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Topoisomerase from *Ustilago maydis* Forms a Covalent Complex with Single-Stranded DNA through a Phosphodiester Bond to Tyrosine†

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ABSTRACT: Highly purified topoisomerase from *Ustilago* breaks single-stranded DNA, forming a complex with protein covalently bound to the DNA. Methods used to detect the complexes include a nitrocellulose filter assay, electrophoresis of the DNA–protein complex in agarose gels containing alkali, and isolation of the complex after removal of all but a small oligonucleotide fragment bound to the protein. The linkage of the *Ustilago* topoisomerase is to the 3′ end of the broken strand of DNA. The DNA–protein complex formed is through a phosphodiester bond to tyrosine.

Topological interconversions of DNA require transient breakage and resealing of DNA strands. Topoisomerases are the enzymes that interconvert topological isomers of DNA. Relaxation of negatively supercoiled DNA by topoisomerases was first observed a number of years ago to occur by repeated breakage and rejoining of DNA with no added cofactor providing energy for rejoining (Wang, 1971; Champoux & Dulbecco, 1972). Wang suggested that the energy of the phosphodiester bond was conserved in a reaction intermediate,

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a transient covalent complex between the topoisomerase and a broken end of DNA. Since the discovery of the archetypal topoisomerases or type I enzymes, a second class of topoisomerases, type II enzymes, requiring ATP as a cofactor was found [for a review, see Gellert (1981a)]. Although there are mechanistic differences between the reactions catalyzed by the two classes, DNA–protein covalent complexes are formed in each case (Wang, 1981; Gellert, 1981b). Examples have been reported of covalent complexes between DNA and type I or type II topoisomerases from phage T4 (Rowe et al., 1984), bacteria (Tse et al., 1980; Gellert et al., 1977; Sugino et al., 1977), yeast (Durnford & Champoux, 1978; Badaracco, 1983), insect (Sander & Hsieh, 1983; Javaherian et al., 1982), and mammalian tissue (Been & Champoux, 1980; Prell & Vosberg, 1980; Halligan et al., 1982; Hsiang et al., 1985). In a few instances, the chemical nature of the linkage was studied in some detail. Phosphotyrosine was identified as the linkage between *Escherichia coli* and *Micrococcus luteus* type I topoisomerase and the 5′ end of DNA (Tse et al., 1980). The linkage of rat liver type I topoisomerase was also established to be through phosphotyrosine, except that complexes were formed at the 3′ end of the DNA (Champoux, 1978, 1981).

The role of type I topoisomerases in cellular processes such as recombination is still uncertain (Bullock et al., 1985). However, one might anticipate from the breakage and rejoining activities observed in vitro that topoisomerases function in genetic recombination. Indeed, the phage λ *int* gene product (Kikuchi & Nash, 1979) and the *E. coli* Tn3 resolvase (Krasnow & Cozzarelli, 1983), which are required for site-specific recombination, both have associated type I topoisomerase activity. Our interest in the biochemistry of genetic recombination in the fungus *Ustilago maydis* has led us to examine the type I topoisomerase from this organism in detail. In a previous study, we reported the partial purification and characterization of the enzyme (Rowe et al., 1981). In this study, we describe a procedure which yields more pure enzyme, report conditions for formation of DNA–enzyme covalent complexes, and identify the chemical nature of the DNA–protein linkage.

Experimental Procedures

Materials

Growth of *Ustilago maydis* was carried out as described before (Rusche et al., 1980). Radiolabeled or unlabeled phage DNAs were prepared as described by Cunningham et al. (1980). Phosphoserine, phenylmethanesulfonyl fluoride, and micrococcal nuclease were from Sigma Chemical Co. Phosphothreonine was from D. Rawlins in T. Kelly's laboratory, The Johns Hopkins School of Medicine. Phosphotyrosine was synthesized according to Rothberg et al. (1978). Constant-boiling HCl was from Pierce Chemical, Co. The radioactive nucleotides [α-^{32}P]dCTP and [γ-^{32}P]ATP were from Amersham. Nitrocellulose filters, type GN-6, were from Gelman Sciences, Inc. Proteinase K was obtained from E. Merck. Bacterial alkaline phosphatase and pancreatic DNase were obtained from Worthington Biochemicals, Inc. *E. coli* DNA polymerase I was obtained from Boehringer Mannheim Biochemicals. T4 polynucleotide kinase, large Klenow fragment of *E. coli* DNA polymerase I, and restriction endonuclease *Sau*96 were obtained from Bethesda Research Laboratories. Exonuclease I was prepared from *E. coli* B according to the procedure of Lehman and Nussbaum (1964), by M. Yarnall of this laboratory. Exonuclease VII was prepared according to the procedure of Chase and Richardson (1974). Calf thymus topoisomerase was a kind gift from Leroy Liu, The Johns Hopkins School of Medicine.

