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A Functional 125-kDa Core Polypeptide of Fission Yeast DNA Topoisomerase II

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We purified fission yeast DNA topoisomerase II (topo II) to apparent homogeneity. It consists of a single 165-kDa polypeptide in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and, upon treatment with a bifunctional reagent, doubles its molecular weight. Limited proteolysis of intact topo II by papain produces a 125-kDa core, which lacks the N-terminal 75 and the C-terminal ~260 amino acids but still contains regions similar to those of bacterial or phage T4 topo II subunits. The core retains relaxing and unknotting activities. Further digestion inactivates the core, cleaving it at the middle of the GyrB-like domain and at the beginning of the GyrA-like domain. Therefore, papain appears to cleave spatially distinct subdomains of topo II. We made *top2* mutant genes deleted of the C-terminal 286 or N-terminal 74 amino acids, which can substitute for the wild-type *top2*$^+$ gene in mitosis and meiosis. However, a mutant containing deletions of both termini cannot rescue the *top2* null mutant, despite the fact that the product is enzymatically active. Therefore, the *top2* product of the doubly truncated gene may not fulfill all of the in vivo requirements for *top2*$^+$ function.

DNA topoisomerase II (topo II) plays an essential role in regulating topological structures of DNA by transient breakage and rejoining of double-stranded DNA (8). In eukaryotes, the enzyme catalyzes the ATP-dependent relaxation of negatively and positively supercoiled DNA as well as the catenation-decatenation and knotting-unknotting of circular DNA. In prokaryotes, the comparable enzyme, called gyrase, has a tetrameric structure (2A2B) made up of two distinct subunits, GyrA and GyrB (14); GyrA binds to DNA, whereas GyrB has ATPase activity. In contrast, purified eukaryotic topo II is homodimeric, consisting of a single polypeptide with a molecular weight (MW) of about 150,000 (17, 18). In higher eukaryotes, topo II is reported to bind to the nuclear matrix in interphase cells and to the chromosome scaffold in mitotic cells (11, 13). In vivo roles of topo II have been extensively studied in budding and fission yeasts by genetic means (reviewed in references 52 and 53). Mutant isolation and gene disruption demonstrated that the topo II gene is essential for viability (10, 19, 47). In temperature-sensitive (ts) mutant cells, mitotic chromosome separation is impaired and abnormal chromosomes are formed during mitosis (22, 47, 48). Analysis of reciprocal temperature shifts using ts and cold-sensitive (cs) mutants established that topo II is required for condensation and segregation of mitotic chromosomes (23, 46). Recent studies using mitotic cell extracts demonstrated the necessity of topo II for in vitro chromosome condensation (2, 50). However, the molecular mechanism of topo II involvement in chromosome condensation and segregation is poorly understood.

Predicted amino acid sequences of eukaryotic topo II genes such as the yeast, fruit fly, and human genes (28, 43, 44, 51) are extensively similar, revealing approximately 50% identity. Furthermore, they show a weak but significant similarity to the amino acid sequences of bacterial gyrase and phage T4 topo II subunits. The N-terminal half of eukaryotic topo II is similar to that of the ATP-binding GyrB or phage gp39 and gp60, whereas the central to C-terminal region is similar to the N-terminal domain of the DNA-binding GyrA or phage gp52 subunit. Thus, the eukaryotic topo II polypeptide consists of domains similar to the prokaryotic topo II subunits arranged tandemly. The C terminus, making up 15% of the entire polypeptide, is nonhomologous and contains a number of charged residues. Thus, eukaryotic topo II appears to have at least three subdomains, namely, the GyrB-like region, the GyrA-like region, and the nonhomologous C terminus. These domains could have distinct functional roles (53).

Does the highly charged C terminus play an important role specifically for eukaryotic topo II, such as interacting with chromatin or another nuclear structure? Do these regions predicted from the sequence comparison exist as spatially distinct substructures in the intact topo II molecule? And is the entire topo II molecule required for chromosome condensation and segregation? Pleiotropic effects of the *top2* mutant in yeast cells suggest that different parts of the topo II molecule might be responsible for different topo II functions. In this study, we address these questions by purifying fission yeast topo II, and we make an initial attempt to dissect the molecule into structural and functional subdomains with the use of biochemical and genetical methods.

MATERIALS AND METHODS

Yeast strains and plasmids. Haploid *Schizosaccharomyces pombe* wild type, ts *top2-191* and cs *top2-250* mutant strains (46, 47) were used. Multicopy plasmid pDB248′ (3, 4) and an *S. pombe* expression vector with the alcohol dehydrogenase (ADH) promoter (pEVP11 [38]) were employed. These constructs contained the *Saccharomyces cerevisiae LEU2* gene, which complements *S. pombe leu1*, as the selectable marker. For preparing bacterial fusion proteins, pAR3038 (41) was used. Transformation of *S. pombe* was performed by the lithium method (26). Procedures for Southern hybridization were described previously (29).

Assay of topo II. ATP-dependent relaxing and unknotting activities were measured by procedures described previously (47), using supercoiled plasmid pBR322 and knotted P4

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DNA as the substrate. When purified topo II was used, bovine serum albumin (BSA; 1 mg/ml) and 10% glycerol were added to the dilution buffer, and BSA (50 μg/ml) was added to the reaction buffer (24). One unit of topo II completely relaxes 0.3 μg of supercoiled pBR322 DNA at 30°C for 30 min. The double-strand cleavage reaction by topo II in the presence of VP-16 was monitored by incubating topo II with supercoiled pBR322 at 30°C for 30 min in reaction buffer containing 20 μM VP-16 (supplied by National Products Branch, Division of Cancer Treatment, National Cancer Institute), after which the reaction was stopped by the addition of sodium dodecyl sulfate (SDS; prewarmed at 30°C; final concentration, 0.5%). Proteinase K digestion (final concentration, 50 μg/ml) was done at 50°C for 40 min.

Gene disruption. To construct a heterozygous diploid carrying one copy of wild-type *top2*$^+$ and one copy of the *top2* disruptant, the one-step gene disruption method (37) was employed, using linear DNA containing the *top2* sequence (6.8-kb *Hin*dIII fragment) interrupted with the *S. pombe ura4*$^+$ gene at the *Hpa*I site of the coding region. This linear DNA was used for transformation of a diploid CM3/CM7 (*h*$^-$/*h*$^+$ *leu1/leu1 ura4/ura4 +/his2 ade6-210/ade6-216*). Stable Ura$^+$ transformants were then obtained, and genomic DNA was isolated and examined by Southern hybridization to determine whether the interrupted *top2* gene was integrated onto the chromosome by homologous recombination. The heterozygous diploid cells thus made were sporulated, and their tetrads were dissected.

