Functional Dissection of the Phosphorylated Termini of Fission Yeast DNA Topoisomerase II

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Abstract. Fission Yeast DNA topoisomerase II (165 kD) consists of an enzymatically active 125-kD core, ~10-kD NH_2-terminal and 30-kD COOH-terminal domains. The question addressed in the present study is what is the role of the topo II termini. Although deletion of either the NH_2 or the COOH terminus is viable, deletion of both termini is lethal; the termini share an essential role for viability. We show here that topo II phosphorylation sites are localized in the terminal domains, but dephosphorylated topo II is still active. The topo II terminal sequences are required for nuclear localization; topo II double terminal deletion mutants are deficient for nuclear targeting, whereas wild-type and single deletion mutant topo IIs are transported into the nucleus with different efficiencies. Functional subdomains in the NH_2 terminus are further dissected; we identified a 15 amino acid nuclear localization sequence (NLS) which is essential for viability and nuclear localization when the COOH terminus is deleted. This NLS could be substituted with SV-40 large T-antigen NLS. Two other functional subdomains were found; a non-essential acidic stretch which is phosphorylated and apparently enhances the nuclear localization and an essential hydrophilic stretch of unknown function. Motifs similar to these three NH_2-terminal subdomains are also found in the COOH terminus. Our results support the possibility that phosphorylation of topo II does not play an essential role in fission yeast.

DNA topoisomerase II (topo II)[1] is an enzyme which alters the topological state of DNA by transient concerted breaking and rejoining both strands of a double-stranded DNA helix (Wang, 1985). In prokaryotes, the enzyme, called gyrase, is composed of two kinds of subunits, GyrA and GyrB, that exists as a tetramer (Gellert, 1981). The former catalyzes the cleavage and religation of DNA whereas the latter binds ATP and hydrolyzes it. On the other hand, eukaryotic topo II exists as a homodimer consisting of a single polypeptide of ~160 kD (Hsieh, 1990). The primary structure of topo II has been determined by cloning and sequencing the genes from various organisms, such as yeast, fruit fly, and human (Giaever et al., 1986; Uemura et al., 1986; Wyckoff et al., 1989; Tsai-Pflugfelder et al., 1988). Sequence comparison suggests that the ATPase domain is located in the NH_2-terminal quarter of eukaryotic topo II and the DNA-binding catalytic domain in the central part (Lynn et al., 1986; Uemura et al., 1986). In Fig. 1, the stretches of amino acid sequence conserved in all eukaryotic topo IIs are shown by open boxes, while those similar to GyrB and GyrA, respectively, are shown by hatched and filled boxes. There are a number of regions conserved only in eukaryotic topo II, suggesting that additional functions are specified for eukaryotic topo II. In fact, eukaryotic topo II has the ability to relax positive supercoils but lacks gyrase activity.

Topo II of fission yeast has been dissected into several structural domains (Shiozaki and Yanagida, 1991). Papain digestion of purified topo II showed that topo II is made up of a 125-kD core and NH_2- and COOH-terminal domains. The core, displaying full activity in relaxing and unknotting, consists of all the regions conserved among eukaryotic topo IIs. The highly divergent NH_2 and COOH termini have 76 and ~260 residues, respectively. Prolonged papain digestion produced smaller fragments (indicated by the arrows in Fig. 1). They contain regions similar to the ATPase domain of GyrB (Wigley et al., 1991) and the GyrA-like region, respectively, showing the presence of spatially distinct subdomains in the core. Similar subdomains have been found in budding yeast (Lindsley and Wang, 1991).

Genetics of topo II have been extensively studied in yeasts (Yanagida and Sternglanz, 1990). Analyses of temperature-sensitive budding yeast mutants indicated that the gene coding for topo II is essential for viability (DiNardo et al., 1984) and that topo II becomes essential in mitosis (Holm et al., 1985). In fission yeast, topo II is also essential for viability, and is required for nuclear division (Uemura and Yanagida, 1984, 1986), chromosome condensation, and disjunction in mitosis (Uemura et al., 1987). Topo II also plays an impor-

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1. *Abbreviations used in this paper*: ADH, alcohol dehydrogenase; CK-II, casein kinase II; NLS, nuclear localization sequence; PAP, potato acid phosphatase; PAS, protein A-Sepharose; topo II, DNA topoisomerase II.

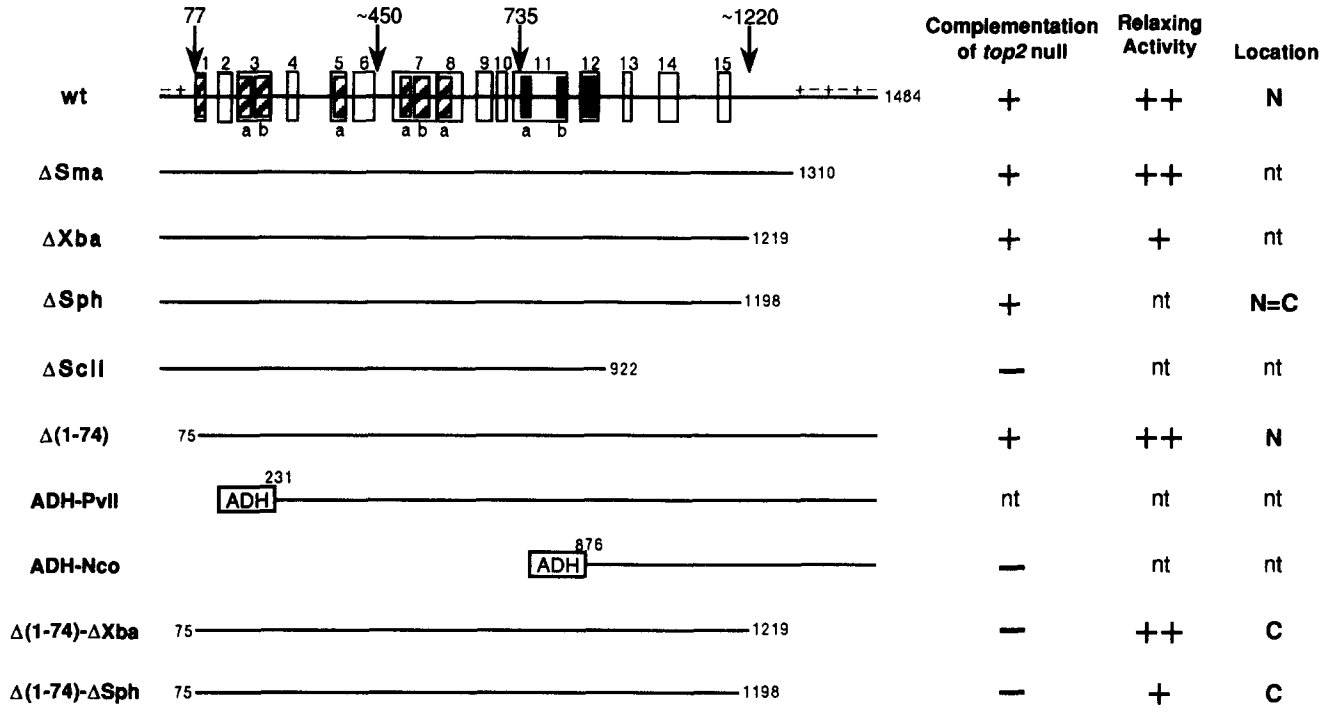


Figure 1. Analysis of fission yeast topo II deletion mutants. The ability to complement the *top2* null mutation and to relax supercoiled circular DNA was examined (+, complementation or relaxation; −, no complementation nor relaxation; *nt*, not tested). The subcellular location of mutant gene products was determined by immunofluorescence microscopy using anti-topo II antibodies. *N*, nuclear; *C*, cytoplasmic; *N = C*, nuclear and cytoplasmic localization. The wild-type (*wt*) topo II contains 1,484 amino acids, and the cleavage sites by papain are indicated by arrows (Shiozaki and Yanagida, 1991). The open boxes represent the regions similar among eukaryotic topo IIs. The striped boxes indicate the domains similar to bacterial GyrB whereas the filled boxes represent the domains similar to GyrA. The PvuII and NcoI restriction sites were used for the NH_2-terminal deletion and subsequent ligation to the *S. pombe* ADH promoter. Construction of other mutant genes was described previously (Shiozaki and Yanagida, 1991).

tant role for organizing the nucleolar structure in conjunction with topoisomerase I (Hirano et al., 1989). In vitro experiments using vertebrate chromosomes implicated topo II in condensation (Wood and Earnshaw, 1990; Adachi et al., 1991). Topo II has been reported to be a major component in the nuclear matrix or chromosome scaffold structure (Berrios et al., 1985; Earnshaw et al., 1985; Gasser et al., 1986). Little has been established as to how topo II interacts in vivo with nuclear chromosomal DNA or how it might alter chromosome structure. We have been interested in this problem and tried to identify topo II protein domains essential for appropriate location of topo II in the nucleus. To this end, we undertook functional dissection of fission yeast topo II (Shiozaki and Yanagida, 1991). As shown in Fig. 1, mutants lacking either the NH_2 or COOH terminus could complement the *top2* null mutant (indicated by + in Fig. 1), but the double deletion mutants (Δ[1–74]-ΔXba and Δ[1–74]-ΔSph) did not, despite the fact that the 125-kD polypeptide produced was enzymatically active. This apparently paradoxical finding prompted us to investigate the properties of topo II terminal sequences.

What kind of essential function can reside in the topo II terminal domains? Terminal sequences may play a role for topo II location in the nucleus and may interact with specific chromosomal DNA. Nuclear proteins synthesized in the cytoplasm have to be transported to the nucleus through the pore complexes (for review see Silver, 1991). For example, the COOH-terminal domain of *Xenopus* nucleoplasmin was shown to be necessary for nuclear transport (Dingwall et al., 1982). Short amino acid sequences rich in basic residues are often used as nuclear localization determinants (Dingwall and Laskey, 1986). In fact, amino acid sequences similar to the nuclear localization sequence (NLS) of nucleoplasmin are present in the NH_2 and COOH termini of *S. pombe* topo II.

Alternatively, the terminal sequences may be involved in regulation of the enzymatic activity. In accordance with this notion, the 125-kD core made by papain digestion has a relaxing activity about fourfold higher than that of intact topo II (Shiozaki and Yanagida, 1991). Modulation of the topo II activity in different steps of cell cycle, growth, and differentiation may be exerted through posttranslational modifications of terminal sequences. Topo II in *Drosophila* Kc, chicken lymphoblastoid, mouse FM3A, HeLa cells, and budding yeast are phosphorylated in vivo (Ackerman et al., 1988; Heck et al., 1989; Saijo et al., 1990; Kroll and Rowe, 1991; Cardenas et al., 1992). Phosphorylation sites have recently been reported for budding yeast topo II (Cardenas et al., 1992). The topo II activity was reported to be enhanced by phosphorylation (Ackerman et al., 1985; Sahyoun et al., 1986; Rottmann et al., 1987).

