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Bacterial DNA Topoisomerase I Can Relax Positively Supercoiled DNA Containing a Single-stranded Loop

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Using heteroduplex molecules formed from a pair of plasmids, one of which contains a small deletion relative to the other, it is shown that bacterial topoisomerase I can relax a positively supercoiled DNA if a short single-stranded loop is placed in the DNA. This result supports the postulate that the specificity of bacterial DNA topoisomerase I for negatively supercoiled DNA in its relaxation reaction derives from the requirement of a short single-stranded DNA segment in the active enzyme–substrate complex. Nucleolytic and chemical probing of complexes between bacterial DNA topoisomerase I and heteroduplex DNA molecules containing single-stranded loops ranging from 13 to 27 nucleotides in length suggests that the enzyme binds specifically to the region containing a single-stranded loop; the site of DNA cleavage by the topoisomerase appears to lie within the single-stranded loop, with the enzyme interacting with nucleotides on both sides of the point of cleavage.

1. Introduction

Through studies made during the last decade, DNA topoisomerases have emerged as a class of enzymes that act in a number of vital processes in which DNA is a key participant, including replication, transcription, and recombination (for reviews, see Cozzarelli, 1980; Gellert, 1981; Wang, 1985). In both prokaryotes and eukaryotes, two mechanistically distinct subclasses of topoisomerases have been found. The type I enzymes transiently break one DNA strand at a time, whereas the type II enzymes make a pair of staggered transitory cuts, one in each strand of a DNA duplex, to permit strand passage through the disjoined strand or strands before the restoration of strand continuity.

Bacterial DNA topoisomerase I, first identified in *Escherichia coli* as the ω protein (Wang, 1971), is a type I enzyme that has been studied extensively. Complete sequencing of the *E. coli* gene *topA* encoding this enzyme reveals a coding sequence for 872 amino acids (Y.-C. Tse-Dinh & J. C. Wang, unpublished results); direct sequencing of the N terminus of the purified enzyme indicates that the first fMet residue is removed (Y.-C. Tse-Dinh, personal communication) to give a protein with a calculated molecular weight of 98,000. The enzyme is known to catalyze four types of DNA topoisomerization reactions: the relaxation of negatively supercoiled DNA (Wang, 1971); the interconversion of simple and knotted single-stranded DNA rings (Liu *et al.*, 1976): the linking of a pair of single-stranded DNA rings of complementary sequences into a covalently closed double-stranded ring (Kirkegaard & Wang, 1978); and the catenation of a pair of double-stranded rings, provided at least one of them contains a pre-existing, single-strand nick (Tse & Wang, 1980; Brown & Cozzarelli, 1981) or gap (Low *et al.*, 1984). Topologically, all reactions require the transient breakage of a DNA strand. There is strong evidence that the enzyme does so by breaking a DNA phosphodiester bond while simultaneously forming a protein–DNA phosphotyrosyl covalent linkage (for reviews, see Wang & Liu, 1979; Gellert, 1981; Wang, 1981, 1985).

Notably missing from the list of topoisomerization reactions that the bacterial enzyme catalyzes, however, is the relaxation of positively supercoiled DNA. Topologically, a transient single-stranded scission is sufficient for the relaxation of positively as well as negatively supercoiled DNA. Indeed, the type I DNA topoisomerase found in all eukaryotic cells has been known since its discovery to relax both positively and negatively supercoiled DNA substrates (Champoux & Dulbecco, 1972). The specificity of the bacterial enzyme for negatively supercoiled DNA is, however, well-

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established (Wang, 1971; Kung & Wang, 1977). Another type I bacterial topoisomerase discovered recently (Dean *et al.*, 1983), DNA topoisomerase III, shares the specificity of bacterial DNA topoisomerase I for negative supercoils (Srivenugopal *et al.*, 1984).

It has been postulated that the active enzyme–substrate complex in the relaxation of supercoiled DNA by bacterial topoisomerase I contains a short segment of single-stranded DNA (Wang, 1971). Because negative supercoiling of DNA favors the unpairing of strands, whereas positive supercoiling has the opposite effect, this postulated mechanism could account for the specificity of the enzyme for negatively supercoiled DNA. A direct prediction of this postulate is that, if a single-stranded region is artificially introduced into a positively supercoiled DNA, it should be relaxed by the enzyme. In this paper, we show that bacterial DNA topoisomerase I from either *E. coli* or *Micrococcus luteus* can indeed relax a positively supercoiled DNA if a short single-stranded loop is placed in the DNA by forming heteroduplex molecules from two plasmids, one of which contains a small deletion relative to the other. This looped DNA substrate also allows us to bind a bacterial topoisomerase I to a particular sequence, thus permitting the chemical and enzymatic probing of the complex at the DNA sequence level. These results are described below.

2. Materials and Methods

(a) *Enzymes*

E. coli and *M. luteus* DNA topoisomerase I were prepared as described by Kung & Wang (1977) and Depew *et al.*, (1978), or in a similar way. All other enzymes were from commercial suppliers.

(b) *DNA substrates*

Plasmid DNAs were prepared as described by Wang & Becherer (1983). Whenever the DNA was to be subjected to denaturation and reannealing, the plasmids were propagated without amplification with chloramphenicol.

The construction of a set of plasmids that differ by small deletions has been described (Wang & Becherer, 1983; Kirkegaard *et al.*, 1984, see also the legend to Fig. 1). For clarity, they are designated Δ4, Δ7, Δ13, Δ18, Δ25 and Δ31 in reference to the sizes of the deletions they bear relative to their parent. Heteroduplex molecules between Δ4 and Δ31 plasmids were used in most of the experiments, and their preparation is described as an example. DNA Δ4 was first linearized with *Bam*HI restriction endonuclease, and labeled at the 5′ ends by a cycle of dephosphorylation with alkaline phosphatase and phosphorylation with bacteriophage T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Before end-labeling, tRNA was removed from the plasmid DNA preparations either by Biogel A-50 (Biorad) chromatography or by selective precipitation of the plasmid DNA with polyethylene glycol (Schleif, 1979). DNA Δ31 was cut at the single *Eco*RI or *Pst*I site away from the *Bam*HI site. Typical denaturing/reannealing reactions to give heteroduplex molecules were performed roughly according to the procedure of Davis *et al.* (1971). To 210 μl of a solution containing 25 μg of 5′ end-labeled *Bam*HI-cut Δ4 DNA and 50 μg of *Eco*RI-cut Δ31 DNA in 0·01 M-Tris·HCl (pH 8), 0·1 mM-EDTA, were added 30 μl of 0·2 M-EDTA and 60 μl of 1 M-KOH, and the mixtures were incubated at 37°C for 10 min. The mixture was gently vortexed while 240 μl of distilled water, 60 μl of a solution containing 1·8 M-Tris·HCl and 0·2 M-Tris base, and 600 μl of formamide (Mallindcrodt) were sequentially added. The formamide had been deionized by stirring overnight with ion-exchange resin X-501-AG (Biorad), followd by filtration. The 50% (v/v) formamide mixtures were left at room temperature for more than 5 h, after which 1·2 ml of 0·6 M-sodium acetate and 6·0 ml of ethanol were added to precipitate the DNA. The DNA was precipitated a second time and redissolved in a solution containing 10 mM-Tris (pH 8), 0·1 mM-EDTA.

Since Δ4 and Δ31 were linearized with restriction enzymes cutting at 2 separate sites, the heteroduplex molecules are in the form of nicked circles, whereas the homoduplex molecules are linear, as shown in Fig. 1(a). Two alternative methods were used to make positively supercoiled heteroduplex rings. In one method, the nicked heteroduplex molecules prepared as described above were isolated by preparative gel electrophoresis, and extracted from the gel by electroelution. The purified nicked heteroparental rings were incubated for 16 h at 0°C in 250 μl of solution containing 10 mM-Tris (pH 7·8), 6 mM-$MgCl_2$, 6 mM-β-mercaptoethanol, 50 μg bovine serum albumin/ml, 0·5 mM-ATP and several hundred units of bacteriophage T4 DNA ligase. Subsequent extraction with phenol and precipitation with ethanol prepared the DNA for further reactions. Another strategy of forming covalently closed heterduplex circles preferentially is to use *E. coli* DNA ligase and its cofactor NAD instead of T4 DNA ligase and ATP in the ligation step. The *E. coli* enzyme joins the nicks in the heteroduplex molecules efficiently, but linear homoduplex molecules are ligated into the closed circular form inefficiently when the linear molecules posess flush ends. In either scheme, covalent closure of duplex rings at 0°C ensures that they will become positively supercoiled at higher temperatures, due to the temperature dependence of the helical twist of DNA (Wang, 1969; Depew & Wang, 1975; Pulleyblank *et al.*, 1975). In some *E. coli* ligase reactions, 0·1 M-ammonium chloride was included in the reaction to increase further the helical twist during ligation (Anderson & Bauer, 1978).

