The essential role of yeast topoisomerase III in meiosis depends on recombination

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Yeast cells mutant for *TOP3*, the gene encoding the evolutionary conserved type I-5' topoisomerase, display a wide range of phenotypes including altered cell cycle, hyper-recombination, abnormal gene expression, poor mating, chromosome instability and absence of sporulation. In this report, an analysis of the role of *TOP3* in the meiotic process indicates that *top3Δ* mutants enter meiosis and complete the initial steps of recombination. However, reductional division does not occur. Deletion of the *SPO11* gene, which prevents recombination between homologous chromosomes in meiosis I division, allows *top3Δ* mutants to form viable spores, indicating that Top3 is required to complete recombination successfully. A topoisomerase activity is involved in this process, since expression of bacterial TopA in yeast *top3Δ* mutants permits sporulation. The meiotic block is also partially suppressed by a deletion of *SGS1*, a gene encoding a helicase that interacts with Top3. We propose an essential role for Top3 in the processing of molecules generated during meiotic recombination.
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Introduction

Topoisomerases play a crucial role in cellular metabolism (for a review see Dugué, 1997), and variations in topoisomerase expression levels result in pleiotropic phenotypes in bacteria and yeast (Kim and Wang, 1989; Wallis *et al.*, 1989; Drlica, 1990). DNA topoisomerases are essential for the replication of DNA molecules (reviewed in Wang, 1996), for chromosome condensation (Adachi *et al.*, 1991; Downes *et al.*, 1994; Castano *et al.*, 1996) and for the proper segregation of chromosomes at mitosis and meiosis (DiNardo *et al.*, 1984; Holm *et al.*, 1985, 1989; Rose *et al.*, 1990). Another fundamental role for topoisomerases involves the relaxation of positive and negative superhelical domains of DNA that are generated by transcription (Liu and Wang, 1987; Giaever and Wang, 1988; Wu *et al.*, 1988; Tsao *et al.*, 1989). Additionally, the regulation of gene expression for many functions is dependent on

the degree of supercoiling found in intracellular DNA (Gangloff *et al.*, 1994a; Wang and Droge, 1996). Recently, a role for topoisomerases I and II in checkpoint control was proposed (Downes *et al.*, 1994; Castano *et al.*, 1996).

In *Saccharomyces cerevisiae*, one mitochondrial and three nuclear topoisomerase activities have been described. Among the nuclear activities, DNA topoisomerases I and II (encoded by the *TOP1* and *TOP2* genes, respectively) are capable of relaxing both negatively and positively supercoiled DNA molecules (Goto and Wang, 1984; Thrash *et al.*, 1984), while DNA topoisomerase III (encoded by the *TOP3* gene) weakly relaxes only negatively supercoiled DNA (Kim and Wang, 1992). Little is known about the recently identified mitochondrial activity (Ezekiel *et al.*, 1994). The genes encoding the nuclear activities (*TOP1*, *TOP2* and *TOP3*) have been cloned (Goto and Wang, 1984, 1985; Thrash *et al.*, 1985; Wallis *et al.*, 1989) and studied extensively (for reviews see Champoux, 1994; Gangloff *et al.*, 1994a; Hsieh *et al.*, 1994; Lima and Mondragon, 1994; Watt and Hickson, 1994; Roca, 1995; Wang, 1996).

The *TOP3* gene is unique in that it encodes a type I-5' topoisomerase in yeast. It is homologous to the *Escherichia coli* *topA* and *topB* genes but not to the *S.cerevisiae* *TOP1* (I-3') or *TOP2* (II) genes. The relationships originally proposed on the basis of sequence comparison have been confirmed biochemically (Kim and Wang, 1992). Purified Top3 protein exhibits many of the properties of TopA and TopB, including relaxation of only negatively supercoiled DNA and a 5'-covalent phosphotyrosine ester linkage between the conserved tyrosine in the active site and the DNA. The enzyme is also proficient in preferentially binding single-stranded DNA and in decatenating single strands (Kim and Wang, 1992). Recently, homologs in man and mouse have been identified, and the gene has been shown to be essential during early mouse embryogenesis (Hanai *et al.*, 1996; Fritz *et al.*, 1997; Li and Wang, 1998; Seki *et al.*, 1998).

The *top3* mutants originally were isolated because they stimulate recombination between repeated sequences (Wallis *et al.*, 1989; Arthur, 1991; Gangloff *et al.*, 1996). This hyper-recombinogenic phenotype is not restricted to direct repeats, since the absence of Top3 also elevates recombination between homologous ectopic genes (Bailis *et al.*, 1992). In addition to the recombination phenotype, *top3Δ* mutants exhibit a cell cycle aberration characterized by an accumulation of cells containing an undivided nucleus in the neck of the bud. This cell cycle delay translates into slow growth that is believed to result from a defect in single-stranded DNA decatenation in an alternative pathway for DNA replication termination (Gangloff *et al.*, 1994a). Among other mitotic phenotypes, *top3Δ* mutants are affected in the transcriptional control of several genes (Arthur, 1991).

A search for suppressors of the *top3Δ* slow growth phenotype led to the isolation of mutations in the *SGS1* gene (Gangloff *et al.*, 1994b). The Sgs1 protein is a member of the helicase family characterized by the *E.coli* RecQ protein (Nakayama *et al.*, 1985; Umezu *et al.*, 1990). Over the past few years, Sgs1 homologs have been identified in many organisms, including yeast and man (Puranam and Blackshear, 1994; Ellis *et al.*, 1995; Puranam *et al.*, 1995; Yu *et al.*, 1996; Stewart *et al.*, 1997; Yan *et al.*, 1998). The absence of Sgs1 suppresses all the mitotic phenotypes caused by the lack of Top3 (Gangloff *et al.*, 1994b), which led to the hypothesis that Top3 is required to act on substrates created by Sgs1. This model is supported by the finding that Sgs1 interacts physically with Top3 in a two-hybrid system, suggesting that Top3 and Sgs1 may work together as part of a complex (Gangloff *et al.*, 1994b).

