly cross-linked due to an unknown type of covalent linkage across each DNA circle;

—the superhelical DNA molecules might have become intramolecularly base-paired, which would then allow partially homologous paired strands to become intertwined topologically via a type of topoisomerase reaction known to be carried out by both the E. coli ω protein (Kirkegaard and Wang, 1978) and the rat liver nicking-closing enzyme (Champoux, 1977);

—the superhelical DNA could have become topologically knotted, for example, into a form resembling a pretzel).

Each of these three possibilities could result in a further compaction of superhelical DNA circles and thus explain the increased mobility of this DNA observed by agarose gel electrophoresis. To help distinguish among them, the early topoisomerase product DNA containing the fast-migrating DNA species (Figure 2A) was cut with several different restriction endonucleases. If these DNA molecules were either intramolecularly cross-linked (first possibility, above) or intertwined topologically through a partial DNA sequence homology (second possibility, above), restriction nuclease treatment should generate linear DNA fragments which migrate differently upon agarose gel electrophoresis than the same restriction fragments derived from the original DNA substrate. When Eco RI restriction nuclease was used to digest the new topoisomerase-generated DNA species, however, all of the DNA product migrated in an agarose gel with exactly the same mobility as the unit-length linear DNA molecules produced by digestion of the control DNA (compare Figures 2C and 2D). Consequently, this information rules out the first two possibilities mentioned above. Furthermore, Hinc II restriction endonuclease treatment produced comparable yields of the same two DNA fragments from both topoisomerase-treated and control DNA samples (compare Fig-

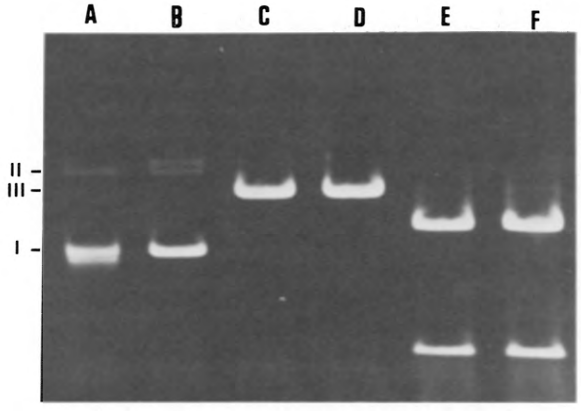

Figure 2. Restriction Enzyme Analysis of the New DNA Species Produced by T4 DNA Topoisomerase Treatment

Knotted, covalently closed pBR322 DNA was prepared as described in Experimental Procedures. Eco RI and Hinc II restriction enzyme digestions were carried out on separate aliquots of both DNAs. For each reaction (25 μl volume and 0.25 μg DNA), either 5 units of Eco RI or 1 unit of Hinc II was added, as indicated. After 30 min at 37°C, each reaction was stopped by addition of 6 μl 5% SDS, 50% glycerol plus 1 mg/ml bromophenol blue and electrophoresed through a 0.8% agarose gel (Liu et al., 1979). (A) Uncut knotted covalently closed pBR322 DNA; (B) uncut normal superhelical form I pBR322 DNA; (C) knotted pBR322 DNA digested with Eco RI; (D) normal pBR322 DNA digested with Eco RI; (E) knotted pBR322 DNA digested with Hinc II; (F) normal pBR322 DNA digested with Hinc II.

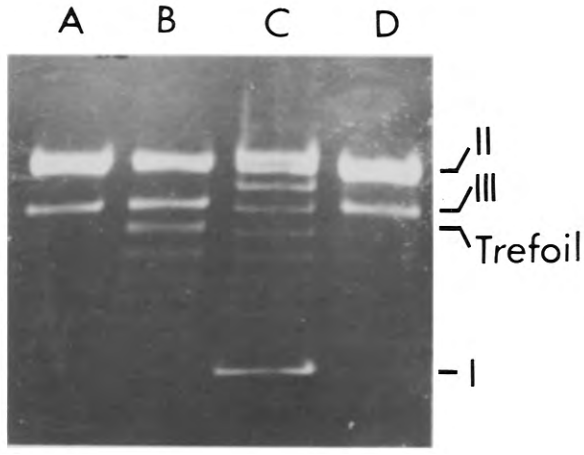

Figure 3. Electrophoretic Analysis of the Properties of the Fast-Migrating, Topoisomerase-Produced DNA Product: Response to DNAase I Nicking and to Treatment with the Topoisomerase in the Presence of ATP

DNA samples were loaded onto an agarose slab gel and electrophoresed as described in the legend to Figure 1. The positions corresponding to form I (superhelical covalently closed circular form), form II (nicked circular form) and form III (unit length linear) DNAs are indicated on the gel margin. The proposed position to which a nicked trefoil DNA migrates (the simplest knotted DNA and the third band from the top in lane B) is also marked.

Lane (A) shows a nicked pBR322 DNA sample prepared by a limited pancreatic DNAase I treatment of pBR322 form I DNA. The sample in lane (B) was prepared by an identical DNAase I treatment of topoisomerase-treated pBR322 DNA (as described for preparation of nicked, knotted pBR322 DNA in Experimental Procedures). For comparison with the sample in (B), lane (C) shows the migration of partially relaxed form I pBR322 DNA, prepared by treatment of normal superhelical pBR322 DNA with a small amount of T4 DNA topoisomerase in the presence of ATP. Lane (D) is the DNA obtained by treating the sample in lane (B) with the T4 DNA topoisomerase in the presence of ATP, as follows: a reaction mixture (20 μl) containing 50 mM Tris–HCl (pH 7.4), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_3EDTA, 16 μg/ml of the "nicked, knotted pBR322 DNA" in lane (B) and 0.3 μg/ml T4 DNA topoisomerase was incubated at 30°C for 30 min and then stopped by addition of 5 μl of 5% SDS, 50% glycerol plus 1 mg/ml bromophenol blue. Note that the number of molecules of T4 DNA topoisomerase used in the unknotting reaction in (D) was smaller than the number of DNA molecules present; similar experiments have shown that an amount of enzyme sufficient to unknot roughly half of the DNA in such samples is able to relax about half of the superhelical turns of a form I DNA (data not shown).

ures 2E and 2F). This result shows that there are no major DNA sequence rearrangements present in the fast-migrating superhelical DNAs produced by the topoisomerase, consistent with the possibility that the new DNA species are topologically knotted.

