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DNA Topoisomerase II Must Act at Mitosis To Prevent Nondisjunction and Chromosome Breakage

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The hypothesis that DNA topoisomerase II facilitates the separation of replicated sister chromatids was tested by examining the consequences of chromosome segregation in the absence of topoisomerase II activity. We observed a substantial elevation in the rate of nondisjunction in *top2/top2* cells incubated at the restrictive temperature for one generation time. In contrast, only a minor increase in the amount of chromosome breakage was observed by either physical or genetic assays. These results suggest that aneuploidy is a major cause of the nonviability observed when *top2* cells undergo mitosis at the restrictive temperature. In related experiments, we determined that topoisomerase II must act specifically during mitosis. This latter observation is consistent with the hypothesis that the mitotic spindle is necessary to allow topoisomerase II to complete the untangling of sister chromatids.

DNA topoisomerase II plays a critical but as yet incompletely defined role in chromosome metabolism in eucaryotes. Two general experimental approaches support the conclusion that topoisomerase II is important in chromosome segregation. First, in vitro studies show that topoisomerase II has the strand-passing abilities expected of an enzyme that can untangle sister DNA molecules that are topologically linked following DNA replication. Second, temperature shift experiments in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* show that topoisomerase II is specifically required at the time that chromosomes are segregating from one another.

Extensive in vitro studies of topoisomerase II demonstrate that the enzyme catalyzes the interconversion between various topological forms of circular DNA molecules (for recent reviews, see references 5, 9, and 39). Although type I topoisomerases also catalyze topoisomerization, their mechanism involves a single-strand nick rather than a double-strand break (26). In contrast, type II topoisomerases act by making a double-strand break, passing another double strand through the break, and then resealing it (2, 21). Thus, in addition to catalyzing the increase or decrease in linking number of a DNA molecule, type II topoisomerases can also catalyze catenation and decatenation of circular DNA molecules, and they can tie and untie knots in them (1, 14, 15, 19–21). The in vitro properties of topoisomerase II are therefore consistent with a role in untangling intertwined DNA molecules in vivo.

Experimental evidence that topoisomerase II is important for chromosome transactions in vivo derives from studies of circular DNA molecules and temperature-sensitive mutants. A variety of circular DNA molecules appear to be intertwined following DNA replication in the absence of topoisomerase II activity (6, 33, 34), although the degree of intertwining may be influenced by the DNA sequence at the termination point of DNA replication (40). The implications of these results are extended by studies of temperature-sensitive topoisomerase mutants. When temperature-sensitive *top2* mutants in *S. cerevisiae* or *S. pombe* are shifted to the restrictive temperature, they rapidly lose viability only if they are passing through mitosis (13, 37). In other words, topoisomerase II activity appears to be essential only at the time of mitosis. Cytological observations in both organisms show that at the time that viability plummets, chromosome segregation is cytologically abnormal (6, 13, 36, 37). The observations are particularly striking in *S. pombe*, in which condensed chromosomes appear to fragment when they segregate in the absence of topoisomerase II activity (35). Taken together, these observations suggest that topoisomerase II activity is required to untangle sister chromatids at the time of mitosis and that cells become nonviable if this untangling does not happen.

Despite the economy of this hypothesis, two questions remain. First, does DNA damage actually occur when chromosomes segregate in the absence of topoisomerase II activity? Second, if such catastrophic damage does occur, why doesn't topoisomerase II untangle replicated sister chromatids well before mitosis? In this report, we bridge the gap between the in vitro data and the in vivo and cytological observations. We demonstrate that chromosomal damage does in fact occur in *top2* strains at the restrictive temperature. At minimum, the frequency of chromosome breakage increases approximately 2-fold and the frequency of nondisjunction increases over 10-fold. We also show that topoisomerase II does not untangle intertwined chromatids prior to mitosis because its function is formally dependent on polymerized microtubules; we suggest that the pulling of the mitotic spindle may be necessary to provide direction for the DNA-untangling activity of topoisomerase II. These experiments tie together the cytological and biochemical data and show that topoisomerase II is critical for chromosome transactions in vivo.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strains used in this study are listed in Table 1. Strains CH1100, CH1101, CH1102, CH1103, CH1114, CH1115, and CH1117 were constructed from strains YF70.2, YF70.4, YPH263, and YRS30, which were generously provided by Phil Hieter. Strains CH1100 to CH1103 were produced by crossing the Hieter strains with

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
CH325	*MATa his4-539*(Am) *lys2-801*(Am) *ura3-52 top2-4*	13
CH326	*MATa his4-539*(Am) *lys2-801*(Am) *ura3-52 top2-5*	13
CH335	*MATa his4-539*(Am) *lys2-801*(Am) *ura3-52 TOP2*$^+$	13
CH336	*MATa his4-539*(Am) *lys2-801*(Am) *ura3-52 TOP2*$^+$	13
CH426	*MATa LEU2 his4 can1-51 URA3 hom3 ade2 lys2 top2-4* *MATα leu2 HIS4*$^+$ *CAN*^{s}*1 ura3-52 HOM3*$^+$ *ADE2*$^+$ *LYS2*$^+$ *top2-4*	This study
CH427	*MATa LEU2 his4 can1-51 URA3 hom3 ade2 lys2 top2-4* *MATα leu2 HIS4*$^+$ *CAN*^{s}*1 ura3-52 HOM3*$^+$ *ADE2*$^+$ *LYS2*$^+$ *TOP2*$^+$	This study
CH602	*MATa his4-539*(Am) *can1-51 URA3 hom3 ade2-101 lys2-801*(Am) *top2-3* *MATα HIS4*$^+$ *CAN*^{s}*1 ura3-52 HOM3*$^+$ *ADE2*$^+$ *LYS2*$^+$ *top2-3*	This study
CH603	*MATa his4-539*(Am) *can1-51 URA3 hom3 lys2-801*(Am) *top2-3* *MATα HIS4*$^+$ *CAN*^{s}*1 ura3-52 HOM3*$^+$ *LYS2*$^+$ *TOP2*$^+$	This study
CH605	*MATa his4-539*(Am) *can1-51 URA3 hom3 lys2-801*(Am) *TOP2*$^+$ *MATα HIS4*$^+$ *CAN*^{s}*1 ura3-52 HOM3*$^+$ *LYS2*$^+$ *TOP2*$^+$	This study
YF70.2	*MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-1* (CF *URA3*$^+$ *SUP11*)[a]	P. Hieter
YF70.4	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-1* (CF *URA3*$^+$ *SUP11*)	P. Hieter
YPH263	*MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$	P. Hieter
YRS30	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$ (CF *TRP1*$^+$ *SUP11*)	P. Hieter
CH1100	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-1* (CF *URA3*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-1*	This study
CH1101	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-1* (CF *URA3*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$	This study
CH1102	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-1* (CF *TRP1*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$	This study
CH1103	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$ (CF *TRP1*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$	This study
CH1114	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-4* (CF *TRP1*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$	This study
CH1115	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$ (CF *TRP1*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TOP2*$^+$	This study
CH1117	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-4* (CF *TRP1*$^+$ *SUP11*) *MATα ade2-101 HIS3*$^+$ *leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 top2-4*	This study