Purification of fission yeast topo II. We developed procedures for purification of *S. pombe* topo II which consist of differential salt extraction, ammonium sulfate fractionation, and phosphocellulose, Mono Q, and hydroxyapatite chromatography. Topo II was detected by ATP-dependent relaxing activity and immunoblotting using anti-topo II antibodies (TP2-F; described below). Topo II was insoluble by 0.1 M NaCl extraction but completely solubilized by 0.4 M NaCl (46). The amount of topo II was found to greatly increase after introduction of a multicopy plasmid carrying the *top2*$^+$ gene into cells. Thus, the *S. pombe* strain *h*$^-$ *leu1 end1 top1* that contained the multicopy plasmid pSPTOP2 (carrying the wild-type *top2*$^+$ gene [44]) was used for subsequent purification. Cells were initially grown in 1 liter of minimal EMM2 (32) medium lacking leucine to a concentration of 10^7/ml, then inoculated into 9 liters of YPD, a rich medium, and further grown for 10 h at 33°C. After the cell concentration reached 4×10^7/ml, the culture was passed through a glass fiber filter (21), and an approximately 50-g wet pellet of cells was collected. All procedures described below were done at 4°C, and ultracentrifugation was run at 25,000 rpm, using a Beckman type 30 rotor. Cells were once washed in 500 ml of TE (50 mM Tris HCl [pH 7.5], 1 mM Na_3EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM 2-mercaptoethanol) containing 20 mM NaCl and 1 mM sodium bisulfite and resuspended in the same buffer. The cells were broken by spraying at a low temperature, using liquid nitrogen in the Cryo Clean Blaster (Taiyo Sanso, Osaka, Japan). The broken cells were centrifuged at $700 \times g$ for 5 min to remove unbroken cells, and then the supernatant was ultracentrifuged. The pellet was resuspended in 100 ml of TEG (10% glycerol added to TE) containing 100 mM NaCl, and the suspension was again ultracentrifuged. The pellet was extracted twice (30 and 20 ml) with gentle shaking in TEG containing 400 mM NaCl and then ultracentrifuged. The collected supernatant was mixed with the saturated ammonium sulfate solution to 35% saturation. The supernatant after ultracentrifugation at 25,000 rpm for 20 min was precipitated by ammonium sulfate (final 50% saturation) with stirring for 30 min on ice. The pellet obtained after ultracentrifugation at 25,000 rpm for 20 min was resuspended in 30 ml of TEG containing 100 mM NaCl. By immunoblot using anti-Top2 antibodies, 60% of topo II was recovered by these procedures. Topo II was fractionated by stepwise phosphocellulose (P11; Whatman) chromatography (25 ml, 2.6 by 4.7 cm). It was not eluted by 0.45 M NaCl but was eluted by 0.75 M NaCl in TEG. The eluant was dialyzed against Q buffer (20 mM Tris HCl [pH 7.5], 0.1 mM Na_3EDTA, 10% glycerol, 0.1 mM PMSF, 1 mM 2-mercaptoethanol) containing 200 mM NaCl. The dialysate was chromatographed with a Mono Q HR5/5 column (Pharmacia) in a 20-ml linear gradient between 0.2 and 0.5 M NaCl in Q buffer. Topo II was eluted at approximately 0.35 M NaCl. The topo II fraction was applied to Bio-Gel HPHT (Bio-Rad) chromatography. The column was washed once with P buffer (0.1 mM Na_3EDTA, 10% glycerol, 0.1 mM PMSF, 1 mM mercaptoethanol) containing 0.2 M sodium phosphate (pH 7.0) and eluted by a 20-ml linear gradient of P buffer containing 0.2 to 0.5 M sodium phosphate. Topo II was eluted at 0.35 M sodium phosphate. Topo II was concentrated by Centrisart I (Sartorius) and dialyzed against the storage buffer (15 mM sodium phosphate [pH 7.5], 100 mM NaCl, 0.1 mM Na_3EDTA, 0.1 mM 2-mercaptoethanol, 0.1 mM PMSF, 50% glycerol) and stored at −20°C. Starting from a 50-g wet pellet of cells, 300 μg of purified topo II with a specific activity 1×10^6 to 3×10^6 U/mg was obtained. The preparation was completely devoid of topo I activity.

Preparation of anti-topo II antisera. About 100 μg of topo II purified from *S. pombe* was used for each immunization injection of a rabbit with complete adjuvant (Difco). Preimmunized serum was obtained before immunization. The rabbit was boosted 2 and 6 weeks after the initial injection. This antiserum against purified topo II was named TP2-P. A different topo II antigen was used for injecting another rabbit. LacZ-*top2* fusion protein was made by using pUC19 and *Escherichia coli* JM109. The expressed protein was insoluble and purified according to the procedures described by Watt et al. (49). One to two milligrams of fusion protein was obtained from 500 ml of culture. Partially purified fusion protein (approximately 1 mg) was run on an SDS-polyacrylamide gel (5 mm thick), stained, destained, and isolated from the band by electroelution (30). Then it was dialyzed against phosphate-buffered saline, and 200 to 250 μg was mixed with complete adjuvant and injected to a rabbit. Boosting was done every 2 weeks for 8 weeks, using incomplete adjuvant. Antibodies against the fusion protein (named TP2-F) were affinity purified by the procedures of Smith and Fisher (40).

Limited proteolysis of purified topo II. (i) Trypsin, chymotrypsin, and V8 protease. Trypsin (type III; Sigma), α-chymotrypsin (type VII; Sigma), or *Staphylococcus aureus* V8 protease (Worthington) (0.05 μg of each) was added to 5 μg of topo II in 40 μl of 0.2 M sodium phosphate buffer (pH 7.0) containing 20% glycerol and incubated at 30°C. Aliquots were taken at intervals within 2 to 240 min and boiled in SDS-polyacrylamide gel electrophoresis (PAGE) buffer for 2 min.

(ii) Papain. Five millimolar cysteine and 1 mM EDTA were added to the reaction buffer, otherwise the same as described above.

(iii) V8 protease in the presence of SDS. The procedure described by Cleveland et al. (7) was followed. V8 protease (0.025 μg) was added to 2.5 μg of topo II in 0.125 M Tris HCl (pH 6.8) containing 0.5% SDS, 10% glycerol, and 0.0001%

bromphenol blue which had been previously boiled for 2 min and incubated at 30°C for 15 to 240 min.

Determination of the NH_2-terminal sequences. Purified topo II (~17 μg) in 10 mM NH_4HCO_3 containing 0.02% SDS was lyophilized, dissolved in H_2O, and subjected to Edman degradation for determining the NH_2-terminal sequence by a protein sequencer (Shimazu model PSQ-1). Papain-digested fragments were isolated by procedures described previously (33). The fragments were separated by SDS-PAGE, electroblotted to a polyvinyl difluoride membrane (IPVH; Millipore), and stained by Coomassie brilliant blue R. The band was excised and applied for determination of the NH_2-terminal sequence by the 473A sequencer (Applied Biosystems).

