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Cloning and sequencing of *Schizosaccharomyces pombe* DNA topoisomerase I gene, and effect of gene disruption

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ABSTRACT

We cloned the structural gene top1$^+$ for Schizosaccharomyces pombe DNA topoisomerase I (topo I) by hybridization. An eight-fold increase of topo I relaxing activity was obtained in S. pombe cells transformed with multicopy plasmid with top1$^+$ insert. Nucleotide sequence determination showed a hypothetical coding frame interrupted by two short introns, encoding a 812 residue polypeptide (M.W. 94,000), 43 residues longer than and 47% homologous to Saccharomyces cerevisiae topo I. We show that the top1 (null) strain made by gene disruption is viable, although its generation time is 20% longer than that of wild type. The top1 locus is mapped in the long arm of chromosome II, using the Leu$^+$ marker integrated with the cloned top1$^+$ sequence. We constructed a double mutant top1 (null) top2 (ts) and found its defective phenotype similar to that of previously obtained top1 (heat sensitive) top2 (ts). The other double mutant top1 (null) top2 (cs), however, was lethal. Our results suggest that top1$^+$ gene of S. pombe is dispensable only if topo II activity is abundant.

INTRODUCTION

DNA topoisomerases are the enzymes that control the topological states of DNA by transient breakage and subsequent rejoining of DNA strands (reviewed in ref. 1). The enzymes are classified in two types: those acting by means of a double-strand break are called DNA topoisomerase II (abbreviated topo II), while DNA topoisomerase I (topo I) makes a single-strand break. These enzymes are thought to play important and distinct roles in folding and organizing eucaryotic and procaryotic chromosomes. In yeasts, topo II is essential in mitosis but topo I appears to be dispensable (reviewed in ref. 2). This is thought to be due to the abundant presence of topo II that can substitute for the relaxing activity of topo I. Consistently, the double mutants top1-top2 that lacked the relaxing activity showed immediate arrest in the cell cycle by blocking RNA and DNA synthesis, suggesting that the relaxing activity is required throughout the cell cycle.

The eucaryotic topo I and II can relax both negative and positive supercoils, whereas procaryotic enzymes relax only negative supercoils (1).

Topoisomerase I enzymes have been purified from various eucaryotic sources and characterized (3-7). Molecular weights range 90,000-135,000 and they appear to exist as monomeric forms (3) while topo II enzymes are homologous dimers. Little is known, however, about the primary structure of eucaryotic topo I, except for Saccharomyces cerevisiae which has been deduced by gene cloning and subsequent nucleotide sequence determination (8).

We report here the cloning and nucleotide sequence determination of the fission yeast Schizosaccharomyces pombe DNA topo I gene (top1$^+$). The predicted topo I polypeptide has been compared with that of S. cerevisiae. To examine the possibility that the complete absence of topo I activity might result in a phenotype differing from that of previously obtained heat-sensitive topo I mutants (designated hs top1; enzyme activity itself is heat-sensitive but growth is apparently normal) (9,10), we investigated effect of the gene disruption on viability of S. pombe and compared defective phenotype between two classes of double mutants top1 (null)-top2 (temperature-sensitive lethal, ts or cold-sensitive lethal, cs) and top1 (hs)-top2 (ts or cs). Ts and cs stand for the phenotype for cell growth while hs represents the heat inactivation of enzyme. We found that the top1 (null) strain is viable like top1 (hs) but the double mutants show distinct growth phenotypes. Our results also suggested that the top1$^+$ gene becomes essential when the activity of topo II is not abundant. By integration of the cloned top1$^+$ sequence with the marker gene, we mapped the chromosomal locus for top1.

MATERIALS AND METHODS

Strains and media

S. pombe haploid strains used were hs top1-710, ts top2-191, -342, -437 (9,10) and the wild type HM123. The type I relaxing activity in the extracts of top1-710 is inactivated at 36°C, but is retained at 20-26°C (9). The residual relaxing activity at 36°C is less than 1% of the wild type. YPD (1% yeast extract, 2% polypeptone and 2% dextrose) and EMM2 (11) were rich and minimal media, respectively. Escherichia coli HB101 was used for preparation of plasmids.

