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DNA Topoisomerase II Is Required at the Time of Mitosis in Yeast

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Summary

We have constructed five new temperature-sensitive DNA topoisomerase II mutations and have analyzed their physiological consequences in yeast. Several lines of evidence suggest that the activity of topoisomerase II is required specifically at the time of mitosis. First, *top2* mutations cause dramatic lethality at the restrictive temperature, but only if the mutant cells are actively traversing the cell cycle. Second, temperature-shift experiments with synchronized cultures show that the onset of inviability coincides with the time of mitosis. Third, fluorescence microscopy reveals that the normal progression of mitosis is disturbed in mutant cells at the restrictive temperature. Finally, inviability at the restrictive temperature is prevented by nocodazole, an inhibitor of tubulin polymerization that prevents formation of the mitotic spindle. These results are consistent with the hypothesis that the essential function of topoisomerase II is to allow the separation of intertwined chromosomal DNA molecules during mitosis.

Introduction

Type II topoisomerases have been well characterized in vitro (Gellert, 1981). They act via a double-strand break in the DNA, and they change the linking number of covalently closed circular DNA molecules in steps of two (Brown and Cozzarelli, 1979; Liu et al., 1980). Type II topoisomerases also catenate and decatenate circular DNA molecules (Baldi et al., 1980; Hsieh and Brutlag, 1980; Kreuzer and Cozzarelli, 1980), and they can tie and untie knots in them (Liu et al., 1980; Liu et al., 1981; Hsieh, 1983). Eukaryotic type II topoisomerases differ in an important way from DNA gyrase, the type II topoisomerase of E. coli. Whereas DNA gyrase can actively introduce negative supercoils into circular DNA molecules (Gellert et al., 1976a), eukaryotic type II topoisomerases cannot (Baldi et al., 1980; Hsieh and Brutlag, 1980; Miller et al., 1981; Goto and Wang, 1982). This difference in activity may reflect different intracellular roles played by the enzymes.

Although in prokaryotes DNA gyrase appears to play an important role in determining the superhelical density of chromosomal DNA (Gellert et al., 1976b; DiNardo et al., 1982; Pruss et al., 1982), in eukaryotes the role of topoisomerase II is much less clear. Results with SV40 and yeast suggest that topoisomerase II activity is required to allow the segregation of circular DNA molecules after replication; in the absence of topoisomerase II activity, replicated circular molecules accumulate as multiply-intertwined, catenated dimers (Sundin and Varshavsky, 1981; DiNardo et al., 1984). However, it is unlikely that the segregation of replicated circular DNA molecules is the sole role of topoisomerase II in the cell.

It has been suggested that topoisomerase II is required for segregation of linear chromosomal DNA molecules as well as of circular plasmid molecules (Hsieh and Brutlag, 1980; Sundin and Varshavsky, 1981; DiNardo et al., 1984). One way of testing this hypothesis is to determine whether topoisomerase II performs its essential function at a particular time in the cell cycle. If topoisomerase II activity is required only to allow segregation of chromosomal DNA molecules after replication, it should act in the cell cycle sometime between DNA synthesis and mitosis. If, instead, it is necessary for other "housekeeping" functions, it might be required at many or all times in the cell cycle.

Hartwell coined the term "execution point" to refer to the time at which a gene product completes its essential function in the cell cycle (Hartwell, 1971). His cell division cycle mutants (*cdc* mutants) appear to have specific defects at certain points in the cell cycle. The execution point for *cdc* gene products is determined by assessing the ability of cells at various points in the cell cycle to complete the cell cycle at the restrictive temperature. If a cell has passed the "execution point", it will complete the present cell cycle; if it has not, it will halt its progression through the cell cycle at a characteristic "terminal morphology." Thus, it is possible, by observing the behavior of individual cells, to determine the execution point for the *cdc* gene products.

Topoisomerase II mutants differ from *cdc* mutants in that exponentially growing cultures do not give rise to cells with a single characteristic morphology when they are incubated at the restrictive temperature. Temperature-sensitive *top2* strains produce cells with two different morphologies in Saccharomyces cerevisiae (DiNardo et al., 1984), and an analogous result is seen in Schizosaccharomyces pombe (Uemura and Yanagida, 1984). Thus, it may not be possible to determine the execution point of topoisomerase II simply by examining the morphology of mutant cells at the restrictive temperature.

In this report, we use a novel approach to determine the execution point for topoisomerase II in Saccharomyces cerevisiae. Instead of following progress into a subsequent cell cycle, we follow the viability of the cells. We find that cells become inviable when they undergo mitosis in the absence of topoisomerase II activity, and that inviability can be prevented by treatments that prevent progress through the cell cycle. Our results support the hypothesis that topoisomerase II is required to allow the normal segregation of sister chromatids at the time of mitosis.

Results

TOP2 Is the Structural Gene for Topoisomerase II

Before beginning in vitro mutagenesis, we confirmed that the structural gene for topoisomerase II identified by Goto and Wang (1984) corresponds to the locus identified by the temperature-sensitive mutation (*top2-1*) of DiNardo et al. (1984). We first determined that the cloned structural gene maps genetically to the *TOP2* locus. DiNardo et al. (1984) found that *TOP2* lies on chromosome XIV near *MET4*, and using the 2-micron plasmid mapping method of Falco and Botstein (1983), we showed that the cloned structural gene for topoisomerase II maps to the same location (see Experimental Procedures). We also showed that the cloned structural gene for topoisomerase II complements the temperature sensitivity of the *top2-1* mutant. When the cloned gene was integrated at the *URA3* locus of a temperature-sensitive *top2-1* strain, 7 of 8 transformants proved to be temperature-resistant. These results confirm that the structural gene for topoisomerase II is the same as the *TOP2* locus, and therefore, that mutagenesis of the cloned structural gene should allow the production of new alleles of *top2*.

Isolation of Temperature-Sensitive *top2* Mutants

New temperature-sensitive *top2* alleles were produced by the method of Shortle et al. (1984). Briefly, the method involves mutagenizing in vitro a plasmid carrying approximately 90% of the *TOP2* gene, and then integrating the mutagenized plasmid at the *TOP2* locus in yeast (Figure 1). Because 10% of the gene is missing from the plasmid, this integration event produces a strain bearing one intact copy of the *TOP2* gene and one disrupted copy; each copy is composed of sequences derived partly from the plasmid and partly from the chromosome. If a recessive mutation resides in the active copy of *top2*, its effect on the cells can be observed immediately. Thus, it was possible to screen transformants directly for the acquisition of temperature sensitivity. Among 15,000 yeast transformants, six transformants were temperature-sensitive on rich medium.

Five of these six temperature-sensitive transformants are temperature-sensitive *top2* mutants by four criteria (for details, see Experimental Procedures). First, all five fail to complement *top2-1*; when crossed with a *TOP2*$^+$ strain, they produce temperature-resistant diploids, but when crossed with a temperature-sensitive *top2-1* strain, they produce temperature-sensitive diploids. Second, crosses with a *TOP2*$^+$ strain show that all five temperature-sensitive mutations are linked to integrated plasmid sequences in the original transformants; in all tetrads examined, temperature-sensitive spores carry the *URA3*$^+$ allele from the plasmid, and temperature-resistant spores do not. Third, when excision of the plasmid from the original transformants is selected, the expected mixture of temperature-resistant and temperature-sensitive strains is produced (Figure 1); at least two temperature-resistant papillae were produced by each of the original temperature-sensitive transformants. Finally, the temperature-sensitive mutations in the strains from which the plasmid