We report in the present paper that among three subdomains identified in the NH_2 terminus of fission yeast topo II, one basic domain is essential for viability and required

for nuclear targeting. Another hydrophilic domain is also essential for viability but its function is unknown. The other domain is acidic, highly phosphorylated, and nonessential for viability, but possibly facilitates nuclear localization.

Materials and Methods

Yeast Strains and Plasmids

Haploid strains of *Schizosaccharomyces pombe* used were HM123 (genotype *h⁻ leu1*), TM101 (*h⁻ leu1 end1 top1*), TM141 (*h⁻ leu1 top2-191*) (Uemura and Yanagida, 1984), and TM147 (*h⁻ leu1 top2-250*) (Uemura et al., 1987). HM123 was used as a wild type strain. A heterozygous diploid in which one of the two *top2*$^+$ genes was disrupted using the *S. pombe ura4*$^+$ gene as the marker was made previously (Shiozaki and Yanagida, 1991). Multicopy plasmid pDB248′ (Beach and Nurse, 1981; Beach et al., 1982) and an expression vector with the alcohol dehydrogenase (ADH) promoter (pEVP11; Russell and Hall, 1983) were used for introducing mutant *top2* genes into *S. pombe*. Transformation of *S. pombe* was performed by the lithium method (Ito et al., 1983).

Construction of top2 Deletion Mutants

A 0.9-kb MluI-BamHI fragment of the *S. pombe top2*$^+$ gene (Uemura et al., 1986) which encodes the NH_2-terminal region of topo II was subcloned into Bluescript (Stratagene, La Jolla, CA) and used for preparing uracil-containing single-stranded template DNA (Kunkel, 1987). Site-directed mutagenesis was performed according to Zoller and Smith (1987) with the following oligonucleotides (from 5′ to 3′, resulting in the mutations in brackets):

1) CTAACACAACCACCCCTAAGAAGAAGCGTAAGGTTGCAAGCACTCCTGA (26-40/SV)
2) TTTTACAAACTATGCCTAAGAAGAAGCGTAAGGTTGCAAGCACTCCTGA (1-40/SV)
3) TTTACAAACTATGCCTAAGAAGAAGCGTAAGGTTGGTACACAGTACC-AGC (1-74/SV)
4) CCAACTTAATTTTACAAACTATGACAGCTTCTGAACAGAT (Δ[1-53])
5) ACTTAATTTTACAAACTATGACACAGTACCAGCGTCTTAC (Δ[1-74])
6) ATTTTACAAACTATGAACGTTTTACCTAAC (Δ[1-17])
7) CCTAACACAACCACCGCAAGCACTCCTGAC (Δ[26-40])
8) CGCGCCAAGAAGGCAACACAGTACCAGCGT (Δ[42-74]).

The sequences of resulting mutant clones were confirmed by nucleotide sequencing (Sanger et al., 1977). AatII-HindIII fragments of wild-type or COOH-terminal deletion mutant *top2* genes, ΔXba and ΔSph which have a termination codon at 1,220th and 1,199th, respectively (Shiozaki and Yanagida, 1991), were ligated to mutagenized fragments in order to re-create the entire gene, for example in the 26–40/SV, 26–40/SV-ΔXba, 26–40/SV-ΔSph series. Then each mutant gene was cloned into pDB248′. The ability of various *top2* mutant genes to complement *top2* ts, cs, and null mutations were tested as described previously (Shiozaki and Yanagida, 1991).

Purification of Fission Yeast Topo II

The procedures were described previously (Shiozaki and Yanagida, 1991). The assay of topo II was done according to Uemura and Yanagida (1984).

In Vivo Labeling and Immunoprecipitation

The procedure for in vivo labeling described by Simanis and Nurse (1986) was followed with modifications. $H_3{}^{32}PO_4$ (2–10 mCi) was added to the 10-ml culture of wild-type *S. pombe* cells grown at 33°C in the synthetic EMM1 (Mitchson, 1970) medium to a cell concentration of 3–4 × 10^6/ml and incubated with shaking for 3–4 h. Cells were washed and broken with glass beads. The L buffer containing inhibitors for phosphorylation/dephosphorylation and proteolysis was used for preparation of extracts: 50 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 10 mM EDTA, 5 mM EGTA, 50 mM NaF, 50 mM β-glycerophosphate, and 0.1 mM Na_3VO_4 containing a cocktail of protease inhibitors (1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 10 μg/ml TPCK). The resulting extract was centrifuged at 14,000 rpm for 20 min, and the supernatant was diluted 3-fold by L buffer, without NaCl but containing NP-40 (final concentration 1%), sodium deoxycholate (0.5%), and SDS (0.1%). Immunoprecipitation was done as described by Harlow and Lane (1988). 10 μl of rabbit anti-topo II serum (TP2-F; Shiozaki and Yanagida, 1991) was incubated with the preswollen protein A-Sepharose (PAS; Pharmacia Fine Chemicals, Piscataway, NJ) in 500 μl RIPA buffer (L buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) at room temperature for 2 h. PAS bound to antibody was washed five times in RIPA buffer. The supernatant of cell extracts (equivalent to 5 × 10^7 cells) was mixed with the PAS–antibody conjugates and incubated at 4°C for 3 h. The PAS antibody–antigen conjugates were washed five times with RIPA buffer, once in 50 mM Tris-HCl, pH 8.0, treated by RNase A (200 μg/ml) for 30 min at 4°C (Moreno et al., 1991), boiled in the SDS-PAGE sample buffer for 2 min, and run in SDS-PAGE.

Phosphoamino Acid Analysis and Two-Dimensional Tryptic Peptide Mapping

^{32}P-labeled topo II was immunoprecipitated and run in SDS-PAGE as described above. After autoradiography, the labeled topo II band was cut out and used for phosphoamino acid analysis (Cooper et al., 1983) or two-dimensional tryptic peptide mapping. The labeled protein was recovered from the gel piece by electroelution for phosphoamino acid analysis. The protein was TCA precipitated and hydrolyzed in 6 N HCl at 95°C for 90 min. After four lyophilization steps to remove residual chloride, hydrolysate was subjected to cellulose thin-layer (AVICEL SF; Funakshi Co., Tokyo, Japan) electrophoresis with pH 3.5 buffer (50 acetic acids: 5 pyridine: 945 H_2O). After electrophoresis at 500 V for 3.5 h, phosphoamino acid markers (o-phospho-L-serine, o-phospho-L-threonine, o-phospho-L-tyrosine; Sigma Chemical Co., St. Louis, MO) were stained by the ninhydrin reagent and the thin-layer plate was subjected to autoradiography. For tryptic peptide mapping, the gel piece was washed with 10% methanol, lyophilized, and treated with performic acid for 1 h on ice. After lyophilization, the sample was digested at 30°C for 24 h in 500 μl trypsin-TPCK (50 μg/ml; Worthington Biochemical Corp., Freehold, NJ) with addition of another 500 μl trypsin solution at 8 and 16 h. After lyophilizing the supernatant twice, the sample was dissolved in electrophoresis buffer (50 *n*-butanol: 25 acetic acid: 900 H_2O: 25 pyridine at pH 4.7). Approximately 500 cpm of phosphopeptides were developed two dimensionally on a cellulose thin-layer plate (20 × 20 cm); 1st, electrophoresis at 1,000 V for 80 min and 2nd, ascending chromatography (75 *n*-butanol: 15 acetic acid: 60 H_2O: 50 pyridine).

Papain Digestion of ^{32}P-Labeled Partially Purified Topo II

^{32}P-labeled *S. pombe* cells were broken and extracted by L buffer containing 0.4 M NaCl. Saturated solution of ammonium sulfate was added to 35% saturation, and the precipitate was discarded. Ammonium sulfate was further added to 50% saturation, and the precipitate was suspended in 0.2 M sodium phosphate at pH 7.0 containing 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 50 mM NaF, and 30% glycerol. RNase A was added to a final concentration of 10 μg/ml, then the sample was dialyzed against the same buffer and digested with papain (1% of total protein) at 30°C. Antipain (final concentration, 10 μg/ml) was added to aliquots taken at 0, 2, 5, 15 and 90 min to stop the reaction. Immunoprecipitation was performed using anti-topo II antibodies as described above, followed by SDS-PAGE and autoradiography.

Phosphatase Treatment of Topo II

Potato acid phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in ammonium sulfate precipitates was dissolved in 20 mM Tris-HCl at pH 7.0 containing 1 mM EDTA, and dialyzed against the same buffer. The dialysate was fractionated by a MonoQ HR5/5 column (Pharmacia Fine Chemicals) in a 20-ml linear gradient between 0–0.3 M NaCl. Fractions containing phosphatase activity were pooled and dialyzed against 10 mM Hepes, pH 7.4, 0.5 mM $MgCl_2$, 0.5 mM DTT, 50% glycerol, and stored at −80°C. ^{32}P-labeled immunoprecipitate or 0.2 μg purified topo II was treated with 90 U of potato acid phosphatase in 40 mM Pipes (pH 6.0), 1 mM DTT, 20 μg/ml aprotinin and 20 μM leupeptin at 30°C for 20 min (Cooper and King, 1986). ATP-dependent relaxing activity of dephosphorylated topo II was assayed by the procedure described previously (Shiozaki and Yanagida, 1991).

Indirect Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed by the method described (Hagan and Hyams, 1988). *S. pombe* cells were fixed in culture me-

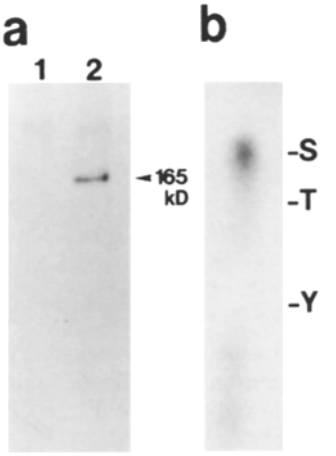


Figure 2. Fission yeast topo II is a phosphoprotein. (*a*) *S. pombe* extracts prepared from cells grown in medium containing ^{32}P were subjected to immunoprecipitation using preimmune sera (lane *1*) or anti-topo II antisera (lane *2*). SDS-PAGE autoradiogram showed that the 165-kD immunoprecipitated topo II band was ^{32}P-labeled. (*b*) ^{32}P-labeled topo II obtained by immunoprecipitation was hydrolyzed in 6 N HCl at 95°C for 90 min followed by cellulose thin-layer electrophoresis. S, T, and Y indicate positions of phosphoamino acid markers, phosphoserine, phosphothreonine, and phosphotyrosine, respectively.

dia containing 3.7% formaldehyde at 33°C for 1 h. The cell wall was digested at 37°C for 70 min with 0.6 mg/ml Zymolyase 100T (Seikagaku Corp., Tokyo, Japan) and 0.1 mg/ml Novozyme 234 (Novo Industry, Bagsvaerd, Denmark), followed by permeabilization with 1% Triton X-100. Primary antibody was TP2-P (anti-*S. pombe* topo II antisera; Shiozaki and Yanagida, 1991) affinity purified by the procedures of Smith and Fisher (1984). Rhodamine-conjugated goat anti-rabbit IgG antiserum (Cappel Laboratories, Malvern, PA) was used as secondary antibody.