Methods

Assays. Topoisomerase activity was measured as described before (Rowe et al., 1981) by monitoring relaxation of superhelical DNA using agarose gel electrophoresis. One unit of activity is that amount of enzyme necessary to convert 1 nmol of superhelical form I DNA to a relaxed form in 10 min at 25 °C. The exonuclease assay measures the conversion of DNA to an acid-soluble form. Reactions with exonuclease I were carried out in 200 μL containing 50 mM glycine, pH 9.5, and 10 mM $MgCl_2$ at 37 °C. After 30 min, the reactions were terminated by adding 0.3 mL of ice-cold carrier salmon sperm DNA (1 mg/mL) and 0.5 mL of 10% trichloroacetic acid. After the tubes stood on ice for 10 min, they were centrifuged in an Eppendorf Microfuge. After 2 min, 0.5 mL of the supernatant was removed and counted in 5 mL of Triton scintillation fluor (New England Nuclear, formula 950 A). Reactions with exonuclease VII were carried out in 200 μL containing 50 mM potassium phosphate, pH 7.9, at 37 °C. After 30 min, the reactions were terminated and processed as described for exonuclease I.

DNA Substrates. Conversion of superhelical fd form I DNA to the linear form III was carried out by digestion with endonuclease *Sau*96. Labeling of φX duplex DNA by nick translation with [α-^{32}P]dCTP was carried out by a modification of the procedure of Rigby et al. (1977). A reaction (55 μL) containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.6, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 25 μM dGTP, 25 μM dATP, 25 μM dTTP, 200 μCi of [α-^{32}P]dCTP (410 Ci/mmol), 3 nmol of φX form I DNA, 1 ng of pancreatic DNase, and 5 units of *E. coli* DNA polymerase I was incubated at 15 °C. After 1 h, the reaction was terminated by adding ethylenediaminetetraacetic acid (EDTA) to 15 mM. The reaction was then phenol extracted 1 time and the aqueous phase loaded onto a Sephacryl S-200 column equilibrated in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The DNA eluted in the void volume and had a specific activity in the range of (0.1–1) × 10^8 cpm/nmol.

Labeling of fd form III DNA at the 5′ end was accomplished by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Prior to kinasing, the DNA was dephosphorylated by using bacterial alkaline phosphatase as described previously (Weiss et al., 1968). A reaction (80 μL) containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 8 nmol of dephosphorylated fd form III DNA, 50 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), and 5 units of T4 polynucleotide kinase was incubated at 37 °C for 90 min. The reaction was then dialyzed against three 250-mL changes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 M NaCl and then three 250-mL changes of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The specific activity of the DNA was 4 × 10^5 cpm/nmol.

Labeling fd form III DNA at the 3′ end was accomplished by using [α-^{32}P]dCTP and the large Klenow fragment of *E. coli* DNA polymerase I (Donelson & Wu, 1972). A reaction (80 μL) containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 μM dGTP, 125 μCi of [α-^{32}P]dCTP (410 Ci/mmol), 25 nmol of fd form III DNA, and 3 units of the large Klenow fragment of *E. coli* DNA polymerase I was incubated at room temperature. After 10 min, the reaction was stopped by adding 12.5 μL of 0.25 M EDTA and 25 μL of 20% sodium acetate, pH 6.0. The reaction volume was then adjusted to 150 μL and dialyzed as described previously for DNA labeled with T4 polynucleotide kinase. The specific activity of the DNA was 1 × 10^6 cpm/nmol.

Denaturation of duplex DNA was carried out by adding NaOH to 50 mM and incubation the DNA at 37 °C. After 10–15 min, the DNA solution was neutralized by adding HCl to 50 mM and Tris-HCl, pH 9.0, to 25 mM. All DNA concentrations are expressed in terms of moles of nucleotide. Forms I, II, and III refer to the duplex forms of viral DNA in the superhelical form, circular form containing at least one nick per molecule, and the full-length linear form, respectively.

Electrophoresis. Analysis of proteins by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis used the method of Laemmli (1970). Electrophoresis of DNA through 1.2% alkaline–agarose gels was carried out according to McDonell et al. (1977).

Results

Formation of Complexes between Ustilago Topoisomerase and Single-Stranded DNA. Highly purified *Ustilago* topoisomerase (Table I) breaks single-stranded circular DNA to form linear DNA molecules with protein tightly attached. We used three different methods to detect such complexes including electrophoresis in both alkaline agarose gels and polyacrylamide gels containing sodium dodecyl sulfate (SDS), and also by use of a nitrocellulose membrane filter binding assay.

The first method followed the procedure described by Been and Champoux (1980). When we reacted topoisomerase with circular DNA of phage fd, a product was formed that migrated slightly slower than full-length linear DNA during electrophoresis in an alkaline agarose gel (Figure 1A). Raising the concentration of topoisomerase in the reaction resulted in a decrease in the amount of intact circular DNA molecules and an increase in the amount of product until eventually DNA molecules migrating faster than full-length linear molecules were produced. Discrete size classes of DNA product were formed, suggesting that topoisomerase cuts the DNA with some specificity.