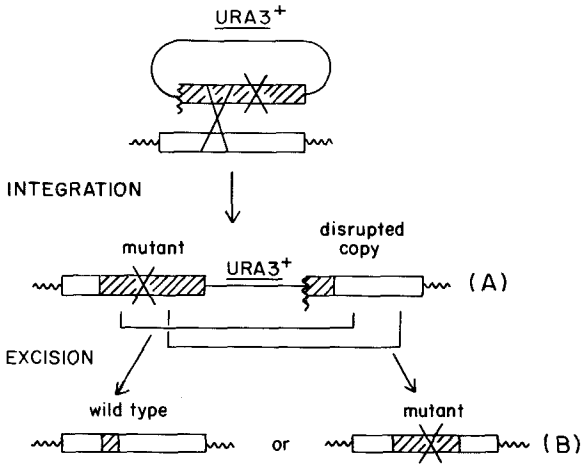

Figure 1. Construction of *top2* Mutants

A plasmid bearing a copy of the *TOP2* gene with a 5′ deletion is mutagenized in vitro (see Experimental Procedures). When it is introduced into yeast by transformation, integration of the plasmid frequently results in a transformant bearing an intact mutant *top2* gene and an inactive disrupted wild-type gene (A). Excision of the plasmid from this transformant can result in either a wild-type or mutant *top2* strain (B), depending on the exact position of the recombination event. Hatched bar represents *TOP2* sequences derived from the mutagenized plasmid; straight line denotes flanking plasmid sequences. Open bar indicates *TOP2* sequences derived from the chromosome; wavy line represents flanking chromosomal sequences.

is excised are tightly linked to *top2-1*; no recombination between *top2-1* and the new *top2* allele was seen in at least ten tetrads for each new mutant. Thus, we produced five new temperature-sensitive *top2* strains by in vitro mutagenesis. Temperature-sensitive strains from which the plasmid has been excised, plus *top2-1* strain SD1-4, were used for phenotypic studies.

Temperature-Sensitive *top2* Mutants Exhibit Cell-Cycle-Dependent Lethality

DiNardo et al. (1984) observed that although a synchronously dividing culture of *top2-1* cells arrests with a fairly uniform morphology at the restrictive temperature, an asynchronous population of cells does not. This behavior is not typical of genes that have been previously identified as being involved at a particular stage in the cell cycle; asynchronous cultures of cells bearing temperature-sensitive "cell division cycle" genes (*cdc* genes) arrest with a single terminal phenotype when held at the restrictive temperature (Hartwell et al., 1973). To test the possibility that this unusual behavior is an allele-specific effect of *top2-1*, we examined the morphology of asynchronous populations of cells bearing each of the six *top2* alleles (*top2-1* to *top2-6*). When held at the restrictive temperature for 3 hr (1.2–1.8 generations), none of the mutants exhibits a uniform terminal morphology; instead, each of the populations is a mixture of approximately equal numbers of unbudded cells and cells with large buds (data not shown). Thus, the mixture of morphologies observed at the restrictive temperature is not an allele-specific effect of *top2-1*.

Although *top2* mutants do not exhibit a uniform arrest

Table 1. Viability of α Factor-Treated Cultures After Incubation at the Restrictive Temperature

First Incubation*	Second Incubation*	CH325 (*top2-4*) Ratio of Viable Cells (35°C/26°C)	CH335 (*TOP2*⁺) Ratio of Viable Cells (35°C/26°C)
26°C	26°C 35°C	0.20	0.88
26°C + αf	26°C + αf 35°C + αf	0.96	0.76

* Each incubation was for 2 hr. "αf" is α factor.

morphology, it is still possible that topoisomerase II is required at a single point in the cell cycle. The heterogeneous arrest morphology of an asynchronous culture could simply be caused by heterogeneity in the cellular response to the failure of a single step in the cell cycle. To examine this possibility, it was necessary first to have some means of assessing the successful completion of the putative topoisomerase II-requiring step in the cell cycle. With *cdc* genes, the successful execution of the gene-product-dependent step is revealed by the ability of the cells to pass the terminal phenotype and traverse an additional cell cycle. This strategy was impractical for *top2* mutants, because they do not exhibit a distinct terminal morphology. Instead, a characteristic lethality proved to be the key in determining the time in the cell cycle at which topoisomerase II is required.

Temperature-sensitive *top2* strains rapidly become inviable when held at the restrictive temperature. Exponentially growing cultures of the six temperature-sensitive mutants and their temperature-resistant counterparts were incubated at 26°C or at 35°C for 3 hr (approximately 1½ generation times), and the cells were plated and incubated on rich medium at 26°C. All six temperature-sensitive *top2* mutants show 90% to 95% lethality when held at the restrictive temperature for 3 hr, whereas the control strains show no loss of viability at 35°C (data not shown). The short-term viability of the cells can therefore be used as a metric of topoisomerase II function in these strains.

Exponentially growing cells are both actively metabolizing and traversing the cell cycle, so the lethality observed in *top2* strains held at the restrictive temperature could be due to a failure of either of these processes. To determine whether lethality is due specifically to the failure of a cell-cycle event, we prevented actively metabolizing cells from traversing the cell cycle with α factor. α factor is a mating pheromone that causes yeast cells to arrest in an unbudded stage early in the cell cycle. The cells continue active metabolism and rapidly resume traversing the cell cycle if the α factor is washed away (Bucking-Throm et al., 1973).

Exponentially growing cells were treated with α factor for 2 hr to cause them to arrest at the beginning of the cell cycle. The culture was then split, and one portion was incubated at 35°C and the other at 26°C; each portion was then plated on rich medium at 26°C. The results in Table 1 show that whereas similar cultures that are not arrested with α factor exhibit 80% inviability, cultures arrested with

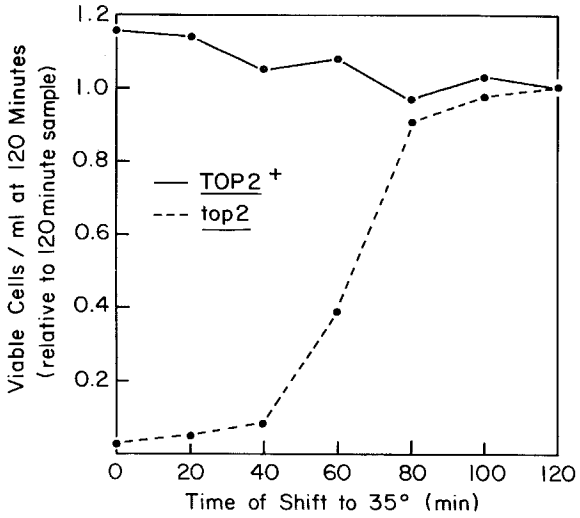

Figure 2. Viability of Synchronous Cultures Shifted from the Permissive to the Restrictive Temperature

Strain CH325 (*top2-4*) or CH335(*TOP2*⁺) was arrested with α factor for 3 hr and then was released into fresh medium at 26°C. Aliquots were shifted to 35°C at the indicated times, and the viability of each aliquot was assessed 120 min after the initial release of the cultures from α factor arrest.

α factor remain completely viable. This result suggests that the lethality caused by failure of topoisomerase II function is specific to the cell cycle, and not due to metabolism in the absence of topoisomerase II activity.

Topoisomerase II Is Required at the Time of Mitosis

To define the time of action of topoisomerase II in the cell cycle, we followed the viability of cells while they synchronously traversed the cell cycle. To determine the earliest time at which topoisomerase II can act to complete its function and prevent lethality, we shifted synchronous cultures from the permissive temperature to the restrictive temperature at various times. To determine the latest time at which topoisomerase II can act to prevent lethality, we shifted synchronously dividing cells from the restrictive temperature to the permissive temperature at various times. In addition, we examined the morphology of the cells from these experiments to correlate a particular biological process with the time of action of topoisomerase II.

