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Novel topologically knotted DNA from bacteriophage P4 capsids: studies with DNA topoisomerases

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ABSTRACT

DNA molecules isolated from bacteriophage P4 are mostly linear with cohesive ends capable of forming cicular and concatemeric structures. In contrast, almost all DNA molecules isolated from P4 tailless capsids(heads) are monomeric DNA circles with their cohesive ends hydrogen-bonded. Different from simple DNA circles, such P4 head DNA circles contain topological knots. Gel electrophoretic and electronmicroscopic analyses of P4 head DNA indicate that the topological knots are highly complex and heterogeneous. Resolution of such complex knots has been studied with various DNA topoisomerases. The conversion of highly knotted P4 DNA to its simple circular form is demonstrated by type II DNA topoisomerases which catalyze the topological passing of two crossing double-stranded DNA segments{Liu, L. F., Liu, C. C. & Alberts, B. M.(1980) Cell, 19, 697-707}. The knotted P4 head DNA can be used in a sensitive assay for the detection of a type II DNA topoisomerase even in the presence of excess type I DNA topoisomerases.

INTRODUCTION

DNA topoisomerases are characterized by their *in vitro* reactions, which interconvert various topological isomers of DNA(for a review, see ref. 1). Recently, two types of DNA topoisomerases have been identified, which differ fundamentally in their reaction mechanisms(2-8, for a review, see ref. 9). Type I DNA topoisomerases are enzymes which introduce transient, single-stranded DNA breaks. *E. coli* topoisomerase I(10), Lambda phage *int* protein (11) and eukaryotic type I topoisomerases(nicking-closing enzymes)(12,13) are examples of such type I enzymes. Type II DNA topoisomerases are enzymes which can catalyze the topological passing of two crossing double-stranded DNA segments, presummably by introducing a transient, enzyme-bridged, double-stranded break on one of the crossing DNA segments(2-9). Although both types of enzymes can alter the linking number of a covalently-closed circular DNA, the linking number change characteristically distinguishes the two types of DNA topoisomerases. Type I DNA topoisomerases can change the DNA linking number in steps of one, whereas type II DNA topoisomerases characteristically alter the linking number of a closed-circular DNA only in steps of two, con-

sistent with the strand passing mechanism(2-9). Recently, Type II DNA topoisomerases have been detected or purified from several eukaryotic organisms (2, 6-8, 14). Topologically knotted double-stranded circular DNA is an ideal substrate for assaying such type II DNA topoisomerases which untie knots efficiently by the strand passing mechanism(2-9). Although a small amount of knotted DNA molecules can be produced either by treatment of the plasmid DNA with highly purified T4 DNA topoisomerase or by an in vitro phage recombination system(5), a more convenient source of knotted DNA is definitely needed.

Recently, highly knotted double-stranded DNA has been isolated from bacteriophage P2 capsids(15). Unfortunately, the large genome size of P2 DNA (33 kb) made it difficult to develop an easy gel electrophoresis assay for the removal of knots. P4 is a satellite bacteriophage of P2(16). P4 DNA (11 kb) is only one third the size of P2 DNA, but has the same 19-base cohesive ends(17). P4 requires all the known head, tail and lysis genes of the P2 helper phage for a productive infection(18). We report here that the DNA isolated from the tailless capsids(heads) of P4 contain highly complex knots. We have developed a simple gel electrophoresis assay for the type II DNA topoisomerase using such a knotted DNA circle.

MATERIALS AND METHODS

Enzymes.

T4 DNA topoisomerase was purified from T4 regA, am N55, am H39-infected E. coli D110 cells as described previously(19). Both forms of type I DNA topoisomerase(100 K and 67 K) and the type II DNA topoisomerase from HeLa cells were prepared as described(13,14). E. coli DNA topoisomerase I was a gift from Professor James C. Wang. Eco RI restriction endonuclease was purchased from Bethesda Research Laboratories.

Nucleic Acids.

Phage PM2 DNA was purified by the published procedure(20). Plasmid pBR322 DNA was purified by phenol deproteinization of a clear lysate followed by CsCl/ethidium bromide equilibrium centrifugation. P4 phage DNA and P4 head DNA were obtained by phenol deproteinization(three times with phenol followed by ether extraction and dialysis into 10 mM Tris·HCl, pH 8.0, 1 mM Na_3EDTA) of the purified mature phages and tailless capsids respectively(15).