Other Biochemical Methods

SDS-PAGE was performed as described by Laemmli (1970). Proteins were detected by Coomassie brilliant blue R250. Immunoblot analysis was done according to the procedure described by Towbin et al. (1979) using peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Cambridge, MA) as secondary antibody.

Results

S. pombe Topo II Is A Phosphoprotein

To determine whether *S. pombe* topo II is phosphorylated like other eukaryotic topo IIs (Ackerman et al., 1988; Heck et al., 1989; Saijo et al., 1990; Kroll and Rowe, 1991; Cardenas et al., 1992), wild-type cells were grown in the synthetic EMM1 medium containing [^{32}P]orthophosphate for 4 h at 33°C (see Materials and Methods). Cells were collected by centrifugation and disrupted with glass beads. Resulting extracts were immunoprecipitated using affinity-purified anti-topo II antibodies bound to protein A–conjugated Sepharose beads. Immunoprecipitates thus obtained were run in SDS-PAGE and subjected to autoradiography. A single 165-kD ^{32}P-labeled band corresponding to the molecular weight of topo II polypeptide was obtained (Fig. 2 *a*, lane *2*). Preimmune serum, on the other hand, did not produce a phospholabeled band (lane *1*). Immunoprecipitates were acid hydrolyzed, run in thin-layer electrophoresis, and phosphoamino acid analysis was performed. An intense spot corresponding to phosphoserine was observed (Fig. 2 *b*).

Dephosphorylated Topo II Is Enzymatically Active

To address the question of whether the enzymatic activity of *S. pombe* topo II is affected by phosphorylation, topo II was treated with potato acid phosphoatase (PAP) and then the enzymatic activity was examined. In vivo–labeled topo II obtained by immunoprecipitation was incubated in the presence or absence of PAP, and run in SDS-PAGE (see Materials and Methods). Autoradiography after the PAP treatment displayed no detectable phospholabeling at the 165-kD band (Fig. 3 *a*, lane *4*; Coomassie blue stain, lane *2*), while an intensely labeled band was obtained without PAP treatment (lane *3*; Coomassie blue stain, lane *1*). Thus most, if not all, phospholabel was removed by PAP under the experimental conditions used.

Chromatographically purified topo II incubated with or without PAP under the same conditions was assayed for the relaxing activity. The results presented in Fig. 3 *b* (lanes *1–5*, without PAP treatment; lanes *6–10*, with PAP treatment; at increasing times of incubation) indicate that the relaxing activity of topo II was retained after PAP digestion. In fact, with PAP treatment there is an apparent twofold increase in the activity (compare lane *3* to *8*, Fig. 3 *b*). Thus the removal of phosphate did not inactivate *S. pombe* topo II. This obser-

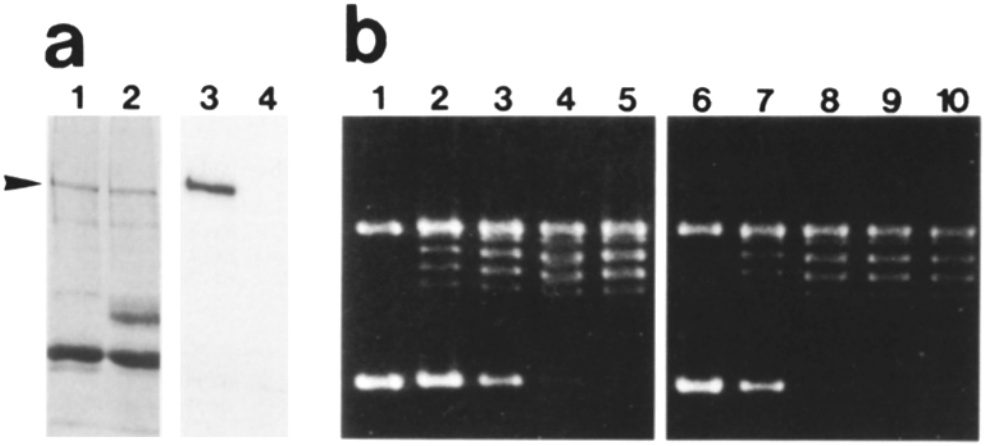


Figure 3. Dephosphorylated topo II retains enzymatic activity. (*a*) ^{32}P-labeled topo II was immunoprecipitated, and incubated with (lanes *2* and *4*) or without (lanes *1* and *3*) potato acid phosphatase (*PAP*) followed by SDS-PAGE. The gels were Coomassie blue stained (lanes *1* and *2*) and then subjected to autoradiography (lanes *3* and *4*). The topo II band is indicated by the arrowhead. (*b*) Relaxing topo II activity after potato acid phosphatase treatment. Purified topo II was incubated with potato acid phosphatase (lanes *6* to *10*) or without (*1* to *5*) at 30°C for 20 min. The the relaxing activity was assayed by adding ATP, Mg^{2+}, and supercoiled plasmid pBR322 to the reaction mixture. At 0 min (lanes *1* and *6*), 1 min (lanes *2* and *7*), 2.5 min (lanes *3* and *8*), 5 min (lanes *4* and *9*), and 7.5 min (lanes *5* and *10*), the reaction was terminated by adding 0.1% SDS and 10 mM Na_3EDTA, and run in agarose gel electrophoresis.

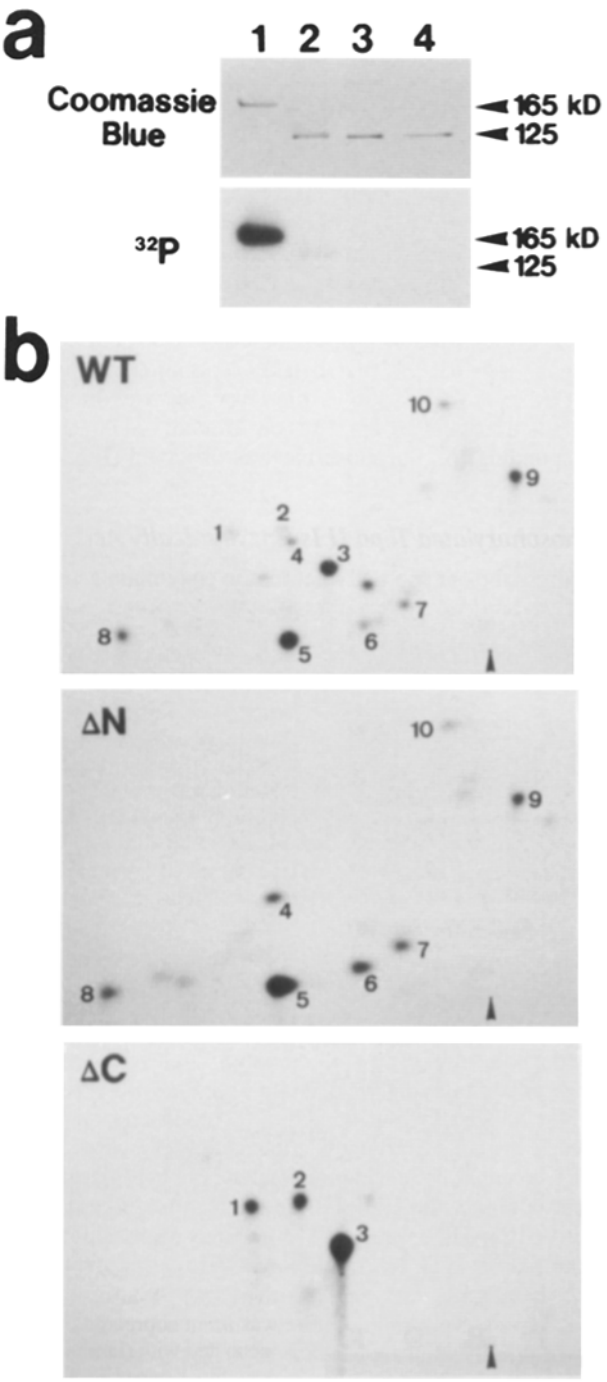


Figure 4. Phosphorylation sites in topo II. (*a*) Papain digestion of ^{32}P-labeled topo II. Partially purified ^{32}P-labeled topo II (lane *1*) was digested with papain, for 2.5 min (lane *2*), 15 min (lane *3*), and 90 min (lane *4*) followed by immunoprecipitation and SDS-PAGE. The upper panel shows the Coomassie blue stained gel while the lower panel shows the autoradiography pattern. (*b*) Tryptic peptide mapping of ^{32}P-labeled topo II. In vivo ^{32}P-labeled wild-type topo II (*WT*), N-truncated ADH-PvII mutant (*ΔN*) deleting the NH_2-terminal 230 residues, and C-truncated ΔSph mutant (*ΔC*) lacking COOH-terminal 286 residues were digested with trypsin and separated in two dimensions (Materials and Methods). The origin in each panel is indicated by arrowhead. Electrophoresis was performed at pH 4.7 in the horizontal dimension (the anode, *left*), followed by ascending chromatography in the vertical dimension. The phosphopeptide spots reproducibly detected in the wild type are numbered (1–10).

vation is different from that reported by Saijo et al. (1990) where inactivation of mouse topo II was seen after dephosphorylation.

Location of Phosphorylation Sites

To roughly determine the location of topo II phosphorylation sites, we first examined whether phosphorylation takes place within the 125-kD core. As shown in Fig. 4 *a*, most if not all, of phospho-labeled amino acid residues were not found in the core fragment. *S. pombe* wild-type cells were in vivo ^{32}P-labeled, and topo II was partially purified as described in Materials and Methods. Topo II was digested with papain at appropriate time intervals, followed by immunoprecipitation using anti-topo II antibodies and SDS-PAGE. The Coomassie blue stained gel shows topo II digested with papain in the upper panel of Fig. 4 *a*. Autoradiography (the lower panel) indicated that phospholabeling was negligible in the 125-kD core. Even a brief digestion by papain (2.5 min) removed almost all the detectable radioactivity in topo II. The faint radioactive band seen at 2.5-min digestion (lane *2*) has a molecular weight slightly higher than that of the 125-kD core band. The result demonstrates that in vivo phospholabeling of topo II occurs in the terminal regions.

Two-dimensional phosphopeptide mapping was applied to further characterize phosphorylated residues. ^{32}P-labeled topo II from a wild-type strain was digested with trypsin for 24 h at 30°C and resulting peptides were separated in two dimensions (Fig. 4 *b*). 10 phospho-labeled spots were reproducibly detected for wild-type topo II (wt). Each spot contained only phosphoserine, shown by acid hydrolysis and two-dimensional electrophoresis of individual phosphopeptides recovered from each spot (data not shown).