Such protein–DNA complexes were also evident as detected by the well-known nitrocellulose membrane filter assay that was devised for studies on the binding of RNA polymerase (Jones & Berg, 1966) and *lac* repressor (Riggs et al., 1968) to DNA. In this assay, labeled DNA bound by protein is retained on nitrocellulose filters. With no added protein, only a few percent of the input radiolabeled DNA was retained. However, when topoisomerase was added, up to 90% of the single-stranded DNA could be retained (Figure 1C). Complex formation was observed over a broad pH range, from pH 6.0 in phosphate to pH 10.0 in glycine with an optimum at pH 8.0 in Tris-HCl. Inhibition of complex formation was observed at salt concentrations greater than 0.1 M. Temperature in the range 0–37 °C had no appreciable effect on complex formation.

By using the nitrocellulose membrane filter assay, we observed that the rate of formation of complexes was fast (Figure 2), in agreement with the kinetics we observed for relaxation of superhelical DNA by topoisomerase (M. J. Brougham et al., unpublished results). The complexes formed could be dissociated by proteolysis. Upon addition of proteinase K to complexes, the level of DNA retained by filters dropped sharply. Dissociation of complexes by proteolysis was confirmed in another way, by electrophoresis of the reaction products in alkaline agarose gels. The mobility of the product generated by reaction of topoisomerase with circular single-stranded DNA was slightly less than that of full-length linear DNA strands (see Figure 2, insert). After proteolysis, the mobility changed, becoming equivalent to that of full-length linear molecules. Thus, there is good correlation between the

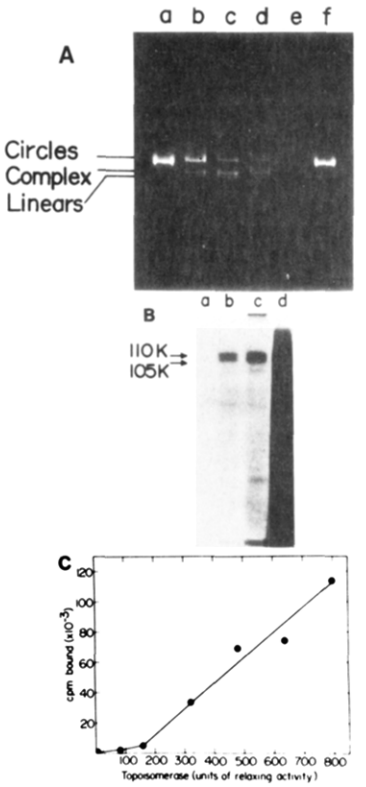

FIGURE 1: Formation of complex between topoisomerase and single-stranded DNA. (A) Analysis of the complex by electrophoresis in an alkaline agarose gel. A series of reaction mixtures (100 μL) containing 40 mM Tris-HCl, pH 9.0, 2.4 nmol of fd phage DNA, and topoisomerase were incubated at 25 °C for 30 min. Reactions were terminated by adding NaOH to 30 mM. Aliquots (50 μL) of each reaction were loaded onto a 1.2% agarose gel containing 30 mM NaOH and 0.5 mM EDTA. Electrophoresis was at 5.5 V/cm for 16 h at 5 °C. Following electrophoresis, the gel was soaked for 10 min in 500 mL of 0.1 M Tris-HCl, pH 7.5, and stained with 1 μg/mL ethidium bromide before being photographed under ultraviolet light. The following amounts of topoisomerase were used: (a) no enzyme; (b) 580 units; (c) 1160 units; (d) 1740 units; (e) 2900 units; (f) no enzyme. (B) SDS–polyacrylamide gel electrophoresis of complex. Reaction mixtures (200 μL) containing 25 mM Tris-HCl, pH 9.0, 50 g/mL bovine serum albumin, 0.2 mM dithiothreitol, and 30 pmol of denatured nick-translated φX DNA (6.2×10^7 cpm/nmol) were incubated at 25 °C with the following levels of topoisomerase: (a) no enzyme; (b) 408 units; (c) 816 units; (d) 1360 units. After 15 min, the reactions were stopped by adding NaOH to 50 mM and incubating for 10 min at 37 °C. The reactions were then made 25 mM in Tris-HCl, and the pH was titrated to 9.0 with HCl. After addition of $CaCl_2$ to a final concentration of 2 mM, 0.1–0.2 unit of micrococcal nuclease was added. Following incubation at 37 °C for 1 h, samples were extracted with 1 volume of phenol. The phenol phase was saved and extracted 6 times with equal volumes of 20 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The protein–oligonucleotide complex was precipitated from the phenol phase by adding 10 volumes of ice-cold acetone and placing the samples in a dry ice–ethanol bath. After 20 min, the samples were centrifuged, and the resulting pellet was resuspended in SDS–polyacrylamide gel electrophoresis sample buffer. Following heating at 100 °C for 5 min, samples were electrophoresed through a 10% SDS–polyacrylamide gel at 5 V/cm for 16 h. After electrophoresis, the gel was stained with Coomassie brilliant blue to locate marker proteins. The gel was then dried down on Whatman 3MM paper and autoradiographed with Kodak X-Omat AR5 film with a Du Pont Lightning-Plus intensifying screen at −70 °C. (C) Binding of DNA–topoisomerase complexes to nitrocellulose filters. A series of reactions (500 μL) containing 25 mM Tris-HCl, pH 9.0, 2.2 nmol of ^{32}P-labeled fd phage DNA (5×10^4 cpm/nmol), and the indicated levels of topoisomerase were incubated at 25 °C for 15 min. Samples were then processed as follows: nitrocellulose filters that had been boiled for 5 min in 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA (binding buffer) were washed with 5 mL of binding buffer. Complexes were then washed through these filters followed by washing with a further 5 mL of binding buffer. Filters were then dried under a heat lamp and counted with 2 mL of Econofluor (New England Nuclear).