Although Top3 is involved in every aspect of DNA metabolism (Wallis *et al.*, 1989; Arthur, 1991; Bailis *et al.*, 1992; Gangloff *et al.*, 1994a, 1996; Kim *et al.*, 1995), its mitotic function is dispensable. In contrast, Top3 function is essential during meiosis, and homozygous mutant diploids are unable to form asci. In this report, we investigate the metabolic alteration responsible for the inability of *top3Δ* strains to sporulate. We show that the *top3Δ* mutants fail to complete reductional division at a step following initiation of recombination and formation of recombinant molecules. Additionally, overexpression of the *E.coli topA* gene restores sporulation, which suggests that a type I-5' topoisomerase activity is essential for processing recombination intermediates generated during meiosis.

Results

The absence of sporulation in *top3Δ* mutants is not due to loss of chromosome III

Diploid cells homozygous for the *top3Δ* deletion fail to sporulate (Wallis *et al.*, 1989; Arthur, 1991; Gangloff *et al.*, 1994a). Light microscopy analysis of 2000 diploid cells homozygous for *top3Δ* indicated that, even 7 days after transfer to sporulation medium, no visible asci could be detected. We previously have observed that diploid cells homozygous for *top3Δ*, at a much higher frequency than wild-type cells, become capable of mating with haploid cells and form colonies on selective medium, an event due to chromosome III loss (Arthur, 1991). In yeast, each chromosome III carries a different *MAT* allele at the mating type locus, and the products of both are required for entry into meiosis. A high frequency of chromosome loss may therefore lead to *top3Δ*'s inability to sporulate. To test this hypothesis, we measured the frequency at which chromosome III is lost in a population of cells ready to enter meiosis. We used a strain (D5-4D×W1193-2C; Table I) heterozygous for *LEU2*, a proximal gene on chromosome III. At the $t = 0$ of meiosis induction, diploid cells were plated out onto rich medium. After 3 days of vegetative growth, the colonies were replicated onto synthetic medium lacking leucine. In those conditions, cells that have lost the *LEU2*-bearing chromosome III prior to the first cell division on the plate become unable to form colonies. This analysis was performed on several thousand colonies and revealed that $<1/3000$ diploid cells

had lost the *LEU2* marked chromosome III. This indicates that the total frequency of chromosome III loss in *top3Δ* mutants is $<1/1500$ cells, and thus cannot account for the inability of *top3Δ* mutants to sporulate.

The absence of TOP3 prevents the cells from undergoing reductional division

To determine at which stage in meiosis *top3Δ* cells are blocked, we next examined the fate of the nucleus of *top3Δ* cells during meiosis. Meiosis was induced, and samples of wild-type and mutant cells were taken at different times, washed, fixed and incubated with 4',6-diamidino-2-phenylindole (DAPI). Analysis by fluorescence microscopy revealed that after 12 h, 5–10% of wild-type cells have already completed both reductional and equational division, and formed four haploid staining bodies. However, in a sample of 2000 *top3Δ* cells, reductional division was never observed. Instead, after 2 days of incubation in sporulation medium, nuclear DAPI-stained material fragmentation is observed with subsequent loss of the intense fluorescent staining of the DNA (Figure 1).

Double-strand breaks (DSBs) are present in *top3Δ* mutants

In *S.cerevisiae*, double-strand breaks (DSBs) initiate meiotic recombination, a process necessary to achieve proper chromosome segregation (for reviews see Kleckner, 1996; Roeder, 1997). To investigate whether *top3Δ* homozygous diploids enter meiosis, the occurrence of DSBs at time $t = 0$, $t = 8$ and $t = 24$ h in sporulation medium was analyzed (de Massy and Nicolas, 1993; de Massy *et al.*, 1994). Interestingly, DSBs at both the *CYS3* and the *ARG4* loci were detected. The levels of breaks as well as their kinetics of appearance and processing are comparable with those observed in wild-type controls (Figure 2). Global analysis of DSB formation along the 340 kb of chromosome III indicated that the pattern of DSBs formed on this chromosome is similar in the *top3Δ* mutants and the wild-type controls, suggesting that the results observed at the *ARG4* and *CYS3* loci reflect a general trend in the genome (data not shown).

Recombinant molecules are formed, but generate a lethal substrate

To analyze the progression of recombination after the introduction of a DSB, we examined the formation of recombinant molecules by using *arg4* heteroalleles that differ from each other by their restriction pattern (Figure 3). The presence of recombinant molecules does not, however, imply that Holliday junctions are resolved actively. In the *arg4-ΔEcoRV/arg4-ΔBgIII* diploid cells, the great majority of the convertants at the *ARG4* locus involve only the *EcoRV* restriction site which lies close to the upstream region of the gene where the DSBs occur. After a double digestion with *EcoRV* and *BgIII*, and in the absence of a pre-treatment with DNA cross-linking agents, passive migration of the junction releases linear DNA fragments. Using this experimental design, the two parental fragments were detected. However, two additional signals corresponding to *arg4* recombinant molecules were also detected by Southern analysis in both wild-type and *top3Δ* mutants (see 1 and 7 kb fragments, Figure 3). Together, these