DNA cloning and sequencing

Restriction enzyme digestion, gel transfer hybridization and other standard methods for recombinant DNAs were followed as described (12). Nucleotide sequences were determined by the dideoxy method (13), using unidirectional progressive deletion and pUC plasmids (14).

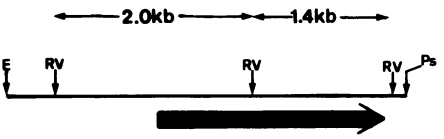


Figure 1. The cloned TOP1 gene of Saccharomyces cerevisiae (8,18) used as the probe for cloning the Schizosaccharomyces pombe $topl^+$ gene. The thick arrow indicates the coding region and the direction for transcription. E, Eco RI; Ps, Pst I; RV, Eco RV.

Assay for DNA topoisomerase I

The whole cell extracts of exponentially grown S. pombe were prepared and the activity of topo I was assayed using supercoiled pBR322 as the substrate (9). The reaction mixture in 25 mM Tris HCl at pH 7.5, 1 mM EDTA, 150 mM KCl and 1 mM 2-mercaptoethanol was incubated for 5 min at 26°C or 36°C, and the reaction was terminated by adding one-fourth volume of 0.5% SDS, 2.5% bromophenol blue and 25% glycerol. The relaxing activity was monitored by electrophoresis in 1.0% agarose gels.

Transformation, gene disruption and genetical methods

The lithium acetate method (15) was used for tranformation of S. pombe. The one-step gene disruption described by Rothstein (16) was followed. Standard genetical procedures for fission yeast were as described by Gutz et al. (17).

RESULTS AND DISCUSSION

Cloning of the DNA topoisomerase I gene

We cloned the S. pombe $topl^+$ gene by screening the cosmid library of the S. pombe genomic DNA probed with ^{32}P-labelled 1.4 kb EcoRV fragment of the S. cerevisiae TOP1 gene (Figure 1, ref. 8 and 18). Two cosmid clones of S. pombe have a common 5.1 kb EcoRI fragment which hybridizes with the S. cerevisiae probe (data not shown). The 5.1 kb EcoRI fragment was isolated and ligated with pUC18 and pUC19 (designated pKM101 and pKM103). The restriction sites in the fragment were determined, as shown in Figure 2a, where the arrow indicates the coding region for the topo I gene (see below). When the genomic DNA was restricted with Eco RI and probed with the Pvu II-Bam HI fragment located within the coding region, a single hybridizing band was obtained, indicating that S. pombe has a single DNA topoisomerase I gene in its genome (data not shown).

