Mutations in Homologous Recombination Genes Rescue *top3* Slow Growth in *Saccharomyces cerevisiae*

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ABSTRACT

In budding yeast, loss of topoisomerase III, encoded by the *TOP3* gene, leads to a genomic instability phenotype that includes slow growth, hyper-sensitivity to genotoxic agents, mitotic hyper-recombination, increased chromosome missegregation, and meiotic failure. Slow growth and other defects of *top3* mutants are suppressed by mutation of *SGS1*, which encodes the only RecQ helicase in *S. cerevisiae*. *sgs1* is epistatic to *top3*, suggesting that the two proteins act in the same pathway. To identify other factors that function in the Sgs1-Top3 pathway, we undertook a genetic screen for non-*sgs1* suppressors of *top3* defects. We found that slow growth and DNA damage sensitivity of *top3* mutants are suppressed by mutations in *RAD51*, *RAD54*, *RAD55*, and *RAD57*. In contrast, *top3* mutants show extreme synergistic growth defects with mutations in *RAD50*, *MRE11*, *XRS2*, *RDH54*, and *RAD1*. We also analyzed recombination at the *SUP4-o* region, showing that in a *rad51*, *rad54*, *rad55*, or *rad57* background *top3*Δ does not increase recombination to the same degree as in a wild-type strain. These results suggest that the presence of the Rad51 homologous recombination complex in a *top3* background facilitates creation of detrimental intermediates by Sgs1. We present a model wherein Rad51 helps recruit Sgs1-Top3 to sites of replicative damage.

FOR every living cell, it is critical to preserve the integrity of genetic material during DNA replication, chromosome segregation, and after DNA damage. Proteins that function in DNA metabolism, such as helicases and topoisomerases, play vital roles in ensuring genome stability. In certain cases, the combination of a helicase and a topoisomerase provides a unique biological function and the interaction between the two is evolutionarily conserved (Duguet 1997; Wu *et al.* 1999). For example, the archaebacterial reverse gyrase contains both a helicase and a topoisomerase domain as part of the same polypeptide (Declais *et al.* 2000; Duguet *et al.* 2001). Whereas no eukaryotic genome encodes a reverse gyrase, in organisms ranging from yeast to humans, a RecQ family helicase interacts with topoisomerase III both physically and genetically (Gangloff *et al.* 1994; Goodwin *et al.* 1999; Bennett *et al.* 2000; Wu *et al.* 2000; Fricke *et al.* 2001). The two proteins and their interaction are critical for maintenance of genome stability (Mullen *et al.* 2000; Wu *et al.* 2000). The RecQ family proteins are so classified because they contain a highly conserved helicase/ATPase domain homologous to that of the *Escherichia coli* RecQ helicase (Aravind *et al.* 1999). Topoisomerase III is a type IA topoisomerase that can relax negatively supercoiled DNA with a strong preference for single-stranded regions and can decatenate interlinked single-stranded DNA (Kim and Wang 1992; Champoux 2001).

In humans, there are five known RecQ helicase homologs: BLM, WRN, RECQL, RECQL4, and RECQL5. Mutations in *WRN* and *RECQL4* cause Werner (WS) and a subset of Rothmund-Thomson (RTS) syndromes, respectively (Gray *et al.* 1997; Lindor *et al.* 2000). Both diseases are associated with a predisposition to certain cancer types, as well as with premature aging (Lindor *et al.* 2000; Nehlin *et al.* 2000; Mohaghegh and Hickson 2001). WS cells exhibit high genome instability in the form of increased deletions, translocations, and illegitimate recombination (Shen and Loeb 2000). Mutations in *BLM* cause Bloom syndrome (BS), a pleiotropic disorder characterized by, among other symptoms, a predisposition to a wide range of cancers (Ellis *et al.* 1995). At the cellular level, a hallmark feature of BS is an increased rate of sister chromatid exchange (SCE; German 1993). The BLM protein physically interacts with an isoform of topoisomerase III, Top3α, and this interaction is critical for its normal function (Wu *et al.* 2000). Cells expressing truncated versions of BLM, which are unable to interact with Top3α, have increased SCE rates, reminiscent of those in BS patients (Wu *et al.* 2000). Although human topoisomerase III has not been implicated in any genetic disorder, deletion of mouse *TOP3*α results in embryonic lethality (Li and Wang 1998). A

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mouse with the *TOP3*β isoform deleted is viable but has a shortened life span reminiscent of that in WS and RTS syndrome patients (Kwan and Wang 2001).

