Identification of the Yeast TOP3 Gene Product as a Single Strand-specific DNA Topoisomerase*

(Received for publication, April 3, 1992)

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The TOP3 gene of the yeast Saccharomyces cerevisiae was postulated to encode a DNA topoisomerase, based on its sequence homology to Escherichia coli DNA topoisomerase I and the suppression of the poor growth phenotype of top3 mutants by the expression of the E. coli enzyme (Wallis, J. W., Chrebet, G., Brodsky, G., Golfe, M., and Rothstein, R. (1989) Cell 58, 409-419). We have purified the yeast TOP3 gene product to near homogeneity as a 74-kDa protein from yeast cells lacking DNA topoisomerase I and overexpressing a plasmid-borne TOP3 gene linked to a phosphate-regulated yeast PHO5 gene promoter. The purified protein possesses a distinct DNA topoisomerase activity: similar to E. coli DNA topoisomerases I and III, it partially relaxes negatively but not positively supercoiled DNA. Several experiments, including the use of a negatively supercoiled heteroduplex DNA containing a 29-nucleotide single-stranded loop, indicate that the activity has a strong preference for single-stranded DNA. A protein-DNA covalent complex in which the 74-kDa protein is linked to a 5' DNA phosphoryl group has been identified, and the nucleotide sequences of DNA-protein covalent complex formation have been determined. These sequences differ from those recognized by E. coli DNA topoisomerase I but resemble those recognized by E. coli DNA topoisomerase III. Based on these results, the yeast TOP3 gene product can formally be termed S. cerevisiae DNA topoisomerase III. Analysis of supercoiling of intracellular yeast plasmids in various DNA topoisomerase mutants indicates that yeast DNA topoisomerase III has at most a weak activity in relaxing negatively supercoiled double-stranded DNA in vivo, in accordance with the characteristics of the purified enzyme.

The TOP3 gene of the yeast Saccharomyces cerevisiae was originally termed EDR1, mutations in which were found to increase the frequency of RAD52-dependent loss of a suppressor rRNA marker flanked by the 5' sequences, short repetitive sequences that are normally present in the terminal repeats of the yeast transpon Ty (Rothstein et al., 1987; Wallis et al., 1989). Cloning and sequencing of the gene showed that it encoded a protein homologous to Escherichia coli DNA topoisomerase I, with 21% identical plus 18% conserved amino acids at corresponding positions (Wallis et al., 1989). Because of this homology, the gene and its product were termed TOP3 and DNA topoisomerase III, respectively.

That the limited homology between the bacterial and yeast gene might be functionally significant was supported by the finding that expression of the bacterial enzyme in yeast top3 mutants appeared to suppress the slow-growth phenotype of the mutants (Wallis et al., 1989). The same study found, however, that the presence of the bacterial enzyme failed to suppress the higher recombination frequency between 5' sequences in top3 strains. The lack of direct evidence that the TOP3 gene encodes a DNA topoisomerase prompted us to purify and characterize its product. In this paper, we show that a 74-kDa protein can be purified to near homogeneity from yeast cells overexpressing a plasmid-borne TOP3 gene. Biochemical characterization of the purified protein shows that it possesses a distinct DNA topoisomerase activity: it partially relaxes negatively but not positively supercoiled DNA; negatively supercoiled heteroduplex DNA containing a single single-stranded loop is readily relaxed by it. A protein-DNA covalent complex in which the 74-kDa protein is linked to the 5' end of a DNA strand has been identified, and the nucleotide sequences at many sites of covalent adduct formation have been determined. The biochemical characteristics of the yeast enzyme suggest that it resembles more E. coli DNA topoisomerase III than E. coli DNA topoisomerase I. In vitro, yeast DNA topoisomerase III is much less efficient than either yeast DNA topoisomerase I or II in the relaxation of supercoiled DNA substrates. In vivo studies also indicate that yeast DNA topoisomerase III has at most a weak activity in relaxing negatively supercoiled intracellular DNA. The plausible biological roles of this enzyme are discussed in light of these findings.