Table I. Strain list

Strain	Relevant genotype	Reference
ORD2130	<i>MATa</i> <i>Inv(Eco-Sn)poly1arg4ΔRV trp1-289 ura3-52 ade2 his3Δ1 CYH2 LEU2</i>	
	<i>MATα</i> <i>Inv(Eco-Sn)poly1arg4ΔBg trp1-289 ura3-52 ADE2 HIS3 cyh^r leu2-3,112</i>	B.de Massy
ORD2184	<i>MATa</i> <i>Inv(Eco-Sn)poly1arg4ΔBg cyh^r his3Δ1 leu2-3,112 trp1-289 ura3-52 top3-4Δ::URA3 ADE2</i>	
	<i>MATα</i> <i>Inv(Eco-Sn)poly1arg4ΔRV cyh^r his3Δ1 leu2-3,112 trp1-289 ura3-52 top3-4Δ::URA3 ade2</i>	B.de Massy
ORD2186	<i>MATa</i> <i>Inv(Eco-Sn)poly1arg4ΔBg trp1-289 cyh^r ura3-52 ADE2 LEU2 TOP3</i>	
	<i>MATα</i> <i>Inv(Eco-Sn)poly1arg4ΔRV trp1-289 cyh^r ura3-52 ade2 leu2-3,112 top3-4Δ::URA3</i>	B.de Massy
W303	<i>MATa</i> <i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	
	<i>MATα</i> <i>ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Thomas <i>et al.</i> (1989)
U739-1A	<i>MATa</i> <i>top3-4Δ::URA3</i>	this study
W1193-2C	<i>MATα</i> <i>top3-5Δ::TRP1</i>	this study
D65-9A	<i>MATa</i> <i>top3-3::LEU2 spo11::HIS3</i>	this study
D51-1A	<i>MATα</i> <i>top3-5Δ::TRP1 spo11::HIS3</i>	this study
D50bis-9D	<i>MATa</i> <i>top3-3::LEU2 spo13::URA3</i>	this study
D65-2B	<i>MATα</i> <i>top3-5Δ::TRP1 spo13::URA3</i>	this study
D5-4D	<i>MATa</i> <i>top3-4Δ::URA3 LEU2</i>	this study
D50-9D	<i>MATa</i> <i>spo13Δ::URA3 top3-3::LEU2</i>	this study
D51-1C	<i>MATα</i> <i>spo13Δ::URA3 top3-5Δ::TRP1</i>	this study
D50-1D	<i>MATa</i> <i>spo11Δ::HIS3 spo13Δ::URA3</i>	this study
D50-3D	<i>MATα</i> <i>spo11Δ::HIS3 spo13Δ::URA3</i>	this study
D52	<i>MATa</i> <i>spo11Δ::HIS3 spo13Δ::URA3 top3-3::LEU2</i>	
	<i>MATα</i> <i>spo11Δ::HIS3 spo13Δ::URA3 top3-5Δ::TRP1</i>	this study
D17	<i>MATa</i> <i>sgs1Δ::HIS3</i>	
	<i>MATα</i> <i>sgs1Δ::URA3</i>	this study
D18	<i>MATa</i> <i>sgs1Δ::HIS3 top3-2::LEU2</i>	
	<i>MATα</i> <i>sgs1Δ::HIS3 top3-5Δ::TRP1</i>	this study
D67-6B	<i>MATα</i> <i>spo13Δ::URA3 sgs1Δ::TRP1</i>	this study
D66-6C	<i>MATa</i> <i>spo11Δ::HIS3 spo13Δ::URA3 sgs1Δ::LEU2</i>	this study
D67-9C	<i>MATα</i> <i>spo11Δ::HIS3 spo13Δ::URA3 sgs1Δ::TRP1</i>	this study
D71-3C	<i>MATa</i> <i>top3-5Δ::TRP1 rad17Δ::LEU2</i>	this study
D70-12D	<i>MATα</i> <i>top3-3::LEU2 rad17Δ::LEU2</i>	this study
D73-2B	<i>MATa</i> <i>top3-2::HIS3 rad24Δ::TRP1</i>	this study
D72-6A	<i>MATα</i> <i>top3-3::LEU2 rad24Δ::TRP1</i>	

All strains are isogenic to W303 (Thomas *et al.*, 1989), except ORD2130, 2184 and 2186.

Fig. 1. DAPI staining during meiosis. Nuclear division and spore formation were examined by fluorescence microscopy. Wild-type (W303) and *top3Δ* (U739-1A×W1193-2C) samples were taken at 0, 48 and 96 h following meiotic induction, and the DNA of ethanol-fixed cells was stained with DAPI. Binucleate (MI) and tetranucleate (MII) cells have completed first and second meiotic divisions, respectively.

Fig. 2. Detection of meiotic DSBs at the *ARG4* and *CYS3* loci. Cells were taken at 0, 8 and 24 h after transfer to sporulation medium. Genomic DNA extracted from wild-type (ORD2130), *top3Δ/TOP3* (ORD2186) and *top3Δ/top3Δ* (ORD2184) meiotic cells was digested with *HindIII* and probed sequentially with DNA corresponding to known meiotic recombination hotspots. Left panel: hybridization with the radiolabeled *EcoRI* fragment encompassing the 3' region of the *CYS3* gene detects the *CYS3* DSBs on chromosome I (see Figure 1 in de Massy *et al.*, 1994). Right panel: in addition to the *CYS3* signal, the radiolabeled *EcoRV-BglIII* (1016 bp) *ARG4* internal fragment (see Figure 3) reveals the *ARG4* DSBs on chromosome VIII. The vertical bars on the sides indicate the positions of the *CYS3* and the *ARG4* meiotic DSBs sites.

results and those presented in the previous section indicate that *top3Δ* cells are progressing through meiosis and that the *Top3* function is not required for the formation of either DSBs or recombinant molecules.