Northern blot hybridization was done using RNA probes synthesized by SP6

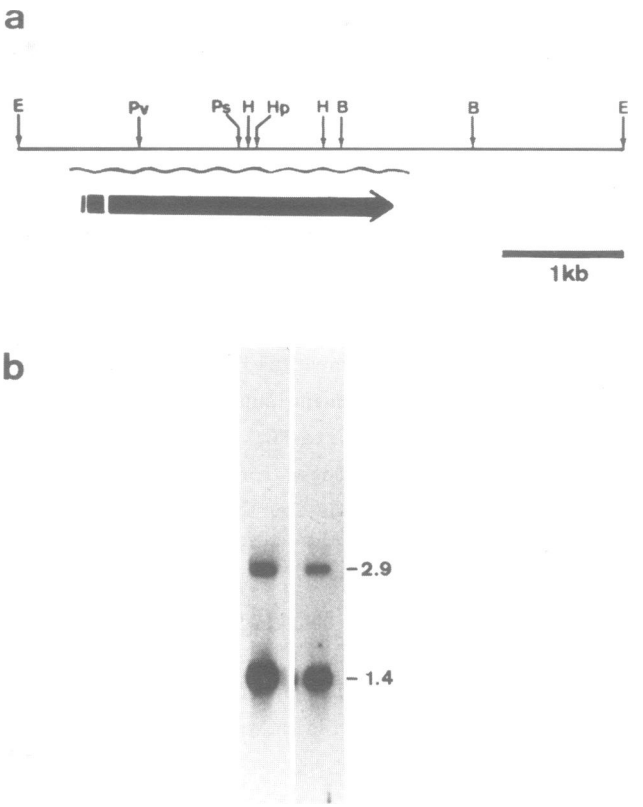


Figure 2. Cloned S. pombe topl$^+$ gene and Northern blot hybridization. (a) Restriction sites of the 5.1 kb Eco RI genomic DNA fragment containing the S. pombe topl$^+$ gene. The thick arrow indicates the coding region and the direction for transcription. Pv, Pvu II; H, Hind III; Hp, Hpa I; B, Bam HI. Other symbols are shown in the caption of Figure 1. The region of which nucleotide sequence was determined is shown by the wavy line. (b) Northern blot hybridization of S. pombe polyA$^+$ RNA using the RNA probes from the topl$^+$ coding region (see text). Probes: 1.0 kb Eco RI-Pvu II (left lane); 0.29 kb Hinc II-Pvu II (right lane).

RNA polymerase from 0.29 kb Hinc II-Pvu II and 1.0 kb Eco RI-Pvu II DNA fragments. The former is located within the coding region (the Hinc II site is at the 5' side of the Pvu II site). Only the RNA probes for the predicted coding direction hybridize with 2.9 and 1.4 kb long polyA$^+$ RNA (Figure 2b). The 2.9 kb transcript seems to be mRNA for the complete topl$^+$ gene because the predicted coding region is 2436 bp long (see below). The appearance of the 1.4 kb band is surprising and not understood; it contains 5' end of the topl$^+$ gene and its amount is significantly higher than that of the 2.9 kb transcript.

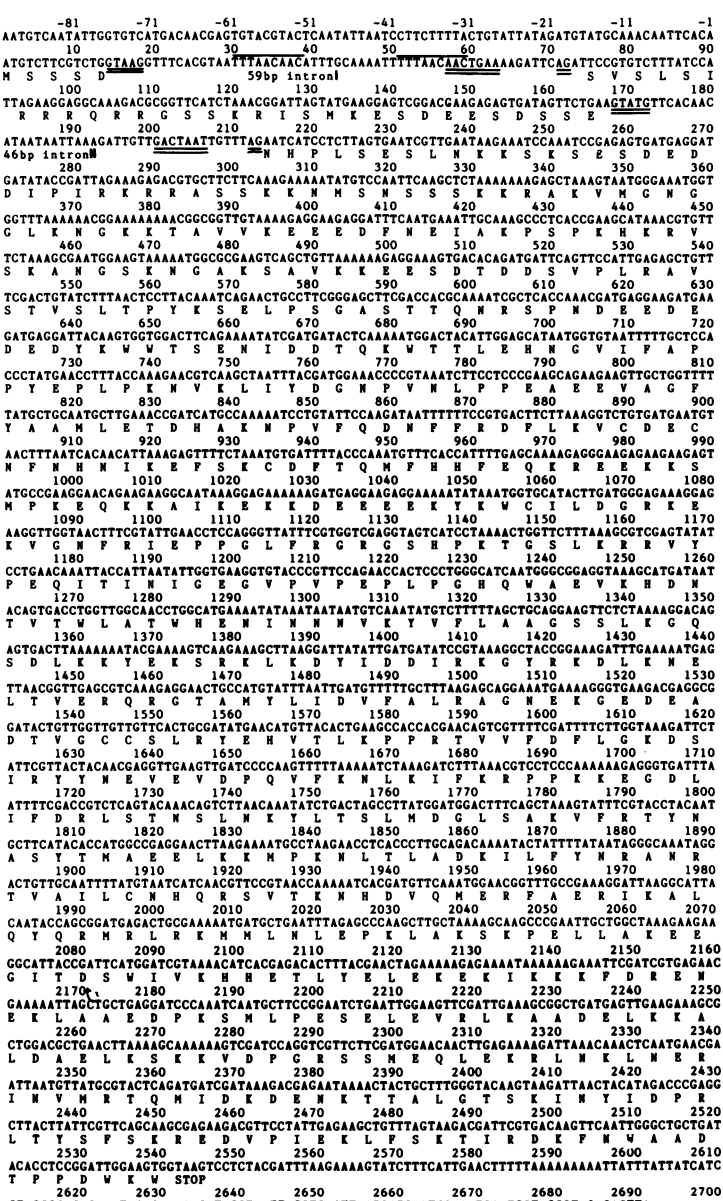
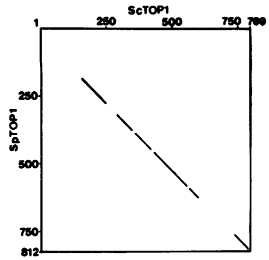