Preparation of P4 phages and P4 tailless capsids.

The procedure is a modification of Barrett et al.(21). P4 vir1 del10(22) was used to infect E. coli C-117(P2)(23) at m. o. i. of about 5 at 37°C in a modified LB broth(21). One hour after infection, EGTA(Ethyleneglycol-bis-

(β-aminoethyl ether) N, N'-Tetraacetic acid) was added to a final concentration of 5 mM to block phage reabsorption. After lysis, the cell debris was removed by centrifugation. The mature phages and the tailless capsids were precipitated by 10%(w/v) PEG-6000(polyethylene glycol) and 0.5 M NaCl. The pellet was collected by centrifugation and then extracted several times with P4 phage buffer(1% NH_4OAc, 10 mM Tris·HCl, pH 7.2, 80 mM $MgCl_2$)(21). Solid CsCl was added to a density of 1.42 g cm^{-3} and the solution was centrifuged to sedimentation equilibrium. The denser band, containing tailless capsids, and the lighter band, containing viable phages, were collected separately and further purified by two cycles of differential centrifugation in P4 phage buffer. P4 phages and P4 heads were then resuspended in P4 phage buffer at about A_{260} of 10.

Electron microscopy.

The aqueous spreading procedure of Davis et al.(24) was used. DNA molecules were visualized in a Zeiss 10B electron microscope.

Topoisomerase assays.

All reactions(20 µl each) contained 50 mM Tris·HCl, pH7.7, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 µg/ml bovine serum albumin, 20 µg/ml of a DNA substrate and various amounts of DNA topoisomerase (1 mM ATP may be present in certain assays as indicated). After 30 min at 30°C, the reactions were terminated by the addition of 5 µl of a SDS-Ficoll stop solution as described before(13). Agarose gel electrophoresis in a horizontal tank was done as described(13).

RESULTS

DNA from P4 tailless capsids is topologically knotted.

Infection of the P2 lysogen(*E. coli* C-117) with bacteriophage P4(P4 *vir*1 *del*10) reproducibly yielded roughly equal amounts of the mature phages and tailless capsids(heads)(21). The majority of P4 DNA from mature phages (phage DNA) appeared normal(linear and circular forms) under the electron microscope, whereas nearly all P4 DNA from the tailless capsids appeared as condensed structures(Fig. 1A). Since DNA from P2 heads is knotted and also appears as condensed structures when examined by electron microscopy(15), we expected the observed condensed structures of P4 DNA to be knotted with the two cohesive ends hydrogen-bonded. The P4 phage DNA and head DNA were further analyzed by agarose gel electrophoresis. Consistent with our interpretation, P4 head DNA migrated in the gel as a smear faster than the simple circular form of P4 DNA(labeled "circle"; see Fig. 2A, lane b). P4 phage

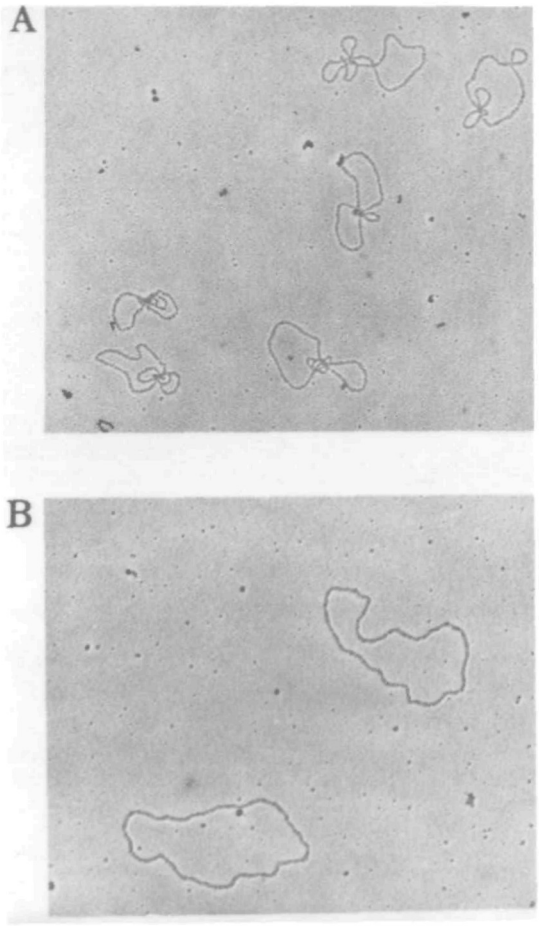

Fig. 1. Electron micrographs of the P4 head DNA before and after type II DNA topoisomerase treatment. (A): P4 head DNA. (B): P4 head DNA treated with HeLa topo II(see legend of Fig. 2).