Exponentially growing *TOP2*⁺ and *top2* strains were treated with α factor to arrest all of the cells as single unbudded cells. After 3 hr the α factor was washed away, and the cells were resuspended in fresh medium at 26°C. Approximately 75% of the cells treated in this way will traverse the cell cycle synchronously (see Experimental Procedures). Every 20 min after release from α factor, a small portion of the 26°C culture was shifted to 35°C. All of the aliquots were plated at 26°C to assess viability 120 min after release from α factor. Figure 2 shows that *TOP2*⁺ cells are unperturbed by this regimen; the number of viable cells per milliliter remains fairly constant, regardless of the time at which a sample is shifted from 26°C to 35°C. In contrast, *top2* cells show a substantial loss of viability if they are shifted to the restrictive temperature earlier than 60 to 80 minutes after release from α factor. This re-

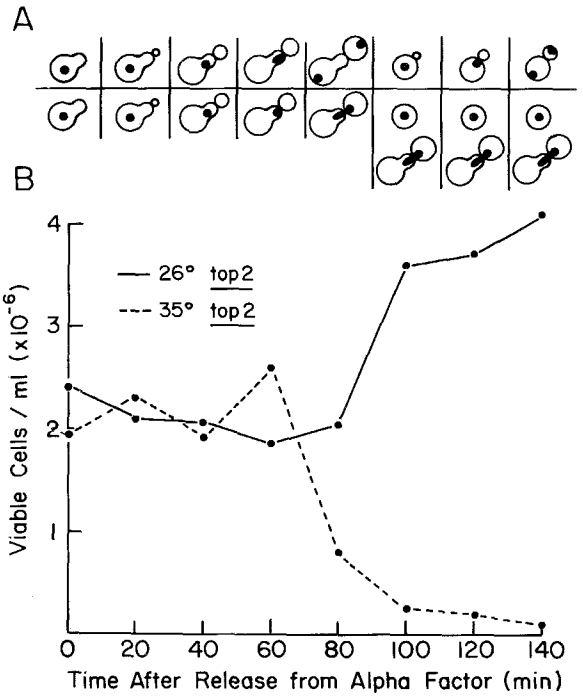

Figure 3. Morphology and Viability of Synchronous *top2* Cultures at the Permissive and at the Restrictive Temperatures

Strain CH325(*top2-4*) was arrested with α factor for 3 hr and then was released into fresh medium at 26°C or 35°C. Aliquots, which were removed every 20 min, were immediately plated for viability and fixed for fluorescence microscopy. (A) Schematic drawings of the most common cell type seen at each time point. (Photographs of representative cells are shown in Figure 4 and in Figure 5). Top: cellular and nuclear morphology of 26°C samples. Bottom: cellular and nuclear morphology of 35°C samples. The parent cells have a characteristic schmoo morphology as a result of their prior incubation with α factor. (B) Viability of cultures. At the indicated times, aliquots of the synchronously dividing cultures were removed and were plated at 26°C to assess viability.

sult implies that the earliest time at which the essential function of topoisomerase II is completed is 60 min after release from α factor.

To determine the latest time at which topoisomerase II can act to prevent lethality, we performed the reciprocal experiment. Cells were arrested for 3 hr in α factor and then were released into fresh medium at 35°C (and at 26°C as a control). Samples were shifted down to 26°C every 20 min, and they were plated immediately to assess viability. (A portion of the culture was also preserved with formaldehyde for later morphological examination). *TOP2*$^+$ cells give exactly the same pattern of viability, regardless of their initial temperature (i.e., 35°C or 26°C), and this pattern is indistinguishable from the pattern exhibited by *top2* cultures at 26°C (Figure 3B). At first the number of viable cells per milliliter stays fairly constant, while the initially unbudded α-factor-treated cells begin to bud. At 100 min, when most of the buds are released from the mother cells, the number of viable cells per milliliter doubles. In contrast, when *top2* cells are incubated at 35°C, their viability decreases rapidly 80 min after release from α factor. This result implies that 80 min after release from α factor is the latest time at which topoisomerase II can perform its essential function in order to prevent lethality.

To examine the biological processes occurring 60 to 80 min after release from α factor, we examined fixed cells from each of the time points in the previous experiment. Overall cell morphology was examined in the light microscope, nuclear morphology was determined by staining with 4′,6′-diamidino-2-phenylindole (DAPI), and microtubule morphology was examined using indirect immunofluorescence with anti-tubulin antibodies (Kilmartin and Adams, 1984). The three control populations (*TOP2*$^+$ at 26°C, *TOP2*$^+$ at 35°C, and *top2* at 26°C) are indistinguishable from one another. Figure 4 shows typical *top2* cells at 26°C from 40 to 100 min after release from α factor. At 60 min the cells are beginning to undergo mitosis; a mixture of cells with slightly elongated, fully elongated, and completely separated nuclei is seen. (Figure 4c shows cells with slightly elongated nuclei.) At 80 min the cells in these control populations appear to have completed mitosis; the population consists almost uniformly of cells with large buds containing completely separated nuclei.

In contrast, *top2* cells at the restrictive temperature do not appear to undergo a normal mitosis (Figure 5). While the cells are morphologically indistinguishable from the control cells for the first 40 min after release from α factor, at 60 min they begin to behave abnormally. Instead of exhibiting the mixture of nuclear morphologies expected during mitosis, at 60 min the mutant cells at the restrictive temperature exhibit a single morphology; they have large buds, and the rounded nucleus is wedged in the neck of the mother cell (Figure 5c). At 80 min, when the cells in the control populations appear to have completed mitosis, the mutant cells at restrictive temperature present an unusual nuclear morphology; the nucleus is partially elongated and somewhat diffuse (Figure 5e). Observation of later time points shows that the nuclei never appear to undergo normal separation; even when the bud is fully as large as the mother cell, nuclear material is still stretched through the neck. Thus, the onset of inviability in *top2* cells is correlated with an aberrant mitosis at restrictive temperature (Figure 3A).

Lethality Is Prevented When Mitosis Is Disrupted

The microtubule-destabilizing drug nocodazole allowed us to address the relationship between the topoisomerase II-requiring step in the cell cycle and the process of mitosis. Like the closely related drug benomyl, nocodazole inhibits assembly of tubulin in vitro (Kilmartin, 1981), and in vivo it causes cells to halt their progression through the cell cycle at mitosis (Zieve et al., 1980; Jacobs et al., 1984); indirect immunofluorescence shows that the mitotic spindle does not form in nocodazole-treated cells (Jacobs et al., 1984). Since the results of the temperature-shift experiments suggest that topoisomerase II also performs its essential function at the time of mitosis, the nocodazole-sensitive step and the topoisomerase II-requiring step should be very close to one another in the cell cycle.

There are two possible relationships between the topoisomerase II-requiring step and the nocodazole-sensitive step, and adding nocodazole to asynchronous

Table 2. Viability of Nocodazole-Treated Asynchronous Cultures After Incubation at the Restrictive Temperature

First Incubation*	Second Incubation*	CH325 (*top2–4*) Ratio of Viable Cells (35°C/26°C)	CH335 (*TOP2*+) Ratio of Viable Cells (35°C/26°C)
26°C	26°C 35°C	0.16	1.2
26°C	26°C + Noc. 35°C + Noc.	1.0	1.1

* Each incubation was for 2 hr. "Noc." is nocodazole.

top2 cells at the time they are shifted to restrictive temperature should distinguish between them. First, the topoisomerase II-requiring step could occur either before the nocodazole-sensitive step or independent of it; in this case adding nocodazole to cells when they are shifted to the restrictive temperature should not improve their survival at the restrictive temperature. Conversely, if the topoisomerase II-requiring step is dependent upon the nocodazole-sensitive step, adding nocodazole to cells when they are shifted to the restrictive temperature should prevent their progression to the topoisomerase II-requiring step; in this case the culture should exhibit very high viability. As can be seen in Table 2, the latter result is observed; even in an initially asynchronous population, the cells survive if they are treated with nocodazole when they are shifted to the restrictive temperature. This result is consistent with the hypothesis that the lethality observed in *top2* strains at the restrictive temperature is the direct result of a topoisomerase II-requiring step in mitosis.