To characterize the fast-migrating DNA species produced by the topoisomerase further, the molecules were treated with DNAase I for increasing times to introduce single-chain scissions into each one. As expected, in a control reaction, superhelical pBR322 DNA (form I) was immediately converted to a simple nicked circular DNA (form II) and, with further nicking, to some linear DNA molecules (form III) by the DNAase I treatment (Figure 3A and data not shown). In contrast, the new fast-migrating DNA species produced by the topoisomerase (representing about 40% of all of the molecules present in this sample) were immediately converted into a group of DNA bands migrating between form III and form I DNAs (Figure 3B), and the mobility of these bands did not change upon further DNAase I treatment until converted directly into form III linear DNA by a prolonged incubation (data not shown; but see Figures 7C and 7E). Upon careful inspection, the nicked DNA bands generated from the topoisomerase-treated sample can be seen to migrate in an agarose gel differently from the bands in a partially relaxed, covalently closed pBR322 DNA control (compare Figure 3C with Figure 3B). While each sample forms a similarly spaced series of bands, the two different groups of bands are not in phase. Moreover, even the most slowly migrating of the circular DNA bands in the nicked topoisomerase-treated sample (the third band from the top in Figure 3B, labeled "trefoil") moves more quickly (and thus is more compact) that the DNA molecules that form the slowest three covalently closed bands in the partially relaxed sample (Figure 3C). As documented below, these unusually fast-migrating DNA molecules which survive DNAase I treatment are the nicked, topologically knotted DNA circles predicted by the third possibility mentioned above.

The New DNA Species Are Topologically Knotted, Double-Stranded Circular DNAs

To determine the structure of the new DNA species, a DNA sample identical to that analyzed in Figure 3B

was examined with the electron microscope. Some DNA structures typical of those observed are shown in Figure 4. Figure 4B shows a DNA structure resembling a trefoil, which is the simplest possible knot. This species would be expected to have a conformation about as compact as that of an unknotted DNA circle with three supercoils, and we believe that it corresponds to the slowest migrating of the bands of nicked, topoisomerase-treated DNA circles (labeled "trefoil" in Figure 3B). Figures 4C and 4D show topoisomerase-treated DNA structures which could represent knots of successively higher complexities; we believed that these correspond to some of the faster migrating DNA bands in Figure 3B.

To quantitate the electron microscopy results, we compared the number of crossovers in topoisomerase-treated and untreated pBR322 DNA circles, both of which had been DNAase I-nicked to remove their supercoiling (Table 1). For the untreated control DNA, 80% of the molecules scored had fewer than two crossovers and thus could not be topologically knotted (the simplest trefoil knot should exhibit a minimum of three crossovers when projected onto a plane). In contrast, for the topoisomerase-treated DNA sample, only 36% of the molecules were found to exhibit fewer than two crossovers, while DNA molecules with more than four crossovers represented 40% of those scored (compared with only 10% in the control sample). The crossovers visible in the control sample are believed to arise from a fortuitous overlapping of DNA strands which occurs when unknotted circular molecules are flattened onto a plane. Considering our other data as well, we conclude that the excess crossovers in the topoisomerase-treated sample are due to a reaction which introduces topological knots into about half of the pBR322 DNA molecules present.

Efficient Unknotting of the Knotted Double-Stranded DNA Circles by the T4 DNA Topoisomerase

When knotted superhelical pBR322 DNA is treated with small amounts of the T4 DNA topoisomerase in the presence of ATP, the knotted superhelical pPB322 DNA is unknotted rapidly, with both relaxation and unknotting proceeding simultaneously. To study this unknotting reaction separately from the relaxation reaction, the knotted superhelical pBR322 DNA was converted into the nicked, knotted form by DNAase I treatment. This nicked, knotted pBR322 DNA (Figure 3B) was then treated with a trace amount of the T4 DNA topoisomerase in the presence of ATP. Electrophoretic analysis showed that the group of bands in Figure 3B was quantitatively converted into normal form II DNA (Figure 3D). This conversion of the nicked, knotted DNA to normal nicked DNA circles was confirmed by electron microscopy. Most importantly, the rate of such an unknotting reaction was found to be comparable to the rate at which a superhelical DNA is relaxed (see legend to Figure 3). The fact that the T4 DNA topoisomerase catalyzes the unknotting of either covalently closed or nicked, knotted DNA about as efficiently as it relaxes the superhelical turns in a closed circular DNA molecule (providing that ATP is present) suggests that the same reaction that unknots a DNA circle removes its superhelical turns.

How Unknotting and Relaxation Reactions Can Occur via the Same Reversible Double-Chain Cleavage of the DNA Helix

Topologically, the knotting or unknotting of a double-stranded circular DNA requires that each of the two DNA strands in the DNA helix be interrupted at least once. Although from a purely topological point of view

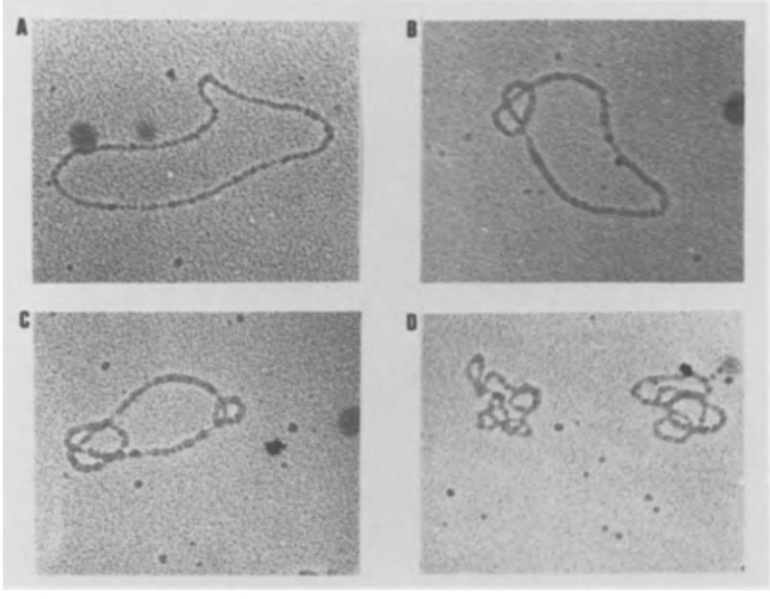

Figure 4. Sample Electron Micrographs of the pBR322 DNA Molecules Observed after T4 DNA Topoisomerase Treatment in the Absence of ATP

Knotted, covalently closed pBR322 DNA was prepared and then nicked with pancreatic DNAase I as described in Experimental Procedures. Electron microscopic analysis of this nicked, knotted pBR322 DNA gave the results presented in Table 1; this figure shows typical molecules with (A) no crossover points; (B) three crossover points; (C) six crossover points; (D) higher topological complexities.