EXPERIMENTAL PROCEDURES

Strains-S. cerevisiae strains CH335 a his4-459 lys2-801 ura3-52 (Holm et al., 1985) and CH1105 a ade2-101 11eu2 lys2-801 1p1 ura3-52 were obtained from Dr. Connie Holm, Harvard University. Strain TG205 a his4-459 lys2-801 1p1:URA3 top2-4 and its ura3 derivative JCW2 have been described previously (Goto and Wang, 1985; Giaever and Wang, 1988). Strain JCW173 a ade2-101 11eu2 lys2-801 1p1:URA3 top2-4 and its ura3 derivative JCW2 have been described previously (Goto and Wang, 1985; Giaever and Wang, 1988). Strain JCW173 a ade2-101 11eu2 lys2-801 1p1:URA3 top2-4 TRP1 was derived from CH105 by first switching the mating-type from a to a, using the method of Herskowitz and Jensen (1991); two cycles of gene transplacement (Rothstein, 1983) were then carried out to introduce the mutations in the topoisomerase genes. The plasmid used in the deletion of TRP1 was the one reported previously (Goto and Wang, 1985). For targeted inactivation of TOP3, a 31-bp segment within TOP3 in pRK480 (see below), bounded by an Nael and a Hpal site, was replaced by an 850-bp segment containing the TRP1 marker. An EcoRI to SaI restriction fragment containing the TRP1 insert and its TOP3 flanking sequences was isolated and used for gene transplacement. Plasmids for the Expression of Yeast TOP3-A partial EcoRI digest of S. cerevisiae strain CH335 (Holm et al., 1985) was used to prepare

* This work was supported by United States Public Health Service Grants GM 24544 and CA47958 and National Science Foundation Grant DMB-8867607. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

1 The abbreviations used are: bp, base pair(s); kb, kilobase(s); kbp, kilobase pair(s); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
a genomic yeast DNA library in phage M13mp18 (Yanisch-Perron et al., 1985). To identify members containing TOP3, a pair of primers 5'-CAGCTACGTAAAGGTG-3' and 5'-CCGTACTCTGTCCATCC-3' were synthesized based on the published TOP3 nucleotide sequence of Wallis et al. (1989), and the polymerase chain reaction method (Mullis and Faloona, 1987) was used to amplify a 2.36-kb fragment bounded by these primer sequences. Following purification of the fragment by agarose gel electrophoresis, it was cloned into the Smal site of pUC18. Gel-purified restriction fragments from the fragment by agarose gel electrophoresis, it was cloned into the fragment bounded by these primer sequences. Following purification the construction of the yeast TOP3 overexpression plasmids, a 7-cycle amplification of a 450-bp segment in the pBluescript-TOP3 plasmid was carried out by the polymerase chain reaction, using a pair of primers 5'-GGGATCCTCTAAGAAATGCTATG-3' and 5'-GGTATCTTGTATCTATGGAGA-3'. The underlined ATG in the primer corresponds to the initiation codon of the TOP3 open-reading frame, and the primer was designed to make this ATG a BspHI site preceded by a BamHI site; the sequence of the other primer contains a BclI site 5'TGATCA3', a unique site in TOP3 and about 430 bp downstream of the initiation codon. The polymerase chain reaction product was used to replace the SmaI-BclI site preceded by a BamHI site; the sequence of the other primer contains a BclI site in TOP3. The polymerase chain reaction product was used to replace the Smal-BglII site of the fragment in pBluescript, yielding pRK480. In the TOP3 overexpression clone pRK485, which was derived from pRK480 and a commercial vector pMAL-c (New England Biolabs), the following sequences were tandemly inserted in-between the NcoI and XbaI sites in the polylinker region of the vector: (a) 5'-GGGGGCAATGGACAAAGGGTCTTGTCCAGAGG-3' and (b) a segment of yeast TOP3 from the initiation ATG codon to an XbaI site in the 3'-untranslated region of the gene. The sequence in parenthesis (a) encodes a domain in human c-myc and is an epitope recognized by a mouse monoclonal antibody MCY1-9E10 (ATCC/NIH Repository Number CRL 1729). The bulk of the (a) sequence ending at the underlined GATGAC BamHI site was derived from a clone pMCT6 (Murro and Felham, 1986), and routine recombinant DNA methodology was used in splicing these segments together to yield pRK485. Nucleotide sequencing of a region in the final clone, from the end of the maltose gene of the vector to the beginning of the yeast TOP3 coding sequences, was carried out to ensure that the functional product contained the desired junction region. The final step described above served the purpose of concentrating protein and reducing the KC1 concentration to 400 mM.

RESULTS

Purification of S. cerevisiae TOP3 Gene Product—Initial attempts to detect the yeast TOP3 protein in crude or fractionated cell extracts as an activity capable of relaxing negatively supercoiled DNAs were unsuccessful: no ATP-independent relaxation activity was detectable in extracts of Δtop1 cells devoid of DNA topoisomerase III or in Δtop1 top2-4 cells lacking DNA topoisomerase I and containing a temperature-sensitive DNA topoisomerase II; fractionation of cell extracts by several procedures commonly employed in the purification of enzymes involved in DNA metabolism, including ammonium sulfate precipitation and step-elution from a phosphocellulose column, also failed to yield an active fraction. These failures led us to adopt a strategy of overexpressing first an immunologically marked TOP3 protein and purifying it without an enzymatic assay; the characteristics of the tagged protein could then serve as a guide in the purification of the untagged protein.

