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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

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We have isolated mutants defective in DNA topoisomerases and an endonuclease from the fission yeast *Schizosaccharomyces pombe* by screening individual extracts of mutagenized cells. Two type I topoisomerase mutants (*top1*) and three endonuclease mutants (*end1*) were all viable. The double mutant *top1 end1* was also viable and, in its extract, Mg^{2+}- and ATP- dependent type II activity could be detected. Three temperature-sensitive (ts^-) mutants having heat-sensitive (hs^-) type II enzymes were isolated, and the ts^- marker co-segregated with the hs^- type II activity. All the ts^- mutations fell in one gene (*top2*) tightly linked to *leu1* in chromosome II. The nuclear division of single *top2* mutants was blocked at the restrictive temperature, but the formation of a septum was not inhibited so that the nucleus was cut across with the cell plate. In contrast, the double *top1 top2* mutants were rapidly arrested at various stages of the cell cycle, showing a strikingly altered nuclear chromatin region. The type II topoisomerase may have an essential role in the compaction and/or segregation of chromosomes during the nuclear division but also complement the defect of the type I enzyme whose major function is the maintenance of chromatin organization throughout the cell cyle.

Key words: DNA topoisomerase/*Schizosaccharomyces pombe*/temperature sensitive mutants/chromatin organization

Introduction

DNA topoisomerases alter the topological state of DNA by transiently breaking and rejoining the strand(s). They have been classified into two groups; type I topoisomerases transiently cut one of the two DNA strands whereas type II topoisomerases catalyze the concerted cleavage and rejoining of both strands (Cozzarelli, 1980a). Both types of topoisomerase activities have been found in procaryotic and eucaryotic cells.

Investigation of the roles *in vivo* of eucaryotic topoisomerases has been hampered by the lack of mutants. In procaryotes, analyses of mutants defective in topoisomerases have shown that the enzymes play crucial roles (reviewed by Gellert, 1981) in DNA replication (Gellert *et al.*, 1976b; Liu *et al.*, 1979), recombination (Kikuchi and Nash, 1979; Krasnow and Cozzarelli, 1983), and transcription (Trucksis and Depew, 1981; Sternglanz *et al.*, 1981). Eucaryotic topoisomerases hitherto isolated and characterizied have features distinct from those of procaryotes. For example, eucaryotic type I topoisomerases can relax both negative and positive supercoils (Champoux and Dulbecco, 1972; Vosberg *et al.*, 1975; Keller, 1975), whereas procaryotic type I enzymes can relax only negative supercoils (Wang, 1971). *Escherichia coli* type II topoisomerase (gyrase) catalyzes ATP-dependent negative supercoiling (Gellert *et al.*, 1976a), but eucaryotic type II enzymes do not have such an activity. They can, however, decatenate or catenate (Baldi *et al.*, 1980; Hsieh and Brutlag, 1980) and unknot or knot (Liu *et al.*, 1981) the entangled ring DNAs as do the procaryotic type II enzymes. These and other differences between eucaryotic and procaryotic topoisomerases may reflect different states of DNA supercoiling in chromatin and nucleoid structures, respectively.

The yeast *Saccharomyces cerevisiae*, a lower eucaryote, has a type II topoisomerase similar to those of higher eucaryotes (Goto and Wang, 1982). We intended to isolate topoisomerase mutants from the fission yeast *Schizosaccharomyces pombe* as the first step to studying the *in vivo* roles of the enzymes. *S. pombe* has a well defined genetical system (Kohli *et al.*, 1977). A number of cell division cycle (*cdc*) genes controlling DNA synthesis or nuclear division have been identified (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981; Toda *et al.*, 1983). Our approach to mutant isolation was to screen extracts of a large number of heavily mutagenized cells. This approach has led to the isolation of *E. coli* mutants defective in various enzymes such as DNA polymerase I (Lucia and Cairns, 1969), polynucleotide phosphorylase (Reiner, 1969) and type I topoisomerase (Sternglanz *et al.*, 1980, 1981). Because the genome of *S. pombe* is only 3-4 times larger than that of *E. coli* (Bostock, 1970; Y.Hiraoka, unpublished data), it seemed feasible to obtain topoisomerase mutants by this mass screening method.

Results

Topoisomerase and endonuclease activities in the extracts of wild-type cells

The isolated nuclei of wild-type *S. pombe* contain a strong DNA-relaxing activity resistant to EDTA and an endonuclease activated by Mg^{2+} (Okada and Yanagida, 1982). These activities were also found in the crude extracts of wild-type cells prepared as described in Materials and methods.

Negatively supertwisted pBR322 DNA (20 μg/ml) in 25 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.15 M KCl was relaxed by incubation with the extract (3 x 10^7 cells/ml) at 30°C (Figure 1, lanes 2-5); the activity was negligible if 0.15 M KCl was omitted (lane 6). In the presence of EDTA, the extract showed little endonucleolytic activity so that the relaxing activity could be monitored. The eucaryotic type I topoisomerases are generally active in the absence of Mg^{2+} and in the presence of chelators, although activity can be obtained in the presence of Mg^{2+} (Keller, 1975; Vosberg *et al.*, 1975).