The above preparations yield labeled heteroduplex molecules of both possible combinations of strands. In order to follow each combination individually, we have modified Δ4 and Δ31 DNA as follows. Each DNA was first cut with *Eco*RI, repaired with *E. coli* DNA polymerase, and *Bgl*II linkers (C-A-G-A-T-C-T-G, New England Biolabs) were ligated to the repaired ends. For Δ4 DNA, the reacted DNA was cut with *Bgl*II after removal of excesss linkers, ligated and cloned. This yields a DNA termed Δ4 (*Bgl*). For Δ31 DNA, the reacted DNA was cut with both *Bgl*II and *Bam*HI, after the removal of excess linkers, and then the *Bam*HI- and *Bgl*II-cut ends were joined by ligation. Upon cloning, a DNA termed Δ31 (ΔR1-*Bam*) is obtained. The new pair of DNAs (see Fig. 1(b)) are used in making the heteroduplex molecules as follows. DNA Δ4 (*Bgl*) is cut with *Bgl*II, labeled at the 5′ ends, and then cut with *Bam*HI. This DNA is then mixed with *Pst*I-cut Δ31 (ΔR1-*Bam*) to give heteroduplex nicked circles as described before; only one particular strand of Δ4 (*Bgl*) is labeled in the heteroduplex. For the specific labeling of the other strand of Δ4, Δ4 (*Bgl*) is first

cut with *Bam*HI, labeled at the 5′ ends, and then cut with *Bgl*II and processed as before.

For probing the topoisomerase-looped DNA complex chemically or nucleolytically, restriction fragments of the heteroduplex or homoduplex molecules labeled at a single 3′ or 5′ end were obtained by standard methods (Maxam & Gilbert, 1980). Restriction fragments containing single-stranded loops are found to have much lower electrophoretic mobilities than double-stranded restriction fragments of the same size without loops. Reduced electrophoretic mobility of partially denatured duplexes has been observed (Fischer & Lerman, 1979, 1980; Lyamichev *et al.*, 1982). We found that the electrophoretic mobilities of fragments containing loops are sufficiently sensitive to the size and base composition of the loops that 2 duplex fragments containing identically sized loops of complementary sequence can be separated on a polyacrylamide gel. Therefore, there is no possibility that the single-stranded loops can reanneal with each other, once the fragments have been purified.

Numbering of nucleotides on both strands of heteroduplex fragments and homoduplex control fragments will be specified in a later section (see the legend to Fig. 4). Restriction sites used in excising and labeling fragments are *Bam*HI at position 1, *Fok*I at position 222, *Rsa*I at position 39 and *Hae*III at position 294. The numbers of one DNA strand (the bottom strand in Fig. 4(b)) are consistently primed. The following DNA fragments were prepared: the *Bam*HI–*Hae*III fragment of Δ4/Δ31′ heteroduplex DNA, 5′ end-labeled at the *Bam*HI site (denoted by 5′ *Bam*HI–*Hae*III Δ4/Δ31′; the same notation is used for other fragments); 5′ *Bam*HI–*Hae*III Δ4; 3′ *Bam*HI–*Hae*III Δ4/Δ31′; 3′ *Bam*HI–*Hae*III Δ31; 5′ *Fok*I–*Rsa*I Δ4′/Δ31; 5′ *Fok*I–*Rsa*I Δ4; 3′ *Fok*I–*Rsa*I Δ4′/Δ31; 3′ *Fok*I–*Rsa*I Δ4; 3′ *Bam*HI–*Hae*III Δ4/Δ31′; and 3′ *Bam*HI–*Hae*III Δ31.

(c) *Enzyme reactions*

Except as indicated, all topoisomerase reactions took place in 100-μl solutions containing, in addition to DNA and enzymes, 10 mM-Tris (pH 7·8), 150 mM-KCl, 5 mM-$MgCl_2$, 100 μg bovine serum albumin/ml and 4% (v/v) glycerol (ω buffer). For each topoisomerase-mediated DNA cleavage reaction, after incubation of the enzyme–DNA mixtures at 37°C for 20 min, 10 μl of 2 M-NaOH were added to each and incubation was continued for 1 min. The mixtures were gently vortexed while 20 μl of a solution containing 1·8 M-Tris·HCl and 0·2 M-Tris base was added, followed by the sequential addition of 100 μl of ammonium acetate stop solution (4 M-ammonium acetate, 100 μg tRNA/ml and 20 mM-EDTA) and 600 μl of ethanol to precipitate the DNA. After centrifugation and removal of the supernatant, the DNA was redissolved in 200 μl of 0·3 M-sodium acetate and precipitated with ethanol again. The samples were then redissolved in a solution containing 90% (v/v) formamide, 0·01 M-KOH, and tracking dyes bromophenol blue and xylene cyanol in preparation for sequencing gel electrophoresis.

DNase I footprinting (Galas & Schmitz, 1978) was performed by adding 1 μl of a 50 μg/ml solution of pancreatic DNase I, freshly diluted from a 5 mg/ml stock solution, to 100-μl reaction mixtures. Staphylococcal nuclease footprints were performed in the same way, except that a 5 mg/ml Staphylococcal nuclease stock solution was diluted to 25 μg/ml before the addition of 1 μl to the reaction mixtures. Low concentrations of both nucleases were used in most reactions to ensure that the DNA fragments sustained less than one cleavage per molecule. Incubation at 37°C was continued for 1 min after the addition of nuclease. Then, 20 μl of 0·2 M-EDTA were added, and the solutions were heated to 65°C for 10 min. This heating step inactivates the topoisomerase and minimizes its cleavage of DNA. After this, 100 μl of ammonium acetate stop solution and 600 μl of ethanol were added, and the samples were treated in exactly the same way as the cleavage reaction samples before sequencing gel electrophoresis. Staphylococcal nuclease cleavage generates 3′-phosphoryl termini, and DNase I cleavage leaves 3′-hydroxyl termini. Thus, fragments cleaved by the 2 nucleases that contain the same number of nucleotides differ slightly in mobility and appropriate corrections were made (Tapper & Clayton, 1981). Comparison of frequencies of nuclease cleavage at specific sequences, and of the reactivities to dimethyl sulfate of guanine and adenine residues in the DNA molecules in the presence and absence of enzyme was performed as described (Kirkegaard & Wang, 1981). Autoradiographs were scanned with a densitometer (Helena) to quantify the patterns.

3. Results

(a) *Preparation of covalently closed circular DNA containing a single-stranded loop*

Figure 1(a) outlines the general method used for the preparation of covalently closed DNA rings each containing a single-stranded loop. The plasmids Δ4 and Δ31 are identical except that the former has a 27 base-pair segment (represented by the filled bar clockwise from the *Bam*HI site) not present in the latter. Plasmid Δ4 is first cut with *Bam*HI and 5′ end-labeled by a cycle of phosphatase and polynucleotide kinase treatments, and plasmid Δ31 is cut with *Eco*RI. The DNAs are mixed, denatured with alkali, and then renatured to give heteroduplex circles labeled at one or the other strand and homoduplexes that are in the linear form.

Three different approaches have been used to obtain preparations of radioactively labeled covalently closed heteroduplex rings that contain few or no labeled homoduplex molecules. In one method, the nicked heteroduplex circles are separated from the homoduplex linear molecules by agarose gel electrophoresis. Ligation of the nicked circles yields covalently closed rings each containing a small single-stranded loop. In the second approach, labeled covalently closed heteroduplex rings are obtained without the gel isolation step by the use of excess *Pst*I-cut Δ31 DNA. The presence of excess unlabeled Δ31 DNA during the renaturation step minimizes the formation of labeled homoduplex Δ4 DNA. In the third approach, the products of renaturation are treated with *E. coli* DNA ligase rather than bacteriophage T4 DNA ligase. The *E. coli* enzyme converts the linear homoduplex molecules to the covalently closed form poorly when the reaction is carried out at 30°C or at higher temperatures, or when Δ4 DNA was first cut with a restriction endonuclease that generates flush ends.