In the budding yeast *Saccharomyces cerevisiae*, loss of topoisomerase III, encoded by the *TOP3* gene, leads to a pleiotropic phenotype of marked genome instability. Characteristic features of *top3* mutants include slow growth, hyper-sensitivity to chemicals that cause DNA lesions or replication arrest, hyper-recombination at the *SUP4-o* and rDNA loci, increased chromosome missegregation during mitosis, and failure to complete meiosis (Wallis *et al.* 1989; Gangloff *et al.* 1994, 1996, 1999). Slow growth of *top3* mutants is suppressed by mutation of *SGS1*, the gene encoding the only RecQ-like helicase in *S. cerevisiae* (Gangloff *et al.* 1994). In a wild-type background, *sgs1* mutants exhibit a spectrum of defects similar to those in *top3* mutants, albeit less severe, as well as a shortened life span (Gangloff *et al.* 1994; Watt *et al.* 1995, 1996; Sinclair *et al.* 1997; Mullen *et al.* 2000). For most of these defects, *sgs1* is epistatic to *top3*, suggesting that the two proteins act in the same pathway (Gangloff *et al.* 1994). This conclusion is reinforced by the finding that Sgs1 and Top3 proteins physically interact (Gangloff *et al.* 1994; Bennett *et al.* 2000; Fricke *et al.* 2001). The genetic and physical interactions between Sgs1 and Top3 have led to the following model (Gangloff *et al.* 1994). In wild-type cells, Sgs1 creates a chromosomal intermediate that Top3 resolves. In *top3* mutants, this intermediate persists or is resolved improperly, leading to slow growth and other aspects of the *top3* phenotype. In *sgs1* mutants this substrate is not created and the need for functional Top3 is alleviated, resulting in suppression of *top3* defects.

Analyses of the cell cycle distribution of *SGS1* and *TOP3* mRNA transcripts and protein products suggest that both Sgs1 and Top3 function during DNA replication. Sgs1 protein levels peak during S phase, decline in G2, and are not detectable during M or G1 (Frei and Gasser 2000). *TOP3* mRNA levels peak in late G1 and decline in late S/G2 (Cho *et al.* 1998; Chakraverty *et al.* 2001). Both Sgs1 and Top3 have been implicated in intra-S-phase checkpoint activation upon DNA damage. For instance, phosphorylation of Rad53, a central *S. cerevisiae* checkpoint signal transductor, is compromised in *top3* mutants and in *sgs1* mutants that also carry mutations in *RAD24*, a gene involved in DNA damage recognition and processing (Frei and Gasser 2000; Chakraverty *et al.* 2001). Further support for the role of these proteins in DNA replication comes from studies in *Xenopus laevis* where inactivation or immunodepletion of either of the Sgs1 homologs, FFA-1 or xBLM, leads to inhibition of DNA replication in egg extracts (Liao *et al.* 2000; C. Y. Chen *et al.* 2001).

Proposed roles of the Sgs1-Top3 complex during S and G2 phases include decatenation of newly replicated sister chromatids, regulation of homologous recombination during DNA replication, and maintenance of stalled replication forks (Gangloff *et al.* 1994, 2000; van Brabant *et al.* 2000). In support of its role in chromosome decatenation and segregation, it was shown that Sgs1 physically interacts with topoisomerase II and functions in the topoisomerase II chromosome segregation pathway (Watt *et al.* 1995). In addition, Harmon *et al.* (1999) found that *E. coli* RecQ protein in combination with *E. coli* or *S. cerevisiae* topoisomerase III can catenate and decatenate double-stranded DNA circles *in vitro*.