[a] CF, Chromosome fragment.

derivatives of these strains that had lost the chromosome fragment. CH1114, CH1115, and CH1117 were made by transforming YPH263 and YRS30 with a plasmid bearing the *top2-4* mutation. Isogenic *top2-4* and *TOP2*$^+$ derivatives were crossed to produce the diploid strains. Standard genetic techniques were used (30).

Media have been described previously (13). Media for chromosome loss experiments were as follows. Complete medium (C) contained the following (per liter): 6.7 g of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.); 20 mg each of adenine, histidine, methionine, threonine, and uracil; 60 mg of leucine; 30 mg of lysine; 20 g of glucose; and 20 g of Bacto-Agar (Difco). C minus methionine (C−Met) is the same as C but with the methionine omitted; C plus canavanine (C+Can) is as C, but supplemented with 50 mg of canavanine per liter. Strains bearing the *hom3* mutation require either homoserine or methionine plus threonine to grow. For red-white colony experiments, medium with limiting adenine was used (12); it contained the following (per liter): 6.7 g of yeast nitrogen base without amino acids; 6 mg of adenine; 20 mg each of histidine, tryptophan, and uracil; 100 mg each of leucine and lysine; 20 g of glucose; and 20 g of Bacto-Agar.

Genetic measurements of mitotic recombinants and monosomic progeny. To measure the production of mitotic recombinants and cells lacking one copy of a chromosome (monosomic progeny) in a diploid strain, we used a modification of the method of Hartwell and Smith (10). These previous experiments were done with cell division cycle mutants, which cease traversing the cell cycle if they are incubated at the restrictive temperature. Thus, they performed their experiments with cultures grown at semipermissive temperatures for many generations. In contrast, *top2* mutants do not arrest immediately when incubated at restrictive temperature. In addition, 90% of the cells become nonviable. Since we wanted to determine what type of damage was occurring during this short incubation, we measured the frequency of mitotic recombinants and monosomic progeny after incubating the cells at restrictive temperatures for only 2 h (approximately 1.5 doubling times).

These experiments were performed with strains CH426 (*top2-4/top2-4*), CH427 (*TOP2*$^+$*/top2-4*), CH602 (*top2-3/top2-3*), CH603 (*top2-3/TOP2*$^+$), and CH605 (*TOP2*$^+$*/TOP2*$^+$). The general results obtained from *top2/top2* strains CH426 and CH602 were the same, so only the results of CH426 are reported in detail here. As mentioned in Results, the results obtained from strain CH602 were more dramatic than those from CH426.

The frequencies of mitotic recombinants and monosomic progeny were determined by the method of Hartwell and Smith (10). The diploid strains were heterozygous for *can*^{r}*1* and *hom3* (Fig. 1). To determine viability for each sample,

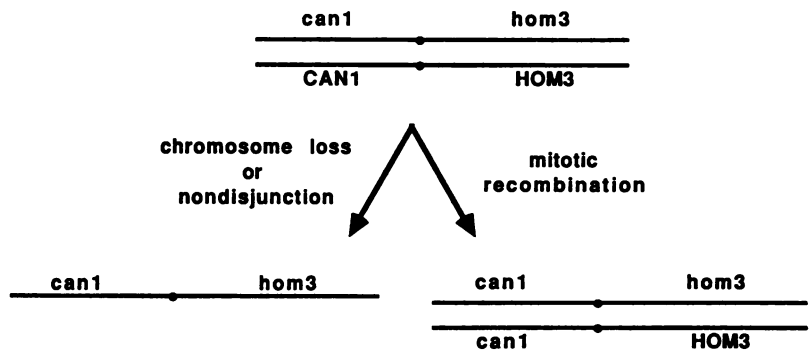

FIG. 1. Genetic scheme for detecting mitotic recombinants and monosomic progeny (10). The diploid strain to be tested is heterozygous for *can*^{r}*1* and *hom3* on chromosome V. Both markers are recessive, so the strain is initially canavanine sensitive and prototrophic. If the bottom copy of chromosome V is missing owing to nondisjunction or simple loss, the aneuploid strain will be canavanine resistant and auxotrophic. If, instead, mitotic recombination occurs between *CAN1* and the centromere, followed by appropriate segregation, the resulting diploid strain will be canavanine resistant and prototrophic. Thus, the frequency of monosomic progeny and mitotic recombinants can be determined by selecting for canavanine-resistant cells and then testing their progeny for homoserine auxotrophy.

cells were sonicated, diluted appropriately, and spread on C plates. To evaluate the frequency of mitotic recombinants plus monosomic progeny, cells were sonicated, diluted appropriately, and spread on C+Can plates. It was possible to determine which of the canavanine-resistant colonies represented mitotic recombinants and which represented monosomic progeny by testing the colonies individually for methionine auxotrophy, which appears if the cells are hemizygous for *hom3*. After 5 days at 26°C, the canavanine-resistant colonies were individually suspended in water and spotted onto C+Can plates. After 1 to 3 days, these spots were replica plated to C+Can, C−Met, and C plates. For strain CH602, there were so many canavanine-resistant colonies that it was impractical to pick all of them. Instead, an unbiased sample of colonies was picked, and the information obtained from the sample was used to calculate the frequencies of mitotic recombinants and monosomic progeny in the original sample. Strain CH430 (*top2-5/top2-5*), which exhibits somewhat impaired growth at the "permissive temperature" of 26°C, exhibited a very high frequency of mitotic recombinants and monosomic progeny even at 26°C. We believe that this result is due to the temperature (26°C) being not truly permissive for this strain. Since the purpose of the present experiment was to look at short-term damage caused by *top2* mutations at restrictive temperature, the high background exhibited by this strain made it unsuitable for these experiments.