For the ADH-PvII deletion mutant (see Fig. 1), which lacked the NH_2-terminal 230 residues, spots 1, 2, and 3 were missing although the seven other spots were detected (ΔN, Fig. 4 *b*). On the other hand, for ΔSph (Fig. 1), which lacked the COOH-terminal 286 residues, spots 1, 2, and 3 were present but spots 4–10 were missing (ΔC, Fig. 4 *b*). Similar experiments were done using other deletion mutants, ADH-Nco, ΔSma, and ΔScII, and consistent phosphopeptide mapping data was obtained. Phosphopeptides present in wild-type and these deletion mutants are summarized in Table I. Phosphopeptides 1–3 appeared to be derived from the NH_2 terminus and 4–10 from the COOH terminus.

Immunofluorescence Microscopy with the Use of Anti-topo II Antibodies

Wild-type *S. pombe* cells were observed by immunofluores-

Table I. Phosphopeptides Identified in Fission Yeast Topo II

Mutant topo II*	Tryptic phosphopeptides
Wild Type	1,2,3,4,5,6,7,8,9,10
ADH-PvII (ΔN)	4,5,6,7,8,9,10
ADH-Nco	4,5,6,7,8,9,10
ΔSma	1,2,3
ΔSph (ΔC)	1,2,3
ΔScII	1,2,3

See also Figure 4.
* Mutant topo IIs are schematically presented in Fig. 1.

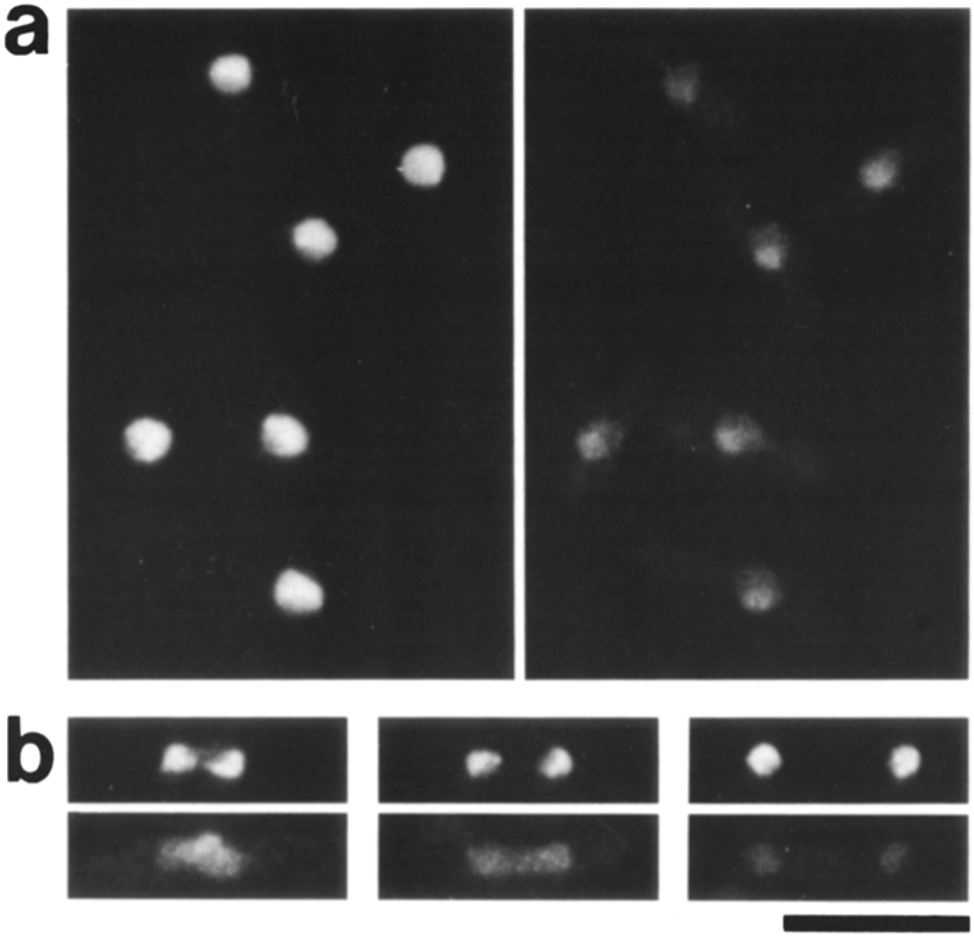


Figure 5. Nuclear localization of wild-type topo II. Indirect immunofluorescence micrographs of wild-type *S. pombe* cells using affinity-purified anti-topo II antibodies (*a*, *right*; *b*, *bottom*) are shown with DAPI stain (*a*, *left*; *b*, *top*). In the interphase cells (*a*), immunofluorescence was prominent in the nonchromosomal nuclear areas (see text) in addition to weaker staining of the chromosomal domain. Cells in nuclear division are shown in *b*. Bar, 10 μm.

cence microscopy using affinity-purified anti-topo II antibodies. As shown in Fig. 5, the nucleus was intensely stained by anti-topo II. No immunofluorescence signal was obtained with preimmune serum (data not shown). Curiously, topo II seemed to be abundantly present in the nucleolar region as well as the chromosomal region of the nucleus. The *S. pombe* interphase nucleus consists of two hemispherical domains, the chromosomal domain stained by DAPI, and the nucleolar domain rich in RNA, containing short protrusions of rDNA repeats (Toda et al., 1981; Yanagida et al., 1986; Hirano et al., 1989; Uzawa and Yanagida, 1992). In interphase nuclei (Fig. 5 *a*), the chromosomal stain by anti-topo II antibodies was less intense than that in the nucleolar region. In the course of nuclear division (Fig. 5 *b*), both domains were stained.

Localizations of Topo II Deletion Mutant Proteins

To determine whether terminal deletions affect subcellular localization of topo II, immunofluorescence microscopy was performed on mutant topo IIs lacking either the NH_2- or COOH-terminal regions or both.

Multicopy plasmids carrying the wild-type and truncated mutant *top2* genes were introduced into *S. pombe* wild-type cells. Because the amount of topo II expressed from the multicopy plasmid was more than 10-fold that of the single-copy genomic *top2*$^+$ (Shiozaki and Yanagida, 1991), immunofluorescence is mostly derived from the product of plasmid gene. As shown in Fig. 6, cells carrying the NH_2-terminal deleted gene, Δ(1–74) (ΔN) revealed nuclear staining as strong as cells carrying the plasmid with the wild-type gene. The N-truncated topo II appeared to retain a strong NLS. However, the cells carrying C-truncated ΔSph which lacked COOH-terminal 286 residues revealed both cytoplasmic and nuclear staining, the cytoplasmic staining as intense as the nucleus (ΔC). Therefore, the C-truncated ΔSph appeared to contain only a weak NLS. We found that cells carrying the doubly truncated gene which lacked both termini showed only cytoplasmic immunofluorescence staining (ΔN-ΔC). Thus the doubly truncated mutant topo II was not transported to the nucleus. These results strongly suggest that both the NH_2- and COOH-terminal regions of topo II contain nuclear localization signals, although the one in the NH_2-terminal region is weak. For the mutant lacking both termini cytoplasmic accumulation of topo II was observed, consistent with its inability to complement the *top2* null mutation.

Construction of N-Deletion Mutants

We further dissected the NH_2-terminal domain to examine its function in nuclear localization. It appears to contain sufficient terminal information for cell viability, as the COOH-terminal deleted *top2* genes (ΔXba and ΔSph in Fig. 1) could complement the *top2* null mutant. It is shorter than the COOH-terminal domain (less than one third its size), facilitating dissection.

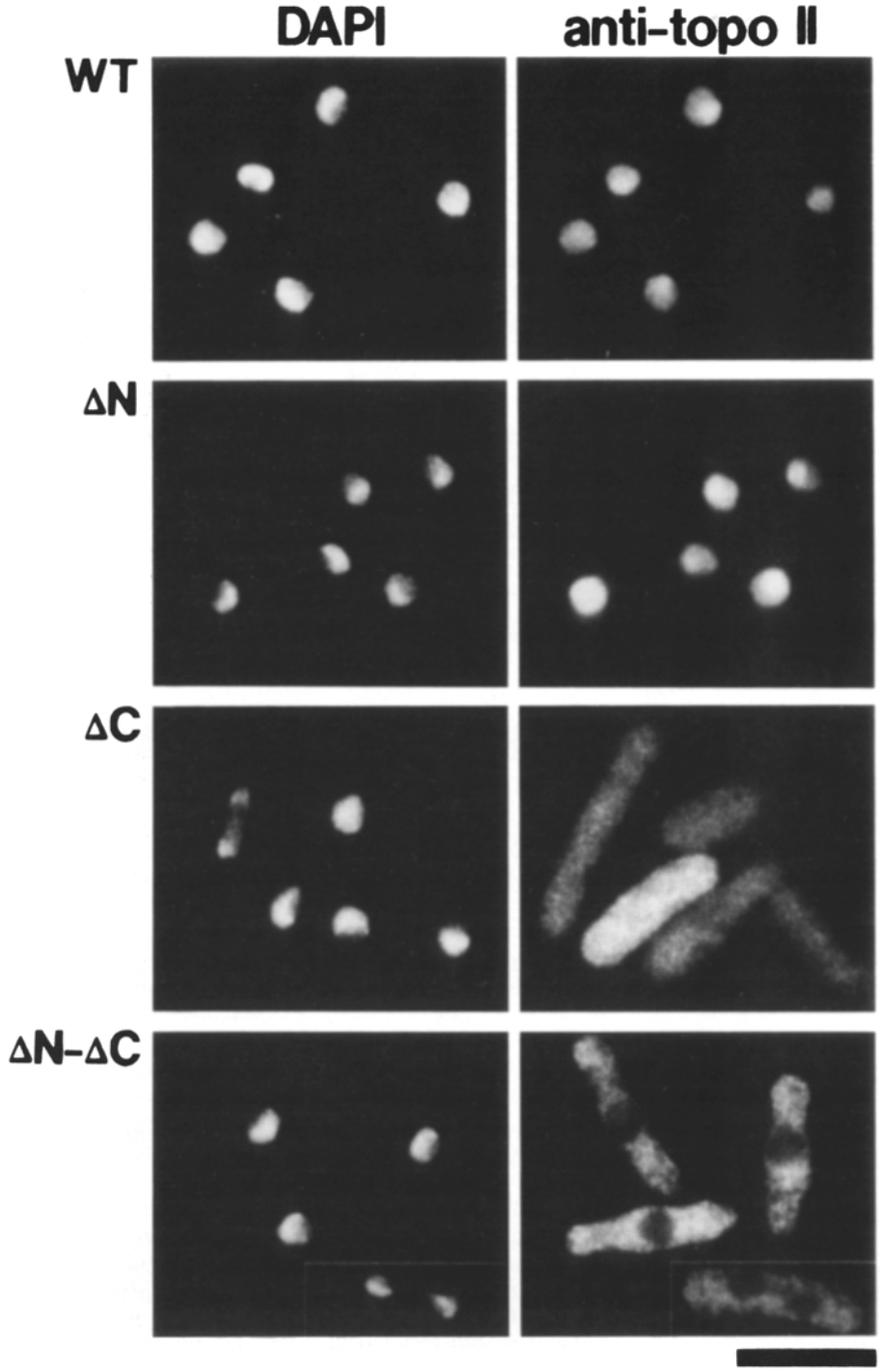


Figure 6. Subcellular localization of N- and C-deleted topo II mutant proteins. (*a*) wild-type; (*b*) Δ(1-74) lacking the NH_2-terminal 74 residues; (*c*) ΔSph lacking the COOH-terminal 286 residues; (*d*) Δ(1-74)-ΔSph lacking both termini. Multicopy plasmids carrying these mutant genes were introduced into *S. pombe* cells. Subcellular localization of the overexpressed topo II was observed by immunofluorescence microscopy using anti-topo II antibodies (*right*). *Left*, DAPI stain. Bar, 10 μm.