Table 1. Electron Microscopic Determination of the Topology of a Circular, Double-Stranded DNA before and after Treatment with T4 DNA Topoisomerase in the Absence of ATP

Crossover Points	Untreated pBR322 DNA	T4 DNA Topoisomerase-Treated pBR322 DNA
<2	99 (80%)	102 (36.3%)
3	3 (2.4%)	27 (9.6%)
4	4 (3.2%)	22 (7.8%)
>4	12 (9.7%)	113 (40.2%)
Dimers	6 (4.8%)	19 (6.0%)
Total molecules scored	124	281

Nicked pBR322 DNA ("untreated DNA") and nicked, knotted pBR322 DNA ("T4 DNA topoisomerase-treated DNA") were prepared under identical conditions and then spread for electron microscopy (see Experimental Procedures). All circular DNA molecules detected were scored by counting the number of crossover points visible in each molecule (see Figure 4 for examples). Note that both DNA samples had been lightly nicked with pancreatic DNAase I (see Figures 3 and 4) to relax them and thereby allow better spreading on the grid.

these two strand interruptions need not occur at exactly the same base pair or at the same instant, it is conceptually simpler to consider that such DNA topoisomerization reactions occur via a simple reversible double-strand break in the DNA helix. This break enables two double-stranded DNA segments to pass through each other in an enzyme-mediated reaction, as illustrated schematically in Figure 5, after which the two broken DNA ends are immediately rejoined. The observed stoichiometry of about one ATP molecule hydrolyzed by the topoisomerase per strand passing event (Liu et al., 1979) suggests that the topoisomerase-induced, transient double-chain breaks are triggered only when a second double-stranded DNA segment is in a position where it can be forced directly through the break by the topoisomerase. Data presented below (Figure 6) reveal that the two DNA ends at such double-chain breaks are not free to diffuse, but instead remain bound to the topoisomerase at all times. (This proposal is also consistent with the fact that this topoisomerase neither acts as a nuclease on linear double-stranded DNAs nor causes rearrangement of a DNA sequence.)

In principle, the transient double-strand break introduced by T4 DNA topoisomerase can cause an interlocking or segregation of two interlocked DNA rings, if the two DNA segments that cross in Figure 5 are each in different DNA circles. If the two DNA segments that cross are in the same DNA circle, various intramolecular DNA topoisomerization reactions (relaxation and/or unknotting) will occur. Figures 5A and 5B show how both DNA unknotting (A) and DNA relaxation (B) can be achieved via such a reversible double-chain cleavage in the DNA. Note also that in the relaxation reaction in Figure 5B, a single passage of

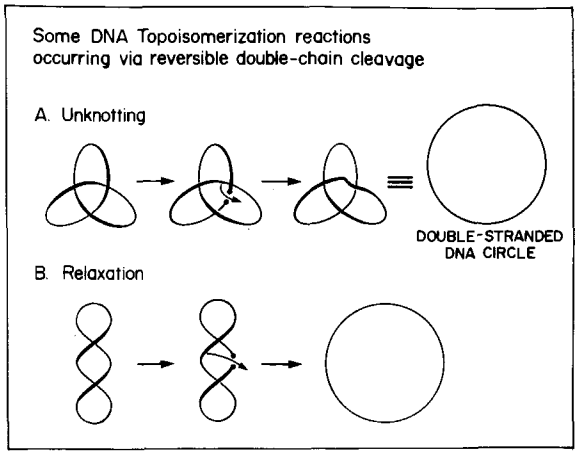

Figure 5. Models for Some DNA Topoisomerization Reactions Occurring via Reversible Double-Chain Cleavage

For type II DNA topoisomerases, both the unknotting of a double-stranded circular DNA (A) and the relaxation of a superhelical DNA (B) are proposed to occur by exactly the same mechanism. Both reactions are presumed to occur via an enzyme-induced transient double-chain break in the DNA. After the helix is broken on one DNA segment, the passage of a second DNA segment through this transient break must occur by some type of "protein-protein gating" mechanism, since the two broken DNA ends must be kept near each other by the protein at all times. After the passage of one DNA segment through the other, the protein conformation changes and the two DNA ends at the break rejoin. For clarity, the double-stranded circular DNA shown in the unknotting reaction (A) is presumed to be nicked (and therefore relaxed), while the double-stranded circular DNA shown in the relaxation reaction (B) is covalently closed. Careful inspection of the *reverse* reaction in (A) will show clearly how a knot forms: the two helices which pass through each other are at the top and bottom of a folded over "figure-eight" molecule, and are thus separated by a twist in the DNA circle.

a DNA segment through a reversible double-chain break has resulted in the simultaneous removal of *two* (rather than one) superhelical turns.

As Predicted, the T4 DNA Topoisomerase Changes the Linking Number of a Superhelical DNA Molecule Only by Multiples of Two

It has been demonstrated mathematically that if a closed ribbon is passed through itself, the value of the linking number jumps by ± 2, irrespective of the detailed passage mechanism (Fuller, 1978). [Restrictions concerning this statement are that those passage events that lead to topological knotting (for example, the reverse of the reaction in Figure 5A) are excluded and that the two ends at the transient break do not rotate relative to each other]. Thus, given a DNA molecule with a unique linking number, treatment with the type of DNA topoisomerase diagrammed in Figure 5 should change the linking number only by multiples of two.

To test this prediction, pBR322 DNA molecules with unique linking numbers were isolated by preparative gel electrophoresis (see Experimental Procedures) and then treated with the T4 DNA topoisomerase in

the presence of ATP. As shown in Figure 6, when a pBR322 DNA sample containing an equal mixture of species with even and odd linking numbers was used as substrate, the typical Gaussian distribution of pBR322 DNA topoisomers with linking numbers differing by one was generated (Figure 6E → 6F). However, when pBR322 DNA molecules with either a unique even or a unique odd linking number served as substrate, only the even or odd linking number species in the Gaussian distribution were obtained, respectively (Figure 6A → 6B, and Figure 6C → 6D). Furthermore, within each population, each species is present in the relative proportions expected at thermodynamic equilibrium (see the legend to Figure 6).

These observations provide strong independent confirmation of the model proposed for the unknotting and relaxation reactions in Figure 5. They also reveal that the two DNA ends at the transient DNA break illustrated in Figure 5 must be constrained rigidly, because even an occasional rotation by 360° between a breakage and rejoining event would change the linking number by one, and thus interconvert the odd and even linking number species.

Bacterial DNA Gyrases Also Break the DNA Double Helix Reversibly

We have proposed previously that the T4 DNA topoisomerase functions as a DNA gyrase at the T4 replication origin(s) (Liu et al., 1979). We therefore expected that bacterial DNA gyrases also might be able to catalyze the unknotting of knotted, covalently closed DNA circles. When nicked, knotted pBR322 DNA was used as the substrate, neither E. coli DNA gyrase nor M. luteus DNA gyrase was capable of changing the knotted DNA topology efficiently. As shown in Figure 7, however, these DNA gyrases can efficiently catalyze the conversion of a covalently closed, knotted DNA molecule to unknotted form I DNA (Figure 7A → 7B).