Discussion

Analysis of temperature-sensitive *top2* mutants shows that topoisomerase II activity is required to allow yeast cells to traverse the cell cycle without incurring irreversible damage. Although 80% to 90% of growing cells become inviable when held at the restrictive temperature for 2 hr, lethality does not occur if the cells are prevented from passing through the cell cycle. Lethality is prevented whether the cells are prearrested with α factor or nocodazole, and these treatments arrest cells at widely separated points in the cell cycle. Thus, it is probably not a particular metabolic state, but rather the prevention of cycling that allows the cells to survive.

Results of experiments with synchronized cells show that topoisomerase II activity is essential only at one point in the cell cycle. Synchronized cells that are shifted from the restrictive to the permissive temperature become inviable if the shift is done more than 80 min after release from α factor, and synchronized cells that are shifted from the permissive to the restrictive temperature become inviable if the transfer is done less than 60 min after release from α factor. Taken together with the observation that mitosis occurs approximately 60–80 min after release from α factor, these results suggest that topoisomerase II performs an essential function at the time of mitosis. The results also show that the inactivation of topoisomerase II at other times in the cell cycle does not cause any irreversible effects, but they do not preclude the possibility that topoisomerase II has other roles in either metabolism or in the cell cycle. It is possible that all six *top2* mutations affect a single domain of topoisomerase II, and that the mutant enzymes perform some functions normally. Also, although *top2-1* strains are temperature-sensitive for topoisomerase II activity in vitro (DiNardo et al., 1984), it is possible that the effects observed in vivo are consequences of a temperature-sensitive structural requirement at the time of mitosis; for example, topoisomerase II could be part of an essential structure that can be assembled only at mitosis, and the mutants could be temperature sensitive for this assembly process. Finally, the conclusions presented here apply only to the product of the *TOP2* gene; any type II topoisomerases that remain to be discovered in yeast could obviously exhibit different properties. With these caveats, however, the evidence nonetheless indicates that topoisomerase II performs an essential function at the time of mitosis.

The temporal correlation between topoisomerase II-induced lethality and mitosis is extended by the observation that a drug that prevents mitosis also prevents lethality. An asynchronous population of cells remains completely viable if it is treated with nocodazole when it is initially shifted to the restrictive temperature. It is possible that this effect is due to secondary effects of the drug, but it is striking that the cells are rescued without prior incubation. This result suggests that the lethality observed in the absence of topoisomerase II activity is dependent upon a process requiring microtubules. An obvious candidate for this process is chromosome segregation.

Evidence that topoisomerase II is required for the segregation of circular DNA molecules has been found in both SV40 and in yeast. Replicating SV40 DNA molecules accumulate as multiply intertwined catenated dimers when cells are grown in hypertonic medium (Sundin and Varshavsky, 1980; 1981), and it has been suggested that this phenomenon is the result of the inactivation of a type II topoisomerase in the cells (Sundin and Varshavsky, 1981). Sundin and Varshavsky proposed that bidirectional replication of SV40 DNA is initially facilitated by a "swivelase" that relieves the positive supercoils that would otherwise accumulate ahead of the replication forks. However, the replication complexes sterically exclude the postulated swivelase when the two replication forks approach within 200 bp of each other opposite the origin of replication on the circular molecule. Thus, the twist of the helix is converted into intertwinings of the daughter molecules as the last 200 bp of DNA are replicated. The results observed with the temperature-sensitive *top2-1* mutant of yeast are entirely consistent with this model (DiNardo et al., 1984); the circular plasmid 2-micron DNA accumulates as multiply intertwined catenated dimers when cells are held at the restrictive temperature.

It has been suggested that long linear chromosomal DNA may be subject to topological constraints similar to those of circular DNA molecules (Hsieh and Brutlag, 1980; Sundin and Varshavsky, 1981; DiNardo et al., 1984). The linear chromosomes might be organized into multiple

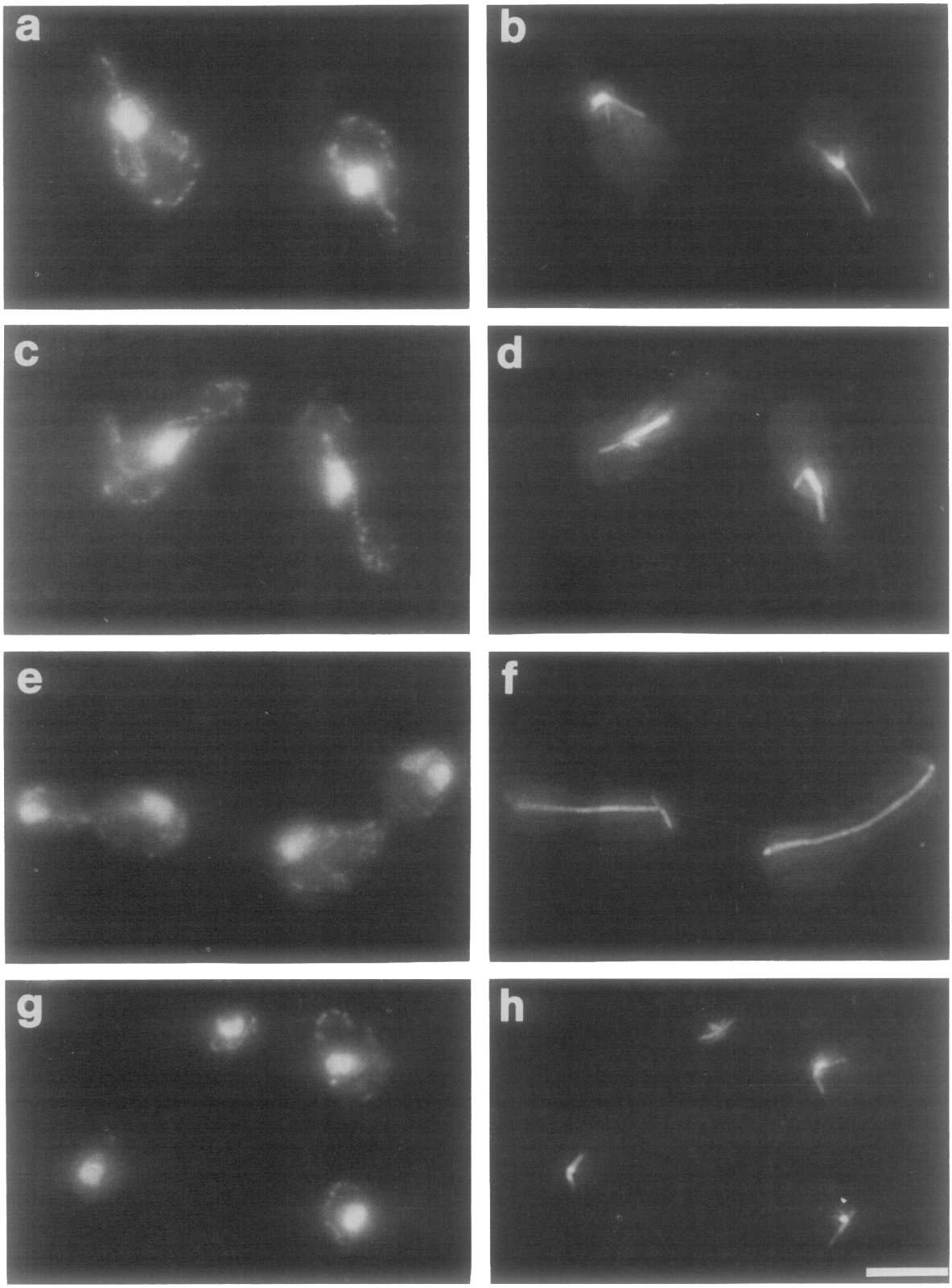

Figure 4. Fluorescence Micrographs of *top2* Cells at the Permissive Temperature

Strain CH325(*top2-4*) was arrested with α factor for 3 hr and then was released into fresh medium at 26°C. Cells were fixed and prepared for immunofluorescence at 40 min (a and b), 60 min (c and d), 80 min (e and f), and 100 min (g and h) after release from α factor. Left (a, c, e, and g), DAPI staining reveals cellular DNA: the large fluorescent mass is the nuclear DNA, and the smaller bright dots are mitochondrial DNA. Right (b, d, f, and h), the same cells were photographed with fluorescence filters set to reveal indirect immunofluorescence of tubulin. Bar represents five microns.