The NH_2-terminal sequence consists of three characteristic components; an initial acidic stretch of 15-amino acid residues, a middle basic stretch of 15 residues, and a hydrophilic stretch of 35 residues adjacent to the core (Fig. 7 *a*). A series of deletion or substitution mutants was made by site-directed mutagenesis. Truncation mutants Δ(1–17), Δ(26-40), and Δ(42–74) lack the residues indicated in the parenthesis. Δ(1–53) starting from Met 54 lacks both the acidic and basic stretches. The basic stretch 26–40 resembles a previously characterized class of NLS that contains two basic clusters (Robbins et al., 1991). In the substitution and deletion mutants 26–40/SV and 1–40/SV, the basic stretch was substituted with PKKKRKV, NLS of SV-40 large T-antigen (Kalderon et al., 1984). The 1–40/SV mutant lacked the acidic stretch, whereas for 1–74/SV, the SV-40 NLS was directly bound to the NH_2 terminus of the core. These NH_2-terminal mutants were combined with COOH-terminal deletions, either ΔXba or ΔSph. The deleted COOH-terminal sequences are shown in Fig. 7 *b*.

In Vivo Function of the NH_2-terminal Subdomains

A heterozygous diploid in which one of the two *top2*$^+$ genes was disrupted was transformed with multicopy plasmid car-

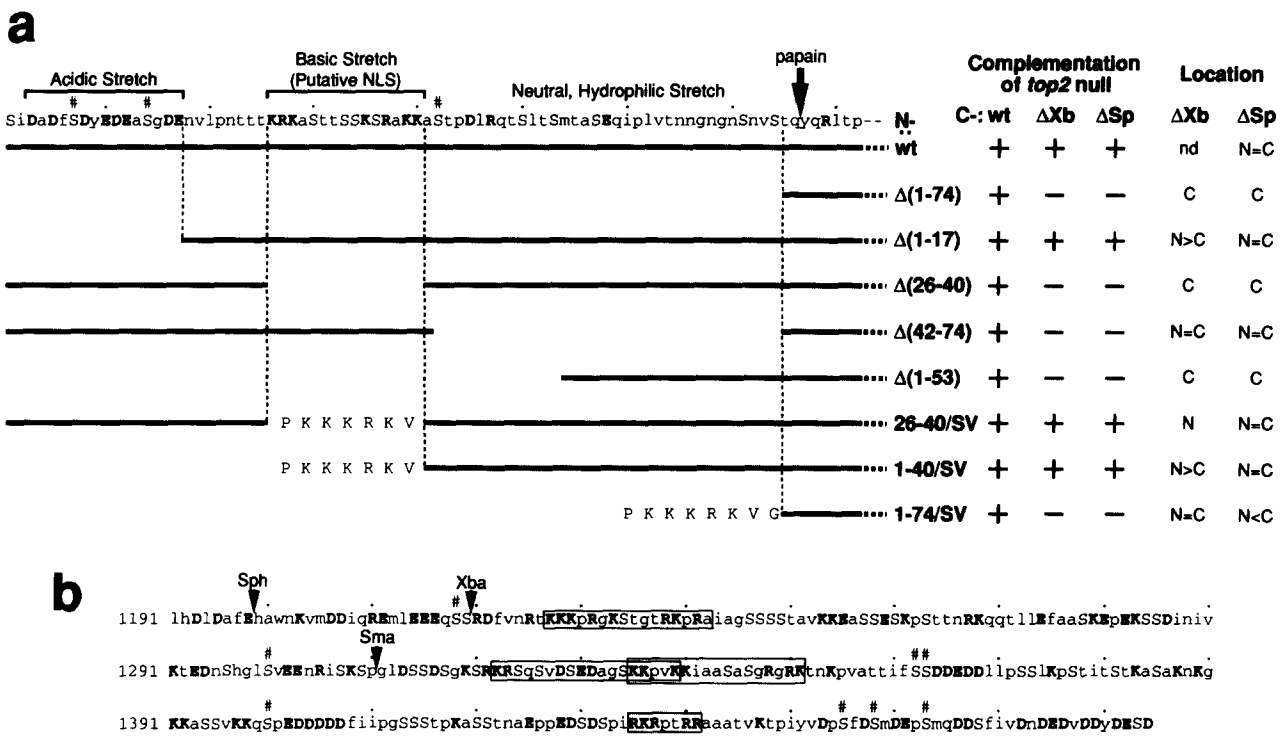


Figure 7. Construction of N-truncated or -substituted and C-truncated topo II mutants, complementation of *top2* null mutant and subcellular localization. (*a*) The amino acid sequence of the fission yeast topo II NH_2-terminus is shown along with schematic representations of mutants constructed. The boundary to the core (cleaved by papain) is indicated by a thick arrow. In the NH_2-terminal region three subdomains exist: an acidic stretch, a basic stretch containing the putative NLS and a neutral, hydrophilic stretch. In the mutants 26-40/SV, 1-40/SV, and 1-74/SV, the indicated residues were substituted with PKKKRKV, the SV-40 large T-antigen NLS (Kalderon et al., 1984). NH_2-terminal mutations were combined with deletions of the COOH-terminal region of wild-type, ΔXba, or ΔSph which deleted the sequences downstream of the XbaI or SphI sites, respectively. The ability to complement the *top 2* null is shown by +. The location of the gene products (see Fig. 8) is also summarized. *N*, nuclear; *C*, cytoplasmic localization of the protein. In the cases where the subcellular location is not exclusively nuclear or cytoplasmic, the pattern of immunostaining is schematically indicated by N > C, N = C, or N < C. nd, not determined. (*b*) Amino acid sequence of the COOH-terminal region of topo II. Arrowheads with the name of restriction enzymes indicate the sites where the deletions were made. The COOH-terminus of the core is near the XbaI site (Shiozaki and Yanagida, 1991). Boxed sequences are putative NLSs. Charged amino acid residues are in bold and serine residues fitting the CK-II consensus for phosphoacceptor sites are indicated by #.

rying *top2* mutant genes constructed above. Transformants were sporulated and haploid segregants were examined to determine whether mutant genes can rescue the *top2* null mutant (Shiozaki and Yanagida, 1991). Results of complementation are shown in Fig. 7 *a*. Wild type, ΔXb, and ΔSp columns indicate the COOH-terminal sequences of the mutant genes. The deletion Δ(1–17) lacking the acidic stretch could fully complement *top2* null in the absence of COOH terminus. Therefore, the acidic stretch is not essential for viability.

However, deletions Δ(26–40), Δ(42–74), and Δ(1–53) did not rescue *top2* null in the absence of COOH terminus, indicating that both basic and neutral stretches are essential for cell viability. The basic stretch 26–40 was functionally substituted with SV-40 large T-antigen NLS; two substituted mutants 26–40/SV and 1–40/SV could complement the *top2* null in the absence of the COOH terminus. The 1–17 stretch was also nonessential for the substitution mutant with the SV-40 NLS. However, 1–74/SV in which the SV-40 NLS was directly ligated to the NH_2 terminus of the core could not rescue the *top2* null, again indicating the essential role of the neutral stretch.

Subcellular Localization of the NH_2-terminal Deletion Mutants

Wild-type cells carrying multicopy plasmids with the mutant *top2* genes described above were observed by immunofluorescence microscopy using anti-topo II antibodies. Immunofluorescence micrographs of cells overexpressing the NH_2-terminal mutant series which also lack the COOH-domain from XbaI are shown in Fig. 8 *a*. Mutants Δ(26–40) and Δ(1–53) lacking the basic stretch showed cytoplasmic localization exclusively. This is consistent with their failure to complement the *top2* null. If, however, the NLS of SV-40 large-T antigen was added, they were able to enter the nucleus and complement the *top2* null; mutant 26–40/SV revealed nuclear staining. Mutant 1–40/SV displayed prominent nuclear staining (indicated by N>C in Fig. 7 *a*). Deletion of the acidic and neutral stretch reduces the fluorescence in the nucleus and increases the cytoplasmic staining (also see 1–74/SV). The absence of nuclear stain correlates with the inability to complement the *top2* null.

Localization of various NH_2-terminal deletion mutant *top2* genes with the COOH-terminal deletion from Sph was also examined, as shown in Fig. 8 *b*. A difference between

the COOH-terminal deletions ΔXba and ΔSph is that nuclear localization in the latter is somewhat diminished, suggesting that an acidic stretch present in the COOH terminus of ΔXba deletions but absent in that of ΔSph deletions has a positive effect for nuclear localization (compare the localization of 26–40/SV-ΔXba and-ΔSph, for example). N-deletion mutants Δ(1–17) and Δ(42–74), respectively, lacking the acidic or neutral stretch, with the COOH-terminal deletion from Sph showed topo II localization both in the nucleus and cytoplasm. Similar patterns were obtained by mutants substituted with the T-antigen NLS (26–40/SV and 1–40/SV). Note that these ΔSph COOH-terminal deletion mutants lacking NH_2-terminal basic or neutral stretches could not rescue *top2* null mutants as was also the case for the ΔXba mutants (Fig. 7 *a*). Mutants lacking the basic stretch revealed nuclear exclusion for anti-topo II staining (Δ[26–40] and Δ[1–53]). It should be emphasized that mutants lacking the neutral stretch produced a product which could enter the nucleus but was not able to complement *top2* null.