In yeast cells, meiotic levels of recombination can be induced before commitment to reductional division in a return to growth (RTG) experiment (Sherman and Roman, 1963). In *top3Δ* mutants, although DSBs and recombinant molecules are observed, no stimulation of recombination could be measured through the formation of *Arg*⁺ prototrophs. At *t* = 0, we recovered four and 20 *Arg*⁺ clones for 10⁶ cells in wild-type and *top3Δ* mutants, respectively. After 24 h in the sporulation medium, 17 000 *Arg*⁺ recombinants were recovered for 10⁶ wild-type cells, compared with only 25 for *top3Δ* mutants. We interpret this result as a failure of *top3Δ* cells to resume vegetative growth following meiotic recombination initiation. This is not due to a general deficiency in DSB repair since mitotic *top3Δ* mutants switch mating type in the presence of the HO endonuclease (S.Gangloff, unpublished result).

Bypassing recombination between homologs restores sporulation

To determine whether the *top3Δ* arrest in meiosis I is related to recombination, we examined whether cells that do not undergo meiotic recombination are capable of sporulating in the absence of *Top3*. The *spo11Δ* mutation abolishes meiotic DSB formation (Klapholz *et al.*, 1985; Bergerat *et al.*, 1997; Keeney *et al.*, 1997) and, thus, recombination. Since *spo11Δ* mutants do not sporulate efficiently and produce mostly inviable spores due to non-disjunction, we tested our hypothesis in strains homozygous for both the *spo11Δ* and the *spo13Δ* mutations. *spo13Δ* mutants bypass meiosis I and produce asci containing two diploid spores (dyads) that are viable even in the absence of recombination (Malone and Esposito, 1981; Klapholz *et al.*, 1985). Isogenic diploid strains were constructed in which the *spo11Δ*, *spo13Δ* and *top3Δ* mutations are homozygous (Table I). These diploid cells were analyzed for their ability to undergo meiosis and

form spores. The results summarized in Table II reveal that *TOP3* is not required to form viable spores in the absence of recombination: while the *top3Δ spo13Δ* cells form no asci, the triple mutant *top3Δ spo11Δ spo13Δ* sporulates and forms dyads with a viability similar to that of *spo11Δ spo13Δ* controls. We conclude that the meiotic arrest of *top3Δ spo13Δ* cells depends on the activity of Spo11 and, therefore, on meiotic recombination. This result suggests that resolution of entangled homologs after recombination is probably responsible for the sporulation defect observed in *top3Δ* mutants. This hypothesis is also in agreement with the observation that nuclear division occurs within the same time frame in the presence or absence of *TOP3* when recombination is abolished. As expected, two DAPI-staining bodies could be observed in <24 h following meiotic induction in the *top3Δ spo11Δ spo13Δ* triple mutant background, while no nuclear division was detected in >2000 cells after 2 days (Figure 4) or even after 6 days of incubation (data not shown) when the *SPO11* controlled initiation of recombination is functional. Furthermore, the timing of dyad formation is independent of *TOP3*, which also suggests that the absence of this topoisomerase does not greatly affect pre-meiotic DNA synthesis.

Overexpression of *TopA* suppresses the sporulation deficiency of *top3Δ* mutants

Wallis *et al.* (1989) have shown that expression in yeast of the *E.coli topA* gene, encoding a functionally related type I-5' topoisomerase, is capable of complementing the slow growth phenotype of *top3Δ* null mutants. Thus, we tested whether expression of *topA* (YE_{topA}-PGAL1) would also complement the sporulation defect of *top3Δ* mutant strains. Diploid cells homozygous for the *top3Δ* deletion and containing the *topA* plasmid were tested for spore formation. The diploid strains were pre-grown on galactose medium to induce the expression of *topA* and then replica-plated to sporulation medium containing 0.1% galactose. Under these conditions, three- and four-spored asci accounted for 20% of the cells present after 3 days

Fig. 3. Physical map of the *ARG4* region and detection of recombinant molecules. **(A)** Physical map of the alleles present at the *ARG4* locus. The *EcoRV* and *BglIII* heteroallelic mutations in the *ARG4* genes are indicated. The position of the *ARG4* *EcoRV*–*BglIII* probe is indicated by the black box, and the *ARG4* open reading frame and direction of transcription are indicated by the horizontal arrow. The sizes of the parental (P1 and P2) and expected recombinant fragments (Rec1 and Rec2) resulting from the *EcoRV* + *BglIII* digest are shown underneath. **(B)** At times 0 and 24 h after meiotic induction, genomic DNA extracted from wild-type (ORD2130), *top3Δ/TOP3* (ORD2186) and *top3Δ/top3Δ* (ORD2184) cells was digested with *EcoRV* and *BglIII* and probed with the *EcoRV*–*BglIII* (1016 bp) *ARG4* internal fragment. As in Figure 2 (right), the intense unlabeled signal corresponds to the *CYS3* parental band.