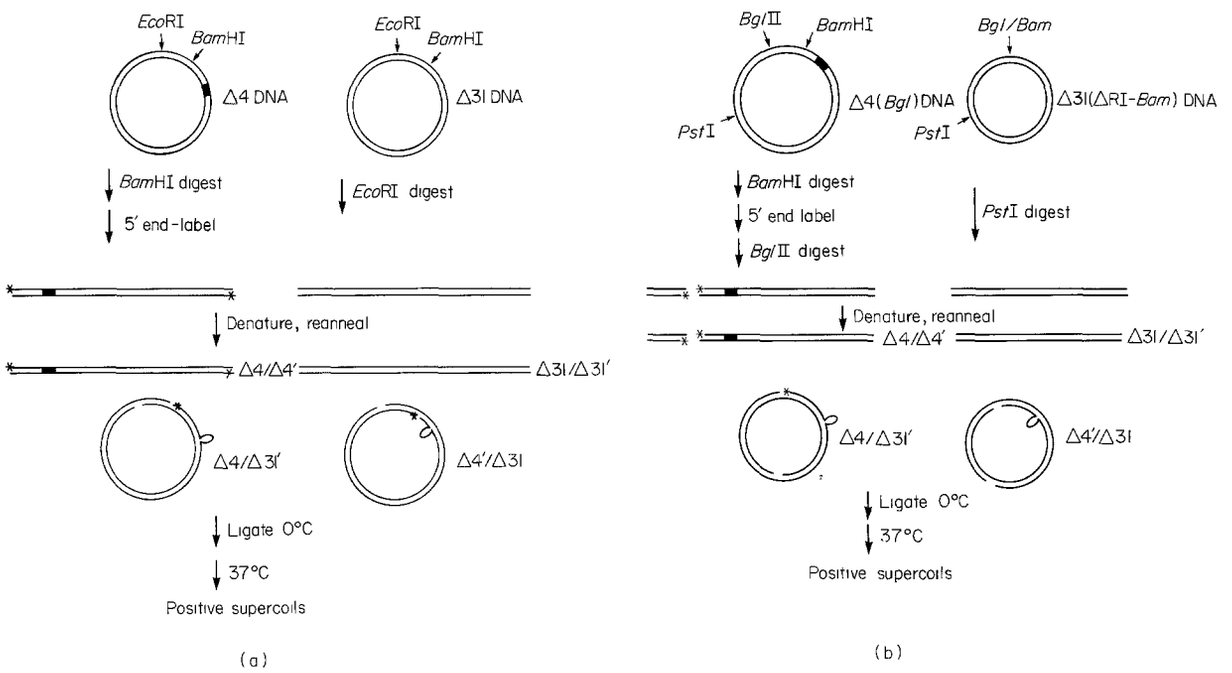


Figure 1. Flow diagrams describing the construction of positively supercoiled duplex rings containing single-stranded loops. Plasmids Δ4 (2201 base-pairs) and Δ31 (2174 base-pairs), in (a), correspond to plasmids pJW225 and pJW243, which have been described (Wang & Becherer, 1983; Kirkegaard *et al.*, 1984). They are identical except for the presence of 27 base-pairs of DNA sequence in Δ4 that is not in Δ31; this is marked by a filled bar in the homoduplex Δ4 molecules, and as a loop in the heterodupexes. Heteroduplex rings are isolated by one of several methods described in Results. The construction of plasmids Δ4 (*Bgl*) and Δ31 (ΔRI-*Bam*) in (b) is described in the text. The preparation of individually labeled heteroduplex Δ4/Δ31′ is outlined in (b). To prepare heteroduplex Δ4′/Δ31, plasmid Δ4 (*Bgl*) is cut first with *Bgl*II, 5′ end-labeled, and then cut with *Bam*HI.

Covalently closed heteroduplex DNA rings prepared as described above contain equal amounts of the two heterologous combinations of Δ4 and Δ31 strands. These two species are termed Δ4/Δ31′ and Δ4′/Δ31 as shown in Figure 1(a) (see also Materials and Methods for strand designations). To test whether the two combinations, which contain single-stranded loops of complementary sequences, might differ in their relaxation by bacterial DNA topoisomerase I, we have designed a scheme to label specifically one or the other strand of Δ4 in the heteroduplex rings. The *Eco*RI site of Δ4 is first converted to a *Bgl*II site, by cutting with *Eco*RI, repairing the staggered ends with *E. coli* DNA polymerase I and inserting a *Bgl*II "linker" in between the repaired ends, to give a plasmid termed Δ4 (*Bgl*). The *Eco*RI site of Δ31 is similarly cut, repaired, and ligated with *Bgl*II linkers. The DNA is then digested with *Bgl*II and *Bam*HI, ligated, and cloned to give plasmid Δ31 (ΔRI-*Bam*). Figure 1(b) outlines the scheme for strand-specific labeling using the new pair of plasmids. To prepare specifically labeled Δ4/Δ31′; Δ4 (*Bgl*) is first cut with *Bam*HI, ^{32}P-labeled at the 5′ ends, and then digested with *Bgl*II. The DNA is then mixed with *Pst*I-cut Δ31 (ΔRI-*Bam*), denatured and renatured to give heteroduplex molecules in which only one strand is labeled. To Δ4′/Δ31 specifically, the order of digestion of Δ4 (*Bgl*) with *Bam*HI and *Bgl*II is reversed to yield heteroduplex rings in which the other strand is labeled. (The products prepared by the scheme shown in Fig. 1(b) differ from the corresponding ones shown in Fig. 1(a) by the deletion of RI-*Bam*HI segment in the original plasmids Δ4 and Δ31. Because this deletion has no consequence for the studies presented here, we have used the same notation, Δ4/Δ31′ and Δ4′/Δ31, for both sets of products.)

(b) *The presence of a single-stranded loop in a positively supercoiled DNA accelerates greatly its relaxation by bacterial DNA topoisomerase I*

Figure 2 depicts an autoradiograph of four DNA samples after agarose gel electrophoresis. Lanes 1 and 2 contain ^{32}P-labeled positively supercoiled Δ4 DNA before and after treatment with *E. coli* DNA topoisomerase I, respectively. The patterns are identical, confirming that the enzyme is ineffective in relaxing positively supercoiled DNA. In this experiment, unlabeled negatively supercoiled Δ4 DNA was present in all reaction mixtures. Ethidium staining of the gel after electrophoresis shows that the negatively supercoiled DNA, which is present in excess over the labeled positively supercoiled DNA, is completely relaxed (results not shown). Lanes 3 and 4 contain, respectively, positively supercoiled heteroduplex DNA, prepared by annealing labeled Δ4 DNA strands and un-