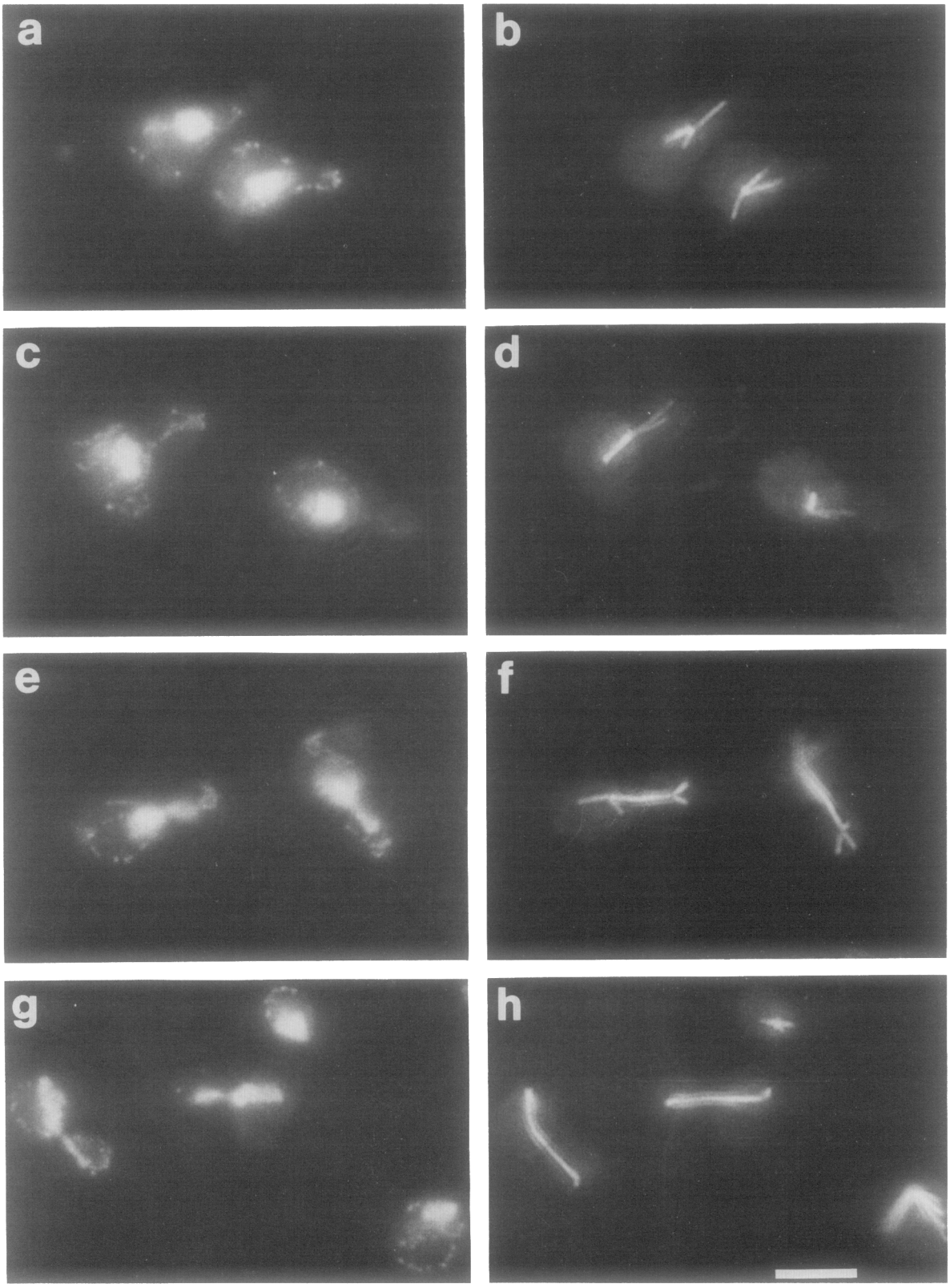

Figure 5. Fluorescence Micrographs of *top2* Cells at the Restrictive Temperature

Cultures of strain CH325(*top2-4*) were treated as in Figure 4, except that they were incubated at 35°C after release from α factor. Samples are from aliquots taken 40 min (a and b), 60 min (c and d), 80 min (e and f), and 100 min (g and h) after release from α factor. Left (a, c, e, and g), DAPI staining reveals cellular DNA. Right (b, d, f, and h), indirect immunofluorescence of tubulin is shown for the same cells. Bar represents five microns.

loops, or the extreme length of the chromosomes may make impossible the rotation of one sister chromatid about the other. Thus, it may be necessary to cleave the DNA backbone to resolve the intertwinings produced at the sites of convergence of multiple replication forks. Topoisomerase II activity may therefore be required at each replicon to disentangle sister chromatids after DNA replication (Sundin and Varshavsky, 1981; DiNardo et al., 1984). As described in the literature, this activity could occur any time in S phase, G2 phase, or early mitosis.

The evidence presented here is consistent with this model, and it further suggests that the essential function of topoisomerase II can be completed only at the time of mitosis. Perhaps there is a process that occurs during mitosis that is required for completion of the action of topoisomerase II. Because topoisomerase II both catenates and decatenates DNA (Baldi et al., 1980; Hsieh and Brutlag, 1980; Kreuzer and Cozzarelli, 1980), it is possible that the structure or movement of the mitotic chromosomes provides the directionality for the reaction. Stated simply, it may not be possible for topoisomerase II to distinguish a reaction that leads to increased intertwining from one that leads to decreased intertwining until chromosome packaging or the spindle itself provides a directional force.

Regardless of the reason that topoisomerase II completes its function late in the cell cycle, it is clear that when cells pass through mitosis without prior activity of topoisomerase II, they incur irreversible damage. The high viability of asynchronous cultures shifted to the restrictive temperature in the presence of nocodazole suggests that as long as a microtubule-mediated process (presumably chromosome segregation) is prevented, lethality is also prevented. Thus, it appears that it is the attempt by the cell to segregate sister chromatids in the absence of topoisomerase II activity that leads to inviability. Such inviability could be due to either chromosome breakage or nondisjunction during anaphase of mitosis. Consistent with this idea, Hartwell (personal communication) has found a 30-fold increase in chromosome loss in the temperature-sensitive *top2-1* mutant at a semipermissive temperature.

The rapid lethality observed in *top2* mutants at the restrictive temperature explains a minor discrepancy of interpretation between the results presented here and those presented by DiNardo et al. (1984). DiNardo et al. (1984) found that when an asynchronous population of *top2-1* cells was shifted to the restrictive temperature, the cells did not exhibit a uniform terminal morphology, and they suggested therefore that topoisomerase II might be required at more than one point in the cell cycle. We, too, observe that when an asynchronous population of temperature-sensitive *top2* cells is shifted to the restrictive temperature, the result is a mixture of unbudded cells and cells with large buds. However, our observations of synchronized cells show that the large budded cells appear first, and we suggest that the unbudded cells derive from a fraction of inviable large budded cells that are nonetheless capable of undergoing a single round of cytokinesis. Observations of micromanipulated cells reveal that the majority of cells of either morphology is incapable of budding even once (unpublished data), and it seems likely that both cell types represent the products of a single lethal event. Therefore, we conclude that topoisomerase II is required at a single point in the cell cycle (mitosis), but that the cells do not respond uniformly to its inactivity.