Table I: Purification of the Enzyme[a]

fraction	volume (mL)	act. (units × 10^{-4})	protein (mg)	sp act. (units/mg)
(I) polyethylene glycol	130	520.0	152.0	3.4×10^4
(II) hydroxyl-apatite	71	142.0	1.22	116×10^4
(III) phospho-cellulose[b]	33	13.2	0.0033[c]	4×10^7

[a] All operations were carried out at 0–4 °C. Fractions were assayed for activity (in 25 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$) by monitoring the conversion of superhelical form I DNA to a relaxed closed form on agarose gels (Rowe et al., 1981). Freshly grown *Ustilago* (710 g wet weight) cells were mixed with 1500 mL of extraction buffer (10 mM potassium phosphate, pH 7.0, 10% glycerol, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride). This cell slurry was crushed by passage through a Manton Gaulin homogenizer (9000 psi) and then spun at 450*g* for 5 min. The resulting supernatant was set aside and the pellet resuspended in 500 mL of extraction buffer. The redissolved pellet was again passed through the Manton Gaulin homogenizer (9000 psi) and centrifuged at 450*g* for 5 min. The resultant supernatant was combined with the supernatant previously set aside and centrifuged at 9500*g* for 60 min. This yielded a pellet which was resuspended in extraction buffer containing 1.0 M KCl. This extract was stirred at 4 °C for 2 h and then spun at 16000*g* for 10 min to remove debris. The supernatant (290 mL) was made 7% with polyethylene glycol. After being stirred at 4 °C for 30 min, the mixture was centrifuged at 16000*g* for 10 min. Of the resulting supernatant (280 mL), 130 mL (fraction I) was carried through the purification, and the rest was stored at −20 °C for later use. Fraction I was loaded directly onto a column of hydroxylapatite (120-mL bed volume). The column was washed successively with extraction buffer containing 1.0 M KCl (1 L) and with extraction buffer alone (500 mL). A 1-L linear gradient (0.01–0.75 M) of potassium phosphate, pH 7.0, in extraction buffer was applied to the column. Topoisomerase activity eluted between 0.2 and 0.3 M potassium phosphate. Topoisomerase pooled from this step is fraction II. Ten milliliters of fraction II was diluted 2-fold in extraction buffer and loaded onto a phosphocellulose column (1-mL bed volume) that was preequilibrated in extraction buffer containing 0.1 M KCl. The column was then washed with 20 mL of equilibration buffer, and protein was eluted in a stepwise fashion with extraction buffer containing 0.2, 0.4, and 0.6 M KCl (5 mL each). Topoisomerase activity eluted in the 0.4 and 0.6 M KCl washes. The 0.6 M KCl pool is fraction III. [b] Total amounts indicated are corrected for the fact that only a portion of fraction II was taken through this step. [c] Protein was estimated from an SDS–polyacrylamide gel that was stained with silver nitrate (Oakley et al., 1980), with reference to a standard curve of bovine serum albumin electrophoresed and stained in the same way.