To measure directly the rate of nondisjunction, we used a modification of the method of Hieter et al. (12). In one set of experiments, diploid strains CH1114 (*TOP2*$^+$*/top2-4*), CH1115 (*TOP2*$^+$*/TOP2*$^+$), and CH1117 (*top2-4/top2-4*) were used; in a second set of experiments, we used strains CH1100 (*top2-1/top2-1*), CH1101 (*TOP2*$^+$*/top2-1*), CH1102 (*TOP2*$^+$*/top2-1*), and CH1103 (*TOP2*$^+$*/TOP2*$^+$). The results from the two sets of strains were similar. As described in Results, these strains carry a single copy of a *SUP11*-bearing chromosome fragment, which causes the colonies to be pink (11, 32). Nondisjunction (2:0 segregation) and chromosome loss (1:0 segregation) can be distinguished on the basis of colony color (white-red and pink-red, respectively). To assess the frequency of these events, cultures were sonicated, spread for single colonies in large (150-mm) low-adenine plates, and scored after 4 to 5 days of growth at 25°C.

Chromosome gels. The apparatus and protocol used for orthogonal-field-alternation gel (OFAGE) electrophoresis were as described by Carle and Olson (3) with minor modifications. All gels were 1.2% agarose, the electrophoresis buffer was 0.5× TBE (1× TBE is 0.089 M Tris, 0.089 boric acid, and 0.002 M EDTA), and the temperature of the buffer was kept at approximately 10°C. Electrophoresis was carried out for 18 h at 280 V with a switch time of 50 s.

Preparation of chromosomal DNA. Yeast strains were grown to the log phase (5×10^6 to 1×10^7 cells per ml) at 23°C. Half of each culture was then shifted to 36°C, and the other half was mock shifted to 23°C. Incubation was continued under these conditions for 2.5 h, cells were harvested by centrifugation, and samples were prepared for electrophoresis as described by Carle and Olson (4). Alternatively, 20 μg of nocodazole per ml in dimethyl sulfoxide was added to portions of the cultures 10 min before shifting; these cultures were then treated as described above. In all cases, the samples were kept at 37°C or higher throughout the DNA preparation so that the mutant strains were not exposed to permissive conditions.

Gel transfer hybridizations. DNA from OFAGE gels were transferred to Zetapor membrane after depurination in 0.25 N HCl for 20 min as described by Southern (31). Hybridizations were performed under conditions described by Wahl et al. (38); washes were done in 2× SSPE at 65°C (1× SSPE is 0.18 M NaCl, 10 mM $(Na_{1.5})PO_4$, and 1 mM Na_2 EDTA, pH 7.0). Probe for the gene *HXK2* (chromosome VII) was made by nick translation of pRB144 (29). Probe for the gene *CAN1* (chromosome V) was made by random hexamer labeling (8) of a restriction fragment of pSK302 (courtesy of S. Kunes). Probe for the gene *PET494* (short arm of chromosome XIV) was made by random hexamer labeling of a fragment of pMC203 (courtesy of T. Fox), and probe for the gene *KAR1* (long arm of chromosome XIV) was made by random hexamer labeling of a fragment of pMR27 (courtesy of G. Fink).

Nocodazole release experiments. Each nocodazole release experiment was performed with strains CH325 (*top2-4*) and CH326 (*top2-5*) with comparable results. The results for CH325 (*top2-4*) are reported in Fig. 4. Mutant and wild-type strains were grown exponentially in YEPD (pH 4) at 26°C (YEPD medium is 10 g of yeast extract [Difco] per liter, 20 g of Bacto-Peptone [Difco] per liter, and 2% glucose). Nocodazole (Sigma Chemical Co., St. Louis, Mo.) was added to 20 μg/ml, and the culture was returned to 26°C. After 3 h (approximately 1.5 doubling times), the cells were rapidly filtered out of the medium, washed twice with medium

TABLE 2. Monosomic progeny and mitotic recombinants at permissive and restrictive temperatures

Temp	No. of monosomic progeny (10^4)[a]		No. of recombinants (10^3)[a]	
	top2-4/top2-4	*TOP2⁺/top2-4*	*top2-4/top2-4*	*TOP2⁺/top2-4*
Before incubation	2.6 ± 0.44	1.2 ± 0.48	1.2 ± 0.43	0.91 ± 0.28
26°C, 2 h	2.0 ± 0.8	0.96 ± 0.55	1.1 ± 0.34	1.1 ± 0.16
35°C, 2h	55.1 ± 9.0	1.3 ± 0.49	2.7 ± 0.32	1.0 ± 0.24

[a] Numerical values indicated are means ± standard deviations for three independent isolates of strains CH426 (*top2-4/top2-4*) and CH427 (*TOP2⁺/top2-4*).

lacking nocodazole, and then resuspended in medium without nocodazole. The culture was split, and half was incubated at 26°C and half at 35°C. Samples were taken from the culture every 20 min for 2 h. Each sample was immediately sonicated, diluted, and plated on three YEPD plates at 26°C. After 3 days of growth, the colonies were counted and used to calculate the number of viable cells in the original liquid culture at the time when the sample was taken.

RESULTS

Aneuploidy and mitotic recombination increase in *top2* strains at restrictive temperature. If sister chromatids are physically intertwined at the time of mitosis, we expect that when they disjoin in the absence of topoisomerase II activity they should behave in many ways like dicentric chromosomes. The behavior of dicentric chromosomes has been examined cytologically and genetically in many eucaryotes, and the behavior of dicentric plasmids has been characterized in *S. cerevisiae* (18, 22, 24). The results of these studies suggest that when the cell begins to separate DNA molecules that are physically joined, there are two major consequences: chromosome breakage and nondisjunction.