Hartwell et al. (1973) suggested that some cell-cycle genes might not yield mutant strains that exhibit the single terminal morphology characteristic of cell division cycle (*cdc*) mutants. The defining characteristic of a cell-cycle mutation is that the gene product is required at a particular time in cell cycle to allow normal progression from that point onward. In the case of *cdc* mutants, the execution point is defined as, "the time in the cell division cycle when the temperature sensitive gene product completes its function at the permissive temperature" (Hartwell, 1971), and the assay for completion of function is the ability of the cell to complete a second cell cycle. Using the same definition, we find that the execution point for topoisomerase II is the time of mitosis; however, we have had to use loss of viability in place of terminal morphology as the assay for completion of function. Similarly, Thomas and Botstein (unpublished data) used production of diploidy in a haploid strain to show that *ndc1* is a cell-cycle mutant. Thus, we propose that any distinctive deviation from normal behavior can be used as an assay for the completion of a cell-cycle-specific event. We believe that such expanded criteria would allow the definition of new types of genes that are specific to the cell cycle.

Experimental Procedures

Strains, Plasmids, and Media

Yeast strains used in this study are listed in Table 3. E. coli strain DB6507 was derived from HB101 and is *leu⁻ pro⁻ thr⁻* r⁻ m⁻ *recA⁻ pyrF74*::Tn5; it comes from this laboratory. HB101(LM1035)MM294 (also called DB1142) was also derived from HB101 and is *leu⁻ pro⁻ thr⁻* r⁻ m⁻ *recA⁻*; it was obtained from Connie Cepko at M.I.T.

Plasmid pCH503 was constructed by inserting the 1.9 kb Hind III fragment of the *TOP2* gene (Goto and Wang, 1984) into YEp24 (Botstein et al., 1979). Plasmid pCH510 was constructed by inserting the 4 kb Bgl II fragment of *TOP2* (Goto and Wang, 1984) into the Bam HI site of YIp5 (Scherer and Davis, 1979).

YEPD liquid medium is 10 g/L yeast extract (Difco), 20 g/L Bactopeptone (Difco) and 2% glucose. For solid medium, 20 g/L Bacto-agar (Difco) are added. For pH 4 liquid medium, the pH is adjusted to 4.0 with HCl. YEPG medium is the same as YEPD, except that the carbon source is 3% glycerol. Selective ("C-ura") plates are 6.7 g/L Yeast Nitrogen Base without amino acids (Difco), 20 mg/L histidine, 30 mg/L lysine, 0.03% charcoal filtered casamino acids (Difco), 2% glucose, and 2% agar. C-ura plates were supplemented when necessary with 20 mg/L uracil. Plates used to select against *URA3*⁺ (Boeke et al., 1984) contained 0.5 mg/ml 5-fluoro-orotic acid, 6.7 mg/ml Yeast Nitrogen Base without amino acids (Difco), 5.5 μg/ml uracil, 30 μg/ml lysine, 18 μg/ml histidine, 2% glucose, and 2% agar.

Mapping the Topoisomerase II Structural Gene to Chromosome XIV

To confirm that the locus *top2* corresponds to the structural gene for topoisomerase II, we mapped the cloned structural gene using the 2-micron plasmid mapping method of Falco and Botstein (1983). Integration of plasmid pCH503 was directed to the structural gene for topoisomerase II by cutting it at the unique Kpn I restriction site (Orr-Weaver et al., 1981). Six haploid transformants of strain DBY703 were each crossed with four well-marked haploid strains (K382-23A, K396-11A, K398-4D, and K399-7D). Diploids were selected, streaked for single colonies on YEPD, and then plated for single colonies on YEPD.

Table 3. Yeast Strains Used in This Study

Strain	Genotype	Source or Reference
CH322	*MATa his4-539 lys2-801 ura3-52 top2-2*	This Study
CH323	*MATa his4-539 lys2-801 ura3-52 top2-3*	This Study
CH325	*MATa his4-539 lys2-801 ura3-52 top2-4*	This Study
CH326	*MATa his4-539 lys2-801 ura3-52 top2-5*	This Study
CH327	*MATa his4-539 lys2-801 ura3-52 top2-6*	This Study
CH335	*MATa his4-539 lys2-801 ura3-52 TOP2*$^+$	This Study
CH336	*MATa his4-539 lys2-801 ura3-52 TOP2*$^+$	This Study
DBY703	*MATα his3 trp1 ura3-52*	M.I.T.
DBY947	*MATα ade2-101 ura3-52 TOP2*$^+$	M.I.T.
DBY1034	*MATa his4-539 lys2-801 ura3-52 TOP2*$^+$	M.I.T.
DBY1480	*MATa his4-539 lys2-801 ura3-52* / *MATα* + + *ura3-52*	M.I.T.
K382-23A	*MATa spo11 ura3 can1 cyh2 ade2 his7 hom3*	Klapholz and Esposito, 1982
K396-11A	*MATa spo11 ura3 ade1 his1 leu2 lys7 met3 trp5*	Klapholz and Esposito, 1982
K398-4D	*MATa spo11 ura3 ade6 arg4 aro7 asp5 met14 lys2 pet17 trp1*	Klapholz and Esposito, 1982
K399-7D	*MATa spo11 ura3 his2 leu1 lys1 met4 pet8*	Klapholz and Esposito, 1982
SD1-1	*MATα ade2 top2-1*	DiNardo et al., 1984
SD1-4	*MATa ade2 ura3-52 top2-1*	DiNardo et al., 1984

Loss of the chromosome arm distal to the structural gene for topoisomerase II was detected by the appearance of an auxotrophy when the diploids were replica-plated to minimal medium. Although uracil auxotrophs were produced by all the diploid strains, only in strains in which K399-7D was one of the parents was there an additional auxotrophy. The 5 Ura$^-$ colonies that were tested from each of the six diploids proved to require methionine. This result shows that the structural gene for topoisomerase II maps to the left arm of chromosome XIV, between *met4* and its centromere. Thus, its map position corresponds to that of *top2* (DiNardo et al., 1984).

TOP2 Is an Essential Gene

To confirm the boundaries of the structural gene for topoisomerase II (Goto and Wang, 1984) and to confirm that it is essential (Goto and Wang, 1984; DiNardo et al., 1984), we disrupted the gene using the method of Shortle et al. (1982b). A plasmid containing deletions of the 5′ and 3′ ends of the gene (pCH503) was constructed by inserting the Hind III fragment of *TOP2* into the Hind III site of YIp5. The plasmid was cut with Kpn I to direct integration to *TOP2*, or with Sma I to direct integration to *ura3*; the cut plasmids were used to transform diploid strain DBY1480 to uracil prototrophy. Integration of the Kpn I-cut plasmid should result in the disruption of one copy of *top2* in the diploid; as expected, when this strain was sporulated, the tetrads exhibited 2:2 lethality that was linked to the *URA3* gene from the plasmid. There were 28 tetrads with 2 viable Ura$^-$ spores, 3 with 1 viable Ura$^-$ spore, and 3 with no viable spores. In contrast, the control integration of the plasmid, which was directed to *ura3*, showed no such effect. There were 22 tetrads with 4 viable spores (2 Ura$^+$: 2 Ura$^-$), 2 with 3 viable spores (4 Ura$^+$ and 2 Ura$^-$), 8 with 2 viable spores (9 Ura$^+$ and 7 Ura$^-$), and 1 with no viable spores. Thus, *top2* is an essential gene, and its 5′ and 3′ boundaries lie outside the Hind III sites.