The idea that the Sgs1-Top3 complex plays a role in homologous recombination is supported by genetic and cell biological studies. Mutation of Sgs1 leads to a severe synthetic growth defect with mutation of another *S. cerevisiae* helicase, Srs2. Gangloff *et al.* (2000) found that inactivation of Rad51 fully rescues the *sgs1 srs2* synthetic defect. This led to the idea that the defects associated with loss of these two proteins are caused by misregulated or "unrestrained" homologous recombination, suggesting that Sgs1 and Srs2 may regulate homologous recombination during DNA replication (Gangloff *et al.* 2000). Furthermore, it was shown that Sgs1 physically interacts with Rad51 and that in human cells BLM physically interacts and partially colocalizes with hRad51 during S phase and after DNA damage (Wu *et al.* 2001). WRN protein was also shown to partially colocalize with RPA and Rad51 in human cells (Sakamoto *et al.* 2001).

Several observations suggest that the Sgs1-Top3 complex function becomes important when replication forks arrest. Replication forks can stall at programmed pause sites or upon encountering transcription machinery or DNA damage (Rothstein *et al.* 2000). In *S. cerevisiae*, both *sgs1* and *top3* mutants exhibit sensitivity to hydroxyurea (HU), a chemical that stops replication progression by depleting cellular dNTP pools (Frei and Gasser 2000; Mullen *et al.* 2000). Inactivation of the only RecQ-like helicase in *Schizosaccharomyces pombe*, Rqh1, also causes HU sensitivity and a defect in recovering from HU-induced S-phase arrest (Murray *et al.* 1997; Stewart *et al.* 1997). In addition, deletions of genes encoding the Mus81-Mms4 heterodimer that can efficiently cleave replication fork-like DNA molecules *in vitro* are inviable in *sgs1* and *top3* backgrounds, suggesting that Sgs1-Top3 and Mus81-Mms4 complexes have partially overlapping roles in replication fork processing (Kaliraman *et al.* 2001). *In vitro*, the Sgs1 protein has $3' \rightarrow 5'$ helicase activity with a preference for forked substrates (Bennett *et al.* 1998, 1999). The Sgs1, BLM, and WRN proteins can efficiently migrate synthetic four-way dsDNA junctions that can represent either Holliday junctions or molecules that form after replication fork reversal (Bennett *et al.* 1999; Constantinou *et al.* 2000; Karow *et al.* 2000).

To gain new insights into the function of the Sgs1-Top3 complex and the causes of genome instability and other defects associated with loss of these proteins, we used a genetic approach. Since *top3* mutants have a striking slow-

growing phenotype, we undertook a comprehensive screen for new suppressors of *top3* slow growth. Thus far, *SGS1* has been the only known gene whose inactivation resulted in alleviation of *top3* defects. New *top3* suppressor mutations uncovered in the screen fall into several classes. This report will focus on the class consisting of genes involved in homologous recombination and on our investigation of genetic and functional relationships between *SGS1*, *TOP3*, and recombination genes. Other mutants identified in the screen will be described elsewhere. Here we show that slow growth of *top3* mutants is suppressed by mutations in *RAD51*, *RAD52*, *RAD54*, *RAD55*, and *RAD57*. Recently, Oakley *et al.* (2002) reported similar results. We also show that *top3* mutants exhibit an extreme synergistic growth defect in combination with deletion of genes encoding the Mre11-Rad50-Xrs2 (MRX) complex, Rdh54, and Rad1.

MATERIALS AND METHODS

Media: Yeast extract-peptone-dextrose (YPD), synthetic complete, and 5-fluoroorotic acid (5-FOA) media were prepared as described (Sherman *et al.* 1986), except twice the amount of leucine was used. Sporulation medium was prepared as described (Klapholz and Esposito 1982).