The Mg^{2+}-activated endonuclease was assayed using supercoiled pBR322 DNA as the substrate. The plasmid when incubated with the wild-type extract in 25 mM Tris-HCl (pH 7.5) containing 6 mM $MgCl_2$ and 1% Triton X-100 was first nicked and then linearized, and then gradually degraded into shorter pieces (Figure 1, lanes 7-10). Triton X-100 much

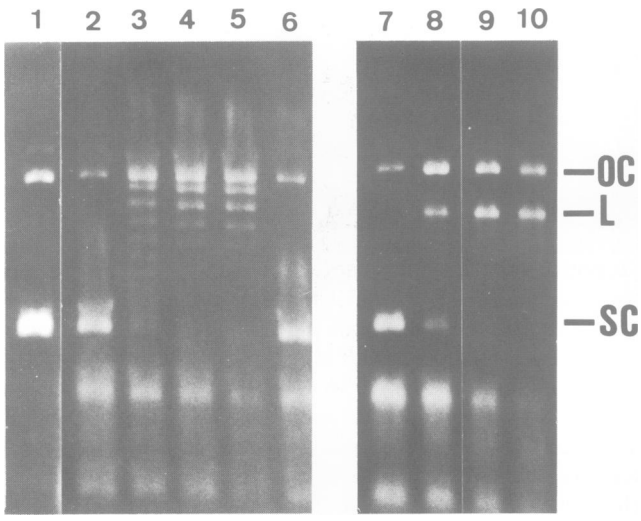

Fig. 1. Assays of type I DNA topoisomerase **(lanes 1–6)** and endonuclease **(lanes 7–10)** activities in the extract of wild-type cells by agarose gel electrophoresis. DNA topoisomerase activity: supercoiled pBR322 DNA **(lane 1)** was incubated with the wild-type extract in 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 0.15 M KCl at 30°C for 0 min **(lane 2)**, 3 min **(lane 3)**, 10 min **(lane 4)** and 60 min **(lane 5)**. The topoisomerase activity was not detected at 30°C for 60 min in the absence of 0.15 M KCl **(lane 6)**. Endonuclease activity: supercoiled pBR322 DNA was incubated with the wild-type extract in 25 mM Tris buffer (pH 7.5) containing 6 mM $MgCl_2$ and 1% Triton X-100 at 30°C for 0 min **(lane 7)**, 2 min **(lane 8)**, 60 min **(lane 9)** and 4 h **(lane 10)**. The electrophoretic bands marked with OC, L and SC are the open circle (relaxed or nicked), linear and supercoiled pBR322 DNA, respectively. The bands below SC are RNAs in the extracts.

enhanced the endonucleolytic activity in the extracts (data not shown).

Isolation of the mutants defective in type I topoisomerase and Mg^{2+}-activated endonuclease

A haploid strain HM123 (*h⁻ leu1*) was heavily mutagenized with nitrosoguanidine, and a large number of surviving colonies were picked. Extracts were made from each and assayed for the enzyme activities; cultures and extracts were prepared in small centrifuge tubes, the enzymes were assayed in microtitre plates and the gel electrophoresis was done in a large, horizontal agarose gel with 96 sample wells.

Out of 1500 mutagenized colonies, two mutants (strains 710 and 1386) defective in the relaxing activity, and out of 1000 mutagenized colonies, four mutants (379, 380, 458 and 872) defective in the endonucleolytic activity were obtained. Topoisomerase activities of both the strains 710 and 1386 were heat-labile (Figure 2a); in the extracts of 710 some topoisomerase activity was detected at 22°C (lane 5), but the activity was negligible at 30° and 36°C (lanes 6 and 7). A similar but less heat-labile activity was found in the extracts of 1386 (lanes 8–10). The wild-type topoisomerase activity was enhanced as the temperature was raised (lanes 2–4). At 36°C the activity of strain 710 was <1% of that of the wild-type (data not shown).

In the extracts of the endonuclease-defective mutants, the substrate DNA was not cleaved, and instead topoisomerase activity was observed (Figure 2b, lanes 3 and 4). This relaxing activity in the presence of 6 mM $MgCl_2$ appeared to be derived from the same type I enzyme monitored in the presence of EDTA, because it was absent in the End1⁻ and Top1⁻ double mutant (see below).

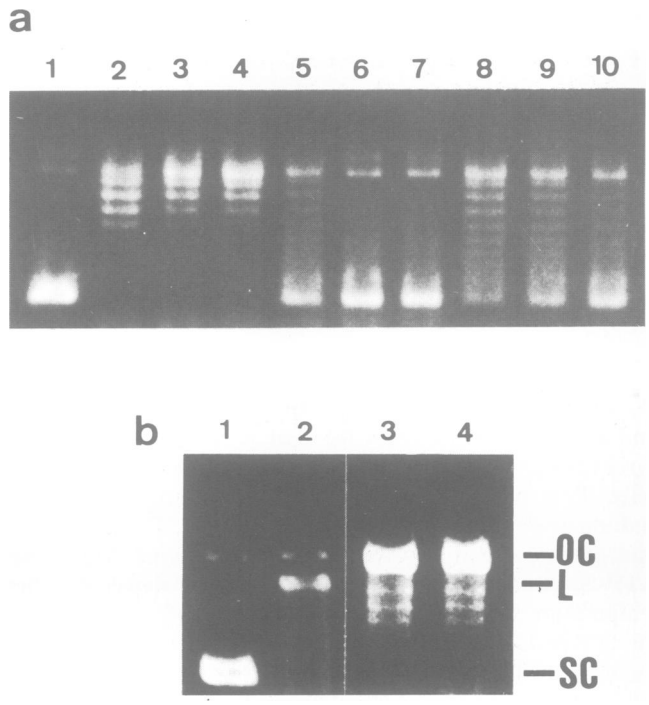

Fig. 2. Defective type I topoisomerase and enconuclease activities in the extracts of isolated mutants. **(a)** Supercoiled pBR322 **(lane 1)** was incubated in the presence of EDTA with the extracts of the wild-type **(lanes 2–4)**, Top1⁻ strain 710 **(lanes 5–7)**, Top1⁻ strain 1386 **(lanes 8–10)** at 22°C **(lanes 2, 5** and **8)**, 30°C **(lanes 3, 6** and **9)** or 36°C **(lanes 4, 7** and **10)**. Topoisomerase activities in the mutant extracts were heat-labile. **(b)** Supercoiled pBR322 DNA **(lane 1)** was incubated with the presence of $MgCl_2$ with the extracts of the parental strain (HM123) **(lane 2)**. End1⁻ strain 379 **(lane 3)** and End1⁻ strain 458 **(lane 4)** at 30°C for 4 h. The mutant extracts were deficient in endonuclease but instead showed the topoisomerase activities.