of induction at 30°C compared with 50% for wild-type diploid controls transformed by YEtopA-PGAL1 plasmid (Table III). When four-spored asci from the *top3Δ/top3Δ* YEtopA-PGAL1 diploids were dissected on either YPD or YPGal, the spore viability was poor. From a total of 71 tetrads dissected, only 110 viable colonies (39%) were recovered, while 89% viability was observed for isogenic wild-type strains containing the *topA* plasmid. Of the 71 *top3Δ/top3Δ* YEtopA-PGAL1 tetrads, only five yielded four viable spores. Among tetrads where only three spores form colonies, some spores were found to grow on medium lacking the two amino acids corresponding to the

auxotrophic markers used to disrupt the two copies of the *TOP3* gene. This implies that the two chromosomes bearing the *top3* deletion did not disjoin and segregated to the same cell. These observations indicate that overexpression of a type I-5' topoisomerase activity can restore sporulation in cells lacking Top3. However, the efficiency of the process is poor, and chromosome non-disjunction may be important in spore inviability. This result suggests that the essential enzymatic activity necessary for performing meiosis is shared with type I-5' enzymes, but absent from the type I-3' or II counterparts.

The absence of *SGS1* allows some sporulation in *top3Δ* mutants

The *SGS1* gene encodes a protein containing a DNA helicase domain conserved from bacteria to man (Rothstein and Gangloff, 1995). Mutations in either *BLM* or *WRN*, two human genes encoding proteins structurally and functionally homologous to Sgs1, lead to genome instability and a high incidence of cancer (Ellis *et al.*, 1995; Yu *et al.*, 1996). We have shown previously that Sgs1 interacts with Top3 in a two-hybrid system (Gangloff *et al.*, 1994b). Interestingly, *TOP3* and *SGS1* also interact genetically, since the absence of *SGS1* suppresses the slow growth, the altered cell cycle distribution and the hyper-recombination phenotype of *top3Δ* mutants (Gangloff *et al.*, 1994b). Because of the potential for Sgs1 and Top3 to act together in a complex (Gangloff *et al.*, 1994b), we analyzed the possibility that unprocessed structures introduced by Sgs1 in the absence of Top3 are responsible for the sporulation defect. As shown in Table II, *top3Δ sgs1Δ* double mutants sporulate very poorly, but produce viable spores. Out of 18 *top3Δ sgs1Δ* tetrads dissected, the overall spore viability was 67%, with five, six, three and four tetrads giving rise to four, three, two and one viable spore, respectively. We found that, unlike *top3Δ* strains, *sgs1Δ* mutant strains complete the meiotic process, but with a long delay in the appearance of four-spored asci and a very low yield (Figure 5). Out of 33 *sgs1Δ* tetrads dissected, the overall spore viability was 74%, with 14, eight, seven and four tetrads giving rise to four, three, two and one viable spore, respectively, indicating that *sgs1Δ* is epistatic to *top3Δ* with respect to spore viability.

We next examined whether the 48 h delay in the meiotic process is related, similarly to the arrest of *top3Δ* mutants, to the recombination process. We therefore constructed *sgs1Δ spo11Δ spo13Δ* triple mutant diploid cells and monitored both nuclear division and spore (dyad) formation. In the *spo11Δ spo13Δ* background, the kinetics of spore formation and the timing of equational division are independent of the presence of *SGS1*, which indicates that Sgs1 is also involved in the meiotic recombination process (Figure 6).

Discussion

The absence of the type I-5' topoisomerase Top3 results in a pleiotropic mitotic phenotype (Wallis *et al.*, 1989; Gangloff *et al.*, 1994a,b, 1996). Vegetative cells are viable but meiosis does not proceed. This indicates that Top3 plays either a specific and essential role in meiosis or that another protein can partially substitute for Top3 during the mitotic but not the meiotic cycle. The experiments

Table II. Sporulation and spore viability

Strain	Strain name or cross	Percentage of asci formed			Spore viability
		2 days	4 days	6 days	
Wild-type	(W303)	45	80	85	95%
<i>top3Δ</i>	(U739-1A×W1193-2C)	0	0	0	0
<i>spo11Δ top3Δ</i>	(D65-9A×D51-1A)	0	0	0	0
<i>spo13Δ top3Δ</i>	(D50bis-9D×D65-2B)	0	0	0	0
<i>spo11Δ spo13Δ*</i>	(D50-1D×D50-3D)	8	15	20	79%
<i>top3Δ spo11Δ spo13Δ*</i>	(D52)	8	15	20	78%
<i>sgs1Δ</i>	(D17)	1	5	30	74%
<i>top3Δ sgs1Δ</i>	(D18)	0	0.1	2	67%
<i>sgs1Δ spo13Δ*</i>	(D66-6C×D67-6B)	0	2	15	ND
<i>sgs1Δ spo11Δ spo13Δ*</i>	(D66-6C×D67-9C)	10	15	20	86%

Diploid cells, homozygous for the mutations listed, were pre-grown on YPD at 30°C (Sherman *et al.*, 1986) and inoculated into 10 ml of SPS medium. When the cells reach stationary phase, they are washed with water and resuspended in 10 ml of 1% potassium acetate medium. Aliquots of 1 ml are taken at various time points, and the proportion of tetrads [or dyads (*) for *spo11Δ spo13Δ* mutants] is determined by dividing the number of four (two)-spored asci by the total number of cells counted. Cell viability was determined after dissection, by dividing the number of spores forming colonies by the total number of spores dissected. For each time point, a minimum of 200 full tetrads (or dyads) were scored. ND, not determined.

Fig. 4. Effect of recombination initiation on *top3Δ* meiotic product formation. The same type of experiment as that described in Figure 1 was performed on *spo11Δ spo13Δ* double mutants (D50-1D×D50-3D) and on *spo11Δ spo13Δ top3Δ* (D52 = D50-3C×D51-3D) triple mutants. Here, binucleate cells correspond to cells that have undergone a single equational division.