Construction of topo II deletion mutants. Various restriction sites present in the *top2*$^+$ coding region were used for the construction of deletion and insertion mutants. To make insertion mutants, a *Bam*HI sequence containing the *S. cerevisiae LEU2* gene was inserted at the restriction sites where the *Bgl*II linker sequence was added. Then the *Hin*dIII fragments were cloned into pDB248′ (4) at the *Hin*dIII site. To make the C-terminal deletion mutants, *top2*$^+$ gene fragments were digested by *Sph*I, *Sac*II, or *Xba*I, blunted by Klenow treatment, and religated. By frameshift mutation, termination codons were introduced just behind those restriction sites. Δ(775-890) was made by using the internal *Acc*I sites; Δ(909-1085) and Δ(1066-1085) were made by using the *Nde*I sites. To make N-terminal deletion mutants, the *top2*$^+$ promoter for the internal truncations or the ADH promoter (38) for upstream deletion was used.

Other biochemical methods. SDS-PAGE was performed as described by Laemmli (27). Proteins were detected by Coomassie brilliant blue R250 or silver stain (Wako). Immunoblot analysis was done according to the procedure described by Towbin et al. (42). Secondary antibodies used were peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Bio-Rad) or [^{125}I]protein A (Amersham).

RESULTS

The NH_2 sequence of purified fission yeast topo II. The fission yeast topo II was purified to apparent homogeneity according to the procedures described in Materials and Methods. By SDS-PAGE, a single polypeptide band with an estimated size of 165 kDa was seen (Fig. 1a, lane 2; the bands at lane 1 were the marker proteins). To examine whether the fission yeast topo II is homodimeric like topo II of budding yeast, fruit fly, and mammalian cells (17, 20, 31, 39), the bifunctional reagent dimethyl suberimidate (9) was used for cross-linking the topo II molecules, followed by SDS-PAGE with a low-concentration acrylamide-agarose gel (2% acrylamide and 0.5% agarose). As shown in Fig. 1b, the intensity of a 340-kDa band increased upon incubation of purified topo II with an increasing amount of dimethyl suberimidate solution. No band higher than the 340-kDa position was observed.

Seventeen micrograms (~100 pmol) of purified topo II was used for protein sequencing (Materials and Methods). The first seven amino acids determined were Ser-Ile-Asp-Ala-Asp-Phe-Ser. This sequence differed from the putative NH_2 terminus previously deduced from the cloned gene (the circled Met residue in Fig. 2a [44]) but was identical to a stretch (boxed) further upstream, which would be in frame if a 56-bp-long intron was assumed, as shown in Fig. 2a (the putative intron had the consensus sequence for fission yeast

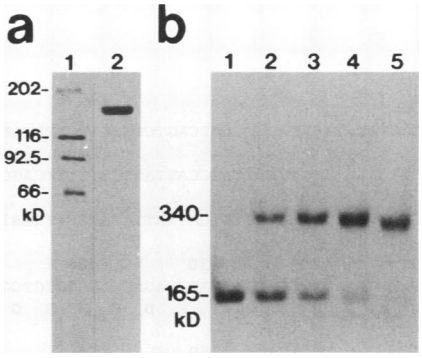

FIG. 1. SDS-PAGE of purified fission yeast topo II. (a) Lanes: 1, MW markers, with positions indicated in thousands; 2, topo II polypeptide purified from the fission yeast *S. pombe*. (b) Assay of purified topo II incubated in different concentrations of dimethyl suberimidate at 22°C for 3 h. Lanes: 1, 0 mM; 2, 0.43 mM; 3, 0.85 mM; 4, 1.7 mM; 5, 3.4 mM. The band at 165 kDa decreased, while the band at 340 kDa increased.

as indicated by the underlines). Consistent with the notion that this seven-amino-acid sequence represents the topo II NH_2 end, it was preceded by Met in the predicted upstream sequence. The region further upstream contains many termination codons and no splice consensus. Thus, *S. pombe* topo II appears to contain 1,484 amino acids (calculated size, 168 kDa), 53 residues larger than that previously reported. The entire amino acid sequence of *S. pombe* topo II is shown in Fig. 2b with the correct NH_2 terminus.

Sequence conservation among different topo IIs. To facilitate the structural descriptions presented below, the regions displaying amino acid sequence similarity among *S. pombe*, *S. cerevisiae*, human topo II, bacterial gyrase subunits A and B, and phage T4 gene products gp39, gp52, and gp60 are schematically aligned in Fig. 2c. Fifteen distinct regions (indicated by the open boxes in Fig. 2b and c) which displayed similarity among eukaryotic topo IIs (44) are numbered. The striped and filled boxes represent strong similarities to prokaryotic GyrB and GyrA subunits, respectively, whereas weak similarities are represented by the open boxes with the broken lines. T4 phage topo II, although consisting of three subunits, is similar to eukaryotic topo II when the subunits of gp39, gp60, and gp52 are arranged in this order. It is of interest that the sequences similar to boxes 10 and 14 are present in T4 topo II but not in bacterial topo II. In this regard, phage topo II is more similar to eukaryotic topo IIs.

Limited proteolysis of topo II by proteases. Purified intact topo II was digested with various proteases (trypsin, chymotrypsin, papain, and V8) and subjected to SDS-PAGE. Digestion invariably produced a ~125-kDa major polypeptide and also smaller bands, which were particularly sharp and stable during the course of papain digestion (Fig. 3a). The ladderlike digestion bands between 165 and 125 kDa indicated that the 165-kDa polypeptide was progressively digested from either or both ends to yield the distinct 125-kDa fragment. This fragment was rather resistant to papain, judging from the time course of digestion. After prolonged incubation by papain, three more bands (82, 46, and 43 kDa) were recognized. The order of appearance suggested that the 82- and 43-kDa bands might be derived from the 125-kDa fragment.