Phosphorylation of the NH_2-terminal Domain

To determine which subdomain in the NH_2 terminus is phosphorylated, the degree of phosphorylation in the NH_2-terminal truncated mutant proteins was investigated. Wild-type *S. pombe* cells transformed with plasmids carrying NH_2- and COOH-terminal truncated *top2* genes were ^{32}P phospholabeled. After disruption of cells with glass beads, topo II was immunoprecipitated using anti-topo II antibodies and analyzed by SDS-PAGE followed by autoradiography. Coomassie blue stained and ^{32}P-labeled bands are shown in Fig. 9, *a* and *b*, respectively. Topo II phosphorylation in cells carrying multicopy wild-type $top2^+$ gene is shown in lane *1*. Cells carrying the C-truncated ΔSph gene (lane *2*) produced two labeled bands at 165 and 139 kD, corresponding to the genomic wild-type and ΔSph proteins, respectively. In contrast, Δ(1–74)-ΔSph (lane *3*) lacking the first 74 amino acids and the COOH terminus downstream from the SphI site showed no detectable phosphorylation at 122 kD. A striking finding was that no phosphorylation was detected for Δ(1–17)-ΔSph (lane *4*) which lacks only the first 17 residues in the NH_2 terminus. However, mutant topo II produced by Δ(26–40)-ΔSph (lane *5*) and Δ(42–74)-ΔSph (lane *6*), which lacked the NH_2-terminal basic stretch and the neutral hydrophilic stretch, respectively, but contained the acidic stretch, were phosphorylated. Consistently, Δ(1–53)-ΔSph (lane *7*) lacking both acidic and basic stretches showed no phosphorylation, while 26–40/SV-ΔSph (lane *8*) in which the acidic stretch was maintained but the basic stretch was substituted with SV-40 NLS was phosphorylated. These results strongly suggest that only the acidic stretch is phosphorylated in the NH_2 terminus.

Discussion

Nuclear Localization Sequences in Topo II

Topo II, being a nuclear protein, has a sequence (NLS) responsible for nuclear localization. We report here that fission yeast topo II contains multiple NLSs which locate in the NH_2 and COOH termini and are essential for viability. Judging from immunofluorescence microscopy of truncated mutants using anti-topo II antibodies, the NLS in the COOH terminus is stronger than that in the NH_2 terminus. The NLSs of many nuclear proteins are highly basic and exist in one to several copies per polypeptide (Dingwall and Laskey, 1986). The SV-40 large T-antigen NLS is relatively simple, the minimal sequence being made up of only seven amino acids, PKKKRKV (Kalderon et al., 1984). More complex NLSs have been found in other proteins, such as *Xenopus* nucleoplasmin or N1 protein. These bipartite motif NLSs consist of two basic residues followed by a spacer of ~10 other residues followed by a second basic cluster in which at least three out of five residues are basic (Robbins et al., 1991). Such NLS-like sequences are present in approximately half of the nuclear proteins present in databases (Dingwall and Laskey, 1991). We identified four sequences similar to such bipartite motifs in the termini of fission yeast topo II, namely, amino acids 26–44, 1227–1242, 1322–1339, and 1335–1351 (Fig. 7). We provide experimental evidence in the present paper that the NH_2-terminal stretch of amino acids 26–44 functions as an NLS in topo II.

Immunofluorescence microscopy using anti-topo II showed that the loss of both terminal sequences causes nuclear exclusion of the topo II protein. This explains our previous finding that mutants deleting both termini were unable to complement the *top2* null mutant although the remaining core region was enzymatically active (Shiozaki and Yanagida, 1991). The 125-kD core exclusively contains the homologous regions to other eukaryotic topo IIs, which span the entire core (see Fig. 1; Shiozaki and Yanagida, 1991). The terminal sequences of eukaryotic topo IIs, however, are highly divergent but similarly rich in hydrophilic and charged residues. Eukaryotic topo IIs possibly acquired the terminal sequences by adding them to the ancestral topo II in evolution. The presence of basic and acidic clusters was noticed in the COOH termini of eukaryotic topo IIs and proposed to have a function specialized to eukaryotic topo II, such as binding to chromatin (Uemura et al., 1986). It is now apparent that at least one of the topo II terminal functions is nuclear targeting.

Three Subdomains in the Topo II Terminal Sequences

Functional dissection using various deletion and substitution mutants revealed three subdomains in the NH_2-terminal domain as schematized in Fig. 10. The acidic stretch (3–17) at the extreme NH_2 terminus is nonessential, but may affect the efficiency of topo II nuclear localization. Furthermore, this stretch contains the sites for phosphorylation. Two other subdomains, namely, the basic (26–40) and hydrophilic (42–74) stretches, are essential for viability if the COOH terminus is also deleted. In the COOH-terminal domain, several copies of sequences similar to these three subdomains are also present.

The deletion of the basic stretch KRKASTTSSKSRAKK (26–40) caused nuclear exclusion in the absence of COOH terminus. The basic stretch was functionally substituted by the large T-antigen NLS. These results strongly suggested that the basic stretch functions as an NLS in topo II. This is the first demonstration that the SV-40 large T-antigen NLS can function in *S. pombe* cells.

Note that the efficiency of nuclear location was significantly enhanced by the NH_2-terminal acidic stretch, when the

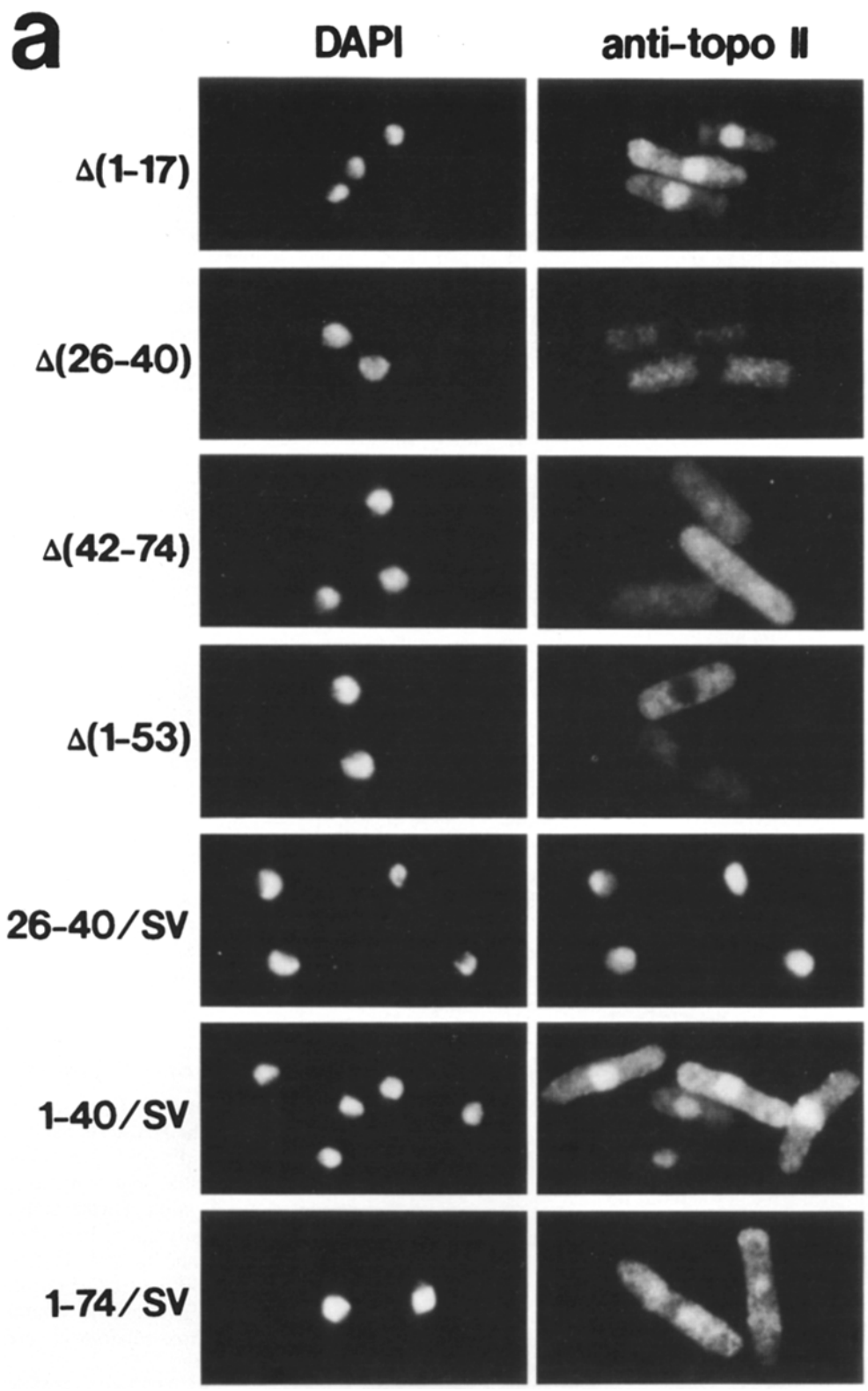
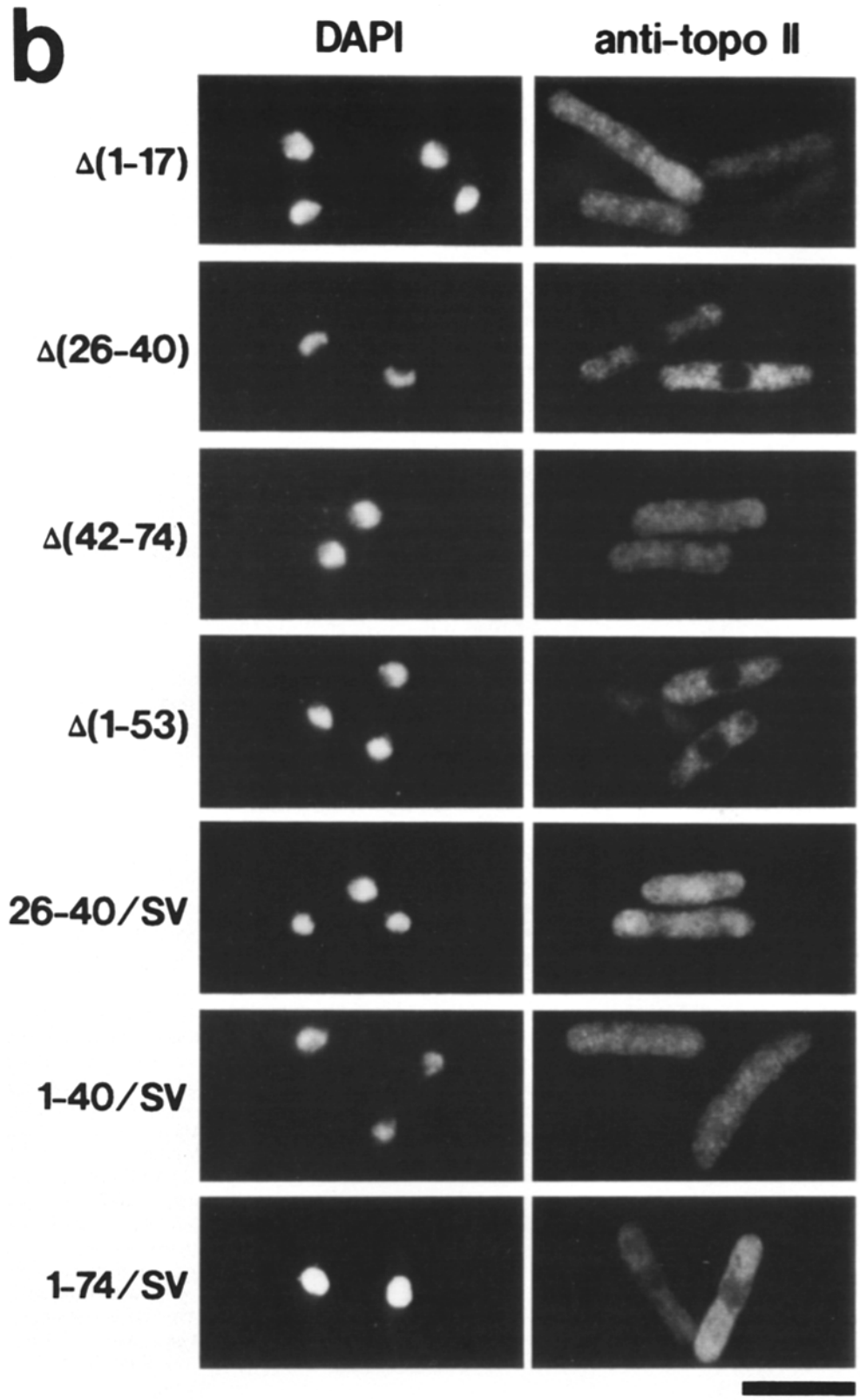