Table III. Effects of TopA overexpression on sporulation

Genotype	Sporulation efficiency	Tetrads analyzed	Four viable spores	Three viable spores	Two and one viable spores	Spore viability
<i>top3Δ + topA</i>	20%	71	5	11	57	39%
wt + <i>topA</i>	50%	46	38	2	6	89%

NB: *top3Δ* strains do not form spores on galactose.

described in this study were designed to address the meiotic role of Top3. Since the Sgs1 helicase interacts with Top3, and since *sgs1Δ* is epistatic to *top3Δ* with

Fig. 5. Sporulation kinetics of wild-type and *sgs1Δ* cells. The percentage of four-spored asci was determined for wild-type and *sgs1Δ* mutants as a function of time following induction of meiosis.

respect to mitotic *top3Δ* phenotypes, we have extended this study to the *sgs1Δ* and *sgs1Δ top3Δ* mutants.

Aberrant gene expression is not a likely cause for the sporulation defect

TOP3 is known to control the expression of several genes (Arthur, 1991). *IME1* and/or *IME2*, which belong to the cascade of regulatory genes involved in the control of entry into meiosis (Sia and Mitchell, 1995), could depend on *TOP3* for their expression. This is not the case since overexpression of these genes was not found to alleviate the sporulation defect of *top3Δ* cells (unpublished results). Although it cannot be formally excluded that *TOP3* controls the expression of other meiosis-specific genes, altered gene expression is not a probable candidate for the sporulation defect since *spo11Δ spo13Δ top3Δ* triple mutants do sporulate similarly to *spo11Δ spo13Δ* strains.

Recombination is responsible for the *top3Δ* meiotic defect

Analysis of *top3Δ* cells at the cellular level indicates that very few, if any, cells are capable of completing the first meiotic division (MI). Since this is the part of meiosis where recombination takes place, several aspects of this process were analyzed. Initiation, as evidenced by formation of DSBs, occurs at a similar frequency in both normal and *top3Δ* cells. Additionally, comparable amounts of

Fig. 6. Effect of recombination initiation on *sgs1Δ* meiotic product formation. Nuclear division and spore formation were examined in the *sgs1Δ* (D47), *spo11Δ spo13Δ sgs1Δ* (D66-6C×D67-9C) and *spo13Δ sgs1Δ* (D66-6C×D67-6B) strains throughout meiosis by staining DNA with DAPI. In the absence of the *SPO13* function, only dyads are obtained. In the *sgs1Δ* mutants, tetrads containing four haploid spores start to appear after 5 days of incubation.

recombinant molecules could be detected in wild-type and mutants, suggesting that *TOP3* is not involved in the formation of these recombinant molecules (Figure 3).

Although detection of intragenic recombinant molecules does not require that the Holliday junctions are resolved, it is still possible that Top3 may affect the recombination process itself. It has been shown previously that meiotic recombinant molecules can be detected by genomic blot analysis in mutants such as *rad51*, *rad52*, *rad55* and *rad57* that are deficient in homologous recombination (Borts *et al.*, 1986; Shinohara *et al.*, 1992; Schwacha and Kleckner, 1997). However, in these studies, the level of recombinant molecules was much lower than in control cells, which is clearly not the case for *top3Δ*.

Cells mutant for *TOP3* appear to proceed into an irreversible phase leading to cell death (Figure 1). In addition, RTG analysis did not indicate any increase in the recovery of Arg⁺ recombinants with *top3Δ*, suggesting a viability loss of the meiotic cells following DSBs. Finally, we used *spo11Δ*, an early meiotic mutation that prevents DSB formation (Cao *et al.*, 1990), to correlate the *top3Δ* defect directly with recombination. Viable diploid spores are obtained if *spo11Δ* is coupled to *spo13Δ*, a mutation that leads to a bypass of the reductional division. While *spo13Δ top3Δ* cells are blocked before MI, the *spo11Δ spo13Δ top3Δ* cells do sporulate, with kinetics of nuclear division similar to the kinetics in *spo11Δ spo13Δ* cells. Furthermore, viable diploid spores were recovered, indicating that the meiotic phenotype of *top3Δ* mutants is due to recombination.

Does the *top3Δ* defect trigger a checkpoint response?

It is still not clear whether the absence of MI division in *top3Δ* meiotic cells relates to a checkpoint activity. Indeed, after a transient delay in prophase, the chromatin appears to be fragmented and the cells die. It is possible, therefore, that some kind of DNA degradation process is activated after a time of arrest with unresolved recombination structures. We tested a possible effect of *rad17Δ* and *rad24Δ*, mutations known to allow MI to occur in mutants such as *dmc1Δ* or *rad51Δ*, affected in intermediate recombination steps (Lydall and Weinert, 1995; Lydall *et al.*, 1996). These mutations had no effect on *top3Δ* cells (unpublished results), in agreement with the proposal that the arrested cells do not contain single-stranded DNA that would activate this checkpoint control. It will be of interest, therefore, to determine if *red1Δ* or *mek1Δ* mutation allows MI division to take place in *top3Δ* cells. These genes have been proposed to monitor the completion of recombination, and to arrest cells until resolution of the recombination structures (Xu *et al.*, 1997).

The *sgs1Δ* mutant also displays a meiotic defect related to recombination

The relationship between *top3Δ* and *sgs1Δ* prompted us to question if *sgs1Δ* diploids display a meiotic defect related to the processing of recombination events.