DNA, as expected, migrated in the gel as linear, circular and various concatemeric forms(Fig. 2B, lane d). About 20% of the P4 phage DNA, however, also migrated as a smear faster than the circle(Fig. 2A, lane d). To prove that these electrophoretic variants(fast-migrating smears) are topologically knotted DNA, both P4 phage DNA and head DNA were treated with Eco RI restriction endonuclease(Fig. 2A, lanes c & a respectively). As expected, Eco RI treatment converted both DNA samples into three identical fragments as judged by gel electrophoresis(Fig. 2A, lanes a & c)(22). It is interesting

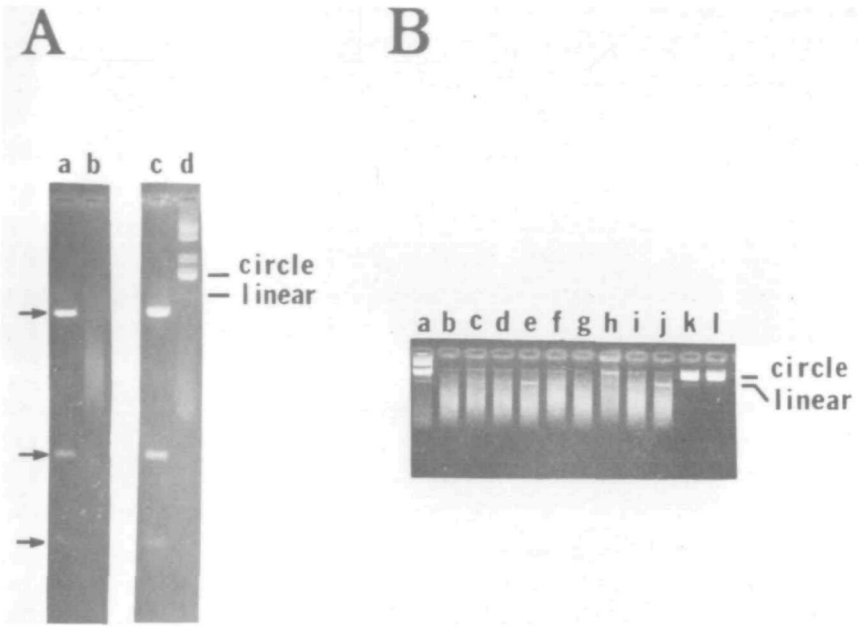

Fig. 2. Gel electrophoretic analysis of the P4 phage DNA and P4 head DNA. (A): P4 head DNA(0.4 μg)(b) and P4 phage DNA(0.3 μg)(d) were each treated with 2 units of Eco RI restriction endonuclease. Lanes a and c show the restricted products of P4 phage DNA(lane d) and P4 head DNA(lane b) respectively. The arrows indicate the DNA fragments generated by the restriction reaction. Samples were run on a 0.7% agarose gel as described in Materials and Methods. (B): P4 head DNA was treated with various DNA topoisomerases; (a) P4 phage DNA control, (b) P4 head DNA control, (c) head DNA + 0.1 μg(22,000 units) of HeLa topo I(100 K form)(13), (d) head DNA + 0.02 μg(4,400 units) of HeLa nicking-closing enzyme(67 K form)(13), (e) head DNA + 2 μg(5000 units) (24) of E. coli topo I, (f) head DNA + 0.5 μg of T4 DNA topoisomerase(19) and (g) head DNA + 0.1 μg of HeLa topo II(14). (h)-(l) were repeat of (c)-(g) in the presence of 1 mM ATP. All reactions were treated with 100 μg/ml of proteinase K at 30°C for 20 min before loading onto a 0.7% agarose gel.

to note that the smear in lane d migrated faster than the smear in lane b, suggesting that knotted P4 phage DNA(about 20% of the total phage DNA) contains more complex knots than the P4 head DNA.

Type II DNA topoisomerases can remove the topological knots of P4 DNA.