For V8 protease, the major digestion products were the

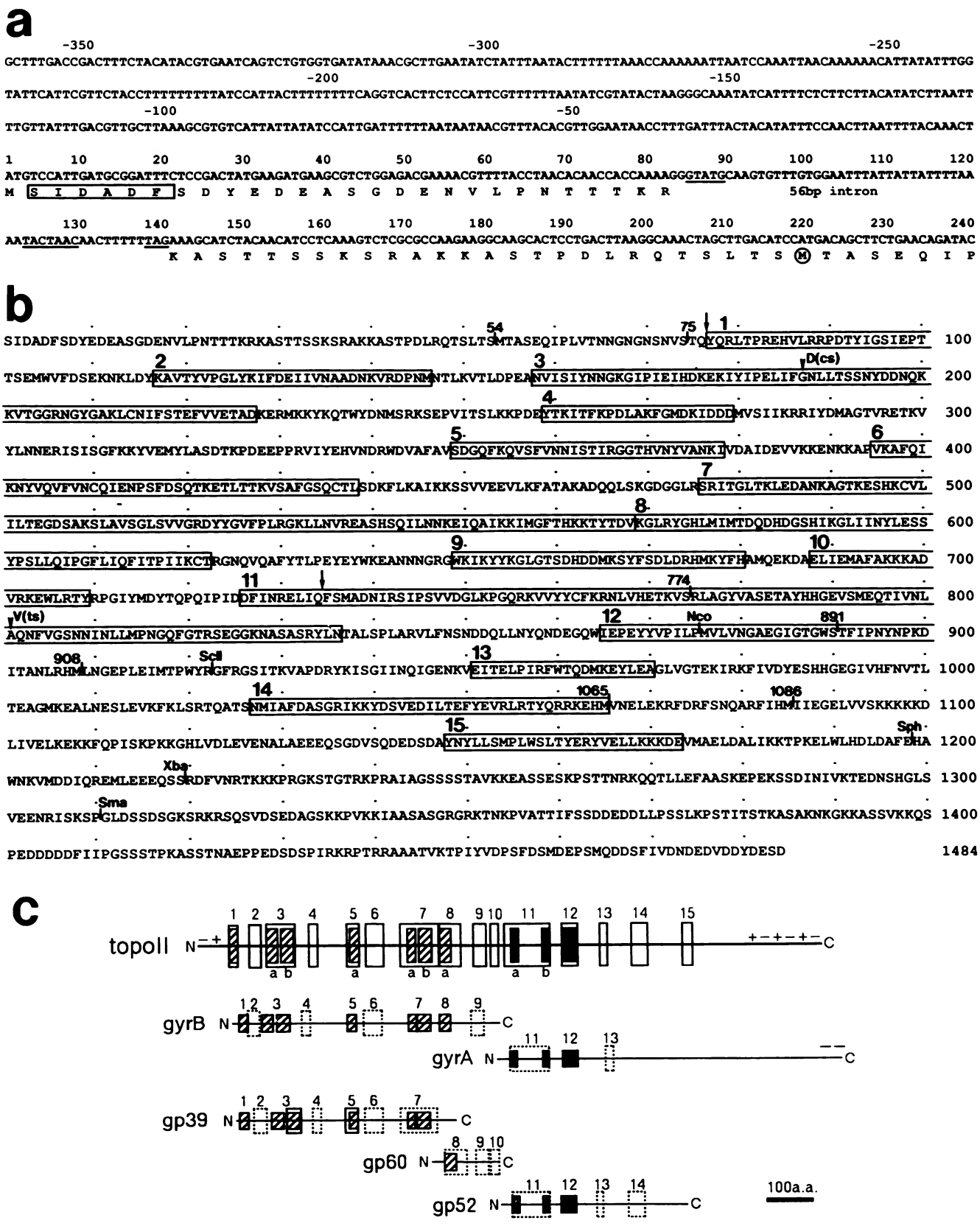
a
b
c
topoII
gyrB
gyrA
gp39
gp60
gp52
100a.a.

FIG. 2. (a) Nucleotide and amino acid sequences for the 5′ upstream and the N-terminal regions of the fission yeast *top2*$^+$ gene. The boxed amino acid sequence was the N terminus of purified topo II determined by protein sequencing. A 56-bp intron is predicted; the *S. pombe* splice consensus sequences are underlined. The circled Met is the previously assigned initiation codon (44). (b) Entire amino acid sequence of the fission yeast topo II. Sequences in the 15 boxes are conserved in eukaryotic topo II. The thick arrows indicate the sites cleaved by papain. The altered amino acids indicated at positions 187 and 801 were found in the cs and ts mutant genes, respectively. The vertical lines are the sites (indicated by the amino acid position or the restriction enzyme cleavage site) where the deletions or insertions were made. (c) Amino acid sequence comparison among eukaryotic topo II, bacterial GyrB and GyrA subunits, and phage T4 topo II gp39, gp60, and gp52 subunits. The 15 boxes are similar among eukaryotic topo IIs. The areas indicated by striped boxes are similar in GyrB, gp39, and gp60. The filled boxes represent segments similar in GyrA and gp52. The boxes with broken lines in bacterial and phage T4 subunits exhibit weaker similarity to eukaryotic topo IIs. Plus and minus signs indicate the amino acid stretches rich in charged residues.

two bands close to ~125 kDa even after prolonged incubation (Fig. 3b). Many cleaved fragments, however, were obtained by V8 protease if topo II had first been denatured in the presence of 0.5% SDS at 100°C for 2 min (Fig. 3c), suggesting that protease digestion of the native topo II was required to produce the ~125-kDa polypeptide. Trypsin and chymotrypsin digestion also displayed a major ~125-kDa band followed by several minor bands in the range of 45 to 100 kDa (data not shown). Thus, a ~125-kDa fragment of topo II is a common, stable product of different proteases, which we designate the core.

The NH_2 sequences of papain-cleaved fragments. The four bands containing the 125-, 82-, 46-, and 43-kDa papain-cleaved fragments were isolated by electroblotting (Materials and Methods). Their N-terminal sequences were determined (Table 1). The 82-kDa fragment was apparently a mixture of N-terminal sequences. The other three fragments showed unambiguous information about the N-terminal sequences. The 125- and 43-kDa fragments had the same N terminus at position 77, whereas the 46-kDa fragment initiated at position 736.

The core fragment showed Tyr77 as the major N terminus. A surprising coincidence was that the conserved amino acid sequence in box 1 (Fig. 2b) started at residue Tyr77. There seems to be a correlation between papain resistance and sequence conservation at the N terminus. The core C terminus has not been determined. Considering the size of the core, its C terminus should fall near box 15 close to position 1220. Thus, papain digestion appeared to remove largely nonhomologous terminal sequences from the intact topo II polypeptide.

The 43-kDa fragment corresponded to the N-terminal domain of the core, because its N terminus was identical to that of the core. Its C-terminal end, judging from the fragment size, would presumably fall in a region near or between boxes 6 and 7. The 46-kDa fragment, on the other hand, appeared to correspond to the C domain of the core, because it initiated at Phe-735 in box 11.