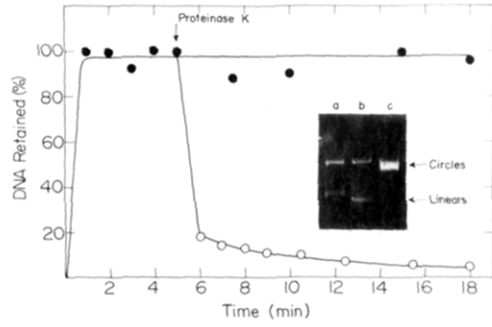

FIGURE 2: DNA–topoisomerase complex dissociated by proteinase K. A 2-mL reaction containing 25 mM Tris-HCl, pH 9.0, 4.4 nmol of ^{32}P-labeled fd phage DNA (2×10^5 cpm/nmol), and 25 μg/mL bovine serum albumin was preequilibrated at 37 °C for 10 min in the absence of topoisomerase. To this equilibrated reaction mixture was then added 1600 units of topoisomerase activity. Incubation at 37 °C was continued, and at the indicated time points, 100-μL aliquots were removed and filtered through nitrocellulose as described in the legend to Figure 1C. At 5 min, a 1-mL aliquot was removed and treated with proteinase K (200 μg/mL). At the indicated times, aliquots (100 μL) were removed form this sample and filtered through nitrocellulose. The effect of proteinase K treatment on DNA–topoisomerase complexes was also analyzed by alkaline agarose electrophoresis. Reactions (40 μL) containing 10 mM Tris-HCl, pH 9.0, 1 mM EDTA, 0.2 mM dithiothreitol, 50 μg/mL bovine serum albumin, 2.4 nmol of fd phage DNA, and either 85 units of topoisomerase (lanes a and b) or no topoisomerase (lane c) were incubated at 25 °C for 20 min. Reactions a and c were terminated by adding 4 μL of 0.3 M NaOH. Reaction b was treated with proteinase K (100 μg/mL) for 30 min at 37 °C before addition of 4 L of 0.3 M NaOH. Aliquots of each reaction (15 μL) were loaded onto a 1.2% alkaline agarose gel and electrophoresed as described in the legend to Figure 1A.

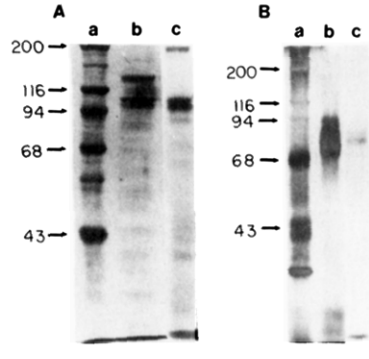

FIGURE 3: Covalent linkage of topoisomerase to nick-translated DNA. Topoisomerases from *Ustilago maydis* and calf thymus were linked to denatured nick-translated DNA as follows: reactions (400 μL) containing 25 mM Tris-HCl, pH 9.0, 0.2 mM dithiothreitol, 20 μg/mL bovine serum albumin, and 75 pmol of denatured nick-translated φX DNA (6.6×10^7 cpm/nmol) were incubated with 1160 units of *Ustilago* topoisomerase and with 1 μg of calf thymus type I topoisomerase at 25 °C for 15 min. Reactions were terminated and processed as described in the legend to Figure 1B. Samples were electrophoresed through 7.5% SDS–polyacrylamide gels as in Figure 1B. Aliquots of the untreated enzyme preparations were also electrophoresed on these gels. Gels were stained for protein, then dried down on Whatman 3MM paper, and autoradiographed with Kodak X-Omat AR5 film with a Lightning-Plus (Du Pont) intensifying screen at −70 °C. Gel A: (a) markers; (b) *Ustilago* protein stained; (c) *Ustilago* protein autoradiographed. Gel B: (a) markers; (b) calf thymus protein stained; (c) calf thymus protein autoradiographed. Markers included myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin.

filter assay and the electrophoretic behavior of complexes during agarose gel electrophoresis.

The third procedure we used to detect protein–DNA complexes was a label-transfer method described by Chow and Pearson (1985). The product formed after reaction of topoisomerase with highly radioactive ^{32}P-labeled DNA was digested with micrococcal nuclease to remove almost all of the attached DNA from the topoisomerase. After phenol extraction to remove oligonucleotides and electrophoresis of the protein in an SDS–polyacrylamide gel, we observed by autoradiography that a small amount of ^{32}P-labeled DNA had become attached to the topoisomerase (Figure 1B).

Two radiolabeled bands of 105000 and 110000 daltons were evident. Analysis by SDS–polyacrylamide gel electrophoresis of the polypeptides in the topoisomerase preparation revealed two major polypeptides corresponding in molecular weight to the radiolabeled bands (Figure 3) We presume the radiolabeled bands arose from linkage of a small DNA oligonucleotide residue to the topoisomerase since a similar result was obtained when we used calf thymus type I topoisomerase as a positive control. The relationship between the two *Ustilago* polypeptides is unclear. However, in a number of topoisomerase preparations, we have consistently observed the two polypeptides in approximately equal amounts. Other workers have suggested heterogeneity in topoisomerase preparations might arise from proteolytic degradation (Liu & Miller, 1981) or modification (Durban et al., 1983; Ferro et al., 1983).