Genetical analyses of Top1⁻ and End1⁻ mutants

Two Top1⁻ (710 and 1386) and four End1⁻ mutants (379, 380, 458 and 872) were each back-crossed with the wild-type 975 h^+. One of the End1⁻ mutants (strain 380) was defective in mating, and therefore not investigated further. In the other crosses, extracts of the cell clones derived from germinated spores were tested for the enzymatic activities; the numbers of tetrads analyzed were 12 (strain 710) and six (strain 1386) for type I topoisomerase, and 11 (strain 379), eight (strain 458) and five (strain 872) for endonuclease. All the tetrads clearly demonstrated 2:2 segregation of the respective enzymatic defects. Therefore, we concluded that a single mutation made each strain defective in the type I topoisomerase or in the Mg^{2+}-activated endonuclease.

A cross was made between the Top1⁻ mutants 710 and 1386, but no Top1⁺ segregant was found among 10 tetrads. Therefore, the two Top1⁻ mutations appeared to be located at the same locus (designated *top1*). Similarly, two crosses were done between the End1⁻ mutants 379 and 458, and between 458 and 872: no End1⁺ segregant was obtained in a total of 20 tetrads, indicating that all three mutations were derived from a single locus (designated *end1*).

These *top1* and *end1* mutants were viable and produced colonies at 22°, 30° and 36°C. Neither their u.v. sensitivity nor their sporulation was altered (T.Uemura, unpublished data).

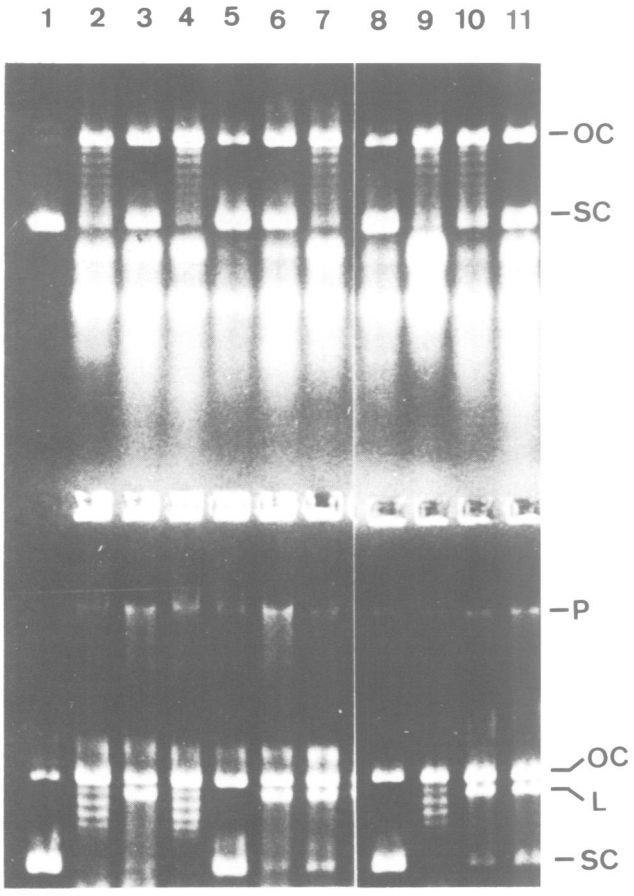

Fig. 3. Construction of Top1⁻ End1⁻ double mutant. The upper lanes represent the assays for type I topoisomerase at 36°C and the lower lanes for endonuclease at 30°C. Supercoiled pBR322 DNA **(lane 1)** was incubated with the extracts of End1⁻ strain 458 **(lane 2)**, Top1⁻ strain 710 **(lane 3)** or the segregants **(lanes 4–11)** from the cross between the h^+ End1⁻ and the h^- Top1⁻ mutants. Results from two tetrads are shown in **lanes 4–7** and **8–11**. **Lanes 5** and **8** represent the expected patterns of the double mutant. Intense bands below SC are RNAs and the bands marked with P are chromosomal DNA in the extracts.

Construction of a double mutant and detection of type II topoisomerase activity in the extract

A double mutant, *top1 end1*, was constructed because, if viable, it could be used to monitor Mg^{2+}- and ATP-requiring relaxing activity (known as one of type II topoisomerase activities). Crossing was done between h^+ *end1*-458 and h^- *leu1 top1*-710. Extracts of the tetrads were made and assayed for type I topoisomerase and endonuclease. A part of the results is shown in Figure 3. The upper lanes of the gel show the assays for type I enzyme in the presence of EDTA, and the lower lanes for endonuclease in the presence of Mg^{2+}. The segregants in lanes 5 and 8 lacked both the activities, and were identified as the double mutant.

The Top1⁻ End1⁻ double mutant was grown normally, producing colonies at 22°, 30° and 36°C. As with the single mutants, no change in u.v. sensitivity or sporulation was observed. The extracts of the double mutant, however, showed a relaxing activity if both $MgCl_2$ (6 mM) and ATP (1 mM) were added (Figure 4; lane 3). No relaxing activity was detected in the reaction mixtures containing only $MgCl_2$ (lane 2), EDTA (lane 4), and ATP + EDTA (lane 5).

The Top1⁻ End1⁻ or End1⁻ mutants also contained ATP-dependent unknotting activity (Figure 5). The substrate, knotted P4 DNA, did not run as a distinct band in gel electrophoresis (lane 3; Liu *et al.*, 1981), whereas, after incubation with the extracts in the presence of ATP, a sharp band was formed at the position of relaxed P4 DNA (lanes 5 and 7). Without the addition of ATP, the unknotting activity could not be detected (lanes 4 and 6). Thus type II topoisomerase activity in the double mutant extract could be easily monitored by ATP-dependent relaxing or unknotting assays.