The 125-kDa core is enzymatically active. The enzymatic activity of topo II was assayed after limited proteolysis. Purified topo II was incubated with papain (1%, wt/wt) at 30°C, and the excess amount of the antipain inhibitor was added at various time intervals. As shown in Fig. 4a, the papain-digested product after 40 min consisted predominantly of the core, whereas the products after 160 min contained the 43- and 82-kDa fragments. The ATP-dependent relaxing activity was then measured by incubating papain-digested topo II (1 ng per lane) with supercoiled pBR322 (0.3 μg per lane) at 30°C for various times. As shown in Fig. 4b, the relaxing activity was still present in the 125-kDa core (contained in the 40-min fraction). The activity of the core appeared to be even higher than that of intact topo II. The activity after 160 min, however, was hardly detected, suggesting that the smaller fragments no longer contained a relaxing activity. Note that the degrees of superhelicity, as evidenced by patterns of topoisomers seen in the ladder, resulting from the relaxing activity of the intact or core topo II were not identical; intermediate topoisomers with high linking numbers were accumulated abundantly by the core enzyme activity (see Discussion). Unknotting activity of the core was also examined by using the knotted P4 phage DNA as the substrate (Fig. 4c). The core also showed

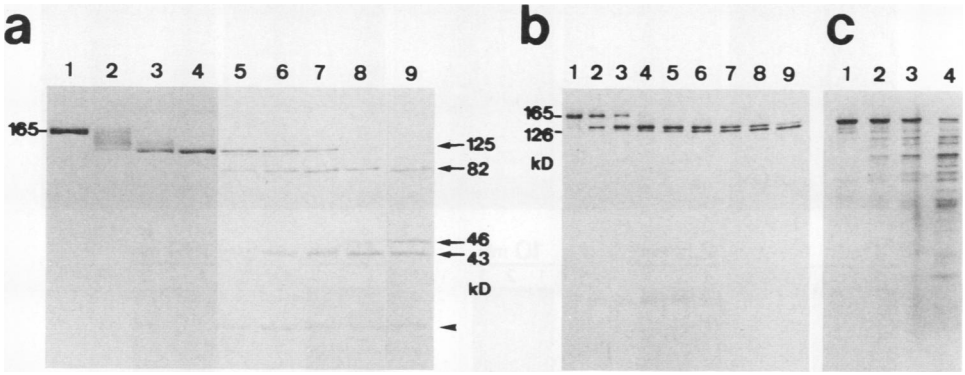

FIG. 3. Limited protease digestion of purified topo II. (a) Time course for papain-digested topo II determined by SDS-PAGE. Lanes: 1, intact topo II before digestion; 2 to 9, samples taken at 2.5 min, 10 min, 40 min, 2.5 h, 5 h, 10 h, 20 h, and 25 h, respectively. Four polypeptides (125, 82, 46, and 43 kDa) were seen during the course of digestion. The band indicated by the arrowhead is papain. Note the ladder bands between 165 and 125 kDa, which appeared to be formed by a progressive digestion by papain. (b) Time course for V8 digestion of native topo II. Lanes: 1, intact topo II before digestion; 2 to 9, samples taken at 2, 5, 15, 30, 60, 90, 120, and 240 min, respectively. (c) Time course for V8 digestion of denatured topo II. Purified topo II was first denatured at 100°C in 0.5% SDS and then digested with V8 protease and subjected to SDS-PAGE. Lanes: 1, intact topo II; 2 to 4, samples taken at 15, 60, and 240 min, respectively.

TABLE 1. N-terminal sequences of topo II and papain-digested fragments

MW (10^3)	N-terminal sequence	Location
165	SIDADFS	1
125	YQRLTP (TQYQRL[a])	77 (75[a])
82	ND[b]	
46	FSMADNIRSIP	735
43	YQRLTP	77

[a] N terminus of the minor fraction of the core.
[b] ND, could not be determined.

strong unknotting activity (40 min), but after cleavage to smaller fragments it had little activity (160 min).

Construction of haploid *top2* null mutant. Although the 125-kDa core was found to be active in vitro, it may no longer contain a part essential for in vivo function. We examined whether terminally truncated topo II could substitute in vivo for the wild-type *top2*$^+$ gene function. To this end, a haploid *top2* null mutant was made. One of the two *top2*$^+$ genes in a diploid was first disrupted by gene replacement using the *ura4*$^+$ marker gene (Materials and Methods). Resulting Ura$^+$ heterozygous diploids were sporulated, and tetrads were dissected. Only two spores were viable, and all of the viable spores were Ura$^-$, indicating that haploid disruptants of the *top2*$^+$ gene were lethal. Genomic Southern hybridization of the heterozygous diploid genomic DNA showed bands of the expected sizes for the disrupted gene (data not shown).

Next, heterozygous diploids were transformed with a multicopy plasmid carrying various truncated *top2* genes with the *S. cerevisiae LEU2* marker (Fig. 5a). Leu$^+$ diploid transformants were sporulated. Complementation of the *top2* null mutant was tested by the ability of the transformant to form viable Ura$^+$ Leu$^+$ haploid spores. As a control, the heterozygous diploid was transformed with the comparable plasmid carrying the wild-type *top2*$^+$ gene and sporulated. Haploid Leu$^+$ Ura$^+$ segregants containing the plasmid with the wild-type *top2*$^+$ gene were viable, showing that the multicopy *top2*$^+$ plasmid could rescue the *top2* null haploid.

Construction of deletion mutants. We constructed a series of C- and N-terminal deletion (designated Δ) or *LEU2* insertion (designated ::) *top2* mutant genes as diagrammed in Fig. 5b. Four C-terminal downstream deletions were prepared by using the restriction sites *Sma*I, *Xba*I, *Sph*I, and *Sac*II. Three internal in-frame deletions, Δ(775-890), Δ(909-1085), and Δ(1066-1085), were made by using the *Acc*I and *Nde*I sites. Two N-terminal deletions, Δ(1-53) and Δ(2-74), were made by using the promoters of the *top2*$^+$ gene by internal deletion. The other N-terminal deletion was made by using the *S. pombe* ADH promoter (pEVP11 [38]) ligated with the N-upstream deletion, ADH-Nco. Furthermore, we constructed two double deletions (75-1219 and 75-1198) which lacked both ends.