Production of New *top2* Mutants

The integrative replacement/disruption method (Shortle et al., 1984) was used to produce five new temperature-sensitive alleles of *top2* in yeast. In this method a plasmid is constructed bearing a gene with a small deletion at either the 5′ or the 3′ end. The plasmid is subjected to in vitro mutagenesis and then integrated into the yeast chromosome. Integration takes place so as to yield one active and one inactive copy of the gene (see Figure 1A), and some fraction of the time the active copy contains mutagenized DNA. Thus, the integrants may be screened directly for the desired phenotype. Once the mutants are found, their mutations may be stabilized by excising the plasmid (see Figure 1B).

Plasmid pCH510, which contains a *TOP2* gene with a small deletion of its 5′ end, was subjected to in vitro mutagenesis before introducing it into yeast. A single random nick was introduced into the plasmid with 0.1 μg/ml Pancreatic DNAase I in the presence of 150 μg/ml ethidium bromide, 50 mM Tris (pH 7.5), and 5 mM $MgCl_2$. The nicks were enlarged into gaps, and the gaps were misrepaired by the misincorporation method of Shortle et al. (1982a). The misrepair reactions and subsequent DNA isolations were done in eight separate pools to ensure that the mutants ultimately recovered would be independent. To check the level of mutagenesis before transforming yeast cells, a sample was taken from each of the eight pools, and the mixture was used to transform E. coli strain DB6507, selecting for ampicillin resistance. The E. coli transformants were then tested by replica plating for the presence of an active *URA3* gene. (The *URA3* gene of the plasmid complements the *pyrF* mutation in DB6507). Uracil auxotrophs appeared among the cells transformed with the mutagenized pool at a frequency of 2.1% (23/1085); none of the cells transformed with control unmutagenized plasmids were uracil auxotrophs (0/1163).

Ten samples of yeast strain DBY1034 were transformed separately with the eight mutagenized pools of pCH510 and with two samples of pCH510 that had not been subjected to mutagenesis; integration was directed to *TOP2* by cutting with Kpn I (Orr-Weaver et al., 1981). Twenty thousand transformants were recovered on 100 C-ura plates at 26°C, and these plates were each replica plated to YEPG(26°C), C-ura(37°C), and C-ura(26°C) plates. After one day, growth on these three plates were compared, and 20 potential temperature-sensitive strains were recovered. Eleven retested as temperature sensitive on C-ura plates; none of these eleven derive from cells transformed with the control unmutagenized plasmid. Five of the 11 temperature-sensitive mutants are temperature-sensitive for uracil prototrophy; they are temperature-sensitive on C-ura plates, but are temperature resistant on YEPD plates or on C-ura plates supplemented with uracil. The six remaining strains, which derive from five separate pools, are temperature-sensitive on both YEPD plates and C-ura plates.

Results of complementation and tetrad analysis show that five of these six temperature-sensitive strains are temperature-sensitive *top2* mutants. All six mutations are recessive to *TOP2*$^+$ (strain DBY947) for temperature sensitivity, and five fail to complement *top2-1* (strain SD1-1). These same five mutations are linked to integrated plasmid sequences, as is expected from their origin if they are *top2* mutations (see Figure 1A); when crossed with *TOP2*$^+$ strain DBY947, uracil prototrophy (from plasmid pCH510) segregates 2:2 with temperature sensitivity in six tetrads from each mutant. The sixth mutant proved to carry an unlinked mutation, and it was not investigated further.

Excision of plasmid sequences was selected by growing the primary transformants on 5-fluoro-orotic acid plates to select against *URA3* (Boeke et al., 1984). As expected for *top2* mutations created by this method (see Figure 1B), the mutant strains produced a mixture of temperature-sensitive and temperature-resistant papillae on 5-fluoro-orotic acid plates. For the various alleles, the number of temperature-sensitive and temperature-resistant papillae produced are as follows: *top2-2*, 5 Ts$^+$/2 Ts$^-$; *top2-3*, 6 Ts$^+$/2 Ts$^-$; *top2-4*, 4 Ts$^+$/2 Ts$^-$; *top2-5*, 5 Ts$^+$/1 Ts$^-$; *top2-6*, 2 Ts$^+$/5 Ts$^-$. (The numbers of papillae tested are too small to allow conclusions to be drawn about the positions of these muta-

tions). One temperature-sensitive and one temperature-resistant papilla were recovered for each transformant; the temperature-resistant strains served as controls for later experiments. The strains produced are listed in Table 3. Strains CH322 and CH323 derive from a single pool of mutagenized plasmid, so they could represent two different isolates of the same allele. We believe that this is unlikely, however, because CH323 is capable of growth at higher temperatures than is CH322 (data not shown).

The final demonstration that the new temperature-sensitive mutations are indeed *top2* mutations is that each is tightly linked to *top2-1*. When crossed with *TOP2*$^+$ strain DBY947, each of the new mutants shows 2:2 segregation for temperature sensitivity, which shows that temperature sensitivity is conferred by a single nuclear gene. (At least 10 tetrads were dissected for each mutant.) When crossed with *top2-1* strain SD1-1, all of the mutants produce tetrads that show 4:0 segregation for temperature sensitivity, which shows that the temperature sensitivity maps to the *top2* locus. (At least 10 tetrads were examined for each mutant.)

Temperature Shift Experiments

For both shift-down and shift-up experiments, strains CH325 (*top2-4*) and CH326 (*top2-5*) were both tested with similar results. Exponentially growing cultures were first synchronized with α factor (Sigma) (Bucking-Throm et al., 1973). Cells were grown with shaking at 26°C in YEPD (pH 4) liquid medium, and α factor was added to a final concentration of 5 μg/ml. After 3 hr (approximately 1½ generations), the cells were rapidly filtered, washed with fresh 26°C medium, and resuspended in medium at 26°C.

For shift-up experiments, the synchronized culture was returned to 26°C after removal of α factor. Every 20 min for the next 2 hr, a 5 ml aliquot was transferred to a flask in a 35°C shaking water bath. At 120 min after release from α factor, each of the aliquots was sonicated briefly to separate the cells, and was then diluted and plated on 3 YEPD plates at 26°C. After 3 days of growth, the colonies on each plate were counted; this number was used to calculate the number of viable cells in the original liquid culture.

For shift-down experiments, after release from α factor, a synchronized culture was split into two portions, one that was placed at 26°C, and one placed at 35°C. Samples were taken from each culture every 20 min for 2 hr. The samples were immediately sonicated, and an aliquot of each sample was made 4% in formaldehyde to preserve it for later observation. The remainder of each sample was immediately diluted and plated on 3 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.

The degree of synchrony in these cultures was assessed in two ways. First, the morphologies of the cells in each sample were examined and were tallied. Over 90% of the cells are unbudded immediately after release from α factor. Twenty minutes later, the cells exhibit sharp points, which can be distinguished as very small buds if the cell fortuitously lies at a good angle for viewing. Forty minutes after release from α factor is the first time at which the presence of a bud can be reliably scored. At this point, 75% of the cells have small buds. This observation suggests that approximately 75% of the cells synchronously begin to traverse the cell cycle.

The degree of synchrony later in the cell cycle can be determined by observing the synchrony with which cytokinesis occurs. If all the cells in the culture divide in unison, then the number of viable cells per milliliter should suddenly increase by 100% immediately after cytokinesis. As can be seen in Figure 3, cytokinesis occurs in these cultures between 100 and 120 min after release from α factor. The average increase in viable counts (from observations of four cultures) in this interval is 74%. Thus, within the limit of the time intervals used in these experiments, approximately 75% of the cells synchronously traverse the first cell cycle after release from α factor.

It is also possible to assess the degree to which incubating cells at 35°C increases the rate of the cell cycle over incubating them at 26°C. By observing the morphology of cells in synchronous cultures split into two portions and incubated at 26°C or 35°C, we find that the cells in the 26°C culture lag only slightly behind the cells in the 35°C culture. By observing the average time of cytokinesis, we estimate that at 100 min after release from α factor the 26°C culture lags at most 10 min behind the 35°C culture. Thus, the error introduced by directly comparing 26°C cultures with 35°C cultures is probably at most 10%.