To test the effect of DNA topoisomerases on such topological variants of P4 DNA, we treated the P4 head DNA with various DNA topoisomerases and analyzed the products by electron microscopy and gel electrophoresis. In the absence of ATP, no significant change of the P4 head DNA(Fig. 2B, lane b)

was observed, even when large amounts of DNA topoisomerase was added to the reactions(Fig. 2B, lanes c-g). In the presence of ATP, both T4 DNA topoisomerase(Fig. 2B, lane k) and HeLa topo II(Fig. 2B, lane l) converted all the knotted DNA into simple circles. None of the type I DNA topoisomerases used (100 K and 67 K forms of HeLa topo I and E. coli topo I, see lanes h-j respectively)(13, 25) changed the knotted DNA topology significantly. These results are consistent with the known properties of type I and type II DNA topoisomerases(2-9). The small amount of unknotting and catenation activity shown in Fig. 2B lane h is due to trace contamination of HeLa topo II in the HeLa topo I preparation(less than one unit of topo II in 22,000 units of topo I).

Fig. 3 shows the time course of unknotting of P4 head DNA by HeLa topo II. The topological knots were gradually removed by HeLa topo II as evidenced by the gradual shift of the smears(Fig. 3A, lanes a-g). To see if the partially unknotted P4 head DNA can be resolved into a ladder of bands corresponding to DNA with different degrees of knotting(2), a time course study using less HeLa topo II and longer electrophoresis time in a 0.4% agarose gel was carried out(Fig. 3B). Indeed, a ladder of bands was found(see top five bands of lanes f-h) which extends from the position of P4 simple circle. Such a characteristic ladder has been identified before for nicked, knotted pBR322 DNA(2). It is interesting to note that P4 knotted DNA migrated in the gel faster than the superhelical PM2 DNA which has the same molecular weight as P4 DNA(see lanes i of Fig. 3A & 3B)(20,22). The topological knots of P4 head DNA thus must be exceedingly complex.

To demonstrate that the knots of P4 DNA are readily accessible to a type II DNA topoisomerase, we compared the unknotting reaction and the relaxation reaction by HeLa topo II under the same conditions. As shown in Fig. 4, HeLa topo II can unknot a knotted DNA as fast as it can relax a superhelical DNA. Since both the relaxation and the unknotting by a type II DNA topoisomerase follow the same strand passing mechanism(2-9), we conclude that the complex topological knots of P4 head DNA are readily accessible to the enzyme.

Assaying of the type II DNA topoisomerase in the crude cell extracts using knotted DNA substrate.

To test whether or not the knotted P4 DNA can be successfully used as a substrate for assaying the type II DNA topoisomerase in crude cell extracts containing excess type I DNA topoisomerase, we made HeLa cell extracts and