Three plasmids were successively constructed for the expression of the TOP3 gene itself or TOP3 gene fused to coding sequences of other polypeptides. The first one, pRK485, was derived from a vector pMAL-c, a commercially available plasmid for the overexpression of fused genes in E. coli (see "Experimental Procedures" for plasmid construc-
tions). In this overexpression clone, the yeast TOP3 gene is fused to the E. coli malE gene (Duplay et al., 1984). Codons for two peptide motifs, those for a tetrapeptide IEGR and those for a decapeptide EQKLISEEDL, are present in between the malE and TOP3 coding sequences. The decapeptide is specifically recognized by a monoclonal antibody MYC1-9E10.2, and its presence provides an immunotag in the fusion protein; the tetrapeptide motif is the recognition sequence of the factor Xe endoproteinase and was carried over from the original pMAL-c cloning vector.

The other two plasmids were constructed for the expression of TOP3 in yeast. In pRK490 (Fig. 1A, top), an open reading frame derived from an NcoI-SalI restriction fragment of pRK485 was placed downstream of an inducible yeast gene promoter. This open reading frame contains, in succession, codons for the following stretches of amino acids: (a) the last 50 amino acids of E. coli malE protein, which begins with an in-frame methionine codon coincident with the NcoI site, (b) some 30 amino acids, including the IEGR and the decapeptide immunotag described above and amino acids encoded by linker oligonucleotides that were introduced during various stages of cloning, and (c) the entire yeast TOP3 polypeptide (Wallis et al., 1989). In pRK500 (Fig. 1A, bottom), the open reading frame downstream from the PHO5 promoter is that of the TOP3 gene.

The first plasmid pRK485 appeared to express the desired TOP3 fusion protein in E. coli, but the protein was unstable and little intact protein could be isolated from cell extracts (results not shown). Induction of the PHO5 promoter in yeast cells harboring the second plasmid, pRK490, produced a protein readily detectable by immunostaining with antibody MYC1-9E10.2. The molecular mass of this protein, as estimated from its electrophoretic mobility in SDS-polyacrylamide gel, is 82 kDa, which is in agreement with that expected from the PHO5 promoter-linked open reading frame containing TOP3.

When cells of a yeast strain JCW173 Δtop1 top3 leu2 were transformed to LEU" with pRK490, the slow growth phenotype of the strain in rich media, which is attributable to the top3 mutation (Wallis et al., 1989), was no longer observed (results not shown). This suggests that the 82-kDa TOP3 fusion protein is functionally similar to the yeast TOP3 gene product itself. In low phosphate media, however, the overproduction of the 82-kDa fusion protein is apparently detrimental to cell growth, and pRK490 transformants of either JCW173 or its top3" parent were found to grow poorly.

The 82-kDa fusion protein was purified to near homogeneity from cells harboring pRK490, using SDS-polyacrylamide gel electrophoresis and immunostaining of the fusion protein to monitor its concentration in various fractions. The purified fractions were found to possess a DNA topoisomerase activity (see below). Together, the genetic and biochemical results provide strong evidence that the 82-kDa TOP3 fusion protein is functional in vivo and possesses the catalytic activity of the TOP3 gene product in vitro.

Similar to results obtained with pRK490, transformation of Δtop1 Δtop3 cells with pRK500 abolishes the poor growth phenotype of the parent cells in rich media. To purify the plasmid-borne TOP3 gene product, JCW173 Δtop1 top3 cells harboring pRK500 were first grown in a rich medium, and cells pelleted centrifugally from the culture were resuspended in a low phosphate medium to induce the PHO5 promoter-linked TOP3 gene. Purification of the TOP3 product from the induced cells was carried out according to the purification scheme for the 82-kDa TOP3 fusion protein (see "Experimental Procedures").

Fig. 1B shows the Coomassie Blue-stained protein bands in the various fractions following their resolution by SDS-polyacrylamide gel electrophoresis. The purification of a protein with an apparent molecular mass of 74 kDa, which is that expected for the yeast TOP3 gene product, parallels that of the 82-kDa malE-TOP3 fusion protein. The 74-kDa protein was purified to near homogeneity following chromatography on single-stranded DNA-agarose embedded in agarose (Fig. 1B, rightmost three lanes). As will be shown below, fractions containing
purified 74-kDa protein contain a DNA topoisomerase activity, and the levels of this activity in fractions from DNA-agarose chromatography correlate well with the intensities of the 74-kDa band in these fractions.