Figure 3. Nucleotide sequence of the S. pombe DNA topoisomerase I gene (top1^{+}) and the predicted amino acid sequence (designated by single letter). The consensus sequences for S. pombe introns are indicated by the double underlines. A 9 bp sequence in the intron I that repeats twice at a 21 bp interval is indicated by the overline.

a



b

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Sp  MSSSDSVSLSIRRRQRRGSSKRISMKESDEESDSSENHPLSESLNKKSKSESDEDDIPIRKRRASSKKNMSNSSSKKRAKVMGNGGLKNGKKTAVVKEEE   100
Sc                                                  MTIADASKVNHELSSDDDDDVPL QTL KR  ASMNSASLQDEAEPYDSD      51

Sp  DFNEIAKPSPKHKRVSKANGSKNGAKSAVKKEESDTDDSVPLRAVSTVSLTPYKSELPSGASTTQNRSPNDEE###DEDEDYKWWTSENIDDTQKWTTLEHNG   200
Sc  AISK S KKT KIKTEPVQS SL#PSPPA  SATSKPKKIK#KEDGD KVKTT K EQENEKKKREEEEEEDKKAKE E E    EK  E   I  V  K    152

Sp  VIFAPPYEPLPKNVKLIYDGNPVNLPPEAEEVAGFYAAMLETDHAKNPVFQDNFFRDFLKVCDECN##FNH#NIKEFSKCDFTQMFHHFEQKREEKKSMPKEQ   300
Sc     P   Q   SHI  Y   K  D   Q        F  L  S          K   N   Q LK SGGPL GIE      R    K  DY QLQK Q  QLTSQE   255

Sp  KKAIKEKKDEEEEKYKWCILDGRKEKVGNFRIEPPGLFRGRGSHPKTGSLKRRVYPEQITINIGEGVPVPEPLPGHQWAEVKHDNTVTWLATWHENINNN   400
Sc    Q RLEREKF  D  F E    R Q    KV   D      A     K      N  D VL LSKDA   PAPE  K G IR      Q   M R   F S   355

Sp  VKYVFLAAGSSLKGQSDLKKYEKSRKLKDYIDDIRKGYRKDLKNELTVERQRGTAMYLIDVFALRAGNEKGEDEADTVGCCSLRYEHVTLKPPRTVVFDF   500
Sc  F  R  N         Y  F  A Q  S   A  RD TRN  SKVML   KAV I           G S                   N  I           455

Sp  LGKDSIRYYNEVEVDPQVFKNLKIFKRPPKKEGDLIFDRLSTNSLNKYLTSLMDGLSAKVFRTYNASYTMAEELKKMPKNLTLADKILFYNRANRTVAIL   600
Sc         F Q     K      T       QP HQL    DPSI    QNY P  T          K  QDQ DLI NKGSV E   K  A            555

Sp  CNHQRSVTKNHDVQMERFAERIKALQYQRNRLRKMMLNLEPKLAKSKPELLAKEEGITDSWIVKHHETLYELEKEKIKKKFDRENEKLAAEDPKSMLPES   700
Sc       T   G AQTV KANN  QE EW KI CKRAI Q DKD L KE KYPEEIDDL KEDEATI KRIIDR I  YQR   V   D RKF K#EEL       654

Sp  ELEVRLKAADELKKALDAELKSKKVD##PGRSSMEQLEKRLNKLNERINVMRTQMIDKDENKTTALGTSKINYIDPRLTYSPSKREDVPIEKLFSKTIRDKF   800
Sc  Q KEW EKV  K QEPEK   TGE ELKSSWN V KIKAQVE  EQ  QTSSI LK  E  SQVS             SVV C KY     I T  L E      756