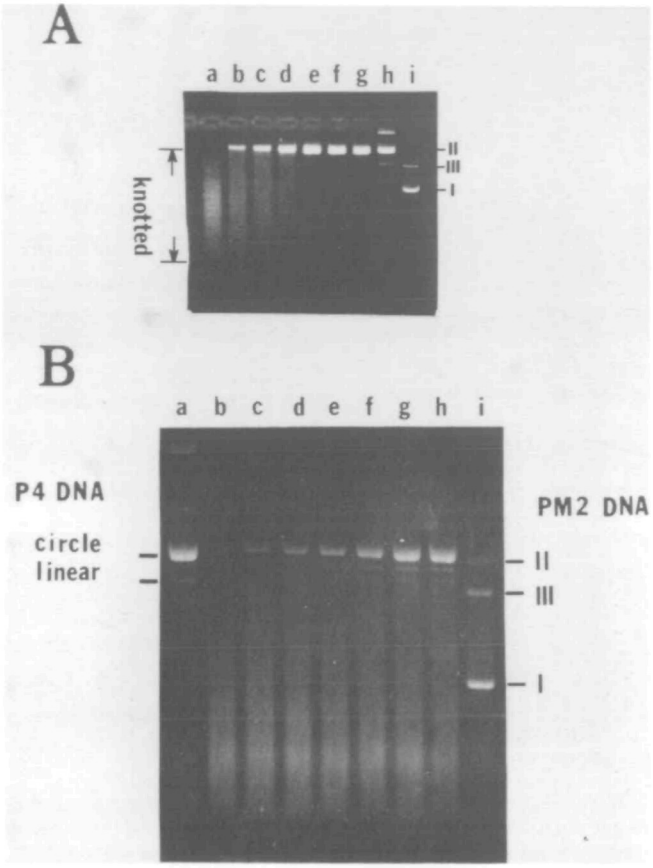

Fig. 3. Unknotting of P4 head DNA by a type II DNA topoisomerase. (A): P4 head DNA was treated with HeLa topo II(25 ng/ml) at 30°C. 1 mM ATP was present in all reactions. 20 µl aliquots of the reaction mixture were withdrawn at each time point and subjected to gel electrophoresis in a 0.7% agarose gel. (a) 0 min, (b) 1 min, (c) 2 min, (d) 4 min, (e) 8 min, (f) 16 min, (g) 32 min, (h) P4 phage DNA(untreated) and (i) PM2 DNA marker. (B): P4 head DNA was treated with HeLa topo II(3 ng/ml) at 30°C. Aliquots were analyzed as described in (A). (a) P4 phage DNA(untreated), (b) 0 min, (c) 30 seconds, (d) 1 min, (e) 2 min, (f) 4 min, (g) 8 min, (h) 10 min and (i) PM2 DNA marker. A 0.4% gel was used for this analysis.

tested the unknotting and relaxation activities of the cell extract in the presence and absence of ATP. When knotted P4 DNA was used as the substrate in the unknotting assay(Fig. 5A), unknotting only occurred in the presence of ATP(Fig. 5A, lanes a-e). In the absence of ATP, no unknotting was detectable(Fig. 5A, lanes f-j). In contrast, the relaxation activity monitored by

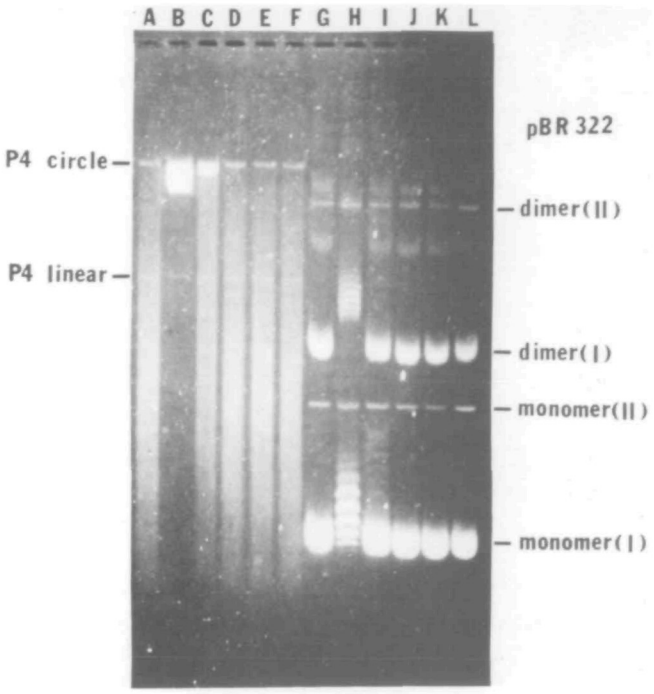

Fig. 4. Comparative study of unknotting and relaxation by a type II DNA topoisomerase. The unknotting of P4 head DNA(lanes A-F) and the relaxation of pBR322 DNA by HeLa topo II were compared. 1 mM ATP was present in each reaction. (A) P4 head DNA control, (B) + 15 ng/ml topo II, (C) + 7.5 ng/ml topo II, (D) + 3.7 ng/ml topo II, (E) + 1.9 ng/ml topo II and (F) + 0.9 ng/ml topo II. (G)-(L) were repeats of (A)-(F) respectively but with pBR322 DNA as substrate.

using pBR322 DNA as substrate was present independent of ATP(Fig. 5B). These results are expected from the known enzymatic properties of type I and type II DNA topoisomerases from eukaryotic cells(9,13,14). Because topo I is present in excess in the crude cell extracts, the relaxation reaction measures only topo I activity, which is ATP independent(12,13). We thus conclude that the P4 knotted DNA can be used as a substrate for assaying type II DNA topoisomerases in crude cell extracts containing excess type I topoisomerases.