Figure 8. Subcellular localization of topo II NH_2-terminal deletions. NH_2-terminal mutations are combined with ΔXba (*a*) and ΔSph (*b*) COOH-terminal deletions. Wild-type *S. pombe* cells were transformed with multicopy plasmid carrying mutant genes, (*a*) Δ(1-17)-ΔXba, Δ(26-40)-ΔXba, Δ(42-74)-ΔXba, Δ(1-53)-ΔXba, 26-40/SV-ΔXba, 1-40/SV-ΔXba, 1-74/SV-ΔXba, (*b*) Δ(1-17)-ΔSph, Δ(26-40)-ΔSph, Δ(42-74)-ΔSph, Δ(1-53)-ΔSph, 26-40/SV-ΔSph, 1-40/SV-ΔSph, and 1-74/SV-ΔSph. Cells were observed by immunofluorescence microscopy using anti-topo II antibodies (*right*) or DAPI stain (*left*). Bar, 10 μm.

T-antigen NLS replaced the topo II basic stretch (compare 26–40/ SV and 1–40/SV). In large T-antigen, an acidic stretch present adjacent to the NLS and containing sites of casein kinase II (CK-II) phosphorylation was reported to promote unclear targeting (Rihs and Peters, 1989; Rihs et al., 1991). In fact, the NH_2-terminal acidic stretch of fission yeast topo II has two CK-II consensus sequences (S/T-X-X-D/E; Krebs et al., 1988, also see Fig. 7, *a* and *b*; putative CK-II sites indicated by #). Interestingly, only this acidic region is phosphorylated in the NH_2 terminus. Jans et al. (1991) recently proposed the CcN-motif which consists of NLS and phosphorylation sites for CK-II and cdc2. There is one potential



cdc2 consensus site (^{43}TPDLR) in the NH_2 terminus of fission yeast topo II. However, phosphorylation at Thr was hardly detected in immunoprecipitated topo II of exponentially growing cells. Note that the COOH-terminal acidic stretch present between Xba and Sph appears to enhance the activity of NLS in the NH_2 terminus. This may be due to homodimeric structure of topo II which enables interaction between the NH_2 and COOH termini.

The NH_2-terminal neutral hydrophilic stretch contains a number of Ser, Thr, Asn, and Gly and is essential for viability. However, its function is unknown. Because mutant topo II lacking the hydrophilic stretch could still enter the nucleus,

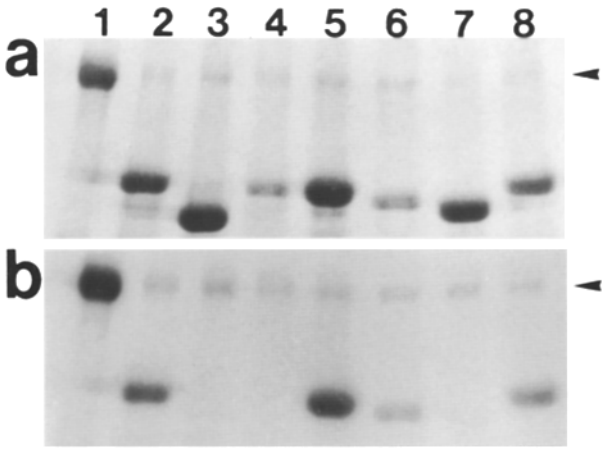


Figure 9. Phosphorylation of N- and C-truncated *top2* mutant polypeptides. Immunoprecipitation of ^{32}P-labeled *S. pombe* cell extracts was performed (see text). Wild-type cell extracts overexpressing wild-type *top2*$^+$ (lane *1*), C-truncated mutant ΔSph (lane *2*), double truncated mutants Δ(1-74)-ΔSph (lane *3*), Δ(1-17)-ΔSph (lane *4*), Δ(26-40)-ΔSph (lane *5*), Δ(42-74)-ΔSph (lane *6*), Δ(1-53)-ΔSph (lane *7*), and 26-40/SV-ΔSph (lane *8*) were immunoprecipitated and run by SDS-PAGE. (*a*) Coomassie blue stain; and (*b*) autoradiography of the gel. The arrowheads indicate the position of the wild-type genomic *top2*$^+$ gene product.

the region is not essential for nuclear location. One hypothesis is that it may be necessary for proper interaction with DNA sequences or chromatin structure. Alternatively, it may act as a spacer between the 125-kD core and NLS. A spacer region may be needed for NLS to interact with the nuclear transport receptor. The other possibility is that the hydrophilic stretch may reduce the inhibition of topo II activity by the basic NLS.

Terminal Phosphorylation of Topo II

Fission yeast topo II is a phosphoprotein like other eukaryotic topo IIs, such as that of *Drosophila* (Ackerman et al., 1988), chicken (Heck et al., 1989), mouse (Saijo et al., 1990), HeLa (Kroll and Rowe, 1991), and budding yeast (Cardenas et al., 1992). Phosphorylated residues in fission yeast topo II are all Ser and are localized in the termini. No phosphorylation was detected in the core. CK-II appears to be responsible for topo II phosphorylation in *Drosophila* (Ackerman et al., 1988) and budding yeast (Cardenas et al., 1992). No phosphorylation of budding yeast topo II was observed in a CK-II mutant strain and the pattern of in vivo phosphorylation is highly similar to that obtained by CK-II phosphorylation in vitro (Cardenas et al., 1992).

Consistently, in fission yeast topo II, there are three and eight putative CK-II sites in the NH_2 and COOH termini, respectively, as shown in Fig. 7 (indicated by #). As mentioned, two sites are found in the NH_2-terminal acidic stretch that is required for phosphorylation of the NH_2 terminus. Purified topo II in vitro phosphorylated by crude fission yeast extracts produced a pattern similar to that in vivo (Shiozaki, K., and M. Yanagida, unpublished data). This phosphorylating activity resembles CK-II, because it was inhibited by heparin and could use GTP as the phosphate donor (Glover et al., 1983; Hathaway et al., 1980). Bovine cAMP-dependent protein kinase also phosphorylated fission yeast topo II, but the phosphorylation peptide patterns were distinct from that of in vivo labeled topo II (Shiozaki, K., and M. Yanagida, unpublished). We have also examined whether cdc2 kinase (Nurse, 1990) phosphorylates topo II; bulk phosphorylation was the same for a wild-type and a *cdc2* mutant strain. Also, *cdc2* mutant extracts could phosphorylate topo II (data not shown). These data suggest that cdc2 is not a major kinase for topo II.

Does phosphorylation of topo II have any physiological significance? Our present results show that fission yeast topo II dephosphorylated by PAP is as active as phosphorylated topo II, and in fact even somewhat higher than the phosphorylated form, indicating that phosphorylation is not essential for the topo II enzymatic activity. This is consistent with the fact that all the phosphorylation sites detected in fission yeast topo II are localized in the terminal regions which were previously shown to be nonessential for the enzymatic activity (Shiozaki and Yanagida, 1991). Consistently, the activity of the 125-kD core is higher than that of intact topo II. An overall effect of phosphorylation seems to be inhibitory for the activity of fission yeast topo II. However, phosphorylation was reported to be essential for the activity or the activation of topo II in mouse FM3A (Saijo et al., 1990) and *Drosophila* (Ackerman et al., 1985). Phosphorylation by CK-II was reported to activate topo II (Ackermann et al., 1985). This apparent discrepancy is not understood. In higher eukaryotes, it is possible that there are more

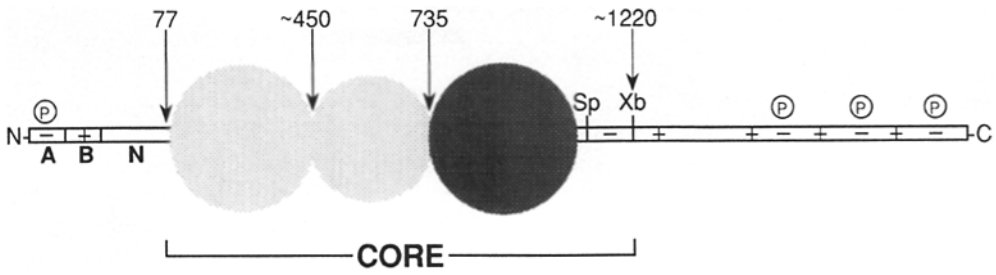


Figure 10. A schematic representation of the fission yeast topo II polypeptide. Papain cleavage sites are indicated by arrows with amino acid positions. The NH_2 terminus consists of an acidic stretch (*A*), a basic stretch (*B*), and a neutral, hydrophilic stretch (*N*). Minus and plus signs represent the clusters of acidic or basic amino acids, respectively. Putative phosphorylated regions are indicated by a circled P. Sp (*SphI*) and Xb (*XbaI*) are the sites used for COOH-terminal deletion.

phosphorylation sites, that may be found in regions essential for activity. Alternatively, fission yeast topo II might be phosphorylated in the enzymatically essential core region, but that phosphorylation was difficult to detect and also difficult to remove by PAP. Another possibility is that the phosphorylation pattern of fission yeast topo II and its role differs from that of topo II in other organisms.

Terminal phosphorylation of topo II of fission yeast might be implicated in the regulation of nuclear location rather than in its activity. SV-40 large T-antigen (Rihs et al., 1991; Jans et al., 1991) and budding yeast *SWI5* protein (Moll et al., 1991) were reported to be controlled by phosphorylated sequences adjacent to and within NLS. In fact, the phosphorylated acidic stretch in the NH_2 terminus appears to enhance the nuclear transport of fission yeast topo II. However, a double deletion mutant gene Δ(1–17)-ΔSph which lacks the COOH-terminal domain and the acidic stretch in the NH_2-terminal domain can complement *top2* null on a multicopy plasmid, although its protein product contains no detectable phosphorylation. Alterations of topo II phosphorylation during the fission yeast cell cycle remains to be determined. It is possible that a particular site of topo II is phosphorylated only for a very short period in the cell cycle and that the phosphorylation site is not detected by the metabolic labeling method of an asynchronous culture used here. Analysis of a site-directed mutant of the putative cdc2 phosphorylation site will be of interest. Considering the essential role of topo II in chromosome condensation and segregation (Uemura et al., 1987; Wood and Earnshaw, 1990; Adachi et al., 1991), an intriguing possibility is that topo II activity is regulated in mitosis by phosphorylation. However, results presented here have led us to support the possibility that phosphorylation of topo II does not play an essential role in fission yeast.