Strains: Standard procedures were used for mating, sporulation, and dissection (Sherman *et al.* 1986). Cells were grown at 30°. Yeast strains used in this study are listed in Table 1. To integrate *LEU2* next to *SGS1*, pWJ1209 was linearized with *Bgl*II and transformed into W1588-4C.

Plasmid construction: Plasmid pWJ1189 was made from pWJ212 (Wallis *et al.* 1989) via several steps, the last two consisting of subcloning a *Bam*HI/*Hin*dIII *TOP3* fragment into *Bam*HI/*Hin*dIII-cut YCp50 and then subcloning a *Bgl*II *ADE2* fragment from pRS417 into *Bam*HI-cut YCp50-*TOP3* plasmid.

Plasmid pWJ1209 was made by amplifying intergenic region iYMR188 with primers F-*Bam*HI-iYMR188 GTGTGTGGATCC TTTCTTAACGTCGCTAGGAGAAGG and R-*Hin*dIII-iYMR188 GCTGCTAAGCTTTCACCCTCCCTTGATATTTCACC, digesting the PCR product with *Bam*HI and *Hin*dIII and subcloning it into the *Bam*HI/*Hin*dIII site of pRS405.

Isolation of *top3*Δ slow growth suppressors: Strain U1619-9D (*top3*::*TRP1 SGS1* pWJ1189 [*CEN-TOP3-URA3-ADE2*]) was subjected to ethyl methanesulfonate (EMS) mutagenesis as described (Lawrence 1991, Chap. 18). Briefly, overnight cultures were washed with phosphate buffer, chilled on ice, sonicated, and resuspended in the buffer to a concentration of 5×10^7 cells/ml. EMS (Aldrich Chemical, Milwaukee) was added to a concentration of 3% and cultures were incubated with shaking at 30° for 30 min. An equal volume of 10% sodium thiosulfate was added to inactivate EMS. Untreated controls were incubated in parallel with the mutagenesis. Cells were counted and plated in triplicate on −ura and YPD plates to estimate mutagen-induced loss of viability. Viability following EMS treatment after three different rounds of mutagenesis was 50, 65, and 80%. Mutagenized cells were split into 5–25 separate cultures and grown in YPD to facilitate plasmid loss. The following day, 200 μl of each saturated culture was plated on 5-FOA/-trp plates to detect colonies that had lost *URA3* expression but had retained the *top3*::*TRP1* disruption. 5-FOA^R colonies were replica plated to −ade medium to detect colonies that had simultaneously lost *URA3* and *ADE2* expression, identifying those that had lost pWJ1189. Growth of colonies that are Ura$^-$ and Ade$^-$ was compared with growth of colonies from an unmutagenized *top3*Δ strain. Isolates that grew better than the *top3*Δ control were identified as putative *top3*Δ slow growth suppressors.

Classifying *top3*Δ suppressor mutations: Some *top3* suppressor mutants were expected to be due to mutation of *SGS1* and have the genotype *top3*Δ *sgs1* and not *top3*Δ *SGS1 sup*. Since homozygous *sgs1*/*sgs1* diploids are sensitive to 0.03% methyl methanesulfonate (MMS), a complementation test was used to identify *top3*Δ slow growth suppressors due to mutation of *SGS1*. All isolates were crossed to an *sgs1*Δ tester strain. The diploids (genotype *top3*Δ/+ +/*sgs1*Δ *sup*/+ or *top3*Δ/+ *sgs1*/*sgs1*Δ) were replica plated to YPD plates containing 0.03% MMS. New mutant alleles of *SGS1* fail to complement *sgs1*Δ MMS sensitivity and produce diploids unable to grow on 0.03% MMS. Since alleles of *SGS1* that suppress *top3* slow growth while remaining MMS resistant in a *TOP3* background also exist, linkage of the other suppressor mutations to *SGS1* was analyzed next (Mullen *et al.* 2000). These suppressors were crossed to a strain containing the *LEU2* marker integrated next to *SGS1*. Suppressors unlinked to *SGS1-LEU2* were backcrossed at least two more times into a nonmutagenized background to remove potential nonspecific mutations induced by EMS. Suppressor mutations in an otherwise wild-type background were analyzed for their sensitivity to HU, MMS, and ionizing radiation (IR). Those that showed severe sensitivity to IR were crossed to strains containing deletions in *RAD51*, *RAD52*, *RAD54*, *RAD55*, or *RAD57* and complementation of the IR^S phenotype was assayed. When a suppressor mutation failed to complement IR sensitivity of a *rad*Δ, the corresponding *RAD* gene was sequenced in the mutant.