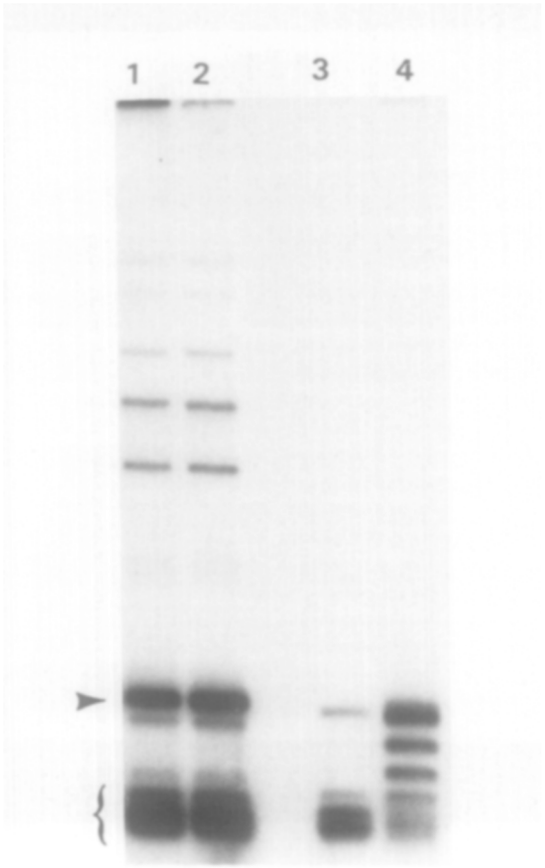


Figure 2. *Escherichia coli* topoisomerase I-catalyzed relaxation of positive supercoils containing single-stranded loops. An autoradiograph is shown of a 0·7% (w/v) agarose gel, electrophoresed at 37°C in TBE buffer (Maniatis *et al.*, 1975) for 14 h at 3 V/cm. The group of bands near the bottom of the gel in each lane consists of homoduplex Δ4 (lanes 1 and 2) or heteroduplex Δ4/Δ31 DNA rings (lanes 3 and 4). Within this group, the slowest migrating band is nicked DNA (marked by an arrowhead to the left of lane 1) and the positively supercoiled topoisomers in the samples before incubation with the topoisomers are bands in the bracketed region indicated in the left margin of the Figure. The bands above this group in lanes 1 and 2 are multimeric circles of Δ4 DNA. Incubation with *E. coli* topoisomerase I was performed as described in Materials and Methods. Lanes 1 and 3 are before incubation with the enzyme; lanes 2 and 4 are after incubation with the enzyme. The positively supercoiled heteroduplex molecules in this experiment are a mixture of Δ4/Δ31′ and Δ4′/Δ31 heteroduplexes, prepared as shown in Fig. 1 by annealing 10 μg of *Bam*HI-cut. 5′-labeled Δ4 DNA with 50 μg of *Eco*RI-cut Δ31 DNA as described in Materials and Methods. Heteroduplex rings were isolated, after agarose gel electrophoresis, by cycles of freezing and thawing of the gel followed by extraction of the centrifuged supernatant with phenol. The nicked rings recovered from the gel were treated with bacteriophage T4 DNA ligase at 0°C for 16 h. Homoduplex rings were prepared by treating 5′-labeled *Eco*RI-cut Δ4 DNA with T4 DNA ligase in the same manner. In other experiments, homoduplex rings were prepared by denaturing and reannealing the DNA before ligation The same results were obtained with the differently prepared homoduplex rings.

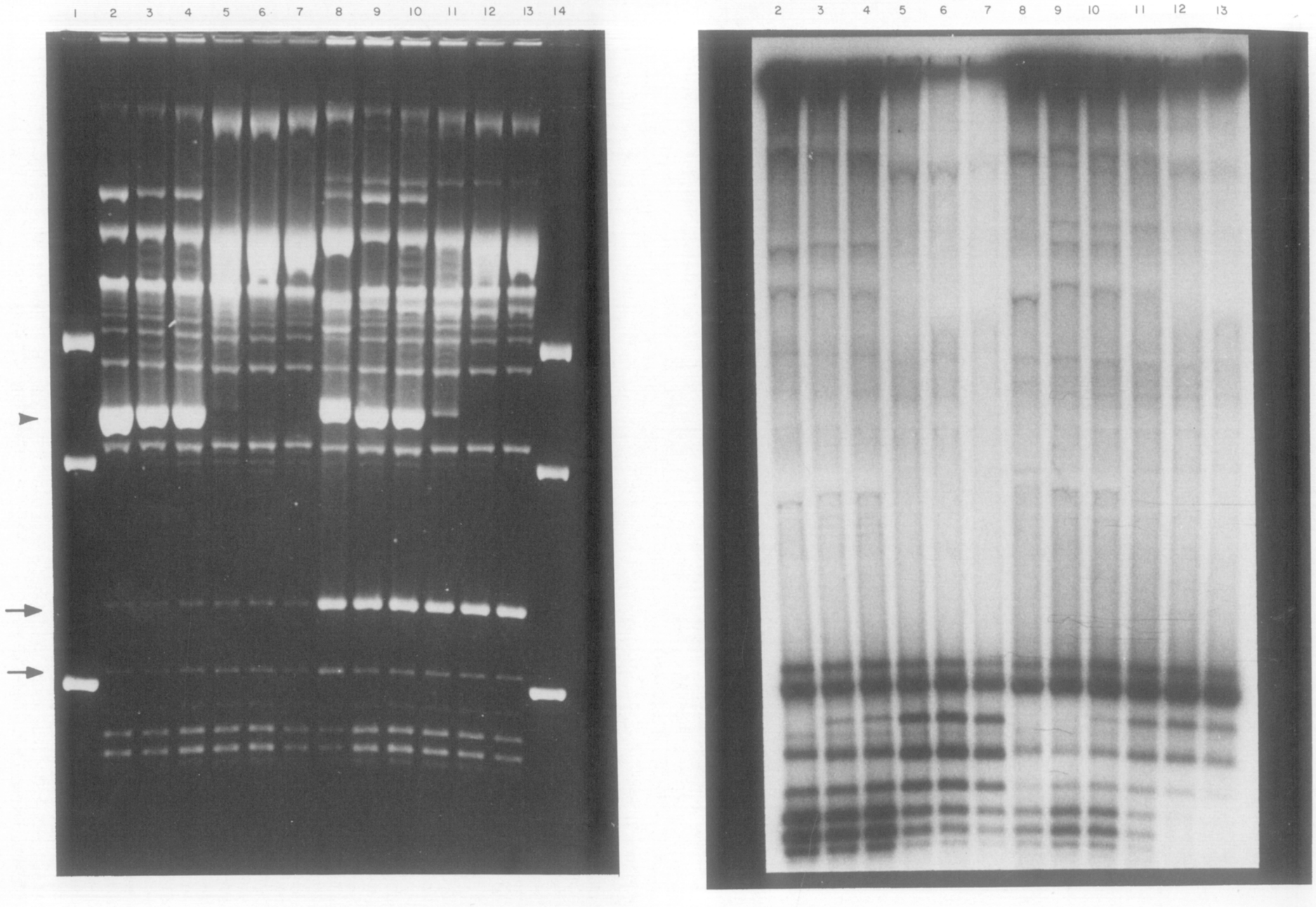
labeled Δ31 strands, as shown in Figure 1(a), before and after incubation with *E. coli* DNA topoisomerase I. Clearly, the positively supercoiled heteroduplex molecules are relaxed by the enzyme.

When the same experiment is carried out with *M. luteus* DNA topisomerase I, similar results are observed except that partial relaxation of the positively supercoiled homoduplex molecules occurs, confirming the previous finding that the *M. luteus* enzyme is less dependent on negative supercoiling than its *E. coli* counterpart (Kung & Wang, 1977).

In the experiment shown in Figure 2, the heteroduplex molecules contain both combinations of strands; thus the single-stranded loop can be of one sequence or of its complement. We have examined whether the rates of relaxation of the two different looped structures differ significantly by using the pair of differentially labeled substrates described in section (a) above. The results are shown in Figure 3. Each reaction mixture contained unlabeled, negatively supercoiled pJW270 carrier DNA (10 μg/ml) and positively supercoiled heteroduplex prepared from Δ4 (*Bgl*) and Δ31 (ΔRI-*Bam*), according to the scheme shown in Figure 1(b). For samples run in lanes 2 to 7 of the ethidium-stained gel shown in Figure 3(a), the strand labeled is the one depicted in Figure 1(b), heteroduplex Δ4/Δ31′; for samples run in lanes 8 to 13 of the same gel, the other strand is labeled, labeling heteroduplex Δ4′/Δ31. For either set of six samples, the amount of *E. coli* DNA topoisomerase I increases approximately linearly from 0 μg/ml (left-most lane) to 2·5 μg/ml (right-most lane), and all samples were incubated for ten minutes at 37°C. Lanes 1 and 14 contained linear DNAs of known lengths, which were used as size-markers.

In the ethidium-stained gel shown in Figure 3(a), the major species in the untreated sample run in lane 2 is negatively supercoiled monomeric pJW270 DNA (marked by an arrowhead in the left-hand margin). Treatment with increasing amounts of bacterial topoisomerase I shifts this band toward the top of the gel, which can be seen clearly in lanes 5 to 7 and 11 to 13. The family of topoisomers below the fastest-moving linear marker in lane 1 are of interest, consisting of positively supercoiled monomeric Δ31 (ΔRI-*Bam*) homoduplex DNA after a cycle of denaturation–renaturation (since unlabeled Δ31 (ΔRI-*Bam*) DNA was present in excess during the preparation of the heteroduplex, covalently closed molecules visible on the ethidium-stained gel are mainly renatured Δ31 (ΔRI-*Bam*) homoduplex DNA, in addition to the higher molecular weight pJW270 DNA. Even at the highest concentration of enzyme used, these positively supercoiled homoduplex molecules are not relaxed, as evidenced by the lack of change of their mobilities across the lanes.