We used a genetic approach to look for the results of chromosome breakage and nondisjunction in *top2* strains incubated at the restrictive temperature. When chromosomes are broken by various methods, the broken ends cause a striking increase in the frequency of recombination (25, 28). Thus, to obtain an initial indication whether chromosomes are broken in *top2* strains at the restrictive temperature, we looked for an increased frequency of mitotic recombinants in diploid strains. Nondisjunction of a particular chromosome produces one daughter cell bearing two copies of the chromosome and one daughter cell bearing no copies of the chromosome (2:0 segregation). As an initial indication of the frequency of nondisjunction in *top2* strains, we assayed the frequency of diploid cells lacking a marked copy of chromosome V (the "0" or "monosomic" [$2n - 1$] class). Of course, monosomic progeny could also arise from simple chromosome loss (1:0 segregation). However, subsequent experiments (see below) showed that monosomic progeny are mainly produced by nondisjunction.

We measured the production of mitotic recombinants and monosomic progeny by the method of Hartwell and Smith (10). Diploid strains were constructed with the genetic composition of chromosome V shown in Fig. 1. Because canavanine resistance (*can^{r}1*) and homoserine auxotrophy (*hom3*) are recessive, the heterozygous diploid strains are canavanine sensitive and prototrophic. When the appropriate copy of chromosome V is missing from a cell (monosomic progeny), the colony it produces is canavanine resistant and auxotrophic. Similarly, if mitotic recombination occurs between *can^{r}1* and its centromere and the appropriate segregation pattern occurs, a clone becomes canavanine resistant and prototrophic. Thus, by selecting for canavanine resistance and screening for prototrophy, it is possible to determine the frequency of mitotic recombinants and monosomic progeny in a population of cells subjected to various conditions.

Chromosome behavior was monitored in diploid strains that were homozygous (*top2/top2*) or heterozygous (*TOP2⁺/top2*) for a temperature-sensitive allele of the structural gene for DNA topoisomerase II (*top2* maps to chromosome XIV). Homozygous *TOP2⁺/TOP2⁺* strains gave results comparable to those of the heterozygous *TOP2⁺/top2* strains, and the results are not shown here. Cells were grown exponentially at 26°C in YEPD and assayed for the frequency of mitotic recombinants and monosomic progeny in the population. Half of each culture was then placed at 35°C; the other half remained at 26°C. After 2 h, the cultures were again tested for mitotic recombinants and monosomic progeny. Table 2 shows that the temperature-sensitive *top2/top2* strain exhibited a 20-fold increase in the frequency of monosomic progeny when it was incubated for only 1.5 generation times at the restrictive temperature. A smaller (twofold) increase in the frequency of mitotic recombinants was also observed. These effects were not seen in the control *TOP2⁺/TOP2⁺* and *TOP2⁺/top2* strains under any conditions, and they were not seen in the *top2/top2* strain at the permissive temperature. Thus, temperature-sensitive *top2* strains incubated at the restrictive temperature exhibit a small increase in mitotic recombinants and a profound increase in the frequency of cells entirely lacking one copy of chromosome V.

These results are consistent with the hypothesis that both chromosome breakage and nondisjunction occur in *top2* strains at the restrictive temperature. However, the interpretation of the results is hampered by the indirectness of the assay. Monosomic ($2n - 1$) progeny could be the result of nondisjunction, but they could also be the result of chromosome loss; elevated mitotic recombination could be the result of chromosome breakage, but it could also be caused by other types of DNA damage. To assess more directly the sources of aberrant progeny, we used the red-white colony color assay (12) to distinguish between nondisjunction (2:0 segregation) and chromosome loss (1:0 segregation), and we used OFAGE gels to examine directly the breakage of chromosomal DNA molecules.

Frequency of nondisjunction increases markedly in *top2* strains at restrictive temperature. We used the red-pink-white color assay (12) to assess directly the rate of nondisjunction in *top2* strains at the restrictive temperature. We began with an *ade2-101/ade2-101* diploid strain carrying a single copy of a fragment of chromosome VII bearing the ochre suppressor *SUP11* (generously provided by Phil Hieter; 32). Because *ade2-101* is an ochre mutation, the red color normally produced in *ade2/ade2* strains is partly suppressed by *SUP11*, and the diploid carrying a single copy of the chromosome fragment gives rise to pink colonies. Cells lacking a copy of the chromosome fragment give rise to red colonies, and cells carrying two copies give rise to white colonies. Thus, nondisjunction (2:0 segregation) in the first

TABLE 3. Nondisjunction and chromosome loss at permissive and restrictive temperatures

Temp	Colony phenotype (per 10^3 colonies)[a]			
	Red	White	Red-white	Red-pink
top2-4/top2-4				
Before incubation (n = 3,362)	3	2	0	0
25°C, 2 h (n = 4,408)	3.2	2	0.2	0
35°C, 2 h (n = 3,695)	16	18	3.0	0.3
TOP2⁺/TOP2⁺				
Before incubation (n = 3,092)	1	2	0	0.3
25°C, 2 h (n = 4,252)	2	1	0	0
35°C, 2 h (n = 4,003)	2.7	1	0.3	0

[a] Most colonies are pink, because they are derived from a cell with one copy of the chromosome fragment. Cells in red colonies lack the chromosome fragment; cells in white colonies possess two copies. Red-white half-sectored colonies derive from nondisjunction (2:0 segregation); red-pink half-sectored colonies derive from chromosome loss (1:0 segregation).

division after plating produces a white-red half-sectored colony, and similarly, chromosome loss (1:0 segregation) gives rise to a pink-red half-sectored colony.