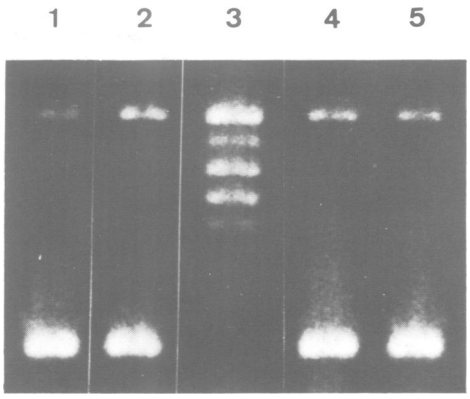

Fig. 4. Mg^{2+}- and ATP-dependent relaxing activity monitored in the extract of the Top1⁻ End1⁻ double mutant (strain 710–458). Supercoiled pBR322 DNA **(lane 1)** was incubated at 36°C with the extract in the Tris buffer (254 mM Tris-HCl, pH 7.5; 0.15 M KCl; 1 mM 2-mercaptoethanol) containing 6 mM $MgCl_2$ **(lane 2)**, 6 mM $MgCl_2$ + 1 mM ATP **(lane 3)**, 1 mM EDTA **(lane 4)** or 1 mM EDTA + 1 mM ATP **(lane 5)**. The relaxing activity was detected only in the presence of ATP and $MgCl_2$.

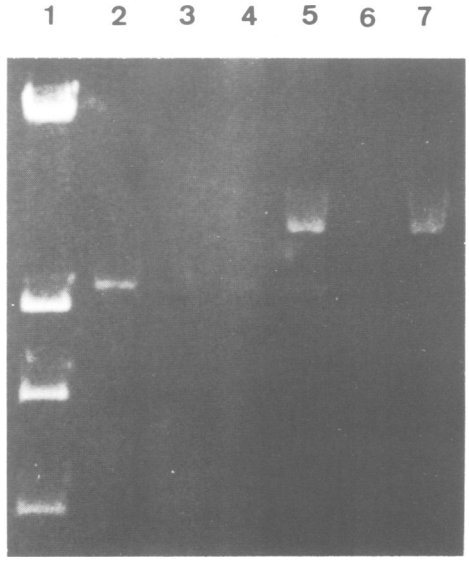

Fig. 5. Unknotting activity monitored in the mutant extracts. **Lane 1,** *Hind*III digest of λ phage DNA. **Lane 2,** linear form of phage P4 DNA. **Lane 3,** knotted P4 DNA not showing a discrete band (Liu *et al.*, 1981). Knotted P4 DNA in the Tris buffer containing $MgCl_2$ was incubated with the extracts of the Top1⁻ End1⁻ double mutant **(lanes 4–5)** and End1⁻ single mutant **(lanes 6–7)**, either in the absence of ATP **(lanes 4** and **6)** or in the presence of ATP **(lanes 5** and **7)**.

Isolation of the mutants defective in type II topoisomerase

587 temperature-sensitive (designated ts⁻) mutants (growing at 26°C but not at 36°C) were isolated by mutagenizing the *top1 end1* double mutant (see Materials and methods). The extracts of ts⁻ mutant cells grown at the permissive tem-

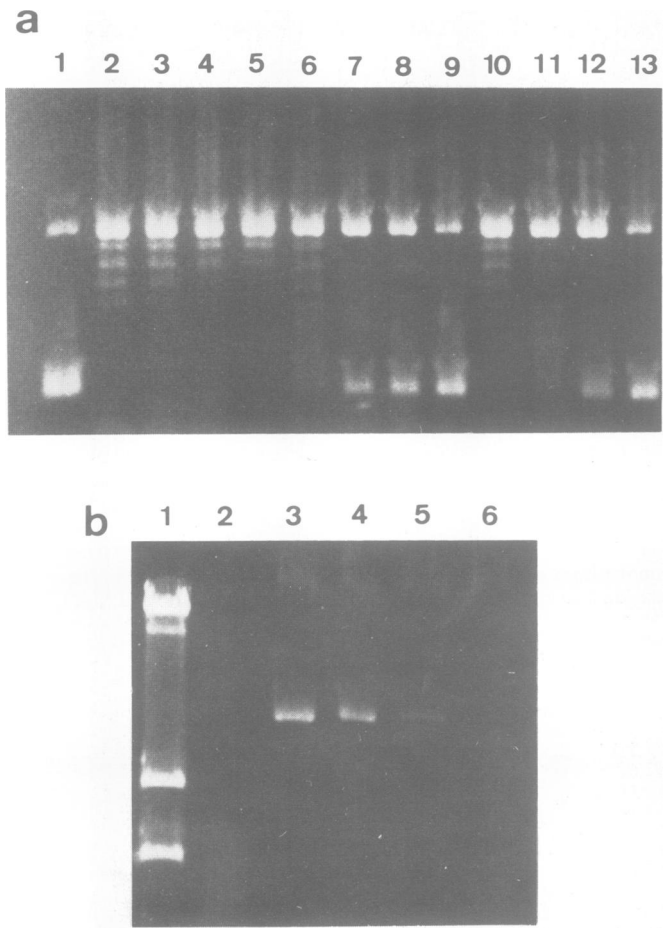

Fig. 6. Heat-sensitive ATP-dependent type II activity in the extracts of temperature-sensitive Top2$^-$ mutants. **(a)** ATP-dependent relaxing activity: supercoiled pBR322 DNA **(lane 1)** was incubated with the extract of the Top1$^-$ End1$^-$ Top2$^+$ mutant (strain 710-458) at 26°C **(lane 2)**, 30°C **(lane 3)**, 36°C **(lane 4)** and 39°C **(lane 5)**; with the extracts of Top1$^-$ End1$^-$ Top2$^-$ mutants (strains D191 and D342) at 26°C **(lanes 6** and **10)**, 30°C **(lanes 7** and **11)**, 36°C **(lanes 8** and **12)** and 39°C **(lanes 9** and **13)**. Results of D437 strain were similar to the patterns shown in **lanes 6–13**. **(b)** ATP-dependent unknotting activity: **lane 1**, *Hind*III digest of λ phage DNA. Knotted P4 DNA **(lane 2)** was incubated with the extract of Top1$^-$ End1$^-$ Top2$^+$ mutant at 26°C **(lane 3)** and at 39°C **(lane 4)**; with the extract of Top1$^-$ End1$^-$ Top2$^-$ mutant (strain D342) at 26°C **(lane 5)** and at 39°C **(lane 6)**.