Assaying sensitivity to DNA-damaging agents: HU and MMS were added to the agar medium prior to pouring the plates. Yeast cells were collected from exponentially growing cultures, sonicated, counted, and plated to a quantity of ~300 cells per plate. After 5 days of growth, HU sensitivity of a given strain was evaluated by comparing the size of its colonies to the wild-type control. To determine the MMS sensitivity of a strain, colonies on YPD and MMS plates were counted after 5 days of growth and the number on YPD was taken as 100%. For spot assays, cells were harvested as above and spotted onto plates in a 10-fold serial dilution series, the most concentrated spot containing 10^5 cells. To assay sensitivity to γ-irradiation, cells were similarly diluted onto YPD plates and exposed to different doses of γ-rays using a Gammacell-220 ^{60}Co irradiator (Atomic Energy, Ottawa).

***SUP4* recombination assay:** Determination of deletion frequencies between *SUP4* repeats has been described previously (Rothstein *et al.* 1987; Wallis *et al.* 1989). Briefly, W303 strains contain mutations in the *ADE2* and *CAN1* genes that are ochre suppressible. The *SUP4*-**o** dominant allele was linked to a selectable *URA3* marker, which was inserted between δ sequences 4 and 5 (see Figure 4A). Upon plating cells onto medium containing 5 μg/ml of adenine and 60 μg/ml of canavanine, it is possible to determine the frequency of cells that have become resistant to canavanine through a recombination event involving the δ sequences. These recombination events lead to the simultaneous loss of the *SUP4*-**o** and *URA3* genes, giving rise to red canavanine-resistant colonies that can no longer grow on medium lacking uracil but can grow on medium containing 5-FOA. The determination of the deletion classes was next performed by probing genomic blots of digested DNA as previously described (Rothstein *et al.* 1987). We also developed a PCR-based assay that could distinguish among the seven known *SUP4* deletion classes. Four PCR primers, A (GCACAAACATAACAGCCATGA), B (GTGCAGAAAA CTTACACGATGG), C (CTTACCGCAGTAGGGGGAG), and

TABLE 1

Strains

Strain[a]	Genotype
W1588-4C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W1588-4A	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
U1619-9D[b]	*MAT***a** *top3::TRP1 pWJ1189 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 lys2*Δ
W3209-3C[b]	*MAT*α *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3326-10B[b]	*MAT*α *sgs1::HIS3 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3209-10C[b]	*MAT*α *rad51*Δ *bar1::LEU2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3210-18B[b]	*MAT*α *rad52::HIS5 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3428-4D[b]	*MAT*α *rad54::LEU2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3211-23B[b]	*MAT*α *rad55*Δ *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 lys2*Δ *met15*Δ
W3212-6D[b]	*MAT*α *rad57::LEU2 RAD52-YFP can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3239-7B[b]	*MAT*α *SGS1-LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3473[b]	*MAT***a** *rad51*Δ *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3472[b]	*MAT***a** *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *rad55*Δ *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ
W3475[b]	*MAT***a** *rad57::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3002[b]	*MAT***a** *rad52::HIS5 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 met15*Δ *MAT*α *sgs1*Δ *top3*Δ *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 lys2*Δ
W3248[b]	*MAT***a** *rad54::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3245[b]	*MAT*α *sgs1*Δ *top3*Δ *rad51::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT***a** *rad50::URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3247[b]	*MAT***a** *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *xrs2::URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3249[b]	*MAT***a** *rdh54::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *sgs1::URA3 top3::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3250[b]	*MAT***a** *mre11::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3251[b]	*MAT***a** *sgs1::HIS3 top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *rad59::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3304[b]	*MAT***a** *rad54-K341A ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3639[b]	*MAT***a** *yku70::K.lactis URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *top3::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3649[b]	*MAT***a** *top3::TRP1 rad51*Δ *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *rad1::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3564[b]	*MAT***a** *top3::TRP1 rad1::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *sgs1*Δ *RAD50::URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*
W3964[b]	*MAT***a** *top3::TRP1 rdh54::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* *MAT*α *rad51::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*