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References

Ackerman, P., C. V. C. Glover, and N. Osheroff. 1985. Phosphorylation of DNA topoisomerase II by casein kinase II: modulation of eukaryotic topoisomerase II activity *in vitro*. *Proc. Natl. Acad. Sci. USA.* 82:3164–3168.

Ackerman, P., C. V. C. Glover, and N. Osheroff. 1988. Phosphorylation of DNA topoisomerase II *in vivo* and in total homogenates of *Drosophila* Kc cells. The role of casein kinase II. *J. Biol. Chem.* 263:12653–12660.

Adachi, Y., M. Luke, and U. K. Laemmli. 1991. Chromosome assembly *in vitro*: topoisomerase II is required for condensation. *Cell.* 64:137–148.

Beach, D., and P. Nurse. 1981. High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. *Nature (Lond.).* 290:140–142.

Beach, D., M. Piper, and P. Nurse. 1982. Construction of a *Schizosaccharomyces pombe* gene bank in a shuttle vector and its use to isolate genes by complementation. *Mol. Gen. Genet.* 187:326–329.

Berrios, M., N. Osheroff, and P. A. Fisher. 1985. *In situ* localization of DNA topoisomerase II, a major component of the Drosophila nuclear matrix fraction. *Proc. Natl. Acad. Sci. USA.* 82:4142–4146.

Cardenas, M. E., Q. Dang, C. V. C. Glover, and S. M. Gasser. 1992. Casein kinase II phosphorylates the eukaryote-specific C-terminal domain of topoisomerase II *in vivo*. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1785–1796.

Cooper, J. A., and C. S. King. 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates $pp60^{c-src}$. *Mol. Cell. Biol.* 6:4467–4477.

Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* 99:387–402.

DiNardo, S., K. Voelkel, and R. Sternglanz. 1984. DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc. Natl. Acad. Sci. USA.* 81:2616–2620.

Dingwall, C., and R. A. Laskey. 1986. Protein import into the cell nucleus. *Ann. Rev. Cell Biol.* 2:367–390.

Dingwall, C., and R. A. Laskey. 1991. Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.* 16:478–481.

Dingwall, C. D., S. V. Sharnick, and R. A. Laskey. 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell.* 30:449–458.

Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu. 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* 100:1706–1715.

Gasser, S. M., T. Laroche, J. Falquet, E. B. d. l. Tour, and U. K. Laemmli. 1986. Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* 188:613–629.

Gellert, M. 1981. DNA gyrase and other type II topoisomerases. *Enzymes.* 14:345–366.

Giaever, G., R. Lynn, T. Goto, and J. C. Wang. 1986. The complete nucleotide sequence of the structural gene *TOP2* of yeast DNA topoisomerase II. *J. Biol. Chem.* 261:12448–12454.

Glover, C. V. C., E. R. Shelton, and D. L. Brutlag. 1983. Purification and characterization of a type II casein kinase from *Drosophila melanogaster*. *J. Biol. Chem.* 258:3258–3265.

Hagan, I. M., and J. S. Hyams. 1988. The use of cell division mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* 89:343–357.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 726 pp.

Hathaway, G. M., T. Lubben, and J. Traugh. 1980. Inhibition of casein kinase II by heparin. *J. Biol. Chem.* 255:8038–8041.

Heck, M. M. S., W. N. Hittelman, and W. C. Earnshaw. 1989. *In vivo* phosphorylation of the 170-kDa form of eukaryotic DNA topoisomerase II. Cell cycle analysis. *J. Biol. Chem.* 264:15161–15164.

Hirano, T., G. Konoha, T. Toda, and M. Yanagida. 1989. Essential roles of the RNA polymerase I largest subunit and DNA topoisomerases in the formation of fission yeast nucleolus. *J. Cell Biol.* 108:243–253.

Holm, C., T. Goto, J. C. Wang, and D. Botstein. 1985. DNA topoisomerase II is required at the time of mitosis in yeast. *Cell.* 41:553–563.

Hsieh, T. 1990. Mechanistic aspects of type-II DNA topoisomerase. *In* DNA topology and its biological effects. N. R. Cozzarelli and J. C. Wang, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 243–263.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163–168.

Jans, D. A., M. J. Ackermann, J. R. Bischoff, D. H. Beach, and R. Peters. 1991. $p34^{cdc2}$-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins. *J. Cell Biol.* 115:1203–1212.

Kalderon, K., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499–509.

Krebs, E. G., R. N. Eisenman, E. A. Kuenzel, D. W. Litchfield, F. J. Lozeman, B. Lüscher, and J. Sommercorn. 1988. Casein kinase II is a potentially important enzyme concerned with signal transduction. *Cold Spring Harbor Symp. Quant. Biol.* 53:77–84.

Kroll, D. J., and T. C. Rowe. 1991. Phosphorylation of DNA topoisomerase II in a human tumor cell line. *J. Biol. Chem.* 266:7957–7961.

Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367–382.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lindsley, J. E., and J. C. Wang. 1991. Proteolysis patterns of epitopically labeled yeast DNA topoisomerase II suggest an allosteric transition in the enzyme induced by ATP binding. *Proc. Natl. Acad. Sci. USA.* 88:10485–10489.

Lynn, R., G. Giaever, S. L. Swanberg, and J. C. Wang. 1986. Tandem regions of yeast DNA topoisomerase II share homology with different subunits of bacterial gyrase. *Science (Wash. DC).* 233:647–649.

Mitchson, J. M. 1970. Physiological and cytological methods for *Schizosaccharomyces pombe*. *Meth. Cell Physiol.* 4:131–165.

Moll, T., G. Tebb, U. Surana, H. Robitsch, and K. Nasmyth. 1991. The role of phosphorylation and the *CDC28* protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor *SWI5*. *Cell.* 66:743–758.

Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* 194:795–823.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature (Lond.).* 344:503–508.

Rihs, H.-P., and R. Peters. 1989. Nuclear transport kinetics depend on phosphorylation-site-containing sequences flanking the karyophilic signal of the Simian virus 40 T-antigen. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1479–1484.

Rihs, H.-P., D. A. Jans, H. Fan, and R. Peters. 1991. The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:633–639.

Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell.* 64:615–623.

Rottmann, M., H. C. Schröder, M. Gramzow, K. Renneisen, B. Kurelec, A. Dorn, U. Friese, and W. E. G. Müller. 1987. Specific phosphorylation of proteins in pore complex-laminae from the sponge *Geodia cydonium* by the homologous aggregation factor and phorbol ester. Role of protein kinase C in the phosphorylation of DNA topoisomerase II. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3939–3944.

Russell, P. R., and B. D. Hall. 1983. The primary structure of the alcohol dehydrogenase from the fission yeast *Schizosaccharomyces pombe*. *J. Biol. Chem.* 258:143–149.

Sahyoun, N., M. Wolf, J. Besterman, T. Hsieh, M. Sander, H. L. III, K.-J. Chang, and P. Cuatrecasas. 1986. Protein kinase C phosphorylates topoisomerase II: topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc. Natl. Acad. Sci. USA.* 83:1603–1607.

Saijo, M., T. Enomoto, F. Hanaoka, and M. Ui. 1990. Purification and characterization of type II DNA topoisomerase from mouse FM3A cells: phosphorylation of topoisomerase II and modification of its activity. *Biochemistry.* 29:583–590.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Shiozaki, K., and M. Yanagida. 1991. A functional 125-kDa core polypeptide of fission yeast DNA topoisomerase II. *Mol. Cell. Biol.* 11:6093–6102.

Silver, P. 1991. How proteins enter the nucleus. *Cell.* 64:489–497.

Simanis, V., and P. Nurse. 1986. The cell cycle control gene $cdc2^{+}$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell.* 45:261–268.

Smith, D. E., and P. A. Fisher. 1984. Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* 99:20–28.

Toda, T., M. Yamamoto, and M. Yanagida. 1981. Sequential alteration in the nuclear chromatin region during mitosis of the fission yeast *Schizosaccharomyces pombe*: video fluorescence microscopy of synchronously growing wild-type and cold sensitive *cdc* mutants by using a DNA-binding fluorescent probe. *J. Cell Sci.* 52:271–287.

Towbin, H. T., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Tsai-Pflugfelder, M., L. F. Liu, A. A. Liu, K. M. Tewey, J. Whang-Peng, T. Knutsen, K. Huebner, C. M. Croce, and J. C. Wang. 1988. Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21–22. *Proc. Natl. Acad. Sci. USA.* 85:7177–7181.

Uemura, T., and M. Yanagida. 1984. Isolation of type I and II DNA topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1737–1744.

Uemura, T., and M. Yanagida. 1986. Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutants: uncoordinated mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1003–1010.

Uemura, T., K. Morikawa, and M. Yanagida. 1986. The nucleotide sequence of the fission yeast DNA topoisomserase II gene: structural and functional relationships to other DNA topoiomerases. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2355–2361.

Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. *Cell.* 50:917–925.

Uzawa, S., and M. Yanagida. 1992. Visualization of centromeric and nucleolar DNA in fission yeast by fluorescence *in situ* hybridization. *J. Cell Sci.* 101:267–275.

Wang, J. C. 1985. DNA topoisomerases. *Ann. Rev. Biochem.* 54:665–697.

Wigley, D. B., G. J. Davies, E. J. Dodson, A. Maxwell, and G. Dodson. 1991. Crystal structure of an N-terminal fragment of the DNA gyrase B protein. *Nature (Lond.).* 351:624–629.

Wood, E. R., and W. C. Earnshaw. 1990. Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J. Cell Biol.* 111:2839–2850.

Wyckoff, E., D. Natalie, J. M. Nolan, M. Lee, and T. Hsieh. 1989. Structure of the *Drosophila* DNA topoisomerase II gene. Nucleotide sequence and homology among topoisomerase II. *J. Mol. Biol.* 205:1–13.

Yanagida, M., and R. Sternglanz. 1990. Genetics of DNA topoisomerases. *In* DNA Topology and Its Biological Effects. N. R. Cozzarelli, and J. C. Wang, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 299–320.

Yanagida, M., K. Morikawa, Y. Hiraoka, S. Matsumoto, T. Uemura, and S. Okada. 1986. Video-connected fluorescence microscopy of large DNA molecules, chromatin, and chromosomes. *In* Applications of Fluorescence in the Biomedical Sciences. Taylor, L., editor. Alan R. Liss, Inc., New York. 321–345.

Zoller, M. J., and M. Smith. 1987. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and single-stranded DNA template. *Methods Enzymol.* 154:329–350.