When the ethidium-stained gel (Fig. 3(a)) is compared with the autoradiograph of the same gel (Fig. 3(b)), it is clear that the positively supercoiled heteroduplex molecules are relaxed under the same



(a) (b)

Fig. 3

conditions in which negatively supercoiled pJW270 DNA is relaxed (compare lanes 5 to 7 and 11 to 13 of the two patterns; lanes 1 and 14 do not show on the autoradiograph, because of the absence of labeled DNA). Furthermore, the two positively supercoiled looped-structures are relaxed at comparable rates (compare lanes 5 to 7 with the corresponding lanes 11 to 13).

The degreee of positive supercoiling of the heteroduplex substrates used in the experiments described above is not high. The data shown in Figures 2 and 3 show a change of about three superhelical turns or −0·015 in specific linking difference (or superhelical density; see Wang, 1980, 1983; Wang *et al.*, 1983) upon relaxation with the topoisomerase. To see if more highly positively supercoiled substrate containing a single-stranded loop can be relaxed, we resorted to the use of the intercalating dye ethidium in the reaction mixture (Wang, 1971). Figure 4 depicts the results of such an experiment. As before, lanes 1 and 8 contained size-markers, and lane 2 contained the control in which the topoisomerase is omitted during incubation. Lanes 3 to 7 in Figure 4(a) show the gel-electrophoretic patterns of samples incubated with *E. coli* DNA topoisomerase I in the absence (lane 3) or presence of increasing amounts of ethidium bromide ranging from 0·3 μg/ml (lane 4) to 19 μg/ml (lane 7).

The negatively supercoiled pJW270 DNA serves as an indicator for the binding of ethidium in this experiment. In the absence of ethidium (lane 3), the covalently closed DNA after incubation with the enzyme runs slightly ahead of the nicked species. The presence of 0·9 μg ethidium/ml during treatment with the topoisomerase gives a product that migrates faster in the gel, signifying the binding of ethidium to the negatively supercoiled DNA during incubation and hence yielding a product with a lower linking number than that produced in the absence of ethidium. This reduction in linking number is more apparent for the sample run in lane 5, which was incubated with the topoisomerase in the presence of 3 μg ethidium bromide/ml. For the samples run in lanes 6 and 7, the amounts of ethidium during incubation were probably higher than that needed to relax the plasmid DNA completely. The DNA was therefore probably positively supercoiled during treatment with the topoisomerase, and hence its linking number is not altered by the topoisomerase. The same is true for the positively supercoiled Δ31 (ΔRI-*Bam*) homoduplex DNA: the presence of ethidium makes it even more positively supercoiled, and hence refractive to the topoisomerase.

The autoradiograph of the same gel shows, however, that relaxation of the positively supercoiled heteroduplex occurs even in the presence of ethidium. From the control shown in lane 2, to the samples run in lanes 3 and 4, the predominating topoisomers shift progressively upward, indicating continued reduction in their linking numbers by the topoisomerase. For the sample shown in lane 5, two new bands are discernible, which are most probably negatively supercoiled topoisomers. Thus it appears that for the looped heteroduplex structure, some relaxation by the bacterial topoisomerase occurs even when ethidium is present to increase the degree of positive supercoiling of the DNA substrate. The presence of high concentrations of ethidium seems inhibitory, however, as evidenced by the samples shown in lanes 6 and 7: more of the starting topoisomers remain unaltered in these samples.

(c) *Nucleolytic probing of the complex of bacterial topoisomerase I and the looped DNA structures*

The demonstration that positively supercoiled heteroduplex DNA containing a short single-stranded loop is relaxed by bacterial DNA topoisomerase I shows that the enzyme can form an active complex with the looped DNA structure. The binding of the enzyme to the looped region is confirmed by nucleolytic and chemical probing of

Figure 3. Relaxation by *E. coli* topoisomerase I of strand-specifically labeled positive supercoils containing single-stranded loops. (a) A photograph taken after ethidium staining and (b) an autoradiograph of the same 1% (w/v) agarose gel, electrophoresed in TBE buffer (see the legend to Fig. 2) at room temperature for 10 h at 4 V/cm, are shown. Lanes 1 and 14 contain unlabeled linear DNA markers. Samples run in lanes 2 to 13 all contained 1 μg unlabeled, negatively supercoiled pJW270 DNA in each reaction mixture as an internal control. Lanes 2 to 7 also contained ^{32}P-labeled, positively supercoiled Δ4/Δ31′ heteroduplexes, and lanes 8 to 13 contained ^{32}P-labeled, positively supercoiled Δ4′/Δ31 heteroduplexes. The position of negatively supercoiled monomeric pJW270 DNA before incubation with the topoisomerase is indicated by the arrowhead to the left of lane 1 in Fig. (a). The 2 arrows below the arrowhead mark the positions of nicked and linear Δ31 (ΔRI-*Bam*) DNA. The group of topoisomers below the marked linear band in (a) are positively supercoiled homoduplex Δ31 (ΔRI-*Bam*) DNA. Bands other than those described above are nicked or multimeric species of the various plasmids and the side-products of the annealing reactions. The samples were incubated with *E. coli* topoisomerase I at the following concentrations: lanes 2 and 8, no topoisomerase; lanes 3 and 9, 0·03 μg/ml; lanes 4 and 10, 0·1 μg/ml; lanes 5 and 11, 0·3 μg/ml; lanes 6 and 12, 1·0 μg/ml and lanes 7 and 13, 2·5 μg/ml. Heteroduplexes Δ4/Δ31′ were made by annealing 4 μg of Δ4 (*Bgl*) DNA that had been cut with *Bam*HI, 5′ labeled, then cut with *Bgl*II and 40 μg of Δ31 (ΔRI-*Bam*) DNA that had been cut with *Pst*I. Heteroduplex Δ4′/Δ31 was made by annealing 4 μg of Δ4 (*Bgl*) DNA that had been first cut with *Bgl*II, 5′ labeled, and then cut with *Bam*HI, and 40 μg of *Pst*I-cut Δ31 (ΔRI-*Bam*) DNA. Since the unlabeled DNA was present in excess during the renaturation step, essentially all the labeled strands were in heteroduplex forms. The mixtures were dialyzed and incubated with *E. coli* ligase in the presence of 0·1 M-ammonium chloride. All other reactions were as described in Materials and Methods.