Sp  NWAADTPP#DWKW                                                                                          812
Sc  K  IESVDEN RF                                                                                          769
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Figure 4. Comparison of topo I between S. pombe and S. cerevisiae. (a) Homology matrix of the amino acid sequences between S. pombe and S. cerevisiae topo I. Sp, S. pombe; Sc, S. cerevisiae. The regions sharing a homology of more than 70% (more than 14 of the 20 residues compared are identical) are indicated. (b) Aligned topo I amino acid sequences of S. pombe (Sp) and S. cerevisiae (Sc). The sequences of Sc begins in the middle of the first row. Identical amino acids are not shown in the sequence of Sc. # indicates deletion.

Nucleotide sequence of the topo I gene

We subcloned the 5.1 kb Eco RI fragment (Figure 2a) into 2.0 kb Eco RI-Pst I and 3.1 kb Pst I-Eco RI fragments. The 1.4 kb Eco RV probe (Figure 1) containing the COOH-region of the S. cerevisiae TOP1 gene strongly hybridized with the 3.1 kb fragment but not with the 2.0 kb fragment. On the other hand, the 2.0 kb Eco RV fragment (Figure 1) containing the NH_2 domain of the S. cerevisiae TOP1 gene did not hybridize with the 3.1 kb fragment but did with the 2.0 kb fragment. Therefore, the PstI site appears to be in the coding region, and the direction for transcription is as indicated by the arrow in Figure 2a.

We determined the 2781 bp long nucleotide sequence (Figure 3; indicated by

the wavy line in Figure 2a) for both strands by the dideoxy method. Although the greater part of the fragment consists of a single reading frame, two putative introns I and II are found in the NH_2 domain at the 5th and between the 36 and 37th codons. They are 59 and 46 bp long, respectively, and have the consensus sequences for S. pombe introns (doubly underlined in Figure 3; refs. 19-21). In intron I, a 9 bp sequence TTTAACAAC (overlined in Figure 3) repeats directly twice in a 21 bp interval (equivalent to the two turns of B-form DNA). RNase mapping (22) was done to determine the presence of introns. Results obtained, however, are inconclusive; the 1.4 kb RNA (described above) as well as the 2.9 kb transcript apparently produces hybridizing fragments so that the interpretation of data is difficult. Thus cloning and sequencing of cDNA clone for $top1^+$ gene is necessary to establish the introns and determine the NH_2 end sequence of S. pombe topo I.

Predicted amino acid sequence of topo I

A hypothetical amino acid sequence for topo I is shown in Figure 3 by a single letter. If translation starts at the presumed initiation codon ATG, it contains 812 residues (M. W. 94,000). This is 43 residues longer than the predicted topo I polypeptide of S. cerevisiae. If translation starts at the second or the third methionine (ATG) codon in the reading frame, the resulting polypeptide would have 788 and 743 residues, respectively.

Topo I has been purified in S. cerevisiae (M.W. 90K) (3), Drosophila melanogaster (135K) (4), avian erythrocyte nuclei (105K) (5), mouse cells (102K) (6) and HeLa cells (100K) (7), but the amino acid sequence is known only for S. cerevisiae topo I which was deduced by nucleotide sequence of the cloned gene.

The overall homology of the predicted topo I polypeptide is 47% between S. pombe and S. cerevisiae. This low value was unexpected but is comparable to the value obtained for DNA topoisomerase II in the two yeasts (23,24). As shown in Figure 4, they are least homologous in the two large domains, namely the NH_2 terminal (1-173th residues in S. pombe) and the near carboxy-end regions (610-760th). Both the NH_2 domains, however, contain high proportions of the charged residues (39% for S. pombe and 48% for S. cerevisiae). The basic and acidic residues cluster and are arranged alternately (Figure 4b; 10-170th in S. pombe and 10-130th in S. cerevisiae). Similar alternating clusters are found in the COOH domains (not in the NH_2 domain) for topo II of two distantly related yeasts (24). The content of serine residues is significantly high (20%) in the NH_2 domain of S. pombe topo I.