(*continued*)

D (TCACAACCGCATAGAAGCC), from regions adjacent to δ 1, 2, 3, and 5, respectively, were designed. PCR products were obtained from two different sets of reactions and were compared both before and after digestion with *Xho*I. For example, primer pairs A/B and C/D amplify fragments of 781 and 1259 bp for class I or II events. Upon digestion with *Xho*I, the C/D product from class I events generates two fragments (303 and 956 bp) while class II events generate three fragments (242, 303, and 714 bp). In a similar fashion, all seven deletion classes could be unambiguously assigned.

RESULTS

Screen for non-*sgs1* suppressors of *top3* slow growth: We performed an EMS mutagenesis to screen for new

TABLE 1

(Continued)

Strain[a]	Genotype
J599[b]	*MAT***a** *SUP4-***o***::URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
J601[b]	*MAT***a** *SUP4-***o***::URA3 top3-2::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1058-11C[b]	*MAT***a** *SUP4-***o***::URA3 sgs1-25 top3-2::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1172-1C[b]	*MAT***a** *SUP4-***o***::URA3 top3-3::LEU2 rad52-8::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1262-12C[b]	*MAT***a** *SUP4-***o***::URA3 rad51::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1262-9B[b]	*MAT***a** *SUP4-***o***::URA3 rad51::LEU2 top3-5::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1266-3C[b]	*MAT***a** *SUP4-***o***::URA3 rad54::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1266-17A[b]	*MAT***a** *SUP4-***o***::URA3 rad54::LEU2 top3-5::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1267-1A[b]	*MAT***a** *SUP4-***o***::URA3 rad55::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1267-8B[b]	*MAT***a** *SUP4-***o***::URA3 rad55::LEU2 top3-5::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1268-8C[b]	*MAT***a** *SUP4-***o***::URA3 rad57::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1268-17B[b]	*MAT***a** *SUP4-***o***::URA3 rad57::LEU2 top3-5::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1292-3C[b]	*MAT***a** *SUP4-***o***::URA3 sgs1-25 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1292-3A[b]	*MAT***a** *SUP4-***o***::URA3 sgs1-25 top3-5::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1594-1A[b]	*MAT***a** *SUP4-***o***::URA3 sgs1::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
W1597-10C[b]	*MAT***a** *SUP4-***o***::URA3 top3-3::LEU2 sgs1::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*

[a] All strains are derivatives of W1588 (Zou and Rothstein 1997), a *RAD5* derivative of W303 (Thomas and Rothstein 1989), unless noted otherwise.

[b] This study.

*top3*Δ slow growth suppressor mutations, including possible hypomorphic mutations in essential genes. We had observed previously that spontaneous mutations (*sup*) that cause faster growth occasionally arise in a *top3* background. When such an event occurs early in a *top3* culture, most cells in the culture are *top3 sup*. To avoid these jackpot events prior to mutagenesis, the *top3* deletion was propagated in a strain containing a *TOP3* plasmid marked with *URA3* and *ADE2*. This serves two purposes: (1) the strain becomes *top3*Δ only upon plasmid loss; (2) the presence of the plasmid can be scored both by 5-FOA sensitivity and by color, as adenine prototrophs yield white colonies. The *top3*Δ *pTOP3-URA3-ADE2* strain was treated with EMS to induce mutations and was subsequently grown under conditions that allow plasmid loss. Growth rates of colonies that had lost both *URA3* and *ADE2* expression were compared with that of an unmutagenized *top3*Δ strain. Putative *top3*Δ slow growth suppressors were identified as growing faster than their unmutagenized counterparts (Figure 1A).