perature (26°C) were made and assayed at 39°C for the ATP-dependent relaxing activity, which was stable at 39°C in the extracts of the parental double mutant (see Figure 6a, lanes 2–5). Among the 587 extracts tested, three (D191, D342 and D437) showed little ATP-dependent relaxing activity at 39°C (and also at 36°C) but had activity at 26°C (Figure 6a, lanes 6–13). Consistently, the ATP-dependent unknotting activity was not present at 39°C but was observed at 26°C (Figure 6b, lanes 5–6).

Co-segregation of the ts$^-$ phenotype with the heat-labile type II activity

To determine the linkage of the ts$^-$ growth phenotype with the heat-sensitive (designated hs$^-$) type II activity, three crosses were done between each of ts$^-$ *top1 end1 leu1* (*h*$^-$) (strains D191, D342 and D437) and the parental ts$^+$ *top1 end1* (*h*$^+$) strains. Ten tetrads were examined for each cross, and they all showed 2:2 segregation of the ts$^-$ phenotype. Extracts of 120 segregants were made, and assayed for ATP-dependent relaxing activity at 26° and 39°C. The results (data not shown) showed the co-segregation of ts$^-$ with hs$^-$; all of the ts$^-$ segregants contained the hs$^-$ enzyme while all the ts$^+$ segregants contained the hs$^+$ enzyme. Therefore, the inactivation of type II topoisomerase must cause the block of cell growth.

In the course of these tetrad analyses we discovered that *leu1* was tightly linked to the ts$^-$ marker of the three mutants; the ratio of PD:NPD:TT was 30:0:0 (<1.6 cM). Consistently the genetic distance between the ts$^-$ marker and the *mat* locus located near the *leu1* was 5.0 cM (PD:NPD:TT = 27:0:3). Furthermore the frequencies of ts$^+$ recombinants were only 0.85×10^{-4} and 2.7×10^{-4}, respectively, from the crosses between D191 and D342, and between D342 and D437. These results show that the ts$^-$ type II-defective mutations were derived from the single locus (designated *top2*) tightly linked to *leu1* that is known to be located in the long arm of chromosome II (Kohli *et al.*, 1977).

Back-crossing of the ts mutants with the wild-type

In the previous section, we showed the co-segregation of the ts$^-$ phenotype with the hs$^-$ type II enzymatic defect. The isolated ts$^-$ mutants, however, contained the additional mutations (*top1* and *end1*), and an uncertainty remained whether the ts$^-$ phenotype depends solely on the hs$^-$ mutation in *top2*. The other mutation(s) already present in the parental *top1 end1* might also be responsible for the expression of the ts$^-$ phenotype. To answer this question, back-crosses of the *top1 top 2 end1 leu1* mutants with the wild-type 975 *h*$^+$ were done. Segregants were tested for ts$^-$ and Leu$^-$ by plating. Extracts of each segregant were prepared, and assayed for type I topoisomerase and endonuclease.

In the two crosses (D191 and 975 *h*$^+$; D342 and 975 *h*$^+$), all the ts$^-$ segregants co-segregated with Leu$^-$ (ratio of PD:NPD:TT was 24:0:0). This confirmed the tight linkage between *leu1* and ts$^-$ described in the previous section. Furthermore, the segregants of Leu$^-$ ts$^-$ were either Top1$^-$ End1$^+$, Top1$^+$ End1$^-$, or Top1$^+$ End1$^+$; the presence or absence of endonuclease and type I topoisomerase did not influence the temperature-sensitive lethality. These results indicated that the *top2* mutations are solely responsible for the ts$^-$ phenotype. The strain D437 contained a suppressor mutation that is currently being investigated. Without the suppressor the ts$^-$ phenotype became leaky; minute colonies were slowly formed at 36°C.

Phenotypic differences between the single top2 and the double top1 top2 mutants

By back-crossing of the three *top1 top2 end1* triple mutants with the wild-type 975 *h*$^+$ strain, we obtained segregants of single *top2* (strains 1913, 3423 and 4373) and double *top1 top2* mutant constitution (strains 1911, 3421 and 4371) as listed in Table I. Although the *top1* mutants grew normally, these single *top2* and double *top1 top2* mutants did not produce colonies at 36°C. Therefore we investigated their defective phenotypes, which proved to be quite different. Two *top1 top2* strains (1911 and 3421) were grown at 26°C, then transferred to 36°C. An aliquot of the cultures was taken at an appropriate time interval and observed under a fluorescence microscope using the DNA-specific fluorescent probe DAPI (Toda *et al.*, 1981, 1983). These two mutants showed the same striking structural transformation in the nuclear chromatin region. The normal hemispherical region seen at