The TOP3 Gene Product Is a DNA Topoisomerase—Purified 74-kDa protein has a distinct albeit weak relaxation activity with negatively supercoiled DNA substrates. Fig. 2A depicts the electrophoretic patterns of a 7.6-kbp negatively supercoiled plasmid DNA upon treatment with the protein under various conditions. Electrophoresis was carried out in a 0.1 M Tris borate buffer containing 5 μg/ml chloroquine. The untreated supercoiled DNA ran as a cluster of positively supercoiled topoisomers ahead of the nicked DNA band in the presence of the intercalating chloroquine (lane 1). Incubation of the DNA with the 74-kDa protein at 37 °C, in 40 mM HEPES buffer (pH 7.5) and 1 mM MgCl2, increased the mobilities of the DNA topoisomers due to increases of their linking numbers (lane 2). When incubation was carried out at 65 °C instead of 37 °C, further relaxation of the negatively supercoiled DNA was observed (lane 3); above 65 °C, the relaxation activity was inactivated (results not shown). At either 37 or 65 °C, relaxation of the negatively supercoiled DNA was incomplete. Under the gel electrophoresis conditions employed, completely relaxed substrate DNA in the reaction buffer ran as a highly positively supercoiled band (lane 4) with a mobility greater than those of the yeast DNA topoisomerase III-treated topoisomer clusters in lanes 2 and 3. When positively supercoiled DNA was used as the substrate, no change in the linking numbers of the topoisomers was detectable (results not shown).

The partial relaxation of negatively supercoiled DNA by the 74-kDa protein was detectable in a range of monovalent counterion concentrations below 150 mM; little difference was observed when NaCl, KCl, potassium acetate, or potassium glutamate was used. The omission of sulphydryl reagents or the presence of 10 mM dithiothreitol similarly showed no effect. Addition of exogenous Mg(II) was not obligatory for the relaxation reaction; as shown in lane 5 of Fig. 2A, incubation of negatively supercoiled DNA with the enzyme in 40 mM HEPES buffer (pH 7.5) resulted in partial relaxation of the DNA (compare the untreated and treated DNA run in lanes 1 and 5, respectively). The extent of relaxation of the DNA in the sample treated in the absence of added Mg(II) was reduced relative to that incubated in the presence of 1 mM MgCl2 (compare samples in lanes 5 and 2). However, when reaction was carried out in 40 mM HEPES plus 1 mM EDTA, no DNA relaxation activity was detectable (Fig. 2A, lane 6). Addition of MgCl2 in molar excess (3 mM) relative to the concentration of EDTA restored activity (Fig. 2A, lane 7). Activity was also readily detected when Mn(II) instead of Mg(II) was added to the HEPES-EDTA buffer, but Ca(II) and Zn(II) were found to be ineffective (data not shown). The optimal Mg(II) concentration for the relaxation of negatively supercoiled DNA by the 74-kDa protein is around 1 mM; increasing the concentration to 5 or 10 mM resulted in less complete relaxation of the negatively supercoiled DNA (Fig. 2A, lanes 8 and 9, respectively).

The results described above show that S. cerevisiae TOP3 gene encodes a 74-kDa protein, which, as predicted by its sequence homology to E. coli DNA topoisomerases I and III (Wallis et al., 1988; DiGate and Marians, 1989), possesses a DNA topoisomerase activity. This activity will be referred to as yeast DNA topoisomerase III.

Yeast DNA Topoisomerase III Is Single Strand-specific—The partial relaxation of negatively but not positively supercoiled DNA by yeast DNA topoisomerase III is reminiscent of the reactions catalyzed by bacterial DNA topoisomerases I and can be interpreted in terms of a requirement for a short single-stranded segment in the DNA substrate (Wang, 1971). This single-stranded segment could either be present in the DNA before the binding of the enzyme, or it could be induced by the binding of the enzyme. In either case, the process is favored by negative supercoiling and disfavored by positive supercoiling of the DNA.

The requirement of a short single-stranded segment in the substrate of yeast DNA topoisomerase III is supported by several experiments described below. As shown in Fig. 2B, the relaxation of negatively supercoiled DNA by yeast DNA topoisomerase III is inhibited by the presence of denatured DNA (lane 3); native double-stranded DNA (lane 4) and tRNA (lane 5) are ineffective as competitive inhibitors. In a second set of experiments, a derivative of phage M13, MP8329, was obtained by deleting 29 nucleotides between the EcoRI and HindIII sites of the M13 derivative MP8. Linearization of the SnaBI site far away from the EcoRI-HindIII region, denatured, and annealed with single-stranded circular MP8329 viral DNA. The nicked circular heteroduplex DNA, which contains a 29-nucleotide-long single-stranded loop, was purified by agarose gel electrophoresis from the linear rena-
tured MP8 homoduplex DNA and any remaining single-stranded species. As shown in lane 3 of Fig. 3, the gel-purified DNA migrated as the nicked circular species. Ligation of the nicked heteroduplex DNA in the presence of ethidium, followed by the removal of ethidium after ligation, converted a significant fraction of the nicked rings to the negatively supercoiled form, which migrated as an intense band near the bottom of Fig. 3, lane 7. Three minor bands are discernible in the lane 7 sample. In the order of increasing mobility, the first minor band is most likely the linear homoduplex DNA, as its mobility is the same as that of linear duplex mp8 DNA (lane 2 of Fig. 3). The next minor band is most likely the single-stranded circular form of MP8s29, the mobility of which is about the same as single-stranded circular MP8 DNA (lane 1 of Fig. 3). The fastest migrating minor band is probably a single-stranded circular DNA from a spontaneous deletion mutant of MP8, which is a contaminant in some of the preparations of MP8 phage or its derivatives.