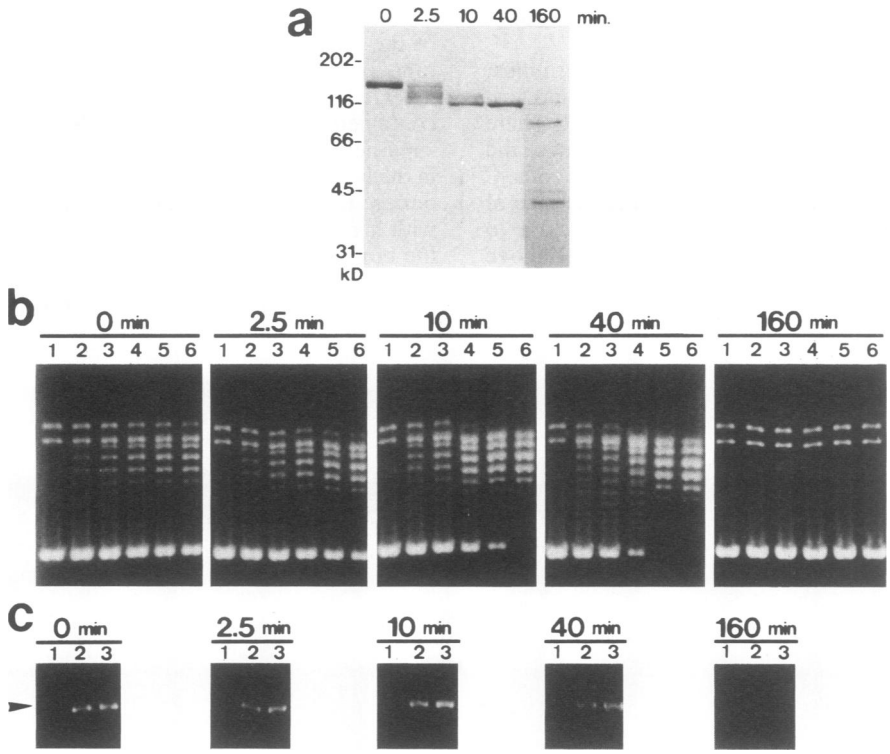

FIG. 4. Enzymatic activity of the 125-kDa core. (a) Degree of papain digestion as monitored by SDS-PAGE. Purified topo II was digested by papain, and digestion was stopped by antipain at different time intervals. (b) Relaxing activity, assayed in the presence of ATP by mixing supercoiled plasmid pBR322 (0.3 μg) and papain-digested (for 0, 2.5, 10, 40, or 160 min) topo II (1 ng) for 0 to 20 min at 30°C. Samples were taken at 0 min (lanes 1), 2.5 min (lanes 2), 5 min (lanes 3), 10 min (lanes 4), 15 min (lanes 5), and 20 min (lanes 6). (c) Unknotting activity. Papain-digested topo II was also mixed with ATP and P4 knotted DNA, and its unknotting activity was assayed. After 0 min (lanes 1), 2.5 min (lanes 2), and 10 min (lanes 3) of incubation at 30°C, the reaction was terminated and analyzed by gel electrophoresis. Unknotted circular DNA is indicated by the arrowhead.

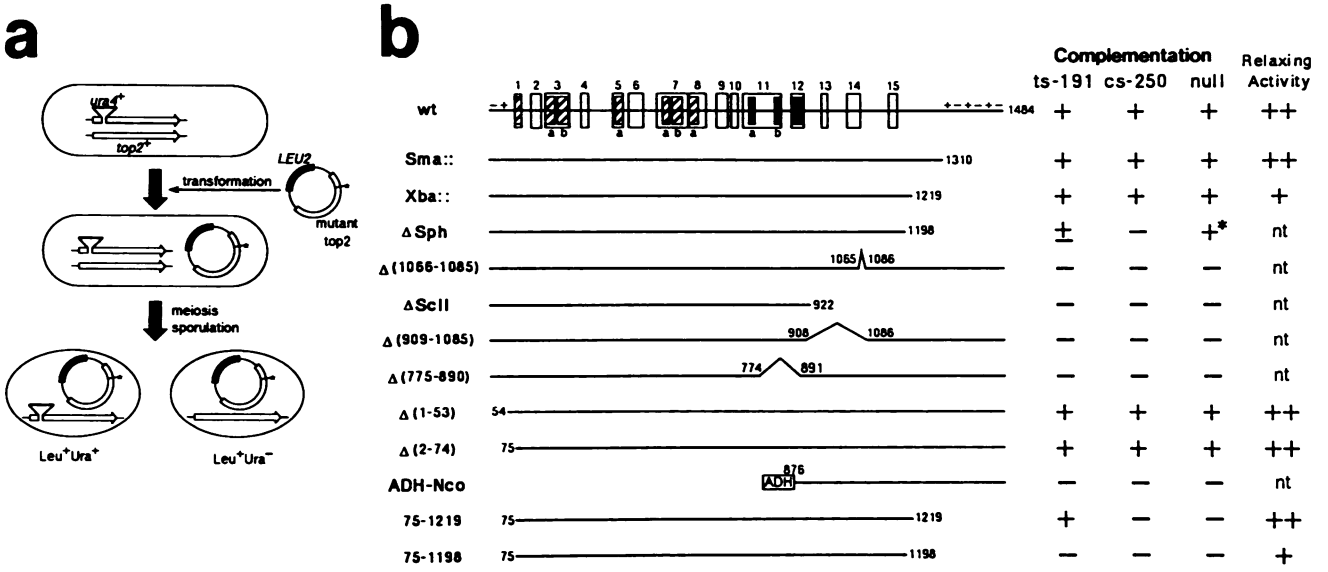

FIG. 5. Complementation of *top2* null mutant by various *top2* mutant genes. (a) Strategy. By one-step gene replacement, a heterozygous diploid was made in which one of the two *top2*$^+$ genes was disrupted by the *S. pombe ura4*$^+$ gene. Multicopy plasmids carrying mutant *top2* genes (indicated by an asterisk) and the *S. cerevisiae LEU2* gene as the marker were used for transformation. Leu$^+$ Ura$^+$ diploid transformants were obtained, sporulated, and analyzed. If haploid Leu$^+$ Ura$^+$ cells were obtained (maximal 50%), the mutant *top2* gene was able to rescue the lethality of haploid *top2* null. (b) Structures of constructs. Restriction site deletions and site-directed mutagenesis were used for construction of various truncated *top2* mutant genes. Δ and :: indicate deletion and truncation by insertion of the *S. cerevisiae LEU2* gene, respectively. The wild-type (wt) *top2*$^+$ protein contains 1,484 amino acids. C-terminal deletions ΔSph and ΔScII contained the termination codon at the position indicated by the number. Similarly, mutants 75-1219 and 75-1198, which are truncated at both their N and C termini, terminate at residues 1219 and 1198, respectively. Sma:: and Xba:: contain additional amino acids at the C terminus. The *Nco*I restriction site was used for the N-terminal deletion and subsequent fusion with the *S. pombe* ADH promoter. Others were internally deleted in the coding region so that they contained the promoter sequence for the wild type *top2*$^+$ gene. + indicates complementation of *top2* ts, cs, or null alleles and relaxing activity of mutant topo II. nt, not tested. *, this strain grows normally at 30 to 33°C but poorly at 22 or 36°C.

Essential and dispensable terminal regions of topo II. The *top2* mutant genes described above were used for transformation of the *top2* null, cs, and ts mutants. Results shown in Fig. 5b demonstrate that the internal deletions Δ(1-53) and Δ(2-74) fully complemented the *top2* null. Therefore, we concluded that the N-terminal 74 amino acids are dispensable.