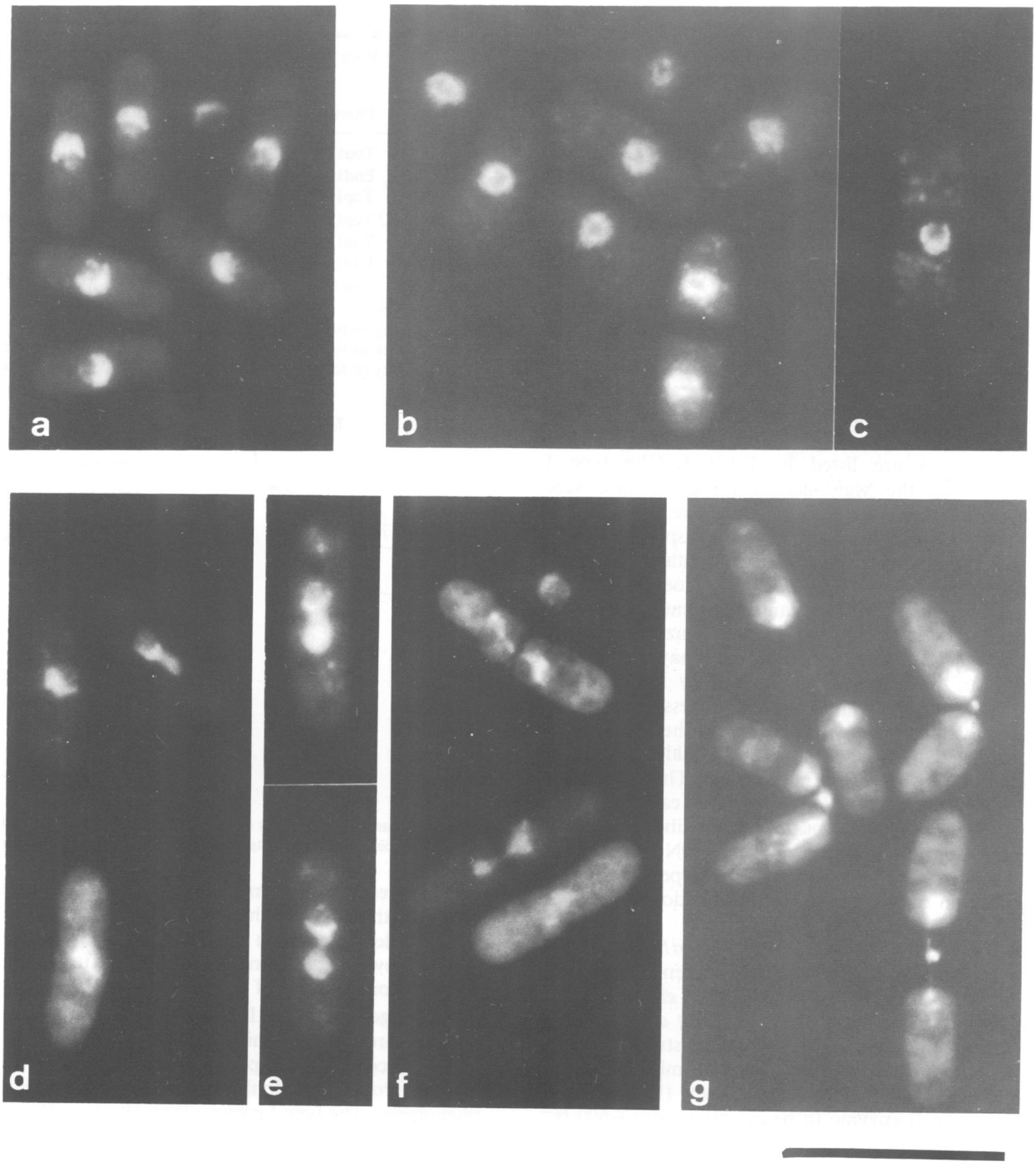

Fig. 7. Fluorescence micrographs of the fission yeast topoisomerase mutants stained with a DNA-specific fluorescent probe DAPI. **(a)** The cells of a double *top1 top2* (strain 3421) mutant were grown at the permissive temperature (26°C) and stained with DAPI. The nuclear chromatin region was hemispherical and indistinguishable from that of the wild type. **(b–c)** The double mutant cells were first grown exponentially at 26°C, then transferred to the non-permissive temperature (36°C) and incubated for 60 min. The cells stained with DAPI showed the ring or the hollow spherical shape of the nuclear chromatin region. **(d–g)** A single *top2* mutant (strain 3423) was first grown exponentially at 26°C, then transferred to 36°C and incubated for 1–3 h. The cells stained with DAPI showed a single nucleus split with the cell plate (**e** and **f**). After cell separation, each of the halved cells contained a part of the damaged nucleus at one end (**g**). The halved cells were occasionally connected with chromatin filaments spilled from the broken nucleus. The bar indicates 10 μm.

the permissive temperature (Figure 7a) rapidly altered to ring-like or horse-shoe structures (Figure 7b–c); 50% of the cells at 15 min and >80% at 60 min after the temperature shift-up showed such altered chromatin regions. The three-dimensional structure of the region must resemble a hollow sphere or a bowl (Figure 8); if the region were ring shaped, it should be seen as a short rod in some viewing angles.

The phenomenon appeared to be independent of the cell cycle because the defective phenotypes appeared so rapidly. The block to cell growth occurred immediately since the cells did not elongate [elongation in cell length at the restrictive temperature is characteristic of the *cdc* mutants of *S. pombe* (Nurse *et al.*, 1976; Toda *et al.*, 1983)]. Furthermore the cells of G2 or late S phase obtained by selection synchrony

(Mitchison and Carter, 1975) also showed the rapid arrest of cell growth and the transformation of the chromatin region.

In contrast, the single *top2* mutants (strains 1913 and 3423) showed a distinct cell cycle dependent phenotype. At the restrictive temperature, nuclear division did not take place normally (Figure 7d–g). The nuclear chromatin region neither condensed nor segregated but a cell plate was formed that cut across the undivided nucleus. After prolonged incubation (longer than 6 h), most cells were split in two, and halved cells with a damaged nucleus at one end were seen (Figure 7g). The frequencies of such split cells were 50% at 3 h and 80–90% at 7 h after the temperature shift-up.