To demonstrate that the superhelical form I pBR322 DNA produced by the DNA gyrase reaction in Figure 7B is indeed in an unknotted topology, DNAase I was used to digest this reaction product. As expected, a slight DNAase I treatment converted this gyrase product first to the nicked form (form II) (Figure 7D) and then to a mixture of form II in a small amount of form III DNA (Figure 7F). As a control, the knotted molecules present in the original covalently closed pBR322 DNA used as substrate were converted by DNAase I to the distinct group of intermediately migrating DNA bands discussed previously (see Figure 3B) using the same treatment (Figure 7A produces 7C and 7E). We

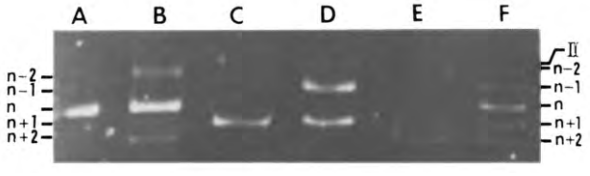

Figure 6. When It Catalyzes DNA Relaxation Reactions, the T4 DNA Topoisomerase Changes the DNA Linking Number by Multiples of Two

Covalently closed pBR322 DNA molecules with different linking numbers were electrophoretically purified from a fully relaxed pBR322 DNA sample on an agarose gel, as described in Experimental Procedures. Each group of DNA molecules was then treated for 30 min at 30°C with 100 units of the T4 DNA topoisomerase in a standard assay mixture with ATP present (Liu et al., 1979). The reactions were stopped by addition of 5 μl of 5% SDS, 50% glycerol plus 1 mg/ml bromophenol blue and electrophoresed on a 0.8% agarose gel in TBE buffer. Lanes (A), (C) and (E) are untreated controls, and contain only half the amount of DNA present in the T4 topoisomerase-treated samples analyzed in lanes (B), (D) and (F). With the most populated topoisomer in the Gaussian distribution of fully relaxed pBR322 DNA topoisomers assigned a linking number of n, the linking numbers of the gel-purified DNA topoisomers used as substrate in each reaction were n for lanes (A) and (B), $n + 1$ for lanes (C) and (D), and a mixture of $n + 1$ plus $n + 2$ for lanes (E) and (F). The Gaussian distribution of the various topoisomers shown in lane (F) is indistinguishable from that in a fully relaxed pBR322 DNA sample produced by treatment of pBR322 form I DNA with excess T4 DNA topoisomerase plus ATP under identical conditions (data not shown). Since excess T4 DNA topoisomerase was present in each reaction, the product pBR322 DNA molecules in (B) and (D) were relaxed to equilibrium and therefore can be seen to fit the same Gaussian distribution observed in a fully relaxed pBR322 DNA sample.

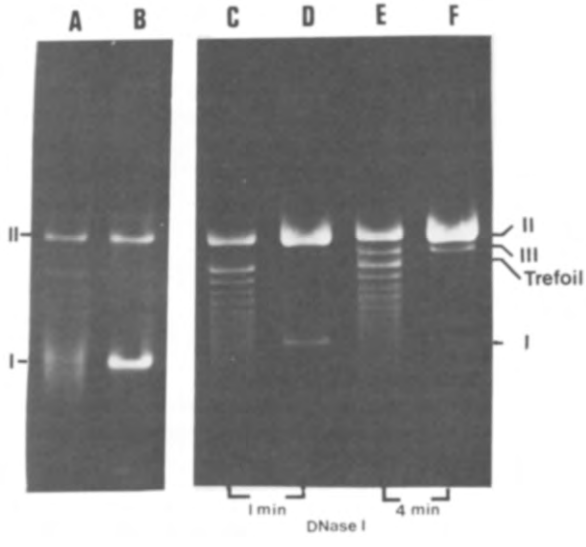

Figure 7. Electrophoretic Analysis of the Unknotting of Knotted, Covalently Closed pBR322 DNA by Bacterial DNA Gyrases

The knotted, covalently closed pBR322 DNA shown in lane (A) was prepared as described in Experimental Procedures. The gyrase reaction was carried out to produce the product in lane (B), using conditions described in the legend to Figure 3, except that knotted, covalently closed DNA (16 μg/ml) and 5 units of M. luteus DNA gyrase served as substrate and enzyme, respectively. (E. coli DNA gyrase gave the same results; data not shown). For analysis, a 50 μl aliquot of the gyrase reaction product in lane (B) was nicked by treatment with 27 ng/ml pancreatic DNAase I at 30°C. Aliquots (25 μl) were withdrawn after 1 min (lane D) and 4 min (lane F). As a control, the knotted, covalently closed pBR322 DNA substrate (lane A) was treated identically with DNAase I for 1 min (lane C) and 4 min (lane E). Note that only after DNA gyrase treatment can the knotted DNA sample be converted to form II DNA by a limited DNAase I nicking; this constitutes proof that it has been unknotted by gyrase.

therefore conclude that bacterial DNA gyrase resembles the T4 DNA topoisomerase, insofar as both enzymes act by making reversible double-strand breaks in the DNA double helix, through which a second DNA helix can pass.

Unknotting of Knotted pBR322 DNA by Other DNA Topoisomerases

Since the superhelicity observed in isolated eucaryotic DNA can be fully accounted for by the changes in linking number created by the wrapping of DNA around nucleosomes (Germond et al., 1975), there is no need for a general eucaryotic DNA gyrase of the bacterial type to maintain the superhelicity of eucaryotic DNA. On the other hand, initiation of eucaryotic DNA replication might well require a DNA gyrase to open up a replication bubble. Since replication bubbles in eucaryotes seem to resemble those created during T4 bacteriophage DNA replication (Huberman and Riggs, 1968; Delius, Howe and Kozinski, 1971), we used an assay for the unknotting of knotted DNA circles to test for a eucaryotic enzyme of the T4 DNA topoisomerase type in 1 hr embryos of Drosophila melanogaster, where the rate of replication bubble initiation is unusually high (Kreigstein and Hogness, 1974).

As shown in Figure 8A, we have been able to detect an ATP-dependent unknotting activity in crude extracts made from such early Drosophila embryos. When the knotted, covalently closed pBR322 DNA in lane B was incubated with the extract, all the DNA molecules present were eventually converted to the group of slower migrating DNA bands in lane C. These bands are characteristic of partially relaxed pBR322 DNA molecules without topological knots. The product DNA molecules in lane C were shown more directly to have lost their topological knots by the DNAase I sensitivity assay used previously (Figure 7) (data not shown).