C-terminal deletions or insertions at position 1310 (*Sma*I), 1219 (*Xba*I), or 1198 (*Sph*I) complemented the haploid *top2* null mutant. The transformants grew and sporulated normally. This complementation did not appear to be due to the high dosage of mutant genes, at least for the 1310 (*Sma*I) insertion. A strain with the *top2* gene replaced with the 1310 (*Sma*I) insertion gene grew and sporulated (data not shown). In contrast, a longer C deletion from 922 (*Sac*II) deleting more than 500 amino acids could not rescue the haploid *top2* null mutant. We concluded that the C-terminal 286 amino acids of topo II are not essential. On the other hand, three internal deletions, Δ(775-890), Δ(909-1085), and Δ(1066-1085), were unable to complement the *top2* null, suggesting that these regions were essential.

We constructed two double deletions, 75-1219 and 75-1198, which lacked both termini and examined whether they could rescue the *top2* null lethality. Unexpectedly, however, the *top2* null was not complemented by these mutant genes. Because single deletions could rescue the null lethality, the termini might have an overlapping function that is essential for viability.

Protein products of topo II deletion mutants. To confirm that the *top2* deletion mutants produced polypeptides with the expected sizes, extracts of the wild-type cells transformed with the mutant plasmids were subjected to SDS-PAGE, and the mutant *top2* proteins were detected by immunoblotting with the use of anti-topo II antibodies (TP2-P; Materials and Methods). As shown in Fig. 6a, mutant *top2* polypeptides of the expected size were identified by immunoblotting. The wild-type topo II product made by the single-copy genomic *top2*$^+$ gene was roughly 10-fold less intense and not seen in these immunoblot patterns. By using the doubly truncated *top2* gene, the size of the core was estimated as shown Fig. 6b. The core produced from papain digestion (lane 4) is similar in size to the 75-1219 mutant but is little larger than the 75-1198 mutant. Its approximate size was estimated to be 125 kDa.

ATP-dependent relaxing activity was assayed in transformant extracts (Fig. 6c). The relaxation activity by the multicopy *top2*$^+$ genes was approximately 10-fold higher than that of a single-copy genomic *top2*$^+$ gene. Extracts containing plasmid with the 1310 (*Sma*I) insertion (lanes 3), Δ(2-74) (lanes 5), and the double deletion 75-1219 (lanes 6) showed a level of activity similar to that with the wild-type gene (lanes 2). The 1219 (*Xba*I) insertion (lanes 4) and the double deletion 75-1198 (lanes 7) displayed a somewhat weaker activity.

Characterization of ts and cs mutations. The ts *top2-191* mutant gene was isolated by the gap repair method (36). The ts gene as well as the cs *top2-250* gene previously isolated (46) was ligated into a multicopy vector and used for transformation of the haploid *top2* null mutant. In both cases, resulting transformants grew at the permissive temperature but not at the appropriate restrictive temperatures,

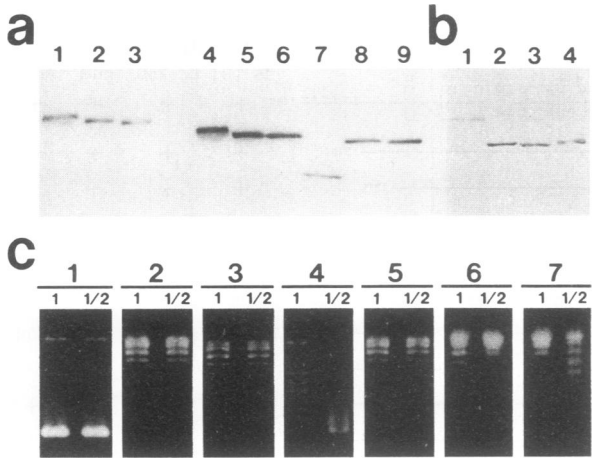

FIG. 6. (a) Detection of protein products of the truncated *top2* genes. Anti-topo II antibody TP2-P was used for immunoblotting of *S. pombe* cell extracts. Cells were transformed with multicopy plasmid carrying wild-type or mutant genes. Lanes: 1, wild type (165 kDa); 2, Δ(1-53) (162 kDa); 3, Δ(2-74) (160 kDa); 4, Sma:: (140 kDa); 5, Xba:: (139 kDa); 6, ΔSph (137 kDa); 7, ΔScII (105 kDa); 8, 75-1219 (125 kDa); 9, 75-1198 (123 kDa). Under the experimental conditions used, the product made by a single-copy genomic $top2^+$ gene was much lower than those produced by a multicopy gene and not detected. (b) Comparison of MWs of truncated *top2* gene products and papain-produced core. Crude extracts of the cells harboring the multicopy wild-type $top2^+$ gene (lane 1), 75-1219 (lane 2), 75-1198 (lane 3), and purified topo II digested by papain (lane 4) were subjected to SDS-PAGE (7.5%) and then detected by immunoblotting. Note that topo II core made by papain has an MW close to that of the 75-1219 product. (c) ATP-dependent relaxing activity of mutant topo II was assayed in crude extracts (1 and 1/2 indicate dilution of the extracts; the assay conditions are described in Materials and Methods). Under these experimental conditions, the relaxing activity in wild-type extract (lanes 1) is negligible, while extracts of cells containing a multicopy plasmid carrying the wild-type $top2^+$ (lanes 2) contain an intense relaxing activity. Sma:: (lanes 3), Δ(2-74) (lanes 5), and 75-1219 (lanes 6) also have strong activity, but Xba:: (lanes 4) and 75-1198 (lanes 7) are somewhat weak.

indicating that the ts and cs properties were retained under the high gene dosage conditions.

By combination of integration mapping and transformation using the chimeric genes of wild-type and ts mutant sequences, the ts *top2-191* allele was found to be located within a short 1.3-kb *Kpn*I restriction fragment in the central domain. Nucleotide sequence determination of the *Kpn*I fragment indicated a change (from C to T) which should cause an amino acid alteration from Ala-801 to Val. This Ala residue is conserved in eukaryotic topo II, bacterial GyrA subunit, and phage gp52. It should be noted that the partially purified ts *191* topo II enzyme was active at 26°C but irreversibly inactivated at 36°C (47).

The cs *top2-250* mutation site was located at residue 187 in box 3 (Fig. 2), causing a substitution from Gly to Asp (46). This Gly is conserved in the budding yeast TOP2 (16) and human topo II (43) but not in phage T4 gp39 (25) and bacterial GyrB (34). We partially purified cs topo II and showed that the enzyme has a reversible cs activity in vitro (46).