Discussion

The simple and rapid procedures for preparing extracts described here facilitated monitoring topoisomerases and endonuclease activities in > 3000 extracts of mutagenized cells. Mutants isolated in the present study and their derivatives obtained by crossing are listed in Table I. The type I topoisomerase and the Mg^{2+}-activated endonuclease were apparently dispensable in fission yeast. We initially anticipated that the endonuclease may be dispensable, and screened the mutant survivors of heavily mutagenized cells: three viable *end1* mutants were obtained. Unexpectedly we also found two viable *top1* mutants. The mutations were derived from the same locus, and the type I enzyme activities in the mutant extracts were heat-labile; *top1* may be the structural gene for the enzyme. The possibility still remains, however, that the type I enzyme plays an essential role in mitotic growth; it may be overproduced in the wild-type so that a residual activity (< 1% of that of the wild-type) may be sufficient for the growth of the mutant cells. The other possibility is that the defect in type I topoisomerase can be replaced by the type II enzyme. We consider this possibility likely. In *E. coli*, mutations in the gene encoding DNA gyrase are known to suppress the inactivation of the type I enzyme or even the deletion of the type I gene (DiNardo *et al.*, 1982; Pruss *et al.*, 1982).

The construction of the double *top1 end1* mutant was necessary to monitor Mg^{2+}- and ATP-dependent relaxing activity. If, however, the knotted DNA (Liu *et al.*, 1981) or the concatemeric kinetoplast DNA (Miller *et al.*, 1981) had been available at the beginning of the present study, contaminations of endonuclease or type I enzyme might not have been a serious obstacle for the assay of type II activity. We expected the type II enzyme to be essential, and ~600 ts^- mutants derived from the mutagenesis of the double *top1 end1* mutant were screened.

Genetical analyses showed that type II topoisomerase is indispensable for mitotic growth: single *top2* as well as multiple mutants with *top1* and/or *end1* were all temperature sensitive. The ts^- lethality co-segregated in tetrads with the hs^- type II enzyme activity. Because the type II activity in the mutant extracts was quickly inactivated at 36°C and 39°C, the mutations most likely occurred in the structural gene of the enzyme. All three $Top2^-$ mutations appeared to reside in the single *top2* locus closely linked to *leu1* in the chromosome II.

A major finding was the striking phenotypic differences between the single *top2* and the double *top1 top2* mutants. The single *top2* mutants showed cell division cycle-dependent arrest at the non-permissive temperature. Nuclear division did not take place normally but the cell plate was formed, cutting across the nucleus. In contrast, the double mutants were quickly arrested by the temperature shift-up, apparently irrespective of the stages of the cell cycle. Furthermore the shape of the nuclear chromatin region was altered to ring or more likely hollow spherical structures. These different phenotypes shed light on the roles of type I and type II topoisomerases.

Table I. Genotype and phenotype of isolated mutants

	Phenotype			
		Growth at		
Genotype	Enzyme	26°C	36°C	Strains
top1	$Top1^-$	+	+	710, 1386
end1	$End1^-$	+	+	379, 458, 872
top1 end1	$Top1^-$ $End1^-$	+	+	710-458
top1 end1 top2	$Top1^-$ $End1^-$ $Top2^-$	+	–	D191, D342, D437
top1 top2	$Top1^-$ $Top2^-$	+	–	1911, 3421, 4371
end1 top2	$End1^-$ $Top2^-$	+	–	1912, 4372
top2	$Top2^-$	+	–	1913, 3423, 4373

$Top1^-$: activity of type I topoisomerase is defective in extracts.
$Top2^-$: activity of type II topoisomerase is heat-sensitive in extracts.
$End1^-$: activity of Mg^{2+}-activated endonuclease is deficient in extracts.

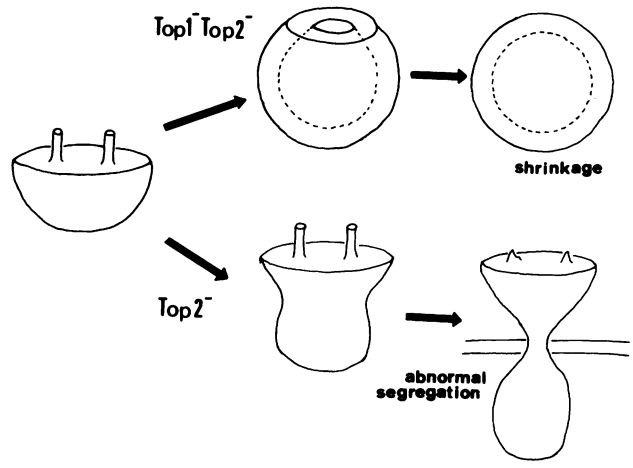

Fig. 8. Diagrams of alterations of the nuclear chromatin region in the cells of DNA topoisomerase mutants after the temperature shift-up (see text).

We suggest that type I enzyme has a role in the maintenance of chromatin organization throughout the cell cycle: as its defect could be complemented with the type II enzyme, only the *top1 top2* double mutants revealed the defective phenotypes. The type I enzyme (and also type II) would continuously remove negative or positive supercoils in DNA introduced during DNA replication and transcription, and nucleosome assembly (reviewed by Gellert, 1981). Its defect, therefore, should increase supercoils in DNA, causing a significant shrinkage of the nuclear chromatin region (illustrated in Figure 8). If the nuclear chromatin is anchored at many sites on the nuclear membrane, the increase in the superhelical density may result in the hollow chromatin structure.

The major role of the type II enzyme may be the resolution of catenated or knotted DNAs, facilitating the transport of duplex DNA segments through each other (Cozzarelli, 1980b). Our finding showed that the compaction and/or segregation of the nuclear chromatin region observed in the normal nuclear division of *S. pombe* (Toda *et al.*, 1981, 1983) failed in the single *top2* mutants, but septum formation and cell separation were not inhibited (Figure 8). These observations were consistent with the notion that the type II en-

zyme is essential in the transport of DNA in condensation or segregation of chromatin.

At the restrictive temperature, only a small fraction (1–5%) of *top1 top2* cells showed phenotypes similar to those of the single *top2* mutants, probably because the arrest in the double mutants by the temperature shift-up took a much shorter time than in the single mutants. The *top2* mutants might also be blocked during the S phase but the block might not be revealed in the temperature shift-up experiments. The G1 phase in *S. pombe* is very short and the G2 occupies two thirds of the cell cycle so that most of the blocked cells would accumulate in the M phase. Understanding the molecular mechanisms of these enzyme functions, however, will require further detailed analyses of the mutants. Recently, Thrash *et al.* (1984) reported the identification of *Saccharomyces cerevisiae* mutants deficient in type I DNA topoisomerase activity. Their mutants grow normally at all temperatures tested, consistent with our findings in the fission yeast *top1* mutants.