Incubation of the heteroduplex DNA run in lane 7 of Fig. 3 with yeast DNA topoisomerase III resulted in the conversion of the negatively supercoiled band to a set of topoisomers extending all the way to the nicked DNA band (lane 6 of Fig. 3). This change is particularly striking in comparison to that observed for negatively supercoiled homoduplex MP8 DNA. The electrophoretic pattern of the homoduplex DNA under the electrophoresis conditions employed was unaffected by incubation with the yeast enzyme (compare the untreated and treated samples run, respectively, in lanes 4 and 5 of Fig. 3).

**Yeast DNA Topoisomerase III Can Link Covalently to a DNA 5’ Phosphoryl Group—** The experiments described below show that similar to the cases with a number of other DNA topoisomerases (reviewed in Wang, 1985), a covalent complex between yeast DNA topoisomerase III and a DNA 5’ phosphoryl group can be trapped by the addition of sodium dodecyl sulfate to the enzyme-DNA complex.

A pair of partially complementary oligonucleotides 5’-GGGCAGGCTTTCATTTTGAATATTTGGTCG-3’ and 5’TCCGAG(CGA(CAAATATGACGAAGCAGCCG)C), which were originally synthesized for the conversion of a DNA with a 5’-TCG overhang to one with a 12-nucleotide phase λ left cohesive end (nucleotides in parentheses in the two sequences are complementary in the text). The labeled oligonucleotides were 32P-labeled at their 5’ ends by treatment with polynucleotide kinase, or, following annealing to allow pairing of the 31-nucleotide-long complementary sequences, at their 3’ ends by repairing with the Klenow fragment of *E. coli* DNA polymerase I.

When yeast DNA topoisomerase III was first incubated with denatured 5’-end-labeled oligonucleotides, treatment of the complexes with SDS yielded no labeled protein-DNA covalent complex (Fig. 4, lane 4). The same experiment with oligonucleotides with 32P-labeled nucleotides at their 3’ ends yielded, however, a cluster of radiolabeled bands following SDS-polyacrylamide gel electrophoresis (Fig. 4, lane 5).

![Fig. 4. Transfer of 32P from labeled DNA oligonucleotides to yeast DNA topoisomerase III.](image-url)

The two partially complementary single-stranded oligodeoxyribonucleotides were annealed and labeled at their 5’ or 3’ ends as described in the text. The labeled oligonucleotides were purified by phenol extraction and exhaustive dialysis, first against 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, and 1 mM NaCl, and then against 10 mM Tris-HCl, pH 8, 0.1 mM EDTA. To form the protein-DNA covalent complex, labeled oligonucleotides were alkali-denatured by treatment with 0.1 M NaOH, chilled, neutralized with an equal volume of 0.1 M HCl and 0.1 mM pH 7.5 Tris-HCl, and desalted by alcohol precipitation, washing, and resuspension in distilled water. Approximately 1.8 pmol of oligonucleotides and 160 ng of yeast DNA topoisomerase III were incubated as described in the legend to Fig. 2, and the reaction mixture was split into two equal portions. CaCl2 was added to one to a final concentration of 2 mM, followed by the addition of 1 unit of staphylococcal nuclease (Worthington). Incubation was continued for 5 min for both samples, and an equal volume of a 2X SDS-loading buffer, which contained 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue, was added to each. Control experiments with the same radiolabeled oligonucleotides were also carried out with *E. coli* DNA topoisomerase I instead of yeast DNA topoisomerase III, following the procedures of Tse et al. (1980). All samples were placed in a boiling water bath for 5 min, and the denatured protein in each was precipitated with 10% trichloroacetic acid. The pellet in each tube was washed twice with 10% trichloroacetic acid and resuspended in 50 μl of the 2X SDS-loading buffer. Several 1-μl portions of 1 M NaOH were gradually added to each sample to neutralize the excess acid in the acid-washed samples; neutrality was gauged by the return of the bromphenol-containing solution to a blue color. Electrophoresis in 3% SDS-10% polyacrylamide gel was carried out as described (Laemmli, 1970) and was terminated when the blue tracking dye ran off the gel. The gel was stained with Coomasie Blue, destained, and dried between two sheets of gel-drying membrane (Promega). Autoradiography of the dried gel was done at room temperature. Lanes 1, 5’-end-labeled oligonucleotides treated with *E. coli* DNA topoisomerase I; lane 2, 3’-end-labeled oligonucleotides treated with *E. coli* DNA topoisomerase I; lane 3, same as in lane 2, except that the reaction product was digested with staphylococcal nuclease following incubation with *E. coli* DNA topoisomerase I; lanes 4–6, same as samples in lanes 1–3, respectively, except that yeast DNA topoisomerase III was used in place of *E. coli* DNA topoisomerase I.