As in the *can*^{r}*1/hom3* experiment, chromosome behavior was monitored in diploid strains that were homozygous (*top2/top2*), heterozygous (*TOP2⁺/top2*), or wild type (*TOP2⁺/TOP2⁺*) for a temperature-sensitive *top2* allele. Heterozygous *TOP2⁺/top2* strains gave results comparable to the homozygous *TOP2⁺/TOP2⁺* strains, and the results are not shown here. Cells were grown exponentially at 25°C and plated for red-pink-white colonies. Half the culture was then incubated at 25°C and half at 35°C, and after 2 h (one generation time), each culture was plated for red-pink-white colonies. Table 3 shows that the frequency of red-white half-sectored colonies increases over 10-fold in the *top2/top2* strain in only one generation time at the restrictive temperature. In addition, the frequency of isolated red and white colonies increases markedly and in equal amounts. These effects are not seen in the *TOP2⁺/TOP2⁺* strain at either temperature nor in the *top2/top2* strain at permissive temperature. Thus, it appears that *top2/top2* strains exhibit a striking increase in the rate of nondisjunction when incubated at the restrictive temperature.

Chromosome breakage can be directly demonstrated in *top2* strains at restrictive temperature. The results of the *can*^{r}*1/hom3* experiment demonstrate that an increase in mitotic recombination is a relatively minor consequence of topoisomerase II inactivity. However, because mitotic recombination is an indirect measure of chromosome breakage, we wished to assess the damage to the chromosomes more directly by visualizing them on chromosome separation gels. The observed minor increase in mitotic recombination suggests that there should be little change in the banding pattern of the chromosomes after *top2* cells are incubated at the restrictive temperature. However, if there are minor breakage products, it should be possible to visualize them by hybridization.

As a control, we first determined that topoisomerase II remained inactive during the preparation of DNA from *top2* cells incubated at the restrictive temperature. To do this, we looked for an unusual 2μm DNA banding pattern, which is expected when 2μm DNA replicates in the absence of topoisomerase II activity and produces catenated daughter molecules (6). *TOP2⁺* and *top2* strains were grown exponentially at 23°C. The cultures were then split, and half was incubated at 23°C and half at 36°C for 2.5 h. DNA was isolated from the cells and subjected to OFAGE. Figure 2B shows the autoradiograph produced when nick-translated 2μm DNA is hybridized to a Southern blot of this gel. As expected, the *TOP2⁺* strain at both 23 and 36°C and the *top2* strain at 23°C all exhibited similar 2μm DNA banding patterns. In contrast, the *top2* strain at 36°C showed a strikingly different pattern. This observation is consistent with previous work reporting the behavior of circular plasmids isolated from *top2* strains at restrictive temperature (6). This result confirms that our method of isolating DNA does not permit DNA topoisomerase II to untangle catenated DNA molecules.

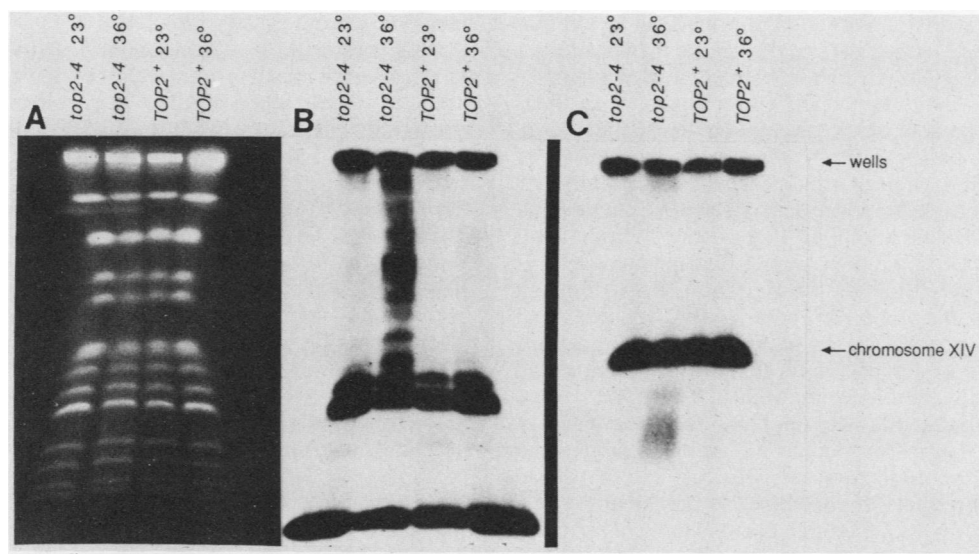

FIG. 2. Chromosomes of *top2* and *TOP2⁺* strains at permissive and restrictive temperatures. Exponentially growing cultures of strains CH325 (*top2-4*) and CH335 (*TOP2⁺*) were split, and half of each was incubated at 23°C and half at 36°C for 2.5 h. DNA was isolated and run on an OFAGE gel for 18 h at 280 V with a switch time of 50 s. (A) Ethidium bromide-stained gel. (B) Autoradiograph of 2μm DNA hybridized to a Southern blot of this gel. (C) Autoradiograph of *KAR1*, a single-copy nuclear gene on the left arm of chromosome XIV, hybridized to a Southern blot of this gel.

Although the migration of 2μm DNA sequences is quite distinct in DNA from *top2* cells incubated at restrictive temperature, the genetic results suggest that there is sufficiently little chromosome breakage that the migration of the bulk of the chromosomes should appear unaffected. Indeed, when the gel used to produce the autoradiograph shown in Fig. 2B was stained with ethidium bromide, the staining patterns produced by *top2* strains and *TOP2*$^+$ strains at 23 and 36°C were virtually indistinguishable (Fig. 2A). The similarity of the ethidium bromide staining patterns suggests that there is not a large amount of chromosome breakage in *top2* strains incubated at the restrictive temperature. This observation is consistent with the genetic results. (The absence of novel "tangled chromosome" bands will be considered in the Discussion.)

Although the genetic results predict that there should be little change in the migration of the bulk of the chromosomes, they also suggest that there is a small amount of chromosome breakage which may be possible to visualize on chromosome separation gels. To test this prediction, we blotted the gel shown in Fig. 2A and hybridized it with various radioactive probes for single-copy genes. Broken chromosomes can be identified as hybridizing material of lower molecular weight (higher mobility) than the original chromosome band. Of course, only the chromosome fragments bearing sequences homologous to the probe will be revealed by this method. Other fragments of the same chromosome will be present in the gel but will not appear on the autoradiograph.