Cell Cycle Experiments

The effects of α factor and nocodazole on the viability of asynchronous cultures at the restrictive temperature were tested as described below. The experiments with α factor (5 μg/ml; Sigma) and nocodazole (20 μg/ml; Sigma) had the same form, so only the nocodazole experiment will be described. Strains CH325(*top2-4*) and CH326(*top2-5*) were both tested and gave similar results.

A culture growing exponentially at 26°C in YEPD (pH 4) was split into two portions, nocodazole was added to one, and both were returned to 26°C. After 2 hr (approximately one doubling time), the nocodazole-containing culture was split into two, and the cells were filtered out of one portion, washed, and resuspended in fresh medium without nocodazole. At the same time, the culture that had been growing without nocodazole for 2 hr was split, and nocodazole was added to one portion. Thus, 2 hr after the beginning of treatment, there were four cultures that had been treated with nocodazole in various combinations. At this point, each of the four cultures was split into two portions, and one was incubated at 26°C and one at 35°C for the next 2 hr. All eight cultures were then sonicated and plated for viability as described above. The viability of cultures at 26°C and 35°C was compared.

Immunofluorescence, Microscopy, and Photography

Treatment of cells for immunofluorescence was essentially as described for formaldehyde fixation by Kilmartin and Adams (1984), except that the cell walls were removed by incubation at 30°C for 1 hr with 50 μg/ml Zymolyase 60000 in 1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5), and 25 mM 2-mercaptoethanol. Monoclonal anti-tubulin antibody was obtained from Accurate Scientific, and rhodamine-conjugated secondary antibody (anti-rat IgG) was obtained from Miles Laboratories. The mounting medium contained 0.2 μg/ml 4′,6′-diamidino-2-phenylindole 2HCl (DAPI).

A Zeiss microscope equipped for epifluorescence was used for viewing the cells and for photography. It contained rhodamine selective filter set 487715-9902 and DAPI selective filter set 487702-7960. A Zeiss Neofluor 100× N.A. 1.3 objective was used for both phase contrast and epifluorescence. Kodak Tri-X Pan film was used for photography; it was developed with Microdol X developer (effective ASA 400).

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References

Baldi, M. I., Benedetti, P., Mattoccia, E., and Tocchini-Valentini, G. P. (1980). In vitro catenation and decatenation of DNA and a novel eucaryotic ATP-dependent topoisomerase. Cell *20*, 461–467.

Boeke, J. D., Lacroute, F., and Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5′-phosphate decarboxylate activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. *197*, 345–347.

Botstein, D., Falco, S. C., Stewart, S. E., Brennen, M., Scherer, S., Stinchcomb, D. T., Struhl, K., and Davis, R. W. (1979). Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene *8*, 17–24.

Brown, P. O., and Cozzarelli, N. R. (1979). A sign inversion mechanism for enzymatic supercoiling of DNA. Science *206*, 1081–1083.

Bucking-Throm, E., Duntze, W., Hartwell, L. H., and Manney, T. R. (1973). Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. Exp. Cell Res. *76*, 99–110.

DiNardo, S., Voelkel, K. A., Sternglanz, R., Reynolds, A. E., and Wright, A. (1982). Escherichia coli DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell *31*, 43–51.

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

Falco, S. C., and Botstein, D. (1983). A rapid chromosome-mapping method for cloned fragments of yeast DNA. Genetics *105*, 857–872.

Gellert, M. (1981). DNA topoisomerases. Ann. Rev. Biochem. *50*, 879–910.

Gellert, M., Mizuuchi, K., O'Dea, M. H., and Nash, H. A. (1976a). DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA *73*, 3872–3876.

Gellert, M., O'Dea, M. H., Itoh, T., and Tomizawa, J. (1976b). Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA *73*, 4474–4478.

Goto, T., and Wang, J. C. (1982). Yeast DNA topoisomerase II: an ATP dependent type II topoisomerase that catalyzes the catenation, decatenation, unknotting, and relaxation of double-stranded DNA rings. J. Biol. Chem. *257*, 5866–5872.

Goto, T., and Wang, J. C. (1984). Yeast DNA topoisomerase II is encoded by a single-copy, essential gene. Cell *36*, 1073–1080.

Hartwell, L. H. (1971). Genetic control of the cell division cycle in yeast. II. Genes controlling DNA replication and its initiation. J. Mol. Biol. *59*, 183–194.

Hartwell, L. H., Mortimer, R. K., Culotti, J., and Culotti, M. (1973). Genetic control of the cell division cycle in yeast: V. Genetic analysis of *cdc* mutants. Genetics *74*, 267–286.

Hsieh, T. (1983). Knotting of the circular duplex DNA by type II DNA topoisomerase from Drosophila melanogaster. J. Biol. Chem. *258*, 8413–8420.

Hsieh, T., and Brutlag, D. (1980). ATP-dependent DNA topoisomerase from D. melanogaster reversibly catenates duplex DNA rings. Cell *21*, 115–125.

Jacobs, C. W., Adams, A. E. M., and Pringle, J. R. (1984). Bud formation in yeast does not require cytoplasmic microtubules. J. Cell Biol. *99*, 43a.

Kilmartin, J. V. (1981). Purification of yeast tubulin by self-assembly in vitro. Biochemistry *20*, 3629–3633.

Kilmartin, J. V., and Adams, A. E. M. (1984). Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. J. Cell Biol. *98*, 922–933.

Klapholz, S., and Esposito, R. (1982). A new mapping method employing a meiotic rec$^-$ mutant of yeast. Genetics *100*, 387–412.

Kreuzer, K. N., and Cozzarelli, N. R. (1980). Formation and resolution of DNA catenanes by DNA gyrase. Cell *20*, 245–254.

Liu, L., Liu, C.-C., and Alberts, B. M. (1980). Type II DNA topoisomerases: enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break. Cell *19*, 697–707.

Liu, L. F., Davis, J. L., and Calendar, R. (1981). Novel topologically knotted DNA from bacteriophage P4 capsids: studies with DNA topoisomerases. Nucl. Acids Res. *9*, 3979–3989.

Miller, K. G., Liu, L. F., and Englund, P. T. (1981). A homogeneous type II DNA topoisomerase from HeLa cell nuclei. J. Biol. Chem. *256*, 9334–9339.

Orr-Weaver, T. L., Szostak, J. W., Rothstein, R. J. (1981). Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA *78*, 6354–6358.

Pruss, G. J., Manes, S. H., and Drlica, K. (1982). Escherichia coli topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell *31*,. 35–42.

Scherer, S., and Davis, R. W. (1979). Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Natl. Acad. Sci. USA *76*, 4951–4955.

Shortle, D., Grisafi, P., Benkovic, S. J., and Botstein, D. (1982a). Gap misrepair mutagenesis: efficient site-directed induction of transition, transversion, and frameshift mutations in vitro. Proc. Natl. Acad. Sci. USA *79*, 1588–1592.

Shortle, D., Haber, J. E., and Botstein, D. (1982b). Lethal disruption of the yeast actin gene by integrative DNA transformation. Science *217*, 371–373.

Shortle, D., Novick, P., and Botstein, D. (1984). Construction and genetic characterization of temperature-sensitive mutant alleles of the yeast actin gene. Proc. Natl. Acad. Sci. USA *81*, 4889–4893.

Sundin, O., and Varshavsky, A. (1980). Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. Cell *21*, 103–114.

Sundin, O., and Varshavsky, A. (1981). Arrest of segregation leads to accumulation of highly intertwined catenated dimers: dissection of the final stages of SV40 DNA replication. Cell *25*, 659–669.

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

Zieve, G. W., Turnbull, D., Mullins, J. M., and McIntosh, J. R. (1980). Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Exp. Cell Res. *126*, 397–405.