**Fig. 3. Relaxation of negatively supercoiled heteroduplex DNA containing a short single-stranded loop by yeast DNA topoisomerase III.** Negatively supercoiled MP8 homoduplex or MP8/MP8s29 heteroduplex DNA containing a 29-nucleotide-long single-stranded loop was incubated with yeast DNA topoisomerase III for 30 min at 37°C in 40 mM HEPES-KOH (pH 7.6 at 20°C) and 1 mM MgCl2. Lanes 1–3 contained marker DNAs: lane 1, single-stranded circular MP8 phage DNA; lane 2, linear double-stranded MP8 DNA generated by *Pst*I digestion of the RF I DNA; lane 3, gel-purified negatively supercoiled heteroduplex DNA containing a single nick, see text for a description on its preparation; lane 4, negatively supercoiled or RF I MP8 homoduplex DNA; lane 5, homoduplex MP8 RF I DNA after incubation with yeast DNA topoisomerase III; lane 6, negatively supercoiled heteroduplex DNA after incubation with yeast DNA topoisomerase III; lane 7, negatively supercoiled heteroduplex DNA before incubation with yeast DNA topoisomerase III. The DNA samples were phenol-extracted and loaded in the sample wells of a 0.8% agarose slab gel. Following electrophoresis in TBE buffer (0.1 M Tris borate, 2.5 mM EDTA), the gel was blotted unto a sheet of filter paper, dried, and destained in 10 mM Tris-HCl and 0.1 M NaCl.

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**Table 1.**

| Lanes | Description |
|-------|-------------|
| 1     | Single-stranded circular MP8 phage DNA |
| 2     | Linear double-stranded MP8 DNA |
| 3     | RF I DNA before incubation with yeast DNA topoisomerase III |
| 4     | RF I DNA after incubation with yeast DNA topoisomerase III |
| 5     | Single-stranded circular MP8 DNA |
| 6     | Negatively supercoiled heteroduplex DNA after incubation with yeast DNA topoisomerase III |
| 7     | Sample before incubation with yeast DNA topoisomerase III |
cluster of bands can be attributed to the attachment of oligonucleotides of varying lengths to the protein. In support of this notion, the slower migrating bands disappeared upon treatment of the products with staphylococcal nuclease (Fig. 4, lane 6). Staining of the same gel with Coomasie Blue prior to autoradiography also showed that the position of yeast DNA topoisomerase III coincided with the front of the radio-labeled cluster of bands (result not shown).

For an oligomer of nucleotides long with $m$ of its 3'-proximal nucleotides radiolabeled, cleavage within the first $(n-m)$ nucleotides from the 5' terminus would give a radiolabeled protein-oligonucleotide covalent complex only if the topoisomerase is linked to a 5'-phosphoryl end generated by the cleavage; cleavage within the last $m$ nucleotides by such an enzyme would, however, give a radiolabeled protein-oligonucleotide covalent complex whether the topoisomerase is linked to a 5'- or 3'-phosphoryl end generated by the cleavage. Thus, based on the results shown in lanes 5 and 6 of Fig. 4 alone, an unequivocal conclusion could not be drawn on whether yeast DNA topoisomerase III is linked to a 5' or 3' phosphoryl group in the covalent complex. However, when these results are combined with the absence of a radiolabeled covalent complex with 5' end-labeled oligonucleotides (Fig. 4, lane 4), it can be concluded that yeast DNA topoisomerase III is linked to a 5'-phosphoryl group in the formation of the covalent intermediate. Further confirmation of this conclusion was obtained through parallel experiments with E. coli DNA topoisomerase I. It is well-established that this enzyme is linked to a 5'-phosphoryl group in the covalent intermediate (Depew et al., 1979; Tse et al., 1980); the labeling patterns of the E. coli enzyme upon incubation with the same oligomers and treatment with SDS are basically the same as those with yeast DNA topoisomerase III (Fig. 4, lanes 1-3).

Nucleotide Sequences of Yeast DNA Topoisomerase III Cleavage Sites Resemble Those of E. coli DNA Topoisomerase III—Based on the finding described above that the yeast enzyme is linked to a 5'-phosphoryl group in the DNA-protein covalent intermediate, DNA strands uniquely radiolabeled at their 5' ends were used to map the cleavage sites: cleavage of such a strand would yield a protein-free labeled strand with a 3'-hydroxy end, and the position of the 3' end could be mapped precisely by electrophoresis in a sequencing gel, using sequencing markers generated by priming the complementary strand with an oligonucleotide identical in sequence with the uniquely labeled end.