Table II: Digestion of Topoisomerase–DNA Complexes with Exonuclease I and Exonuclease VII[a]

	acid solubility (%)		circular
	Exo I	Exo VII	DNA (%)
control	5	9	>95
topoisomerase	6	58	41

[a] To determine whether the topoisomerase was linked to DNA at the 3′ or 5′ end, complexes formed between fd phage [^{3}H]DNA (2×10^4 cpm/nmol) and topoisomerase were treated with exonuclease I (Exo I) or exonuclease VII (Exo VII). A 75-μL reaction containing 10 mM Tris-HCl, pH 9.0, 0.2 mM dithiothreitol, 50 μg/mL bovine serum albumin, 5 nmol of fd phage [^{3}H]DNA, and 85 units of topoisomerase was incubated at 25 °C. After 10 min, three 20-μL aliquots were removed and analyzed as described under Experimental Procedures. One aliquot was diluted into a 200-μL reaction containing 0.8 unit of exonuclease I. Another aliquot was diluted into a 200-μL reaction containing 6 units of exonuclease VII. The third aliquot was electrophoresed through a 1.2% alkaline agarose gel as described in the legend in Figure 1A. A control reaction containing no topoisomerase was processed in the same manner. The percent circular DNA present was determined from tracing the photographic negative of the agarose gel using a Joyce-Loebl densitometer.

Ustilago Topoisomerase Is Linked to the 3′ End of the Broken DNA Strand. Type I topoiosmerases from mammalian tissues have been shown to form a covalent linkage to the 3′ end of DNA after cleavage (Prell & Vosberg, 1980; Been & Champoux, 1980; Halligan et al., 1982; Liu et al., 1983; Hsiang et al., 1985), in marked contrast to *E. coli* topoisomerase I which links to the 5′ end (Depew et al., 1978). We showed that *Ustilago* topoisomerase was linked to the 3′ end of the broken strand of DNA by two methods.

In the first approach, ^{3}H-labeled circular single-stranded fd DNA, which had been cleaved with topoisomerase to form the complex, was treated with *E. coli* exonuclease I, an exonuclease with 3′ to 5′ specificity, or with *E. coli* exonuclease VII, an exonuclease active from either end. If breakage of the DNA by the topoisomerase produces a free 3′-hydroxyl terminus, the broken DNA should be susceptible to digestion by both exonuclease I and exonuclease VII. However, if breakage produces a free 5′-hydroxyl terminus, the broken fragment of DNA should only be susceptible to digestion by exonuclease VII. The result of this experiment was that DNA broken by the *Ustilago* topoisomerase was digestible by exonuclease VII but not exonuclease I (Table II). This indicates that breakage of the DNA by the *Ustilago* topoisomerase produces a free 5′ terminus, suggesting that the topoisomerase is linked to the 3′ side of the DNA break. Since complexes are formed without alkali or detergent treatment, this result also points out that cleavage of DNA by the topoisomerase occurs under normal reaction conditions. This is in contrast to *E. coli* type I topoisomerase which requires the use of detergents or alkali to trap the complex (Liu & Wang, 1979).

The attachment of the topoisomerase to the 3′ end of a broken DNA strand was confirmed in the following way. Full-length linear duplex of fd DNA, produced by digesting fd form I DNA with the restriction endonuclease *Sau*96, was labeled with [γ-^{32}P]ATP at its 5′ ends using T4 polynucleotide kinase. The DNA was denatured and reacted with the *Ustilago* topoisomerase. Electrophoresis of the reaction products on an alkaline agarose gel revealed several major bands arising presumably by specific cleavage (Figure 4, lane B). If the reaction products were treated with proteinase K prior to electrophoresis, the mobilities of these bands were shifted (Figure 4, lane C). This shift is what would be expected if the topoisomerase was linked to the 3′ end of the broken DNA strand. When the same experiment was carried out with DNA labeled at its 3′ ends with [α-^{32}P]dCTP using the large fragment of *E. coli* DNA polymerase I, no shift in the mobility of the reaction products was observed following treatment with proteinase K (Figure 4, lanes E and F). The results from this study in combination with the exonuclease digestion results provide strong evidence for the attachment of the *Ustilago* topoisomerase to the 3′ end of the broken DNA strand.