The other least homologous domains near the COOH-ends share a common

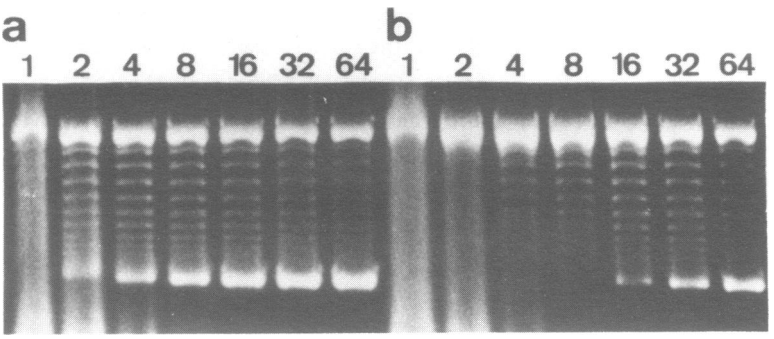

Figure 5. Increase of topo I activity in the S. pombe cells introduced by multicopy plasmids carrying the topl$^+$ gene. The cells of the strain h$^-$ leul endl transformed with the vector pDB248 or with pTOP1 containing the topl$^+$ gene were grown at 33°C to 3x10^6/ml and disrupted by glass beads. Each type I relaxing activity in a series of the diluted extracts (xl extract equivalent to 10^9/ml) was measured using supercoiled pBR322 as the substrate. (a) h$^-$ leul endl/pDB248. (b) h$^-$ leul endl/pDB(TOP1). The numbers indicate the extent of dilution.

property of high hydrophilicity and helical content according to the methods of Kyte and Doolittle (25) and Chou and Fasman (26). Approximately 50 residues in the COOH-terminal domains are well conserved in the two sequences. Thus the S. cerevisiae and S. pombe topo I appear to have a similar overall protein architecture although amino acid sequence homology is approximately 50%. We have not found any significant homology between procaryotic and eucaryotic topo I which greatly differ in their substrate specificities (27). Eucaryotic topo II, however, is significantly homologous to procaryotic topo II (24,28).

Expression of the cloned topl$^+$ gene

An S. pombe strain topl leul was transformed by the plasmid pTOP1 containing the S. pombe topl$^+$ in a multicopy shuttle vector pDB248 which consists of the S. cerevisiae LEU2 gene, 2μ DNA and pBR322 DNA. Extracts of the transformed cells were prepared, and the topo I relaxing activity was assayed at 26°C and at 36°C in the presence of EDTA. The heat resistant topo I activity was detected at 36°C, whereas the topl cells containing only the vector pDB248 showed no detectable activity (data not shown). The heat resistant activity obtained at 36°C was approximately 8-fold higher than that of the wild type extract, indicating that the level of topo I enzyme is increased by the multicopy plasmid with topl$^+$ insert (Figure 5).

Disruption of the topl$^+$ gene

As described above, S. cerevisiae and S. pombe carrying hs topl are

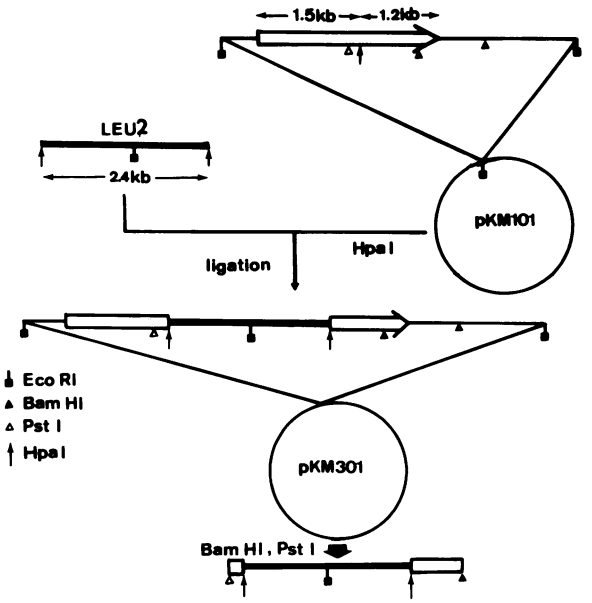

Figure 6. Construction of the plasmid used for the gene disruption of S. pombe $topl^+$ gene. The S. cerevisiae LEU2 gene was ligated with pKM101 that was previously cleaved at the Hpa I site. Resulting plasmid pKM301 was doubly digested with Pst I and Bam HI. The linearized fragment was used for transformation.