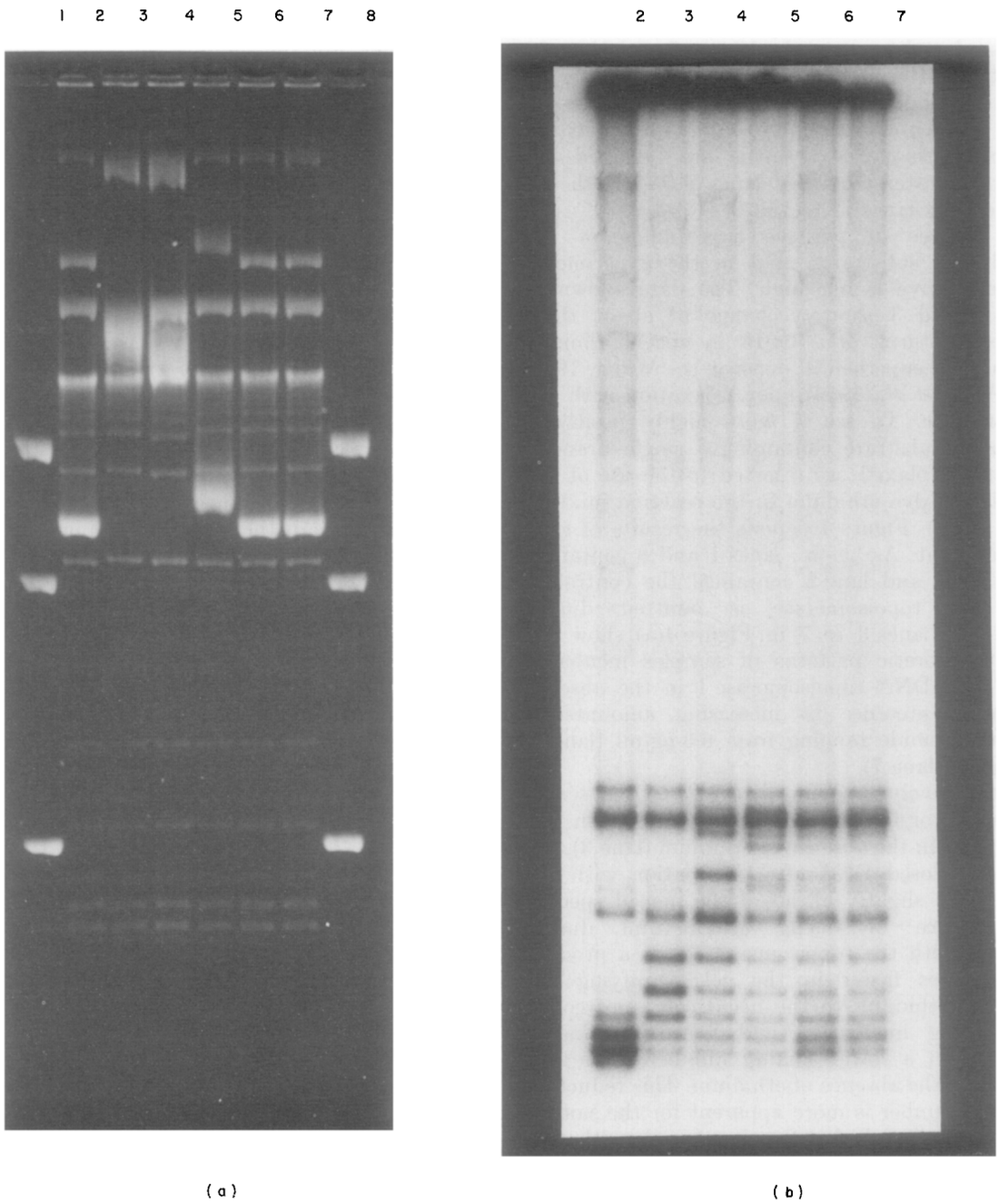


Figure 4. Relaxation of positively supercoiled Δ4/Δ31′ heteroduplexes by *E. coli* topoisomerase I in the presence of ethidium bromide. (a) A photograph of a 1% (w/v) agarose gel, taken after ethidium staining. (b) An autoradiograph of the same gel. Electrophoresis was done in TBE buffer for 10 h at 4 V/cm. Lanes 1 and 8 contain unlabeled linear size markers. Lanes 2 to 7 display samples that contained 2 μg of unlabeled, negatively supercoiled pJW270 plasmid DNA and ^{32}P-labeled, positively supercoiled Δ4/Δ31′ heteroduplexes, prepared as described in the legend to Fig. 3. Samples 3 to 6 were incubated with 2·5 μg *E. coli* topoisomerase I/ml in the presence of the following concentrations of ethidium bromide: none (lane 3), 0·3 μg/ml (lane 4), 0·9 μg/ml (lane 5), 3·0 μg/ml (lane 6), and 9·0 μg/ml (lane 7).

uniquely end-labeled restriction fragments containing the looped region.

Figure 5(a) depicts the patterns of cleavage by staphylococcal nuclease of uniquely end-labeled homoduplex and heteroduplex molecules in the presence and absence of bacterial DNA topoisomerase I. For the samples shown in lanes 1 to 4, both strands of the Δ4/Δ31′ heteroduplex are examined. That is, the *Bam*HI–*Hae*III restriction fragments derived from the Δ4/4′ homoduplex and the Δ4/Δ31′ heteroduplex with the Δ4 strand labeled at its 5′ *Bam*HI end are compared; for the samples shown in lanes 5 to 8, *Bam*HI–*Hae*III fragments derived from the Δ31/Δ31′ homoduplex and the Δ4/Δ31′ heteroduplex with the Δ31′ strand labeled at its 3′ *Bam*HI end are compared (see Fig. 5(b) for the sequences of the fragments used).

Clearly, staphylococcal nuclease can detect the

presence of the looped structure. Comparing the patterns shown in lanes 1 and 3 shows that cleavage at a number of sites in the Δ4 strand is much enhanced in the Δ4/Δ31′ heteroduplex relative to the Δ4/Δ4′ homoduplex. These enhanced sites fall both within the 27 nucleotide section in Δ4 that is absent in Δ31 (demarcated in between lanes 2 and 3 in Fig. 5(a)) and immediately outside this mismatch in an A+T-rich sequence. The nuclease also cleaves preferentially in the Δ31′ strand across the 27-nucleotide insert (compare the patterns of lanes 5 and 7).

When the homoduplex Δ4/Δ4′ or Δ31/Δ31′ is incubated first with bacterial DNA topoisomeraase I, the pattern of cleavage by staphylococcal nuclease is unaltered (compare lanes 1 and 2, and lanes 7 and 8), indicating the absence of a specific complex. With the heteroduplex, however, the binding of the topoisomerase gives a distinct footprint (Galas & Schmidtz, 1978) in both strands (compare lanes 3 and 4, and lanes 5 and 6).

The cleavage experiments with staphylococcal nuclease and heteroduplex Δ4/Δ31′ are summarized in Figure 5(b). For simplicity. the 27-nucleotide appendix of the Δ4 strand in the heteroduplex is displayed as a simple loop. In the absence of the topoisomerase, the concentration of staphylococcal nuclease cleavage sites within this loop and in the A+T-rich region near its base is consistent with the known preferential cleavage of single-stranded nucleic acids by this nuclease (Laskowski, 1971; Drew, 1984). The unique footprint of the topoisomerase in this region confirms the preferential binding of the enzyme to the looped region. Topoisomerase binding increases the susceptibility of some bonds to staphylococcal nuclease cleavage; these enhancements are also shown in Figure 5(b). The entire footprint falls in a region from nucleotides 75 to 129, as numbered in Figure 5(b). Similar experiments were carried out with the Δ4′/Δ31 heteroduplex and the results are summarized in Figure 5(c).

The preferential binding of bacterial DNA topoisomerase I to looped structures is seen also by pancreatic DNase I footprinting of a family of heteroduplexes containing loops ranging from 13 to 27 nucleotides in length (Fig. 6(a)). DNase I cuts duplex DNA more readily than single-stranded DNA (Laskowski, 1971), and cuts poorly within or near single-stranded loops (Drew, 1984). This specificity can be seen in Figure 6(a). All heteroduplex fragments used in this experiment are labeled at the 5′ end of the longer strand. Because of the particular way the set of plasmids is constructed, the sequences of the 5′ side of the loops are identical. The sequences of the 3′ side of the loops vary among the heteroduplexes. Relative to the Δ4 loop shown in Figure 5(b), nucleotides 116 to 118 are deleted in Δ7, 110 to 118 are deleted in Δ13, and 105 to 118 are deleted in Δ18. For the ease of comparing the patterns shown in Figure 6(a), a prominent DNase I cleavage site between nucleotides 131 and 132 in all structures is connected by lines between the lanes. The formation of a specific complex between *M. luteus* DNA topoisomerase I and the heteroduplex structures is evident from the footprints shown in Figure 6(a). The common 5′ side sequences of the loops are similarly protected for the family of four heteroduplexes. Although the size of the single-stranded region varies in this family, the approximate size of the region protected by topoisomerase binding does not change. A summary of the DNase I footprinting results for both strands of the Δ4/Δ31′ structure is shown in Figure 6(b).