We learned subsequently that an ATP-dependent enzyme activity that could catalyze the relaxation of superhelical DNA circles and decatenation of interlocked DNA rings had already been partially purified from Drosophila embryos by T. Hsieh and D. Brutlag at Stanford University (personal communication). The experiment shown in Figure 8B demonstrates that their purified Drosophila enzyme is also capable of unknotting a knotted pBR322 DNA molecule, and that this unknotting reaction occurs at a rate comparable to the rate of its relaxation of DNA supercoils. Like the T4 DNA topoisomerase, their Drosophila enzyme can unknot both nicked and covalently closed, knotted molecules, and the unknotting is ATP-dependent (data not shown).

Using the same assay, we have recently been able to demonstrate the existence of similar ATP-dependent unknotting activities in extracts of Xenopus laevis eggs and in extracts of nuclei isolated from a

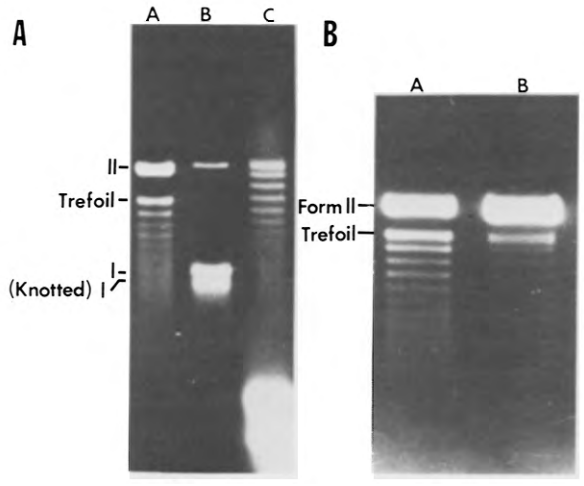

Figure 8. Electrophoretic Test for the Unknotting of Knotted pBR322 DNA by an ATP-Dependent DNA Topoisomerase from D. melanogaster

(A) Reaction of crude extract enzyme. Nicked, knotted pBR322 DNA (lane A) and knotted, covalently closed pBR322 DNA (lane B) were electrophoresed as standards. To obtain the DNA product in lane (C), 20 μl of a reaction mixture containing 50 mM Tris–HCl (pH 7.4), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.5 mM Na_3EDTA, 20 μg/ml of knotted, covalently closed pBR322 DNA (see lane B) and 1 μl of Drosophila embryo extract (see Experimental Procedures) were incubated at 30°C for 30 min and then analyzed by gel electrophoresis as described in the legend to Figure 1. The unknotting activity of this extract is at least partly ATP-dependent. In the absence of ATP, the superhelicity in the knotted, covalently closed pBR322 DNA is relaxed by the extract (probably due to the nicking-closing enzyme of Drosophila), but the product DNA still contains a prominent DNA band at the trefoil DNA position, indicating that most of the topological knots have not been removed (data not shown).

(B) Reaction of purified enzyme. The nicked, knotted pBR322 DNA in lane A (prepared as described in Experimental Procedures) was treated with an ATP-dependent DNA topoisomerase purified from 1 hr Drosophila embryos (a gift from T. Hsieh and D. Brutlag) in a reaction mixture (20 μl) containing 50 mM Tris–HCl (pH 7.4), 200 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_3EDTA, 0.5 mM ATP, 30 μg/ml human serum albumin, 16 μg/ml of the DNA and 1 unit of the enzyme (arbitrarily defined as the amount of activity required to remove half of the superhelical turns in pBR322 under exactly the same conditions). After 30 min at 30°C, the reaction was stopped and the DNA was electrophoretically analyzed in lane (B), as described in the legend to Figure 2. Note that most but not all of the knots have been removed by this treatment.

Chinese hamster ovary (CHO) cell line (data not shown). It is therefore clear that enzymes of this type are widespread throughout nature.

Procaryotic ω proteins and eucaryotic nicking-closing enzymes are known to introduce reversible single-chain breaks, rather than double-chain breaks, into a DNA helix (Champoux, 1978; Wang and Liu, 1979). We would therefore expect that neither type of enzyme would be capable of catalyzing the unknotting of double-stranded DNA rings. Indeed, when purified E. coli ω protein (Eco DNA topoisomerase I) and a Drosophila nicking-closing enzyme preparation were tested in our assay, neither enzyme showed unknotting activity. The unknotting enzymes reported above thus constitute a new class of DNA topoisomerases

(proposed to be designated as type II DNA topoisomerases), whose mechanism is fundamentally different from that of both the procaryotic ω proteins and the eucaryotic nicking-closing enzymes (proposed to be designated as type I DNA topoisomerases).

Discussion

Mechanisms of DNA Knotting by T4 DNA Topoisomerase

Any random cyclization of a linear polymer can result in the formation of a knotted circle. The first such knotted polymer was reported by Liu et al. (1976), who demonstrated that knotted single-stranded DNA circles were formed as a result of treating a circular single-stranded DNA with E. coli ω protein (Eco DNA topoisomerase I). The distribution of the various knotted species was shown to reach an equilibrium in the presence of the enzyme. A statistical-mechanical treatment of such random cyclization of a polymer chain has been developed by Frank-Kamenetskii, Lukashin and Vologodskii (1975). Using computer simulation, the probability of knot formation during DNA cyclization has been shown to be a linear function of the number of DNA statistical segments (Kuhn segments). For example, random cyclization of bacteriophage lambda DNA (molecular weight 32×10^6 daltons, containing about 150 Kuhn segments) is predicted to produce 40% knotted molecules. Since most plasmid DNAs are much shorter than lambda DNA, random cyclization of a linearized plasmid DNA is not expected to yield a significant proportion of knotted molecules. Indeed, when linearized double-stranded φX174 DNA (molecular weight 3.6×10^6 daltons, containing about 17 Kuhn segments) was cyclized and ligated, no knotted DNA circles were detected (U. Hibner, personal communication).

A knotted DNA of average plasmid size (<10 kb) is thus at a higher free energy state than its unknotted form. How then does one explain the formation of knotted DNA when negatively superhelical DNA is treated with excess T4 DNA topoisomerase in the absence of ATP? A clue comes from the study of bacteriophage P2 DNA in the phage head. Using several tailless P2 phage mutants, it was shown that when P2 DNA cyclizes (via its sticky ends) inside the phage head, the formation of highly knotted P2 DNA results (unpublished results of J. C. Wang, L. F. Liu and R. Calendar). It seems intuitively reasonable that the restriction of the volume of a rigid chain, such as DNA, should be equivalent to reducing the length of its statistical segments (thus increasing the number of statistical segments per molecule). Such a restriction in volume should therefore greatly favor knot formation. Our present views of the knotting reaction are that both the superhelical structure of the DNA molecules and the excess topoisomerase protein binding serve to reduce the effective statistical segment length of the DNA, thus favoring knot formation.