viable at 36°C. Residual topo I activity in hs topl might be sufficient for growth, although such possibility is rejected in S. cerevisiae (8). To determine whether the complete absence of topo I might affect growth of S. pombe, a method for gene disruption developed for S. cerevisiae (16) and applicable for S. pombe (29) was employed. We constructed a plasmid pKM301 consisting of pUC18 and disruped $topl^+$ gene, that is, the $topl^+$ gene cleaved at the Hpa I site and ligated with the S. cerevisiae LEU2 gene (Figure 6). The plasmid was doubly digested with PstI and Bam HI. The linearized fragment containing the disrupted $topl^+$ gene was used for transformation of a haploid h^- leu1 topl. If the $topl^+$ gene is non-essential, transformants with the disrupted $topl^+$ (designated topl (null)) should be obtained.

Two Leu^+ topl (null) transformants were obtained. They grow at 22°-36°C with the doubling time at 26°C, 20% longer than that of wild type. When crossed with h^+ leu1, the tetrads showed the segregation $Leu^+ : Leu^- = 2 : 2$, indicating that the disrupted $topl^+$::LEU2 was integrated on the chromosome. To confirm the gene disruption, genomic DNAs of the segregants in two tetrads were isolated, restricted and probed with the 5.1 kb EcoRI fragment. As shown

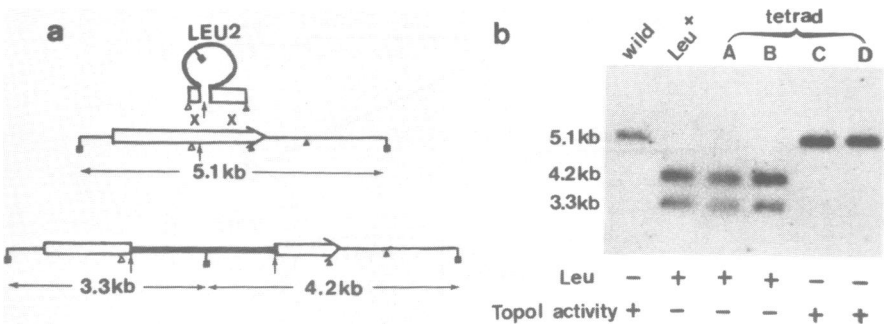

Figure 7. Schematic representation of the disruption of the S. pombe $top1^+$ gene (a), and genomic Southern hybridization of the wild type, the Leu^+ transformant and the segregants A, B, C, and D with or without the gene disruption (b). Genomic DNAs were restricted with Eco RI. The symbols used for restriction sites are described in Figure 6. The probe used was the S. pombe $top1^+$ gene. The predicted structure for the disrupted $top1^+$ gene would produce 3.3 and 4.2 kb Eco RI hybridizing fragments whereas the wild type DNA should produce the 5.1 kb band.

in Figure 7, the hybridization patterns of the Leu^+ segregants showed the bands (at 4.2 kb and 3.3 kb) expected for the disrupted gene, whereas the Leu^- segregants showed the patterns for the non-disrupted wild type gene (5.1 kb band). Extracts were prepared for each segregant and their topo I relaxing activity was assayed. Segregants with the disrupted gene lacked the activity (indicated by - in Figure 7b), while the remaining segregants had normal levels of activity (indicated by +).

The results described above are consistent with the previous finding (9) that S. pombe hs top1 mutants grow normally. In S. cerevisiae, top1 mutants (heat sensitive or null) are also viable (8,18,30). These previous and present results show that $top1^+$ is not essential for viability in the two yeasts. Relaxing activity of the topo II is thought to support the viability of top1 by substituting for the topo I function in the mutant cells (9,10).