We found that *sgs1Δ* decreases sporulation efficiency by approximately two-thirds, reduces spore viability (Table II) and exhibits a slower progression through MI (Figure 5). However, the rate of intragenic recombination among viable spores studied at different loci is unaffected (data

not shown) and confirms previously reported results (Watt *et al.*, 1995, 1996). The MI division is also delayed by ~48 h, indicating that the cells are held up transiently in prophase. This delay was likewise seen in a *spo13Δ sgs1Δ* double mutant, but not in *spo11Δ spo13Δ sgs1Δ* cells, indicating that the effect is due to recombination (Figure 6). It is conceivable that a recombination checkpoint is activated in *sgs1Δ* cells, leading to the arrest of the majority of the meiotic cells, while delaying progression through meiosis of the unarrested cells.

Top3 and DNA replication

Although Top3 is dispensable for vegetative growth, its function is required for the completion of MI. Previous work has shown that mitotic and meiotic DNA replication initiate at the same origins and terminate non-specifically (Collins and Newlon, 1994). The absence of Top3 in mitosis leads to the accumulation of cells with an undivided nucleus in the neck of an elongated bud (Gangloff *et al.*, 1994b). The associated cell cycle delay at the G₂-M transition is consistent with an impediment of chromosome segregation, suggesting that Top3 is involved directly in the separation of replicated chromatids or, alternatively, in the resolution of topological structures resulting from either recombination during replication or collapsed replication forks (Young *et al.*, 1984; Kawasaki *et al.*, 1994; Zou and Rothstein, 1997). Because no noticeable difference in the timing of diploid spore formation could be detected when meiotic levels of recombination are abolished, we do not believe it likely that Top3 plays a meiotic-specific role in the DNA synthesis process.

Topoisomerase activities and recombination

Of the three nuclear DNA topoisomerases characterized in yeast, the activity of Top2 is essential in mitosis and meiosis, while that of Top1 is dispensable in both (Holm *et al.*, 1985, 1989; Rose *et al.*, 1990; Wang, 1996). On the other hand, Top3 is non-essential during vegetative growth but cells mutant for the Top3 topoisomerase do not achieve the first meiotic division. This result indicates that Top3 performs a function in meiosis that cannot be replaced by Top1 or Top2. Different models can account for this observation.

Top3 may exhibit a substrate specificity or may perform a specific biochemical activity. During the early steps of the meiotic process, DNA replication and recombination take place (reviewed in Kleckner, 1996; Roeder, 1997). The observation that MI does not take place in the absence of Top3 when recombination is proficient suggests that physically connected homologs cannot be disentangled efficiently. It has been shown that meiotic recombination intermediates evolve to form double Holliday junctions that need to be resolved to complete the meiotic process (Schwacha and Kleckner, 1994). Failure to resolve the junctions prevents the cells from executing a correct anaphase I, and it has been postulated that a type I topoisomerase could serve as a backup mechanism to process persisting double Holliday junctions or derived hemicatenane structures in which the DNA duplexes are intertwined at a single point (Schwacha and Kleckner, 1994; Gilbertson and Stahl, 1996; Duguet, 1997). Top3 could fulfill such a function. Sequence analysis and biochemical studies have shown that Top3 resembles its

bacterial type I-5' homologs TopA or TopB, in that it binds preferentially single-stranded DNA and is proficient in decatenating DNA molecules (Wallis *et al.*, 1989; Kim and Wang, 1992). In the context of a double Holliday junction, unwound single-stranded DNA is available for Top3 to bind to and to untwine. Prokaryotic and eukaryotic enzymes are all capable of relaxing negatively supercoiled DNA, but only type I-5' enzymes exhibit an extremely strong preference for single-stranded DNA (for review see Wang, 1996). When bound to ssDNA, type I-5' enzymes become very potent decatenases. Support for a specific topoisomerase type I-5' activity in the resolution of recombination intermediates derives from the observation that ectopic expression of the *E.coli* *topA* gene allows *top3Δ* mutants to form viable meiotic products. An alternative to the previous models relies on interactions with proteins capable of specifically targeting the topoisomerase to its substrate. The Top3 topoisomerase has been shown to associate physically with the Sgs1 helicase (Gangloff *et al.*, 1994b). It is therefore conceivable that Sgs1 or another protein could deliver the activity of Top3 to a region of the genome that is not accessible to Top1 or Top2.

Top3 and Top2

Previous work that focused on the role of Top2 in the meiotic process indicates that *top2* mutants fail to undergo both reductional division and the mitosis-like equational division (Holm *et al.*, 1985; Rose *et al.*, 1990). The reductional defect is caused by recombination events, suggesting that Top2 is required to resolve intertwined sister chromatids distal to the site of recombination (Rose *et al.*, 1990).

Two hypotheses can explain the requirement for Top3 in MI. Schwacha and Kleckner (1995) echoed earlier suggestions that topoisomerases are involved in the resolution of Holliday junctions, and it is therefore possible that Top2 and Top3 are involved in this process. During vegetative growth, a limited number of recombination events involving homologous chromosomes occur (Fabre *et al.*, 1984; Kadyk and Hartwell, 1992), which largely limits the role of Top2 to disentangling replicated chromatids (Holm *et al.*, 1985) and that of Top3 to participating in replication termination (Gangloff *et al.*, 1994b). In meiosis, however, connections between homologous chromosomes are stimulated greatly (for a review see Petes *et al.*, 1991). Top2 activity may, therefore, be required, not only to decatenate replicated chromatids to allow recombining chromosomes to segregate, but also directly to resolve recombination intermediates. If Top2 is limiting, it is possible that Top3's function may become essential for resolving recombination intermediates in MI. In the second alternative, Top2 is not involved primarily in the resolution of recombinant structures and Top3 may represent a major activity capable of resolving recombination intermediates involving homologous chromosomes, a structure that is rare in vegetative cells.