We examined whether the cs and ts topo II enzymes could cleave the double-stranded DNA in the presence of VP-16 or VM-26 (6). These drugs inhibit topo II from rejoining the

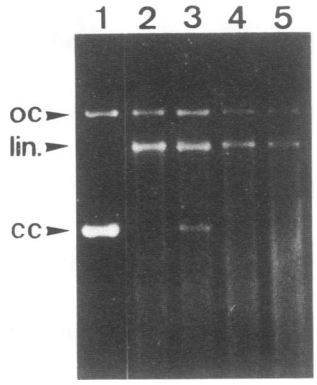

FIG. 7. DNA cleavage by wild-type and cs topo II in the presence of VP-16. Supercoiled pBR322 plasmid (lane 1) was incubated, in the presence of VP-16, with purified wild-type topo II (lanes 2 and 3, at 36 and 20°C, respectively) and cs mutant topo II (lanes 4 and 5, at 36 and 20°C, respectively). cc, supercoiled plasmid; lin., linear plasmid; oc, open circular plasmid. Linearized DNA was produced by both wild-type and cs topo II.

DNA after cleavage of double-stranded DNA, resulting in the linear intermediate form, which can be distinguished from the circular forms of the plasmid in terms of electrophoretic mobility. As shown by accumulation of the linear form in Fig. 7 (lanes 2 to 5), supercoiled circular plasmid pBR322 was cleaved at 20°C by the purified cs enzyme in the presence of VP-16 a little more efficiently than by the wild-type topo II. This efficient DNA cleavage is an interesting property of cs topo II enzyme. A ts *top2-191* enzyme, on the other hand, was unable to cleave DNA at the restrictive temperature (data not shown).

DISCUSSION

The core of topo II. In this study, we developed a procedure for purification of fission yeast topo II and showed that limited digestion of topo II by papain removed large terminal portions, leaving a stable and enzymatically active core. In the core, 76 and ~260 amino acids were lost from the N and C termini of intact topo II, respectively. This core was found to retain activity in an in vitro topo II assay. Thus, approximately one quarter of topo II seems to be dispensable for in vitro activity. The N-terminal end of the core is the first residue in a conserved sequence between topo II of various organisms, displaying a correlation between protease resistance and sequence conservation. Because the amount of purified topo II was limited, the C-terminal end of the core was not determined. It is likely to be near residue 1220, as the MW of core was very similar to that of the 75-1219 polypeptide by SDS-PAGE. All sequences conserved among eukaryotic, bacterial, and phage topo IIs were contained in the core. The fact that the lethal deletions and conditional-lethal point mutations were located inside the core was consistent with the notion that the core might represent a minimal structure required for topo II enzyme activity.

In vivo function of truncated topo IIs. We constructed genetically engineered, truncated *top2* genes and found that the mutant genes deleting either the N-terminal 75 or C-terminal 286 residues could substitute in vivo for the $top2^+$ gene in mitosis and meiosis. However, we found that the gene deleting both the N- and C-terminal amino acids could not substitute for the $top2^+$ gene. Because the core topo II had relaxing and unknotting activity, the inability to com-

plement the *top2* null might not be due to the lack of enzymatic activity. The loss of both terminal sequences might result in the loss of another essential function. We are currently investigating the cause of the inability of the double truncation to complement the *top2* null mutant.

Papain-sensitive sites within the core. Further papain digestion produced smaller inactive fragments, of sizes that indicated that the cleavage occurred at specific sites. There appear to be at least two cleavage sites within the core. One is at the N-terminal end of the 46-kDa fragment, that is, the amino acid 735 at the start of the box 11a (Fig. 2c). This fragment should contain most of the sequences similar to GyrA. The other is at the C-terminal end of the 43-kDa fragment. Because its N-terminal end was identical to that of the core, the C-terminal end should be in a central region of the GyrB-like domain, perhaps between boxes 6 and 7 (Fig. 2). Judging from the order of fragment appearance, the core may be cleaved first at the middle of the GyrB-like domain and then near the N-terminal end of the GyrA-like domain. Hence, papain appears to recognize spatially distinct subdomains of topo II.

Previous reports (5, 15) showed that a 50-kDa proteolytic fragment could be obtained from the 90-kDa bacterial GyrB subunit. The complex of this 50-kDa fragment and the GyrA subunit lacks both the DNA-supercoiling and DNA-dependent ATPase activities of intact DNA gyrase. However, the complex can relax both positive and negative supercoils. Interestingly, the presumed C-terminal end of the 43-kDa fragment is very close to the cleavage site for the GyrB subunit (residue 394) determined by Adachi et al. (1). The overall conformation of the GyrB subunit may be similar to that of the GyrB-like domain in eukaryotic topo II.

Dispensable termini of topo II. Eukaryotic topo II commonly contains a long C-terminal sequence which is hydrophilic, rich in charge, and not conserved. We initially supposed that the region might be required for binding to chromatin but found that it is a dispensable domain. The N terminus is also nonessential and variable in length: 76, 33, and 11 residues, respectively, for *S. pombe*, human, and *S. cerevisiae* topo IIs. These head and tail regions of topo II appear to contain a number of protease-sensitive sites, judging from the ladder formation between 165 and 125 kDa during digestion.

The terminal sequences might affect, quantitatively or qualitatively, the relaxation reaction. The relaxing activity of the core was somewhat higher than that of the intact topo II. The core may have a weaker affinity for supercoiled DNA so that the core tends to be released from the substrate DNA more quickly, resulting in high-linking-number topoisomers. The termini of topo II might be involved in regulating the activity. This view is compatible with our recent finding that the terminal regions of topo II are highly phosphorylated (39a). We found that the core had unknotting activity with use of the knotted P4 DNA as the substrate, and so the involvement of the termini for knotting and unknotting DNA is unlikely.

Are each of the terminal regions dispensable under all conditions? They might become essential if there were an additional mutation in another gene. We examined whether the C terminus would still be nonessential in the absence of the $top1^+$ gene. The $top1^+$ gene encodes a topo I enzyme and is nonessential for growth (45, 47). The $top1^+$ gene was disrupted by one-step gene replacement (37). Transformants obtained by integration were selected by resistance to camptothecin (12, 35). Disruption of the $top1^+$ gene was confirmed by Southern hybridization (data not shown). The strain was then crossed with the Ura^+ Leu^+ *top2* null strain carrying the *top2 Sma*I insertion mutant gene on plasmid. The Ura^+ Leu^+ Cpt^r segregants grew normally, indicating that the C terminus is not essential in the *top1* null strain. We also examined the phenotype of the *top1* null strain when the $top2^+$ gene was replaced with the *Sma*I insertion mutant gene; since mitotic growth rate and sporulation were normal, interaction between topo I and the C terminus of topo II is unlikely.

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