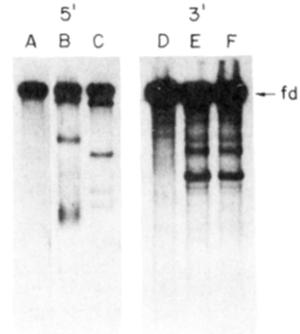

FIGURE 4: Topoisomerase linked to the 3′ end of the broken DNA strand. Duplex linear fd DNA, prepared by digesting fd form I DNA with the restriction endonuclease *Sau*96, was labeled with ^{32}P at the 3′ or 5′ end and then denatured as described under Experimental Procedures. Reactions (40 μL) containing 10 mM Tris-HCl, pH 9.0, 0.2 mM dithiothreitol, 50 μg/mL bovine serum albumin, and 0.3 nmol of denatured 5′ end labeled DNA (lanes A–C) or 3′ end labeled DNA (lanes D–F) were incubated with topoisomerase at 25 °C for 30 min. Reactions were terminated by adding 40 μL of 0.3 M NaOH. Prior to addition of NaOH, control samples were treated with proteinase K (100 μg/mL) for 30 min at 37 °C. Aliquots of 20 μL of each sample were analyzed by electrophoresis through a 1.6% alkaline agarose gel and processed as described in the legend to Figure 1A. The gel was then dried down on Whatman 3MM paper and autoradiographed as described in the legend to Figure 1B: (A) no topoisomerase; (B) topoisomerase added; (C) topoisomerase plus proteinase K; (D) no topoisomerase; (E) topoisomerase added; (F) topoisomerase plus proteinase K.

Table III: Stability of the Topoisomerase–DNA Linkage[a]

treatment	^{32}P made acid soluble (%)
none	<5
1 M NaOH, 5 h	<5
1 M HCl, 5 h	10
0.25 M Tris-HCl, pH 7.5, 1 mM I_2, 5 min	<5
3.3 M NH_2OH, pH 4.75, 1 h	<5
0.2 M NH_2OH, pH 7.5, 1 h	<5
proteinase K (100 μg/mL), 1 h	100

[a] A 2-mL reaction containing 20 mM Tris, pH 8.0, 0.2 mM dithiothreitol, 50 μg/mL bovine serum albumin, 0.42 nmol of denatured nick-translated φXDNA (1×10^8 cpm/nmol), and 5 μg of *Ustilago* topoisomerase was incubated at 25 °C for 15 min. The sample was then processed as described in the legend to Figure 1B up to and including the acetone precipitation step. Following centrifugation, the pellet was redissolved in 1 mL of 10 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA. Reactions (200 μL) were then set up with 100-μL aliquots of the redissolved pellet and the various reagents described in Table II. After incubation at 37 °C for the times specified in Table II, bovine serum albumin was added to 100 μg/mL and trichloroacetic acid to 5%. The samples were then incubated in a dry ice–ethanol bath for 1 h. Centrifugation removed acid-precipitable material, and radioactivity in the supernatant was determined by scintillation counting with Triton X-100 fluor.

Linkage of Ustilago Topoisomerase to DNA Is through Tyrosine. Topoisomerases from rat liver, *E. coli*, and *M. luteus* have been shown to form covalent complexes with DNA involving a phosphodiester bond with tyrosine (Champoux, 1981; Tse et al., 1980). In these studies, stability of protein–DNA complexes to treatment with various reagents was taken as a diagnostic measure for the amino acid linkage involved. We therefore tested the stability of the *Ustilago* topoisomerase–DNA linkage by treating such complexes with

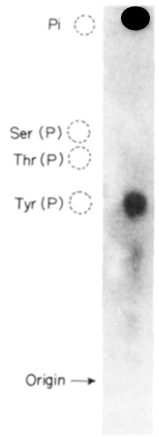

FIGURE 5: Topoisomerase–DNA linkage through phosphotyrosine. Complexes were prepared with *Ustilago* topoisomerase and nick-translated DNA as described in the legend to Figure 3. Samples (40 μL) were sealed in Eppendorf tubes under a nitrogen atmosphere and then hydrolyzed in constant-boiling HCl at 105 °C. After 2 h, the samples were cooled, and the HCl was removed in vacuo. The resulting residue was redissolved in 30 μL of water containing 100 nmol each of phosphotyrosine [Tyr(P)], phosphoserine [Ser(P)], and phosphothreonine [Thr(P)] and 500 nmol of sodium phosphate. The hydrolysate was analyzed by high-voltage paper electrophoresis at 2000 V for 75 min in water/pyridine/acetic acid (945/5/50) on Whatman 3MM paper. The positions of the marker compounds represented by dashed circles were located by spraying the paper with 0.3% ninhydrin in 1-butanol followed by heating to 100 °C for several minutes. Inorganic phosphate was detected by spraying the paper with a solution of 0.42% ammonium molybdate in 1 M sulfuric acid/10% ascorbic acid (6/1). Autoradiography was as described in the legend to Figure 1B.

various reagents and then following the fate of the labeled oligonucleotide by trichloroacetic acid precipitation (Table III). Treatment of the complex with proteinase K resulted in the release of the oligonucleotide into the supernatant, demonstrating that the oligonucleotide released is not precipitated with 5% trichloroacetic acid. However, the enzyme–oligonucleotide linkage was stable to all the other treatments shown in Table III. The stability to acidic hydroxylamine ruled out a phosphoamide bond (Gumport & Lehman, 1971) as the linkage, while stability to neutral hydroxylamine ruled out a mixed anhydride (Berg, 1956). Stability to 1 M NaOH at 37 °C for 5 h made it unlikely that the linkage is either a serine or a threonine phosphoester (Shabarova, 1970; Desiderio & Kelly, 1981). Stability to 1 mM I_2 at neutral pH ruled out a thiol phosphate as the linkage (Pigiet & Conley, 1978). The only remaining possibility was phosphotyrosine, and this was determined by direct identification after acid hydrolysis.