To reveal such subtle changes in chromosome banding patterns, we hybridized various cloned chromosomal genes to Southern blots of gels containing DNA from *TOP2*$^+$ and *top2* strains that had been incubated at 23 and 36°C. Figure 2C shows the results with *KAR1*, a single-copy nuclear gene located on the long arm of chromosome XIV. The main chromosome XIV band is identical in all strains under all conditions. However, a novel diffuse band appears in the *top2* strains incubated at the restrictive temperature. This new band migrates to a position consistent with it being somewhat greater than one-half the size of chromosome XIV. Consistent results were observed when chromosome gels were hybridized with *URA3* to determine the location of chromosome V and with *HXK2* to determine the location of chromosome VII; a novel diffuse band of lower intensity appeared at a position characteristic of a molecule approximately one-half the size of the original chromosome.

If these novel bands actually represent single arms of chromosomes that have been broken, then hybridization with a probe that maps to the opposite arm of a given chromosome may reveal a different band. In Fig. 3, the hybridization pattern of *PET494* to the short arm of chromosome XIV is compared with the hybridization pattern of *KAR1* to the long arm of chromosome XIV. In both cases, the identical full-size chromosome XIV band is revealed. It is apparent, however, that the two probes hybridized to different novel diffuse bands of lower molecular weight. The *KAR1* probe hybridized to a band of lower mobility than the *PET494* probe. Since two genes carried by the same chromosome hybridized to different DNA bands, we conclude that these bands represent products of chromosome breakage.

According to our working model, chromosome breakage is caused by the action of the mitotic spindle on intertwined chromosomes in the absence of topoisomerase II activity. This hypothesis is consistent with the results described above, and it also predicts that chromosome breakage should not occur if the mitotic spindle is depolymerized prior to the inactivation of topoisomerase II activity. Thus, if a microtubule-destabilizing drug is added to *top2* cells before they are shifted to the restrictive temperature, topoisomerase II will no longer be required to untangle sister chromatids to prevent their breakage. As predicted, when *top2* cells were treated with the microtubule-destabilizing drug nocodazole prior to the shift to restrictive temperature, the presumptive chromosome breakage band was not produced (data not shown). This observation is consistent with the hypothesis that the action of the mitotic spindle causes the observed chromosome breakage.

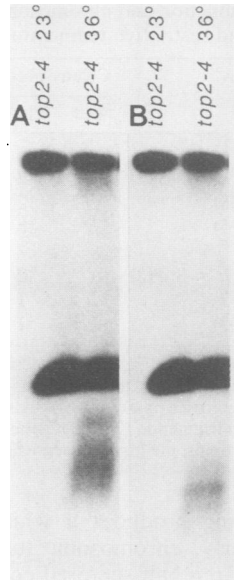

FIG. 3. Hybridization of minor bands to genetic probes from different arms of the same chromosome. Probes from the right arm (*PET494*) and the left arm (*KAR1*) of chromosome XIV were hybridized to the filter described in the legend to Fig. 2. (A) *KAR1* probe. (B) *PET494* probe. As expected, the major intact chromosome XIV band to which both probes hybridize is identical. In contrast, it is clear that the two probes hybridize to different minor bands.

DNA topoisomerase II cannot complete its essential function in the absence of microtubules. Given the catastrophic damage that occurs when chromosomes remain tangled during segregation, it is surprising that topoisomerase II does not complete its essential function well before this time (13); it would seem more prudent to untangle the chromosomes immediately after replication and allow an extra temporal margin of safety prior to chromosome segregation. The relatively late activity of topoisomerase II could be explained in either of two ways. First, intertwined sister chromatids may be produced during the S phase, and 20 to 30 min may be required for topoisomerase II to resolve a large number of tangles. In this case, the requirement for topoisomerase II at mitosis would simply reflect the finite amount of time required for topoisomerase II to complete its essential function. Alternatively, topoisomerase II may be required specifically at the time of mitosis because it is constrained to complete its essential function specifically at that time. This might be the case if the untangling of sister chromatids was influenced in some way by the mitotic spindle.

To distinguish between these hypotheses, we treated *top2* mutants with the microtubule-depolymerizing drug nocodazole for several hours prior to shifting them to restrictive

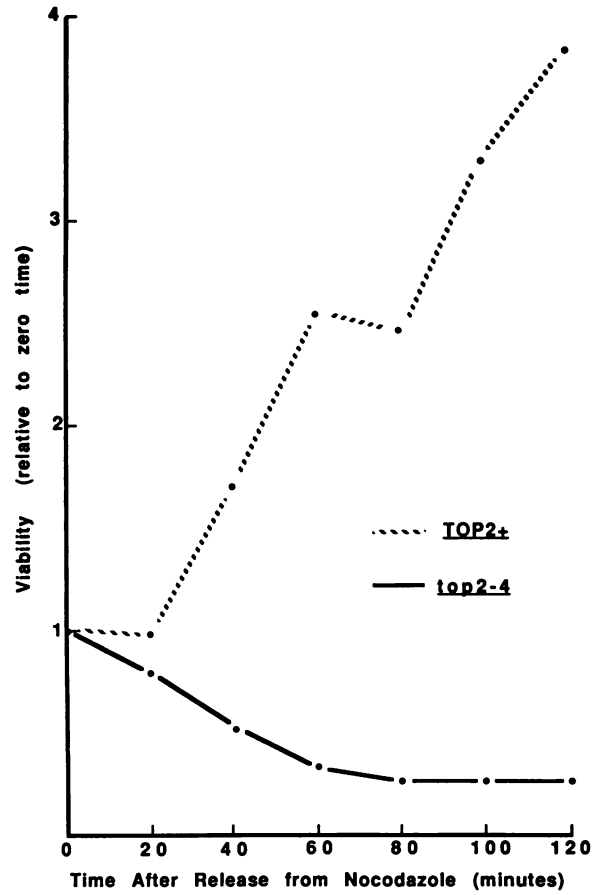

FIG. 4. Topoisomerase II function is dependent on the nocodazole-sensitive step. Exponentially growing cultures of strains CH325 (*top2-4*) and CH335 (*TOP2*$^+$) were treated with nocodazole for 3 h at 26°C. The nocodazole was washed away, and the cultures were split. Half of each was incubated at 26°C, half at 35°C. Samples were plated for viability every 20 min for 2 h. The results obtained from cultures incubated at 35°C are shown here. Results for CH325 (*top2-4*) at 26°C and CH335 (*TOP2*$^+$) at 26°C (not shown) are virtually identical to the results for CH335 (*TOP2*$^+$) at 35°C.