Table I lists the nucleotide sequences of 30 yeast DNA topoisomerase III cleavage sites that have been mapped in two single-stranded DNA fragments. One was the viral strand of a restriction fragment of the replicative form of phage φX174, which was radiolabeled at its 5' end generated by cutting with restriction endonuclease AvaII (nucleotide 5042). The other was a pBR322 restriction fragment from the HindIII site (nucleotide 29) to the BamHI site (nucleotide 375), with its BamHI end radiolabeled. All sequences listed in Table I are in the 5' to 3' direction, and the internucleotide bond between positions -1 and +1 is the site of cleavage in each sequence.

The distributions of bases at various positions relative to the sites of cleavage are also tabulated at the bottom of Table I. These distributions appear to be nonrandom. At position -3, for example, there appears to be a strong preference for the presence of an A. The very strong preference for a C at position -4 of the cleavage sites of E. coli DNA topoisomerase I (Tse et al., 1980; Dean et al., 1983) is not observed in the cleavage sites of yeast DNA topoisomerase III. The particular φX174 DNA fragment was chosen because its sequence overlaps with one used previously in mapping the cleavage sites of E. coli DNA topoisomerase III: the first five sites listed in Table I are in a region within which the sites of cleavage by E. coli DNA Topoisomerase III had been mapped previously (Dean, 1984). Significantly, with the exception of the second sequence from the top of the list, the four sites were all found to be cleaved by both yeast and E. coli DNA topoisomerase III.

Relaxation of Supercoiled DNA by Yeast DNA Topoisomerase III in Vivo—In yeast cells expressing E. coli DNA topoisomerase I, it has been shown that plasmid DNA becomes positively supercoiled upon inactivation of both DNA topoisomerases I and II (Giaever and Wang, 1988). This phenomenon has been attributed to the preferential removal of negative supercoils by the bacterial enzyme from oppositely supercoiled domains generated by transcription or other processes involving the translocation of macromolecular assemblies along DNA (Liu and Wang, 1987). The very fact that positive supercoils accumulate in intracellular yeast DNA under these conditions shows that no DNA topoisomerase other than DNA topoisomerases I and II can efficiently remove positive supercoils in vivo. Furthermore, because the expression of the E. coli enzyme is needed for the accumulation of positive supercoils in the absence of yeast DNA topoisomerases I and II, it appears that no other DNA topoisomerase in yeast can efficiently and preferentially relax negatively supercoiled intracellular DNA (Giaever and Wang, 1988).

The present finding that purified yeast DNA topoisomerase III has a distinct albeit weak relaxation activity for negatively supercoiled DNA raises again the issue whether this enzyme might have a similar activity in vivo. Examination of the endogenous 2-μm plasmid in yeast strain JCW2 top1 top2-4 cells harboring YEpTOP4-PGAL1, a plasmid expressing E. coli DNA topoisomerase I from the glucose-repressible and galactose-inducible yeast GAL1 gene promoter, confirmed the published results that 2-μm plasmid would become positively supercoiled.

| Table I | Preferred sites of cleavage of single-stranded DNA by yeast DNA topoisomerase III |
|---------|-----------------------------------|
| Nucleotide sequences of 30 sites were mapped. Cleavage occurs at the internucleotide bond between -1 and +1; the number of occurrences of each base at each position is tabulated at the bottom of the table. |
FIG. 5. Positive supercoiling of extrachromosomal ribosomal DNA rings in a yeast Δtop1 top2-4 mutant upon thermal inactivation of the mutant DNA topoisomerase II. Yeast strain JCW2 Δtop1 top2-4 harboring YEptopA-PGAL1, a plasmid in which the E. coli topA gene encoding E. coli DNA topoisomerase I is expressed from a galactose-inducible and dextrose-repressible GAL1 gene promoter (Giaever and Wang, 1988), was initially grown at a permissive temperature of 26°C in minimal media containing either 2% galactose or dextrose. The cultures were subsequently heated at 35°C for 2 h to heat-inactivate the top2-4 encoded mutant DNA topoisomerase II. Following isolation of DNA from the cultures, two-dimensional electrophoresis of the DNA samples in a 0.8% agarose slab was carried out as described previously (Giaever and Wang, 1988), using TBE buffer containing 0.6 and 5 μg/ml chloroquine in the first and second dimension analysis, respectively. The gel was blotted onto a nylon membrane and hybridized successively with radioactive probes derived from either yeast rDNA or 2 μm plasmid sequences. Only the autoradiogram obtained through the use of the radiolabeled rDNA probe is shown in the figure. The sample in lane 1 contained DNA from cells grown in the dextrose medium, in which the E. coli topA gene is repressed; the sample in lane 2 contained DNA from cells grown in the galactose medium, in which the E. coli gene is induced. In each case, the diagonal line is due mostly to sheared rDNA-containing chromosomal DNA and nicked circular and linear derivatives of extrachromosomal rDNA rings.