The trace amount of topoisomerase activity required to make the transient double-strand breaks needed for knotting (for example, see reverse of reaction in Figure 5A) presumably comes from a charged form of some of the T4 DNA topoisomerase molecules, each of which may drive a single cycle of relaxation in the absence of ATP (Liu et al., 1979). In this view, the absence of ATP is required merely to limit the reaction rate so that the superhelical structure of the DNA molecules (which is important for knot formation) is maintained.

If the above view is correct, addition of small amounts of the T4 DNA topoisomerase with ATP present should be able to cause the knotting reaction (or a related DNA topoisomerization reaction such as catenation) in the presence of DNA condensing agents such as synthetic polycations, polyamines (Gosule and Schellman, 1976) or histone H1 (Cole, Lawson and Hsiang, 1978). Our preliminary study has shown that T4 DNA topoisomerase can indeed induce catenation of DNA rings in the presence of ATP and a trace amount of the polycation polymin P or histone H1. Furthermore, T4 DNA topoisomerase is also capable of decatenating interlocked DNA rings in the presence of ATP (T. Hsieh and D. Brutlag, personal communication; L. Liu, unpublished result). As we would expect, the unknotting activities detected in extracts of Xenopus eggs and Chinese hamster ovary cells can catalyze an ATP-dependent catenation and decatenation of DNA as well (L. Liu, unpublished results).

Mechanism of ATP Utilization by Type II DNA Topoisomerases

Since bacterial DNA gyrases are also able to unknot the topologically knotted, covalently closed pBR322 DNA (Figure 7), the ATP-dependent negative supercoiling reaction that these enzymes catalyze is likely to involve a transiently broken DNA double helix as an intermediate. P. Brown, K. Kreuzer and N. Cozzarelli have obtained independent evidence for such a mechanism for the E. coli DNA gyrase (personal communication). Figure 9 shows a possible model for this type of DNA gyration.

In our view, DNA gyration requires that two DNA double helices be brought together, with a double-chain break made in one of the two DNA helices by the topoisomerase. An ATP-driven, unidirectional 360° swiveling of one of the two broken ends around the unbroken double-stranded DNA is topologically equivalent to the passage of a DNA segment through the transient double-strand break, and thus can generate two superhelical turns into the DNA loop created between the two separate helical regions bound by the topoisomerase (see discussion above). If an en-

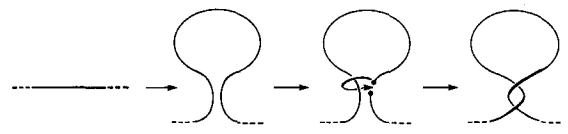

Figure 9. A Possible Model for the ATP-Driven Induction of DNA Supercoiling by Type II DNA Topoisomerases

The model shown requires that two DNA double helices be held together by the DNA topoisomerase, so as to restrict their relative rotation. By introducing a double-chain break and passing one of the broken ends around the other double helix in the indicated direction, negative superhelical turns are introduced into the DNA loop. Note that for each 360° rotation in a cycle, the topological equivalent of one double-strand passing event occurs, and thus two superhelical turns will be generated. In this model, unidirectional protein movement is required and net work is performed; this reaction must therefore be driven by the ordered allosteric changes in protein conformation made possible by the hydrolysis of enzyme-bound ATP (Hill, 1969).

Note that a local topological domain is thought to be created when the T4 DNA topoisomerase binds at the stem region of the loop, so that local DNA supercoiling can occur even in a linear DNA (Liu et al., 1979). For the reasons indicated in the text, however, bacterial DNA gyrases may occupy the entire looped region, rather than binding only at the stem. In this case, circularity of the DNA (or some other constraint) becomes necessary for the introduction of DNA supercoiling. Despite this possible difference between the T4 DNA topoisomerase and bacterial DNA gyrase, the two enzymes appear to act similarly in our unknotting reaction when supercoiling is no longer permissible (that is, in the absence of the hypothetical origin DNA sequences for the T4 DNA topoisomerase and with a highly negatively supercoiled DNA substrate for the bacterial DNA gyrase).

zyme that acts processively repeats this process, large numbers of negative twists can eventually be accumulated in a covalently closed DNA circle. A topoisomerase that acts in this way can also generate supercoiling in a local region of a linear DNA molecule. Such local DNA supercoiling has been postulated to explain how the T4 DNA topoisomerase could act as an origin-specific DNA gyrase (Liu et al., 1979).

How can we reconcile this postulated activity with the demonstrated ability of the T4 topoisomerase to remove either positive or negative supercoils from DNA circles with about equal efficiency, while requiring ATP hydrolysis for the strand-passing events which underlie both of these topological interconversions (Liu et al., 1979)? According to our present view, the reactions measured thus far for the T4 topoisomerase represent a "nonspecific" reaction, in which the topoisomerase binds any two crossing double-stranded DNA segments. Triggered by recognition of a DNA cross, the binding and hydrolysis of ATP presumably drive the enzyme through an ordered series of conformational changes (Hill, 1969), thereby generating the force necessary to push one DNA double helix through the other. For energetic reasons, in a highly negatively supercoiled DNA circle most such strand crosses formed will be right-handed, while in a highly positively supercoiled DNA circle most such strand crosses will be left-handed (when viewed in an interwound conformation; see Bauer and Vinograd, 1968). Since the T4 topoisomerase recognizes only the region of the strand cross itself, it does not differentiate between right- and left-handed crosses on a nonspecific DNA sequence. Consequently, both positive and negative DNA supercoils are removed efficiently, with an approximately stoichiometric hydrolysis of ATP.

As an extension of the above reaction, the T4 DNA topoisomerase may also recognize two specific DNA sequences, one on each side of a T4 replication origin. By binding especially tightly to both of these sequences, the topoisomerase could create a constrained DNA loop containing the origin, with a right-handed DNA strand cross at its base. By catalyzing repeated cycles of strand passing events through this specific cross (topologically equivalent to the repeated, unidirectional swiveling of one broken DNA end around the intact DNA double helix; see Figure 9), negative supercoiling could in principle be created in the loop. In such a view, the T4 DNA topoisomerase would act both as a highly processive, site-specific DNA gyrase, and as a nonprocessive enzyme capable of relaxing DNA supercoils (and catalyzing other related DNA topoisomerization reactions such as knotting:unknotting and catenation:decatenation) on nonspecific sequences.

In the case of bacterial DNA gyrases, it is tempting to imagine that the DNA loop shown in Figure 9 is wound tightly around the enzyme, to account for the fact that a ~145 bp length of DNA is partly wrapped around the gyrase surface in a positive sense (Liu and Wang, 1978a, 1978b). The swiveling indicated in Figure 9 would then enable these gyrases to pump negative supercoils into circular DNAs (a closely related model for gyrase has been proposed independently by Brown and Cozzarelli, 1979 and Cozzarelli, 1980). Note, however, that the induction of supercoiling into local domains, as proposed for the T4 topoisomerase, would then be unlikely to be catalyzed by bacterial gyrases without the existence of additional factors which hold regions of helix together and thus introduce topological constraints (see the legend to Figure 9).