temperature. Cells treated with nocodazole cease traversing the cell cycle after completing DNA synthesis and prior to mitosis (16, 27). In addition, topoisomerase II remains active in nocodazole-arrested cells (17). Thus, if *top2* cells are incubated in nocodazole at permissive temperature for a full generation time, then topoisomerase II has ample opportunity to untangle the replicated sister chromatids if it is possible to do so in the absence of microtubules. On the other hand, if the completion of topoisomerase II function is dependent on microtubules, then the cells incubated in nocodazole should still require topoisomerase II activity to undergo mitosis successfully. The requirement for topoisomerase II activity can be assessed by incubating temperature-sensitive *top2* cells at the restrictive temperature and measuring viability. If they require topoisomerase II activity, they will lose viability; if they no longer require topoisomerase II activity, they will retain viability.

To ascertain the dependence of topoisomerase II function on microtubule function, we incubated *top2* and *TOP2*$^+$ cells for 3 h (approximately 1.5 generation times) in nocodazole at the permissive temperature. If it was possible for topoisomerase II to complete its essential function in the absence of microtubules, it should have done so during this incubation. The nocodazole was then washed away, and the cells were incubated at the permissive temperature (26°C) or the restrictive temperature (35°C). Samples of the cultures were tested for viability at various times. *TOP2*$^+$ cells at both temperatures and *top2* cells at the permissive temperature all remained fully viable after the nocodazole was washed away (Fig. 4). Furthermore, an increase in the number of viable cells showed that the *TOP2*$^+$ cells successfully underwent mitosis and cytokinesis by 60 min after release from nocodazole. In contrast, *top2* cultures incubated at the restrictive temperature began to lose viability within 40 min after they were removed from nocodazole (Fig. 4).

Because about 20% of the cells survive incubation at restrictive temperature following nocodazole arrest, it is possible that topoisomerase II is functioning in a fraction of the cells even in the absence of microtubule function. Alternatively, some cells may simply not recover from the nocodazole arrest quickly enough to pass through mitosis at the restrictive temperature in the short high-temperature pulse given in this experiment; they would then survive when plated at the permissive temperature. That the latter explanation is probably correct is shown by two observations. First, morphological observations reveal that 5 to 10% of the cells remain arrested for 2 h after release from nocodazole (data not shown). These cells may nontheless go on to form colonies when subsequently plated on YEPD and incubated for 3 days at permissive temperature. Second, *top2* cells incubated in nocodazole at the restrictive temperature give viability curves indistinguishable from those shown here for nocodazole incubation at the permissive temperature (data not shown). Since having inactive topoisomerase II during the nocodazole incubation does not decrease the survival chances of a cell, it seems unlikely that topoisomerase II is performing an essential function in any substantial fraction of the cells during this time.

In summary, these results show that even when cells are incubated for over a generation time in G2 with full topoisomerase II function, they still require topoisomerase II activity to undergo mitosis successfully. Thus, the topoisomerase II-requiring step is formally dependent on microtubule function.

DISCUSSION

We showed that temperature-sensitive *top2* mutants exhibit a marked increase in the rate of nondisjunction and a small increase in the rate of mitotic recombination when incubated at the restrictive temperature. It appears that the observed increase in mitotic recombination accurately reflects an increase in chromosome breakage, because it is correlated with the appearance of a new half-sized chromosome band in OFAGE gels. Elevations in the rates of nondisjunction and chromosome breakage are consistent with the hypothesis that replicated sister chromatids are to some degree intertwined at mitosis. The results of experiments in which cells are treated with nocodazole suggest that the intertwining cannot be fully resolved before mitosis. Thus, it appears that topoisomerase II can only complete its essential function specifically at mitosis.

To evaluate the hypothesis that sister chromatids are tangled at mitosis, it is useful to compare the behavior of chromosomes from *top2* strains with that of dicentric chromosomes, whose centromeres are known to be physically connected. In *S. cerevisiae*, dicentric chromosomes exhibit both a high probability of nondisjunction and an elevated frequency of chromosome breakage (7, 18, 22, 24). While

exact rates are difficult to specify, measurements of frequencies at a single division suggest that dicentric minichromosomes in *S. cerevisiae* exhibit rates of nondisjunction and chromosome breakage that are of approximately the same order of magnitude (18). (This result may be peculiar to *S. cerevisiae*, which has an unusually low number of microtubules.) Similarly, when *top2* cells are incubated at the restrictive temperature, they exhibit an increased frequency of nondisjunction and chromosome breakage. As with dicentric plasmids, the results of the *can*^{r}*1/hom3* experiment suggest that these increased frequencies are of approximately the same order of magnitude. Furthermore, the putative chromosome breakage band that appears on OFAGE gels in *top2* strains at the restrictive temperature is similar in migration to the novel band that initially appears when known dicentric chromosomes undergo mitosis (K. Bloom, personal communication). Finally, cytological observations in *S. pombe* show that condensed chromosomes appear to rip apart when they are segregated in the absence of topoisomerase II activity (35). Thus, chromosomes in *top2* strains at the restrictive temperature share many characteristics with chromosomes that are known to be physically connected. We take this as supporting evidence for the physical intertwining of mitotic chromosomes.