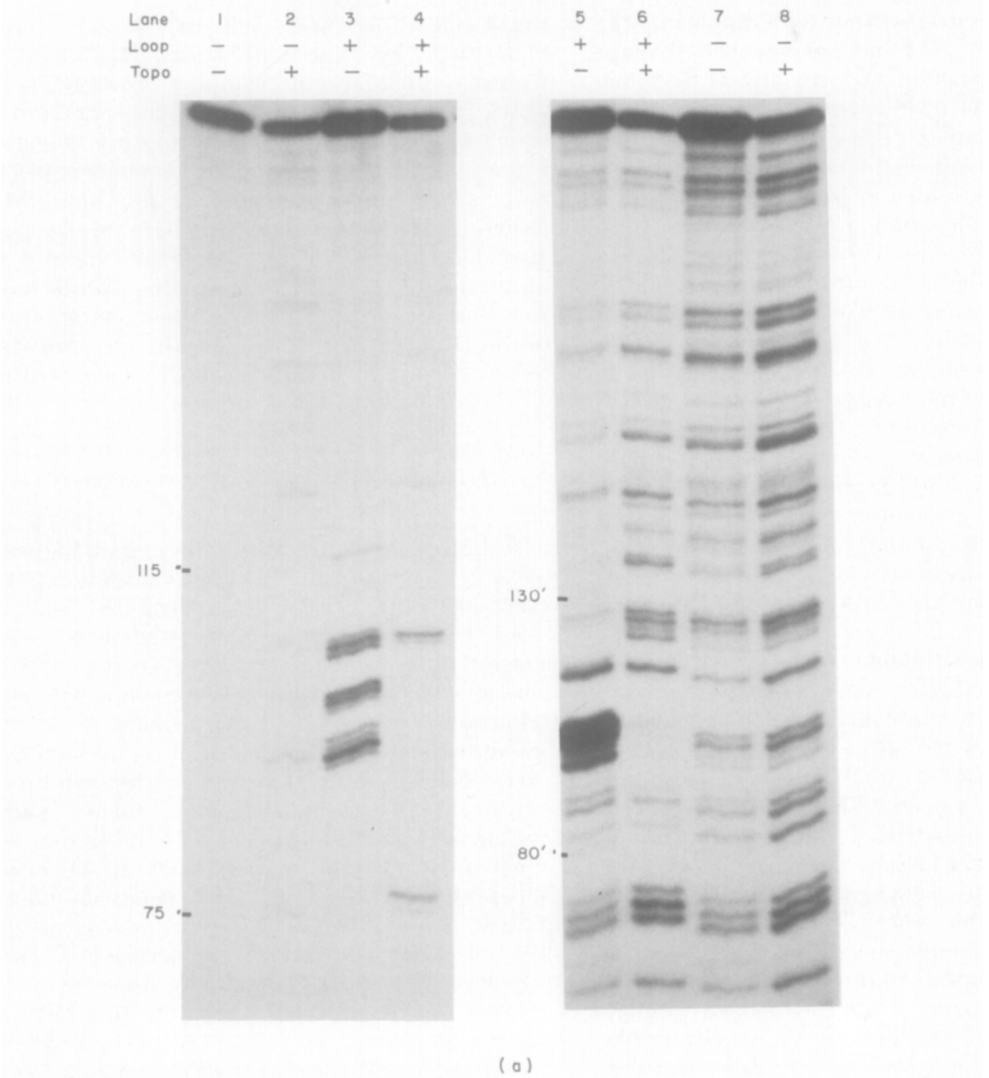
(d) *Chemical probing of complexes of looped Δ4/Δ31 heteroduplex structures and bacterial DNA topoisomerase I*

Although in Figure 5(b) and (c) and Figure 6(b) we have depicted the 27-nucleotide section of Δ4 in the heteroduplex as a single-stranded loop, it is likely that the loop is more structured. This is suggested by probing the accessibility of the N^3 position of the cytosines with dimethyl sulfate (Peattie & Gilbert, 1980; Kirkegaard *et al.*, 1983). As shown before, the N^3 position of cytosine is preferentially methylated by dimethyl sulfate if the cytosine is in a single-stranded region, and the preferential cleavage of the DNA backbone at the sites of N^3-cytosine methylation can be effected. When such experiments are carried out with the Δ4/Δ31 heteroduplexes, few cytosine residues in the 27-nucleotide mismatch are accessible. In the presence of bacterial DNA topoisomerase, the two cytosine residues at positions 109 and 110 of the structure shown in Figure 5(b) become more accessible to this chemical probe, suggesting that the binding of the topoisomerase alters the structure of the loop. For the looped structure shown in Figure 5(c), again few cytosine residues in the 27-nucleotide loop are accessible, and the addition of the topoisomerase enhances the reactivity of cytosine 113′. These and the staphylococcal nuclease footprinting experiments indicate that the region containing the 27-nucleotide appendix in each heteroduplex, though not structureless, is readily perturbed to exhibit single-stranded characteristics.

The probing experiments with dimethyl sulfate reveal also that the reactivities of two guanine residues at positions 91 and 102 in the structure shown in Figure 5(b) and the guanine at position 98′ in the structure shown in Figure 5(c) are diminished by the binding of *M. luteus* DNA topoisomerase I. No other changes in the dimethyl sulfate reactivities of guanines or adenines on either strand of either of the two looped molecules were detected in the presence of topoisomerase.

4. Discussion

Several observations have been made that suggest that the active complex between bacterial


Lane 1 2 3 4 5 6 7 8
Loop – – + + + + – –
Topo – + – + – + – +
115
75
130'
80'
(a)

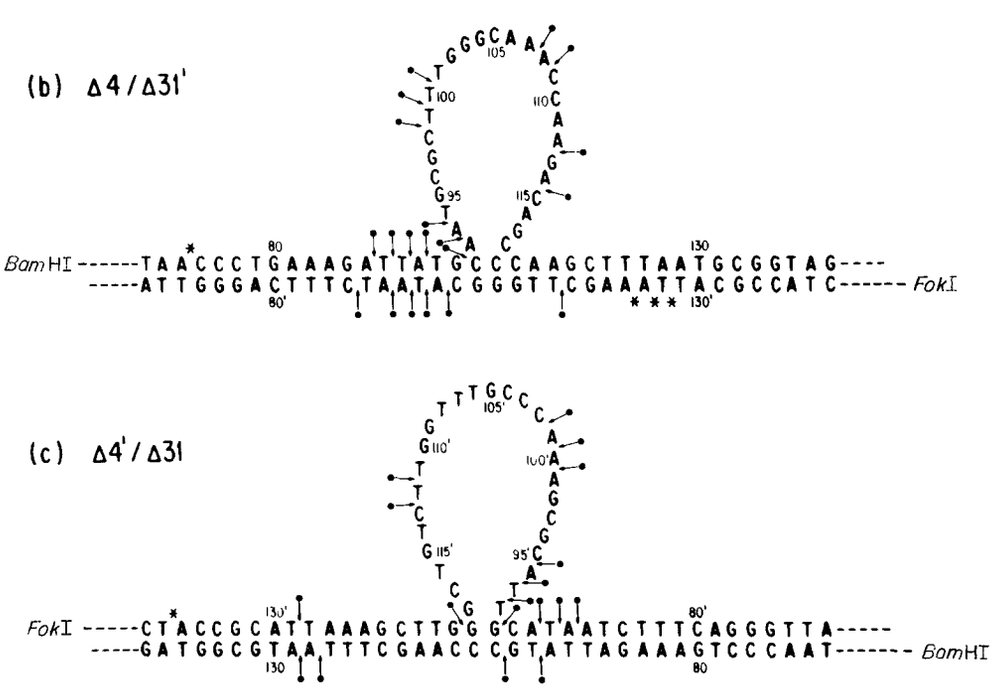
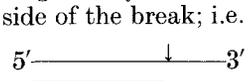

(b) Δ4/Δ31'
BamHI
FokI
(c) Δ4'/Δ31
FokI
BamHI

Fig. 5

DNA topoisomerase I and its substrate might contain a short DNA segment in a single-stranded conformation. The specificity of the enzyme in relaxing negatively supercoiled DNA, the strong inhibition of this reaction by single-stranded DNA fragments (Wang, 1971), and the knotting and linking of single-stranded DNA rings (Liu *et al.*, 1976; Kirkegaard & Wang, 1978), nicked double-stranded DNA rings (Tse & Wang, 1980; Brown & Cozzarelli, 1981) and gapped duplex rings (Low *et al.*, 1984) are all consistent with this view. Furthermore, studies of bacterial DNA topoisomerase I-mediated cleavage of a family of gapped DNA molecules of defined sequences indicate that the site of cleavage is located in the single-stranded region adjacent to double-stranded DNA on the 5′ side of the break; i.e. for a structure:



the cleavage site tends to be near the position marked by the arrow (see Kirkegaard *et al.*, 1984).

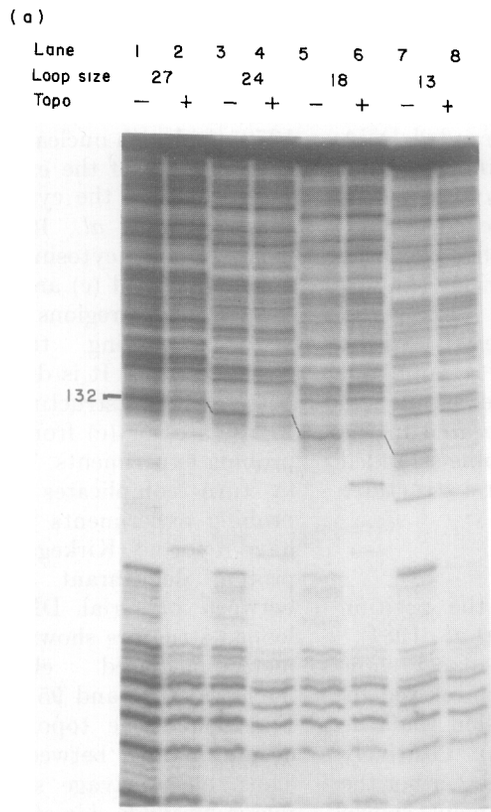
The experiments described in this paper show that positively supercoiled DNA can be relaxed by bacterial DNA topoisomerase I, provided that the DNA contains a single-stranded loop. Positively supercoiled heteroduplex rings containing either the loop shown in Figure 5(b) or its complement, shown in Figure 5(c), are relaxed at rates comparable to those for the relaxation of negatively supercoiled homoduplex rings. These experiments provide strong support for the hypothesis that the specificity of the bacterial topoisomerase for negatively supercoiled DNA is due to the disruption of a short DNA segment in the activated enzyme–substrate complex (Wang, 1971). An alternative interpretation of the specificity of bacterial DNA topoisomerase I for negatively supercoiled DNA is that the enzyme recognizes the sign of crossovers of duplex DNA (Wang, 1971). This interpretation is inconsistent with the results presented here and with the finding that a racemic mixture of double-stranded DNA knots is generated when nicked duplex DNA rings are treated with the enzyme under appropriate conditions (Krasnow *et al.*, 1983).