Possible Biological Functions of Type II Topoisomerases

T4 DNA topoisomerase has been shown to be coded for by the T4 genes 39, 52 and 60 (Liu et al., 1979). Genetic and biochemical studies of these T4 bacteriophage mutants have suggested that the T4 DNA topoisomerase is involved in T4 DNA synthesis and probably in the initiation of DNA replication (Yegian et al., 1971; McCarthy et al., 1976; McCarthy, 1979). If the T4 DNA topoisomerase functions as a site-specific DNA gyrase that catalyzes ATP-dependent negative supercoiling in a local domain at the T4 replication origin(s), it would facilitate the helix opening neces-

sary for formation of a replication bubble (Liu et al., 1979).

It is tempting to propose that the initiation of DNA replication in a eucaryotic organism follows a similar mechanism to that proposed for bacteriophage T4. Both the T4 DNA topoisomerase and the unknotting activity detected in the nuclear extract of CHO cells are inhibited by high novobiocin concentrations (unpublished results of L. Liu). Interestingly, Mattern and Painter (1979) have shown that novobiocin at about this concentration (200 μg/ml) inhibits the initiation of CHO cell DNA replication without blocking replication fork movement. It thus seems quite possible that the type II DNA topoisomerase we have detected in CHO cells is the target protein for the drug, and that the eucaryotic type II DNA topoisomerases described in this report function in initiating replication forks, as suspected by analogy with bacteriophage T4.

Many other biological processes, including the termination of DNA replication (for example, segregations of interlocked DNA rings), phage packaging, site-specific genetic recombination, DNA transposition and chromosome condensation and decondensation might also require DNA double-chain breakage and rejoining events. In addition, it is important to recognize that the existence of such type II DNA topoisomerases means that all large DNA molecules (such as those in bacterial and eucaryotic chromosomes) would be expected to be highly knotted, especially in their condensed forms (Frank-Kamenetskii et al., 1975). Since any DNA knot can be readily tied and untied by double-strand passing events, however, such knots need not have a harmful effect on genetic processes. It is interesting to speculate that an organized pattern of DNA knots might even be useful in helping to hold together complex structures such as eucaryotic chromosomes.

We propose that the ability to catalyze a double-strand passing reaction (such as that illustrated schematically in Figure 5) be used to define a topoisomerase of type II, thus distinguishing such enzymes from the more classical type I topoisomerases which reversibly break and rejoin only one DNA strand at a time (Champoux, 1978; Wang and Liu, 1979). This previously unrecognized class of type II topoisomerases is undoubtedly widespread in nature, and is likely to be of great importance for many fundamental genetic processes.

Experimental Procedures

Enzymes and Nucleic Acids

T4 DNA topoisomerase was purified from T4 *regA*, *am*N55, *am*H39-infected E. coli D110 cells as described previously (Liu et al., 1979). The topoisomerase preparation used in this study had a specific activity of ~10^6 U/mg (Liu et al., 1979). The enzyme had been stored at −20°C in 50% (w/v) glycerol, 30 mM potassium phosphate (pH 7.2), 10 mM β-mercaptoethanol and 0.5 mM Na_3EDTA for more than six months without loss of activity. Purified E. coli DNA gyrase, subunits of M. luteus DNA gyrase and E. coli ω protein were obtained from the laboratory of J. C. Wang. Eco RI restriction enzyme was purified in this laboratory by P. Bedinger. Restriction enzymes Hinc II and Hae II were purchased from New England Biolabs and Bethesda Research Inc., respectively. Drosophila nicking-closing enzyme purified from tissue culture (Kc) cells and the ATP-dependent DNA topoisomerase from Drosophila were obtained from T. Hsieh and D. Brutlag. Proteinase K was purchased from E. M. Laboratory. Plasmid pBR322 DNA was purified by phenol deproteinization of a clear lysate followed by CsCl/ethidium bromide equilibrium centrifugation.

Electron Microscopy

The formamide spreading technique described by Davis, Simon and Davidson (1971) was used. The grids were shadowed with either tungsten or platinum and viewed with a Philips EM201 or EM301 electron microscope.

Analytical Ultracentrifugation

CsCl density gradient equilibrium centrifugation was performed in 12 mm double-sector cells by spinning at 40,000 rpm for 48 hr at 20°C in a Spinco Model E analytical ultracentrifuge.

Preparation of Knotted, Covalently Closed pBR322 DNA

Knotted pBR322 DNA was typically prepared as follows. 1 ml of a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM Na_3EDTA, 0.5 mM dithiothreitol, 30 μg/ml human serum albumin (Worthington), 20 μg/ml pBR322 DNA (form I) and 33 μg/ml T4 DNA topoisomerase was incubated at 30°C for 3 min and then stopped by extracting twice with neutralized phenol. After four extractions of the aqueous layer with ether, the mixture was dialyzed into 10 mM Tris–HCl (pH 7.8), 0.1 mM Na_3EDTA and stored at −20°C.

Normally, about half the pBR322 DNA (form I) can be converted to the knotted form under the above conditions. The amount of the knotted form in each preparation varies with the DNA and/or enzyme concentration. At higher DNA concentration and/or lower enzyme concentration, the amount of knotting decreases and pBR322 DNA (form I) is relaxed without being knotted, even at the initial stages of reaction.

Preparation of Nicked, Knotted pBR322 DNA

The knotted, covalently closed pBR322 DNA described above was converted into nicked, knotted pBR322 DNA by a limited DNAase I treatment as follows. 1 ml of knotted, covalently closed pBR322 DNA (~20 μg/ml) was treated with 16 ng/ml pancreatic DNAase I (Worthington) in a reaction mixture containing 10 mM Tris–HCl (pH 7.8), 4 mM $MgCl_2$, 1 mM Na_3EDTA and 40 μg/ml human serum albumin. After incubation at 30°C for 10 min, the reaction was quenched by phenol extractions. After removing the phenol from the aqueous phase with ether extractions, the DNA was dialyzed into 10 mM Tris–HCl (pH 7.8), 0.1 mM Na_3EDTA for storage at −20°C.