Phenotypes of top1-top2 double mutant

We constructed double mutants top1 (null)-top2 (ts) by crossing top1 (null) with ts top2 alleles (-191, -342, -437; ref. 9) and compared their defective phenotype at non-permissive temperature with that of the previously obtained top1 (hs)-top2 (ts). The morphological phenotype in the two classes of the double mutants is similar. DAPI staining of the double mutants top1 (null)-top2 (ts) at restrictive temperature showed that the cells are arrested irrespective of cell cycle stage and the nuclear chromosomal regions are altered to a ring-like structure (data not shown). The ring-like chromosomal

TABLE 1 Growth phenotype of various topoisomerase mutants

Strain	Temperature	Growth[1]
top1 (hs)	20-36°C	+++
top1 (null)	20-36°C	+++ (2)
top1 (hs)-top2 (ts)	20-26°C	+++
	36°C	-
top1 (null)-top2 (ts)	20-26°C	+ (3)
	36°C	-
top1 (hs)-top2 (cs)	20°C	-
	30°C	+
	36°C	-
top1 (null)-top2 (cs)	20-36°C	lethal (4)
top2 (ts)	20-26°C	+++
	36°C	-
top2 (cs)	20-26°C	-
	30°C	+
	36°C	+++

(1) Estimated by the size of colonies on YPD plates.
(2) Generation time at 26°C is 20% longer than the wild type.
(3) Minute colonies are formed.
(4) Spores of top1 (null)-top2 (cs) are incapable of germination at 20-36°C.

domain was previously shown to be characteristic of the double mutants top1 (hs)-top2 (ts) that block DNA and RNA synthesis (9,10).

A significant difference between two classes of the double mutants, however, is found in their growth properties. At permissive temperature (20-26°C), all top1 (null)-top2 (ts) double mutants produced minute colonies. This is in contrast to normal sized colonies formed by top1 (hs)-top2 (ts) at 20-26°C. The complete absence of topo I relaxing activity combined with reduced topo II activity in ts mutants at permissive temperature may have caused poor growth (see below). The significant difference between hs and null top1 mutants was also observed when they were combined with cs top2 mutation. The cs topo II enzyme appears to contain an amino acid substitution in the NH_2 domain and exhibits cold-sensitive ATP-dependent relaxing activity (31). The activity of cs topo II enzyme is considerably low even at permissive temperature (36°C). The double mutant top1 (hs)-top2 (cs) grows poorly at 30°C, but becomes lethal at 20°C and 36°C; it becomes ts as well as cs. On the other hand, top1 (null)-top2 (cs) turns out to be lethal even at the permissive temperature. This was shown by a number of tetrad analyses of the cross between top1 (null) and top2 (cs). No viable spore carrying both alleles

could be obtained. Growth properties of various topoisomerase mutants in regard to the relaxing activity are summarized in Table 1.

These observations are consistent with a hypothesis that the total level of the relaxing activity by the topo I and II enzymes determines the growth rates of cells. The cells appear to grow poorly or become lethal when their level of relaxing activity is below a certain critical level. The *topl*$^+$ gene of *S. pombe* appears to become essential when the activity of topo II relaxing activity is not abundant.

Mapping of the topl locus

The Leu$^+$ marker integrated on the chromosome together with the cloned *topl*$^+$ gene (described above) was used for chromosomal mapping of the *topl* locus. Leu$^+$ cosegregates with *topl* in all the tetrads examined, indicating that the cloned gene has been derived from the *topl* locus. The intact *topl*$^+$ gene was disrupted as shown in Figure 7. Therefore, the integration must have taken place at the *topl* locus by homologous recombination.

Tetrad analyses indicated that the Leu$^+$ marker is linked to the *nucl* locus (32) which locates in the long arm of chromosome II. The distance between *nucl* and *topl* is 14cM (PD: NPD: TT= 13: 0: 5). The precise location of *nucl* has recently been determined: it is distal to *adel* (T. Hirano et al., unpublished). The *topl* locus is not linked to *top2* (9).

ACKNOWLEDGEMENTS

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