DISCUSSION

Our results indicate that DNA molecules isolated from P4 tailless capsids(heads) are highly topologically knotted. Such DNA knots can be effi-

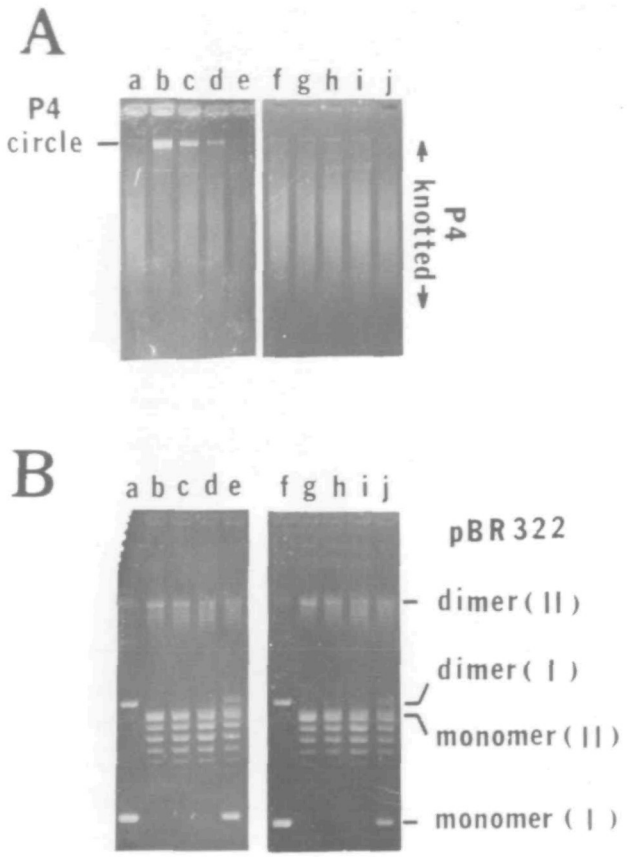

Fig. 5. P4 head DNA as a substrate for the type II DNA topoisomerase assay in crude cell extracts containing excess type I DNA topoisomerase. HeLa cell extract(PEG supernatnat) was prepared as described(13). (A): unknotting assays of the type II DNA topoisomerase using P4 head DNA as substrates. (a) P4 head DNA control, (b) - (e) were each treated with 1 μl of the extract diluted to various extents; (b) 1 : 10, (c) 1 : 30, (d) 1 : 90, (e) 1 : 270. (f) - (j) were the same as (a) - (e) except that 1 mM ATP was omitted in the reactions. (B): relaxation assays using pBR322 DNA as substrates. (a) - (j) were treated the same as the corresponding lanes in (A).

ciently removed by a type II DNA topoisomerase. About 20% of the DNA molecules isolated from mature P4 phages also contain very complex knots. The knots of the phage DNA seem more complex than the knots of the head DNA as suggested by electrophoretic analyses. The knotted DNA molecules are monomeric P4 DNA with their two cohesive ends hydrogen-bonded.

The reason for knot formation of P4 DNA is at present unclear. It has

been demonstrated previously that P2 DNA molecules from the tailless capsids contain highly complex knots(15). It was suggested that in a tailless capsid either the cohesive ends of a DNA are free to join, or are in close proximity so that their joining occurs rapidly upon the disruption of the capsids by phenol(15).

Knotted double-stranded DNA molecules have been successfully used for assaying type II DNA topoisomerases from eukaryotic cells(2). The previous methods for the preparation of such knotted DNA involve either treatment of plasmid DNA with highly purified T4 DNA topoisomerase(19) or *in vitro* site-specific recombination of a plasmid DNA containing phage and bacterial attachment sites(5). P4 heads offer a much more convenient source for highly knotted double-stranded DNA. We have demonstrated that the knots of P4 head DNA are quickly removed by a type II DNA topoisomerase. Furthermore, knotted P4 head DNA serves as an ideal substrate for assaying eukaryotic type II DNA topoisomerases in crude cell extracts containing excess type I DNA topoisomerases.

Recently, it has been demonstrated that *E coli* topo I and the rat liver topo I(both are type I DNA topoisomerases) can catenate nicked circular DNA (26,27). The absence of unknotting activity of *E. coli* topo I and HeLa topo I (see Fig. 2) on P4 head DNA which is in a nicked circular form is thus quite unexpected. We have further tested the ability of type I DNA topoisomerases to unknot P4 head DNA by introducing multiple nicks to the P4 head DNA with pancreatic DNase I. When P4 head DNA was multiply nicked, *E. coli* topo I indeed can unknot some P4 head DNA(unpublished result). HeLa topo I, however, does not unknot P4 head DNA under all conditions we have tested. It seems possible that the eukaryotic topoisomerase I and the bacterial topoisomerase I have quite different mechanism of action.

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