Isolation of pBR322 DNA with a Unique Linking Number

7 μg of pBR322 DNA (form I) were relaxed to completion by treatment with T4 DNA topoisomerase (500 units) in the presence of ATP. The relaxed, covalently closed pBR322 DNA was then electrophoresed through a preparative 0.8% agarose gel at room temperature in a buffer consisting of 40 mM Tris base, 90 mM boric acid (pH 8.3) and 3 mM Na_3EDTA (TBE). After electrophoresis, the agarose gel was sliced into small horizontal strips, each of which contained an individual DNA band (as judged by staining vertical gel slices with ethidium bromide). Each gel strip was transferred to a dialysis bag containing 200 μl of the electrophoresis buffer diluted 4 fold, plus 14 μg/ml of yeast tRNA as carrier. The DNA molecules were eluted from the gel by placing the dialysis tubing into the dilute electrophoresis buffer and electrophoresing for 1 hr at 100 V and 100 mA in a 20 × 10 cm electrophoresis box. The polarity of the electrodes was reversed for 40 sec just before removing the dialysis bags. The solid contents of the dialysis bags were removed by centrifugation, and the supernatants were precipitated by adding 10 μg of yeast tRNA carrier and

67% ethanol in the presence of 0.1 M sodium acetate for 15 min in a dry ice bath. After ethanol precipitation, the DNA precipitates were each dissolved in 30 μl of the buffer used for the T4 DNA topoisomerase reactions (see above).

Preparation of Drosophila Embryo Extract

Freshly deposited D. melanogaster eggs were collected from population cages at 1 hr. Embryos were rinsed extensively in a saline-detergent solution (0.7% NaCl, 0.02% Triton X-100), dechorionated in half-strength Chlorox for about 60 sec and then rinsed thoroughly with saline-detergent solution on a 116 mesh nylon screen (Nitex). About 1 g of embryos was immediately resuspended in 5 ml of 50 mM potassium phosphate (pH 7.3) and broken by 10 strokes of a Dounce homogenizer. The homogenate was filtered through Nitex and centrifuged at 10,000 × g for 10 min. The supernatant was used immediately for the assay.

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References

Baase, W. A. and Wang, J. C. (1974). Biochemistry *13*, 4299–4303.

Bauer, W. R. and Vinograd, J. (1968). J. Mol. Biol. *33*, 141–172.

Bauer, W. R., Ressner, E. C., Kates, J. and Patzke, J. V. (1977). Proc. Nat. Acad. Sci. USA *74*, 1841–1845.

Brown, P. O. and Cozzarelli, N. R. (1979). Science *206*, 1081–1083.

Champoux, J. J. (1977). Proc. Nat. Acad. Sci. USA *74*, 3800–3804.

Champoux, J. J. (1978). Ann. Rev. Biochem. *47*, 449–479.

Champoux, J. J. and Dulbecco, R. (1972). Proc. Nat. Acad. Sci. USA *69*, 143–146.

Cole, R. D., Lawson, G. M. and Hsiang, M. W. (1978). Cold Spring Harbor Symp. Quant. Biol. *42*, 253–263.

Cozzarelli, N. R. (1980). Science, in press.

Davis, R. W., Simon, M. and Davidson, N. (1971). In Methods in Enzymology, *21*, part D, L. Grossman and K. Moldave, eds. (New York: Academic Press), pp. 413–428.

Delius, H., Howe, C. and Kozinski, A. W. (1971). Proc. Nat. Acad. Sci. USA *68*, 3049–3053.

Frank-Kamenetskii, M. D., Lukashin, A. V. and Vologodskii, A. V. (1975). Nature *258*, 398–402.

Fuller, F. B. (1978). Proc. Nat. Acad. Sci. USA *75*, 3557–3561.

Gellert, M., Mizuuchi, K., O'Dea, M. H. and Nash, H. A. (1976a). Proc. Nat. Acad. Sci. USA *73*, 3872–3876.

Gellert, M., O'Dea, M. H., Itoh, T. and Tomizawa, J. (1976b). Proc. Nat. Acad. Sci. USA *73*, 4474–4478.

Germond, J.-E., Hirt, B., Oudet, P., Gross-Bellard, M. and Chambon, P. (1975). Proc. Nat. Acad. Sci. USA *72*, 1843–1847.

Germond, J.-E., Rouviere-Yaniv, J., Yaniv, M. and Brutlag, D. (1979). Proc. Nat. Acad. Sci. USA *76*, 3779–3783.

Gosule, L. C. and Schellman, J. A. (1976). Nature *259*, 333–335.

Hill, T. L. (1969). Proc. Nat. Acad. Sci. USA *64*, 267–274.

Huberman, J. A. and Riggs, A. D. (1968). J. Mol. Biol. *32*, 327–341.

Keller, W. and Wendel, I. (1975). Cold Spring Harbor Symp. Quant. Biol. *39*, 199–208.

Kikuchi, Y. and Nash, H. A. (1979). Proc. Nat. Acad. Sci. USA *76*, 3760–3764.

Kirkegaard, K. and Wang, J. C. (1978). Nucl. Acids Res. *5*, 3811–3820.

Kreigstein, H. J. and Hogness, D. S. (1974). Proc. Nat. Acad. Sci. USA *71*, 135–139.

Liu, L. F. and Wang, J. C. (1978a). Proc. Nat. Acad. Sci. USA *75*, 2098–2102.

Liu, L. F. and Wang, J. C. (1978b). Cell *15*, 979–984.

Liu, L. F., Depew, R. E. and Wang, J. C. (1976). J. Mol. Biol. *106*, 439–452.

Liu, L. F., Liu, C.-C. and Alberts, B. M. (1979). Nature *281*, 456–461.

McCarthy, D. (1979). J. Mol. Biol. *127*, 265–283.

McCarthy, D., Minner, C., Bernstein, H. and Bernstein, C. (1976). J. Mol. Biol. *106*, 963–981.

Mattern, M. R. and Painter, R. B. (1979). Biochim. Biophys. Acta *563*, 306–312.

Peebles, C. L., Higgins, N. P., Kreuzer, K. N., Morrison, A., Brown, P. O., Sugino, A. and Cozzarelli, N. R. (1979). Cold Spring Harbor Symp. Quant. Biol. *43*, 41–52.

Shapiro, J. A. (1979). Proc. Nat. Acad. Sci. USA *76*, 1933–1937.

Smith, C. L., Kubo, K. and Imamoto, F. (1978). Nature *275*, 420–423.

Stetler, G. L., King, G. J. and Huang, W. M. (1979). Proc. Nat. Acad. Sci. USA *76*, 3737–3741.

Sumida-Yasumoto, C. and Hurwitz, J. (1977). Proc. Nat. Acad. Sci. USA *74*, 4195–4199.

Wang, J. C. (1971). J. Mol. Biol. *55*, 523–533.

Wang, J. C. (1973). In DNA Synthesis in Vitro, R. D. Wells and R. B. Inman, eds. (Baltimore: University Park Press), pp. 163–174.

Wang, J. C. and Liu, L. F. (1979). In Molecular Genetics, Part III, J. H. Taylor, ed. (New York: Academic Press), pp. 65–88.

Yegian, C. D., Mueller, M., Selzer, G., Russo, V. and Stahl, F. W. (1971). Virology *46*, 900–919.