The specific binding of bacterial DNA topoisomerase I to the looped structures is demonstrated by the probing of the enzyme–DNA complex with dimethyl sulfate (Gilbert *et al.*, 1975; Johnsrud, 1978), and with nucleases (Galas & Schmidtz, 1978). Examination of the extents of methylation at the N^3 position of the cytosines in the looped regions (Kirkegaard *et al.*, 1983), however, suggest that most of the cytosines in the loops shown in Figure 5(b) and (c) are probably in paired regions, though such regions are readily perturbed by protein binding to exhibit single-stranded characteristics. It is difficult, however, to deduce a better-defined structure of the loop depicted in Figure 5(b) or (c) from the nuclease and chemical probing experiments. This uncertainty in structure in turn complicates the interpretation of the probing experiments with the topoisomerase. We have reported (Kirkegaard *et al.*, 1984) that when a protein denaturant is added to the complex between bacterial DNA topisomerase I and the looped structure shown in Figure 5(b), the topoisomerase-mediated cleavage occurs between nucleotides 94 and 95. For the structure shown in Figure 5(c), the topoisomerase-mediated cleavage occurs mainly between nucleotides 93′ and 94′. Thus, one cleavage site has an adjacent duplex region on the 5′ side of the break, and the other has an adjacent duplex on the 3′ side of the break. These locations are difficult to explain without considering the secondary structures of the loops.

Nevertheless, the chemical and nuclease protection patterns shown in Figure 5(b) and (c) and Figure 6 suggest that the topoisomerase is interacting with the duplex regions flanking a loop as well as the loop itself, and that the site of breakage–rejoining falls within a single-stranded loop. This in turn suggests that when the DNA is in a transiently cleaved state. the topoisomerase is bridging both sides of the cleavage site *via* a covalent linkage to the 5′ end and non-covalent interactions with both the 3′ and 5′ sides of the broken strand. This type of enzyme bridging has been postulated and discussed (Brown & Cozzarelli, 1981; Wang, 1983; Kirkegaard *et al.*, 1984). The

Figure 5. Staphylococcal nuclease footprints of topoisomerase I bound to loop-containing DNA fragments. The 2 autoradiographs in (a) display staphylococcal nuclease digestion patterns of 3 different DNA fragments. Lanes 1 and 2 show nuclease digestions in the presence and absence of *M. luteus* DNA topoisomerase I of a 255 base-pair homoduplex *Bam*HI–*Hae*III fragment of Δ4 DNA, 5′ labeled at the *Bam*HI site. Lanes 3 and 4 show the same digestions of the heteroduplex Δ4/Δ31′ *Bam*HI–*Hae*III fragment, 5′ labeled at the *Bam*HI site to show the looped Δ4 strand. Lanes 5 and 6 also show the Δ4/Δ31′ heteroduplex fragment, now 3′-labeled at the *Bam*HI site to display the unlooped Δ31′ strand. Lanes 7 and 8 display the 226 base-pair homoduplex Δ31 *Bam*HI–*Hae*III fragment, 3′-labeled at the *Bam*HI site to show the Δ31′ strand. Incubations of DNA fragments with *M. luteus* topoisomerase I, nuclease treatment and sequencing gel electrophoresis are described in Materials and Methods; the samples shown in lanes 5 to 8 were digested with a 5-fold higher concentration of staphylococcal nuclease than the samples in lanes 1 to 4.

The staphylococcal nuclease footprint of topoisomerase I bound to the Δ4/Δ31′ loop is summarized in (b). Arrows show the sites of enhanced nuclease cleavage in the looped structure, compared to the analogous homoduplex fragment. Filled circles designate phosphodiester bonds that are protected from nuclease cleavage by topoisomerase binding Asterisks show phosphodiester bonds that become more susceptible to staphylococcal nuclease cleavage in the presence of topoisomerase. Nucleotides are numbered from the *Bam*HI site; nucleotides 92 to 118 are deleted in the strand derived from Δ31 DNA. Similar experiments performed with the Δ4′/Δ31 looped fragment are summarized in (c). Sequence hyphens have been omitted for clarity.



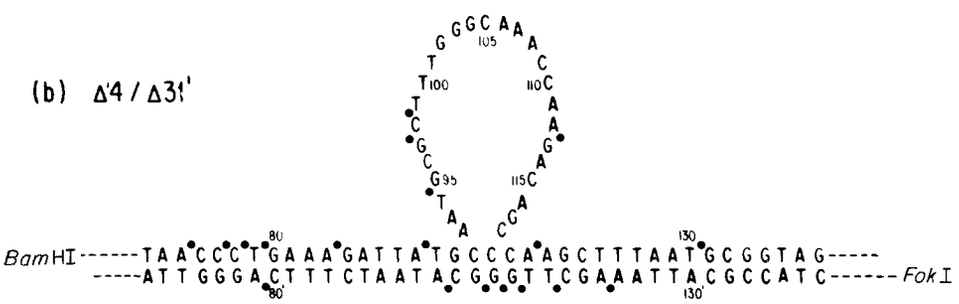


Figure 6. Pancreatic DNase I footprints of topoisomerase I bound to loop-containing DNA fragments. DNase I digestions of 4 different looped DNA fragments, in the presence and absence of *M. luteus* topoisomerase I. are shown in the autoradiograph in (a). All 4 DNAs are *Bam*HI–*Hae*III fragments, 5′ labeled at the *Bam*HI site to show the looped strand. Lanes 1 and 2 display nuclease cleavage of the Δ4/Δ31′ heteroduplex; lanes 3 and 4, Δ7/Δ31′; lanes 5 and 6, Δ13/Δ31′; and lanes 7 and 8, Δ18/Δ31′. Relative to Δ4, nucleotides 116 to 118 are deleted in Δ7 DNA, 110 to 118 are deleted in Δ13 DNA. 105 to 118 are deleted in Δ18, and 92 to 118 are deleted in Δ31 DNA. The sizes in base-pairs of the mispaired regions in the heteroduplexes are shown in the Figure.

The DNase I footprint of *M. luteus* topoisomerase I bound to the Δ4/Δ31′ loop is summarized in (b). Phosphodiester bonds that are relatively protected from nuclease cleavage in the presence of the topoisomerase are marked by filled circles. Sequence hyphens have been omitted for clarity.

interpretation of our present chemical and nuclease probing results is complicated, however, by the possibility that more than one enzyme molecule might be bound to a looped structure. The similar sizes of topoisomerase binding regions in fragments containing different-sized loops suggests that the same number of enzyme molecules, possibly one or two, is bound to each structure.

The results presented in this paper add strong evidence that bacterial DNA topoisomerase I prefers partially single-stranded substrates, and that the facilitation of unpairing a short segment of

DNA by negative supercoiling determines the specificity of the enzyme in its relaxation of supercoiled DNA. *Escherichia coli* DNA topoisomerase III, the other known type I bacterial topoisomerase (Dean *et al.*, 1983; Pastorcic, 1982), also relaxes only negatively supercoiled DNA and the relaxation reaction is inhibited by single-stranded DNA (Srivenugopal *et al.*, 1984). By analogy then, it is most likely that this enzyme also acts preferentially on partially single-stranded DNA. Finally, since single-stranded DNA is present at replication forks, and in recombination and repair intermediates, the preference of type I bacterial topoisomerases for partially single-stranded substrates might be biologically significant.

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