We had hoped to visualize intertwined sister chromatids as novel bands on OFAGE gels. However, although we took care to run long and short gels and to include the contents of the sample wells in Southern transfers, no major shifts of chromosome banding pattern occurred in any of the samples. There were only minor, apparently lower-molecular-weight bands, which appeared to represent broken chromosomes. In retrospect, it is probably not surprising that novel "intertwined chromosome" bands are not seen, because prior to electrophoresis the DNA is subjected to protease and detergent treatments, which should remove the protein and membrane attachments that may constrain the free movement of the molecules. Furthermore, the DNA is subjected to a variety of pulling and pushing forces during electrophoresis. One would expect molecules that are not constrained by covalent bonds to be separated during this treatment. Thus, it is not surprising that the structure of tangled chromosomes is not preserved on OFAGE gels.

A surprising result from the OFAGE gels is that the products of chromosome breakage appear as relatively compact bands rather than as a diffuse smear beginning directly below the intact chromosome band. In part, the compactness of the band is imposed by the way in which the experiment was done. Broken chromosomes were visualized by hybridization to individual DNA probes; thus, chromosome XIV fragments would not be visualized if they contained only material distal to *KAR1* or *PET494* or proximal to both. This rationale explains the absence of relatively small breakage products. However, it should be possible to visualize larger breakage products if they exist. Nonetheless, for several different chromosomes, the largest chromosome fragments migrate to a position consistent with their being approximately one-half the size of the intact chromosome. Thus, we suggest that intertwined chromosomes undergoing mitosis may tend to break near the middle. Such localization could be due simply to the mechanical forces placed on the chromosomes, or it could reflect a localization of the intertwinings remaining at the time of mitosis. Since most yeast chromosomes are metacentric, one possibility is that intertwinings near the centromere are the last to be resolved or the first to cause mechanical interference when the chromosomes are segregated.

Although we were not able to observe tangled chromosomes directly, we were able to observe the elevated frequency of nondisjunction that one would expect from them. It is significant that this result was obtained with linear chromosome fragments; although a similar result with a circular minichromosome was highly suggestive (17), it is difficult to extrapolate with confidence from the behavior of circular minichromosomes to the behavior of linear chromosomes because of the topological constraints that circularity imposes. It is particularly striking that the frequency of red-white half-sectored colonies increases 10-fold in the mutant incubated at restrictive temperature; red-white colonies arise specifically when a cell undergoes nondisjunction and then is plated on the low-adenine plate prior to completing cytokinesis. Any cell undergoing nondisjunction that had completed cytokinesis before plating would be represented by two discrete colonies, one red and one white. Thus, it is also significant that the frequency of red and white colonies increased markedly in the *top2/top2* strain during the brief incubation at the restrictive temperature. Although it is possible that the red and white colonies are produced by two separate phenomena, the fact that they are equal in number suggests that they derive from the same event. It is heartening to note that the frequency of red colonies, which lack the chromosome fragment, is comparable to the frequency of monosomic progeny observed in the *can*^{r}*1/hom3* experiment. Thus, we conclude that nondisjunction is relatively frequent among *top2/top2* cells incubated at the restrictive temperature.

A high rate of nondisjunction could easily lead to the nonviability observed in *top2* strains incubated at the restrictive temperature. However, it is difficult to calculate precisely the overall rate of nondisjunction in *top2/top2* strains at the restrictive temperature. In the present experiment, we determined rates of nondisjunction among the survivors of a shift to restrictive temperature. We have assumed that the nonsurvivors are nonviable because they suffered a more severe version of the problems exhibited by the surviving cells. However, it is possible that the survivors survive precisely because they incur a different sort of damage than the rest of the cells do. The economy of a single mechanism favors the former assumption, as does circumstantial evidence from the red-white experiment. When *top2/top2* cells were incubated at the restrictive temperature, the survivors exhibited heterogeneous growth rates. This observation suggests that it is most reasonable to view the population as a continuum, running from dead to almost dead to barely alive to healthy. Significantly, a high rate of nondisjunction is seen among the sick survivors of a shift to restrictive temperature. We conclude that chromosome loss is probably the major cause of the nonviability observed in *top2* strains at the restrictive temperature.

In every way we are able to measure, chromosomes segregated in the absence of topoisomerase II activity share important genetic and physical characteristics with the other physically connected chromosomes we can study, dicentric chromosomes. They exhibit a high rate of both nondisjunction and chromosome breakage. The most economical interpretation for these observations is that replicated sister chromatids are physically intertwined to a certain extent. Thus, for ease of discussion, we will refer to sister chromatids at mitosis as intertwined or tangled sister chromatids.

Since segregating still-tangled chromatids has dire consequences for a cell, it initially seems odd that topoisomerase II does not complete its essential function substantially before mitosis. This anomaly is explained by the results of

the nocodazole experiments, which show that topoisomerase II cannot complete its essential function when microtubules are destroyed by nocodazole. In the simplest model, topoisomerase II is unable to remove the final intertwinings of sister chromatids in the absence of microtubules. This observation can be explained because DNA topoisomerase II is a reversible enzyme: it catalyzes both the catenation and decatenation of DNA (1, 15, 19). While the initial untangling of sister molecules may be enhanced by torsional stress (23), there may be no driving force for untangling once the initial stress is relieved. Prior to anaphase of mitosis, then, while the sister chromatids are still in close apposition to one another, at least near their centromeres, topoisomerase II may function but perform a futile cycle. As long as the sister chromatids remain in close physical proximity, topoisomerase II may sequentially tangle them, untangle them, and then tangle them up once more. Perhaps it is only at anaphase of mitosis that the substrates of this reaction will be removed by the pulling of microtubules. This idea explains why topoisomerase II can complete its essential function only at the time of mitosis.

Cytological observations are also consistent with the hypothesis that topoisomerase II completes its untangling function only when a direction is imposed on its action. Elegant studies by Uemura et al. (35) show that even fully condensed chromosomes require topoisomerase II activity to be separated without structural damage. In another experiment, these studies also showed that topoisomerase II is required for full condensation of chromosomes. One interpretation of these observations is that the condensation of sister chromatids can, in part, give direction to the topoisomerase II untangling reaction. This interpretation would explain why condensed chromosome arms in colcemid-treated cells appear to move freely. Finally, taken together, these studies all suggest that topoisomerase II is an important enzyme for transactions involving the gross movement of large chromosomal DNA molecules at mitosis.

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