Top3 and Sgs1

We have shown previously that the Sgs1 helicase interacts genetically and physically with the Top3 topoisomerase (Gangloff *et al.*, 1994b). Epistasis analysis has suggested that the Sgs1 helicase creates the structures that Top3 acts

on. We have proposed, by analogy with the bacterial TopB activity with which Top3 shares many features (Kim and Wang, 1992), that Top3 and Sgs1 may play an important role in an alternative pathway of DNA replication termination. The absence of Sgs1 fully suppresses all the mitotic phenotypes of *top3Δ* mutants, and meiosis is the first instance where the activities of the complex can be uncoupled. The tetrad yield in the *top3Δ sgs1Δ* double mutant is extremely low (~1–2%), compared with that found in *sgs1Δ* mutants (~30%), although *sgs1Δ* viability is retained. This suggests that, in meiosis, Top3 can act on substrates that are not created by the Sgs1 helicase. This view is in agreement with the idea that Top3 and Sgs1 may work together in replication termination (both in mitosis and in meiosis), and with the hypothesis involving Top3 alone in the resolution of some meiotic double Holliday junctions.

Human genes and perspectives

Finally, it is interesting to note that *TOP3* and *SGS1* are present in humans. Mutations in *BLM* or *WRN*, the *SGS1* homologs, are responsible for cancer-prone disorders characterized by genomic instability, a phenotype shared with *sgs1Δ* mutants (Ellis *et al.*, 1995; Rothstein and Gangloff, 1995; Watt *et al.*, 1995; Yu *et al.*, 1996). Two human genes homologous to *TOP3* have also been identified (Hanai *et al.*, 1996; Kawasaki *et al.*, 1997). Disruption of *TOP3α* in mouse revealed that this gene is essential during early embryogenesis (Li and Wang, 1998). It is therefore tantalizing to speculate that mammalian Top3 may both be involved in the resolution of recombination intermediates present during early development, and play a more general role in replication termination in association with Blm or Wrn proteins.

Materials and methods

Strains and genetic manipulation methods

Standard yeast genetic methods were employed for analysis of strains and crosses (Sherman *et al.*, 1986). All strains used in this study are isogenic to W303 (Thomas and Rothstein, 1989), except for ORD2130, 2184 and 2186, and are listed in Table I. The *rad17Δ::LEU2* and *rad24Δ::TRP1* mutants were kindly provided by T. Weinert, the YEPtopA-PGAL1 plasmid is a gift from J.C. Wang, and the *spo11Δ* and *spo13Δ* strains were a kind gift from R.E. Esposito.

Monitoring nuclear divisions

Meiotic cultures were stained with DAPI and examined by fluorescence microscopy for one, two (MI) and four (MII) staining bodies. In meiotic time course experiments, 1 ml aliquots from the sporulation medium were taken at 2 h intervals for the first 12 h, then every day for the next 6 days. The cells were resuspended in 0.5 ml of methanol:glacial acetic acid (3:1 v/v) and fixed for 1–24 h at 4°C. Following fixation, cells were washed and resuspended in distilled water, and stored at 4°C until the time of examination. Then 10⁸ cells were resuspended in 50 μl of distilled water, and 1 ml of ethanol and 1 μl of the DAPI (10 mg/ml) solution were added. After 5 min of incubation at room temperature, the cells were washed several times with distilled water and resuspended at 10⁸ cells/ml for microscopy (Sherman *et al.*, 1986).

Commitment to meiosis

Cells were grown to saturation overnight in YPD (Sherman *et al.*, 1986), diluted into SPS (yeast extract 0.5%, bacto-peptone 1%, yeast nitrogen base 0.17%, potassium acetate 1%, ammonium sulfate 0.5%, potassium phthalate 50 mM pH 5.0) and allowed to grow until they reached the stationary phase. At this time, the cells were washed with distilled water and resuspended at a concentration of 10⁷ cells/ml in sporulation medium (1% potassium acetate) and incubated at 28°C with vigorous agitation.

The proper meiotic characteristics of the strains were analyzed systematically by dissection of the tetrads (or dyads) followed by a test for viability and segregation of the available markers.

Double-strand break analyses

For DSB detection, the *rad50S* mutation commonly is used because it prevents the metabolism of the broken ends and thus leads to DSB accumulation (Cao *et al.*, 1990). We could not use this mutation because *top3Δ rad50S* double mutants are extremely sick. *ARG4* and *CYS3* DSBs were analyzed using the standard techniques previously described (de Massy and Nicolas, 1993). Recombinant molecules present at the *ARG4* locus were analyzed quantitatively on a Molecular Dynamics PhosphorImager with the ImageQuant v1.1 software. Distribution of meiotic DSBs on yeast chromosome III was established using the procedure described by Baudat and Nicolas (1997).

Return to growth experiment

Commitment of the wild-type (ORD2130), *TOP3/top3Δ* (ORD2186) and *top3Δ/top3Δ* (ORD2184) strains to meiotic recombination is followed throughout sporulation. After transfer to sporulation medium, aliquots were taken at different times (Sherman and Roman, 1963). The cells were plated after appropriate dilutions on YPD plates and on plates lacking arginine, allowing determination of the frequency of Arg⁺ prototroph recombinants per viable cell.

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