Materials and methods

Strains and genetical procedures

The fission yeast *S. pombe* HM123 (h^- *leu1*) was employed as the parental strain for the isolation of mutants defective in type I topoisomerase and endonuclease. Standard genetical procedures of *S. pombe* were followed as described in Gutz *et al.* (1974) and Kohli *et al.* (1977). *E. coli* HB101 was used for the isolation of the pBR322 plasmid.

Media and culture conditions

The complex YPD and the minimal SD media were used for the cultures of *S. pombe*. YPD contains 1% yeast extract, 2% polypeptone and 2% glucose. SD contains 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. All plates contained 1.5% agar. Exponentially growing cells of *S. pombe* were prepared as follows. An overnight culture (~1 x 10^8) was diluted 100-fold with the fresh YPD. The cells were grown with shaking at an appropriate temperature, until the number of cells reached 1 x 10^7 cells/ml.

Mutagenesis

For the isolation of mutants defective in endonuclease and type I topoisomerase. The procedure of mutagenesis was as described in Adelberg *et al.* (1965) with some modifications. 0.1 ml of overnight culture was added to 10 ml fresh YPD and shaken at 30°C for ~10 h. When the concentration of the cells reached 1 x 10^7 cells/ml, they were harvested by centrifugation. After the cells were washed with 50 mM Tris-maleic buffer at pH 6.0 (TM), an aliquot of the cells was suspended in 1 ml TM. N-Methyl-N′-nitrosoguanidine (NTG) was added to the cell suspension (final concentration 100–300 μg/ml), and the mixture was incubated at 30°C for 30 min. Then the cells were centrifuged, washed three times with TM and once with YPD. The washed cells were suspended in 5 ml YPD, incubated at 30°C overnight and plated on YPD. When the lower concentration of NTG (100 μg/ml) was employed, the above procedures were repeated once again, to increase mutations.

The survival ratios determined by counting colonies on YPD plates were 0.01–0.15. The frequencies of auxotrophs estimated by comparing colonies on SD containing 2 mg leucine with those on YPD were 3–11% of survivors. 1500 NTG-mutagenized strains (101–1600) were taken and used for screening mutants defective in endonuclease and type I topoisomerase.

For the isolation of mutants defective in type II topoisomerase. A procedure slightly modified from above was employed. A *top1 end1* (710–458) double mutant was treated with 100 μg/ml NTG. After the first NTG treatment at 30°C, the second one was carried out at 26°C. The auxotrophs were 5% of the survivors. Cells grown in the liquid YPD were plated on YPD at 26°C. After 5 days, the colonies were replica plated and incubated at 36°C overnight. Those unable to grow at 36°C were 2% of the total colonies. In total, 587 temperature-sensitive (ts^-) strains were selected (strains D101–D687).

Mass screening

Preparation of extracts. Each mutagenized strain was inoculated into 1 ml YPD in a small Eppendorf centrifuge tube with a sterile tooth-pick. The inoculated tubes were incubated for 24 h at 26°C (for ts^- strains) or at 30°C. The cells were harvested with centrifugation, washed once with 20 mM Tris-HCl (pH 7.5), and resuspended in 0.1 ml of the same buffer containing 1 mM phenylmethylsulfonylfluoride (PMSF). An equal volume of glass beads (0.5 mm in diameter) were added to the cell suspension. The tubes kept in ice in a container were shaken rapidly (200–400 strokes/min) for 30 min with a shaker (Iwaki Co., Tokyo). By these procedures, ~50% of the cells were disrupted. The extracts were kept with glass beads at −20°C.

Reaction conditions. The buffers used were: (i) 25 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 0.15 M KCl for assaying type I topoisomerase; (ii) 25 mM Tris-HCl (p.H 7.5) containing 6 mM $MgCl_2$ and 1% Triton X-100 for endonculease; (iii) 25 mM Tris-HCl (pH 7.5) containing 6 mM $MgCl_2$, 1 mM ATP, 0.15 M KCl and 1 mM 2-mercaptoethanol for type II topoisomerase. 9 μl of a buffer containing 0.2 μg supercoiled pBR322 DNA was placed in each well of a microtiter plate. Then 3 μl of a series of extracts were added to the wells, and the plates were sealed in order to avoid evaporation. The microtiter plates were incubated at 30°C for 60 min for the assays of type I topoisomerase and for 4 h for endonuclease. In the assays of type II topoisomerase, plates containing the DNA-buffer mixture were pre-warmed at 39°C, and after the addition of extracts, the plates were incubated at 39°C for 180 min. Reactions were stopped by the addition of 12 μl stop solution (0.5% SDS, 25 mg/ml bromphenol blue and 25% glycerol).

Gel electrophoresis. An electrophoretic apparatus with a 30 cm x 20 cm horizontal gel (5 mm in thickness) having 96 sample wells was made. Electrophoresis was carried out in 0.7% agarose gel with 89 mM Tris/89 mM boric acid/2.5 mM Na_2EDTA for 5 h at 2 V/cm at room temperature. Gels were stained with ethidium bromide (1.0 μg/ml), illuminated with u.v. light and photographed with type 667 Polaroid film.

Assay for ATP-dependent unknotting activity

ATP-dependent unknotting activity was assayed by using knotted P4 DNA (Liu *et al.*, 1981) and 0.4% agarose gel for electrophoresis. The knotted P4 DNA was kindly provided by Dr A.Kikuchi.

Fluorescence microscopy. Cells grown in YPD medium were washed twice with 20 mM Tris-HCl (pH 7.5), stained with DAPI (0.1–10 μg/ml) and observed under a fluorescence microscope as described in Toda *et al.* (1981, 1983).

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