Discussion

That the TOP3 gene of S. cerevisiae might encode a DNA topoisomerase was previously postulated based primarily on its sequence homology with E. coli DNA topoisomerase I (Wallis et al., 1989). The findings described under “Results” show clearly that the protein indeed possesses a DNA topoisomerase activity; based on these results, the yeast TOP3 gene product can be formally termed yeast DNA topoisomerase III.

In comparison with yeast DNA topoisomerases I and II, yeast topoisomerase III is rather ineffective in the relaxation of supercoiled DNAs. In vitro, positively supercoiled DNA is refractory to the enzyme, and negatively supercoiled DNA is only partially relaxed by it. In vivo, the enzyme has at most a weak activity in the removal of negative supercoils. These results suggest that, in contrast to the earlier postulate based on the suppression of the poor growth phenotype of yeast top3 mutants by E. coli DNA topoisomerase I (Wallis et al., 1989), the biological function of yeast DNA topoisomerase III is unlikely to be related to its relaxation of supercoiled double-stranded DNA.

Yeast DNA topoisomerase III is more avid with DNA substrates containing single-stranded regions. Relaxation of negatively supercoiled DNA by the yeast enzyme is more effectively inhibited by single-stranded DNA than by double-stranded DNA or tRNA. More strikingly, the introduction of a 29-nucleotide-long single-stranded DNA loop in a negatively supercoiled DNA converts it into a much better substrate for yeast DNA topoisomerase III (Fig. 3).

This preference for single-stranded DNA is reminiscent of the properties of both bacterial DNA topoisomerases I and III. It has been shown previously that bacterial DNA topoisomerase I preferentially relaxes negatively supercoiled DNA, that the relaxation reaction is strongly inhibited by exogenous single-stranded DNA fragments, and that positively supercoiled DNA is a very poor substrate unless the DNA contains a short single-stranded region (Wang, 1971; Kung and Wang, 1977; Kirkegaard and Wang, 1985). Several properties of E. coli DNA topoisomerase III also indicate that its action requires the presence of single-stranded regions in its substrates (Dean et al., 1983; DiGate and Marians, 1988).

The common nucleotide sequences of the preferred sites of DNA cleavage by yeast DNA topoisomerase III indicate, however, that the yeast enzyme is more closely related to E. coli DNA topoisomerase III than to E. coli DNA topoisomerase I. Both yeast and E. coli DNA topoisomerase III also exhibit a stronger preference for single-stranded regions than E. coli DNA topoisomerase I, and both lack a carboxyl-terminal domain corresponding to the carboxyl-terminal domain of E. coli DNA topoisomerase I. Significantly, mutations in the gene topB encoding E. coli DNA topoisomerase III have recently been observed to stimulate recombination between repetitive sequences. The molecular mechanism underlying the hyper-recombination phenotype of yeast top3 or E. coli topB mutants is unclear. One plausible interpretation previously proposed is that such an enzyme might be a component of a cellular assembly involved in the reduction of pairing of DNA strands of complementary sequences (Wang et al., 1990; Wang, 1991). The reduction of recombination by enzyme systems has been raised before (see for example, Radman, 1991), and there is substantial genetic and biochemical evidence that various DNA topoisomerases are involved in the maintenance of genome stability (Christman et al., 1988; Kim and Wang, 1989; Wang et al., 1990).

In a purified system for the replication of pBR322, E. coli

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2 J. H. Miller, personal communication.
DNA topoisomerase III has been shown to be a key component in the unlinking of parental strands at the terminal stage of replication (DiGate and Marians, 1988). It is plausible that yeast DNA topoisomerase III may function similarly in vitro and in vivo, either by itself or as a component of a more complex assembly. Because of the multiplicity of bidirectional replication origins in eukaryotic cells, inefficient unlinking of intertwined parental DNA strands near the terminal stage of replication might affect more the growth of eukaryotic than prokaryotic cells.

Finally, DNA topoisomerases have emerged as important targets of antimicrobial and anticancer agents (reviewed in Liu, 1989). Because most of the DNA topoisomerase-targeting agents interfere with the DNA rejoining step catalyzed by these enzymes and thus convert normal cellular entities into prokaryotic cells, the efficacy of a drug does not depend on it target being an essential enzyme. The identification of agents that interfere with the DNA rejoining step catalyzed by DNA topoisomerase thus adds a potential target in the search of new therapeutics of this type.

Acknowledgments—We thank many of our colleagues for stimulating discussions during the course of this work, and we are grateful to Jia Liu, Wendy Raymond, Stanley Shaw, Ryo Hanai, Joachim Roca, and James Berger for providing many of the reagents used in this work. We also acknowledge Paul Caron for assistance in computer graphics and Simon Lynch for assistance in DNA sequencing.

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