Determination of the linkage involved in complexes formed by *Ustilago* topoisomerase and DNA was carried out by reacting the enzyme with denatured nick-translated φX174 DNA as described in the legend to Figure 3. The purified protein–oligonucleotide complex was hydrolyzed in HCl at constant-boiling 105 °C for 2 h, and the products of hydrolysis were analyzed by high-voltage paper electrophoresis (Figure 5). Under these conditions, protein is reduced almost completely to amino acids. We observed a ^{32}P-labeled residue that comigrated with authentic phosphotyrosine. We conclude that the linkage of *Ustilago* topoisomerase with DNA is through tyrosine.

Discussion

We show in this paper that the *Ustilago* topoisomerase can break single-stranded DNA and that breakage is accompanied by simultaneous formation of a protein–DNA covalent bond. Like the complexes formed between DNA and type I topoisomerases from mammalian cells, the *Ustilago* topoisomerase links to the 3′ end of DNA after cleavage. Only bacterial type I topoisomerases have been demonstrated to link to the 5′ end of DNA. The covalent complex formed between the *Ustilago* topoisomerase and DNA is through a tryosine residue as is the case with topoisomerases from rat liver, *E. coli*, and *M. luteus*. Thus, the chemical nature of the complex formed by enzymes from organisms representing the kingdoms (Whittaker, 1969) Animalia, Monera, and Fungi is identical.

Estimates of the size of eukaryotic topoisomerases have been revised upward since it was realized that these enzymes were particularly labile to proteases. When care was taken to avoid proteolysis, the molecular weight of topoisomerases from HeLa cells (Liu & Miller, 1981), *Drosophila* eggs (Javaherian et al., 1982), and wheat germ (Dynan et al., 1981) was found to be greater than 100 000 as determined by SDS gel electrophoresis. Similarly, when we prepared enzyme from freshly harvested cells and used a protease inhibitor in buffers, we found the molecular weight of the *Ustilago* topoisomerase to be greater than 100 000. The strong similarities in physical and chemical properties that the *Ustilago* topoisomerase shares with other eukaryotic topoisomerases indicate how much this enzyme has been conserved throughout evolution.

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The *dna*B Protein of *Escherichia coli*: Mechanism of Nucleotide Binding, Hydrolysis, and Modulation by *dna*C Protein†

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ABSTRACT: The mechanism of nucleotide binding and hydrolysis by *dna*B protein and *dna*B·*dna*C protein complex has been studied by using fluorescent nucleotide analogues. Binding of trinitrophenyladenosine triphosphate (TNP-ATP) or the corresponding diphosphate (TNP-ADP) results in a blue shift of the emission maximum and a severalfold amplification of the fluorescence emission of the nucleotide analogues. Scatchard analysis of TNP-ATP binding indicates that TNP-ATP binds with a high affinity (K_d = 0.87 μM) and a 8.5-fold enhancement of fluorescence emission of the nucleotide. Only three molecules of TNP-ATP or TNP-ADP bind per hexamer of *dna*B protein in contrast to six molecules of ATP or ADP binding to a *dna*B hexamer. TNP-ATP and TNP-ADP are both competitive inhibitors of single-stranded (SS) DNA-dependent ATPase activity of *dna*B protein. TNP-AMP neither binds to *dna*B protein nor inhibits the ATPase activity. Formation of *dna*B·*dna*C complex by *dna*C protein results in diminution of the TNP-ATP fluorescence enhancement and a concomitant decrease in the SS DNA-dependent ATPase activity. Kinetic analysis of the ATPase activity of *dna*B·*dna*C complex indicates that the decrease in the ATPase activity on complex formation is due to a reduction of the maximal velocity (V_{max}). The *dna*B protein hydrolyzes both TNP-ATP and dATP, however, with an extremely slow rate in the presence of single-stranded M13 DNA. The 2′-OH group of the nucleotide most likely plays an important role in the hydrolysis reaction but not in the nucleotide binding.

The *dna*B protein is a major component of the chromosomal DNA replication in *Escherichia coli* (Kornberg, 1980). The *dna*B protein, a hexamer of 52000-dalton identical subunits (Arai et al., 1981a; Reha-Krantz & Hurwitz, 1978a,b; Nakayama et al., 1984), is required in the initiation (Baker et al., 1986), priming, and elongation stages of DNA replication (Arai & Kornberg, 1981b; Arai et al., 1981b; Zyskind & Smith, 1977; Wechsler & Gross, 1971; McMacken & Kornberg, 1978). In vitro DNA replication studies with DNA from

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