Until this report, *SGS1* had been the only known gene whose inactivation resulted in suppression of *top3* slow growth (Gangloff *et al.* 1994). Moreover, all 25 spontaneous *top3* slow growth suppressors identified in our laboratory were shown to be tightly linked to *SGS1* (our unpublished data). To investigate whether the new suppressor mutations were allelic to *SGS1*, complementation tests and genetic crosses were performed (see materials and methods for details). Mutations that complemented *sgs1*Δ MMS sensitivity and were unlinked to *SGS1* were analyzed further (Figure 1B). Overall, 53 *top3* suppressors were analyzed, 34 of which were due to mutations in *SGS1*.

Mutations in homologous recombination genes suppress *top3* slow growth and sensitivity to DNA-damaging agents: The phenotype of 19 suppressor mutations unlinked to *SGS1* was analyzed in an otherwise wild-type background. Since both Sgs1 and Top3 function in DNA metabolism and genome stability, it was possible that new *top3* suppressor mutations would also identify genes involved in these processes. Thus, HU, MMS, and IR sensitivities of the new mutants were tested. We observed that nine strains bearing suppressor mutations were sensitive to all three of these agents. Their sensitivity to IR indicated that they are defective in double-strand break (DSB) repair. Thus, standard complementation tests were used to determine whether the IR-sensitive mutants were allelic to members of the *RAD52* epistasis group. By this analysis, we identified 1 *rad51*, 1 *rad52*, 3 *rad54*, 3 *rad55*, and 1 *rad57* mutants. For each mutant, the appropriate *RAD* gene was sequenced; Table 2 lists the mutations isolated in this screen. In addition to the *rad* mutants, 10 other non-*sgs1* mutants were identified that rescue *top3* slow growth. These mutants will be described in a separate article.

To examine whether deletion of these *RAD* genes has the same effect on *top3* slow growth as the point mutations isolated in the screen, the corresponding *rad* deletion mutants were crossed to a *top3*Δ strain. The diploids were sporulated and the tetrads were dissected. Growth rates of *top3*, *top3 rad* mutants and appropriate controls were examined in liquid YPD medium and the results are summarized in Figure 2A. A wild-type strain has a doubling time of ~90 min, while an isogenic *top3* strain has a doubling time of ~260 min. Deletion of *SGS1* in a *top3*Δ background reduces the doubling time to 113 min, which is similar to that of an *sgs1 TOP3* strain, illustrating that *sgs1* is epistatic to *top3* for growth. We

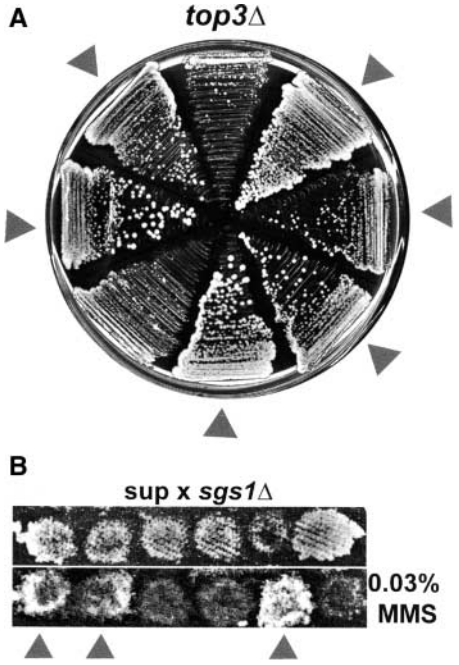

FIGURE 1.—Isolation of non-*sgs1* suppressors of *top3*Δ slow growth. (A) After EMS mutagenesis, colonies that lost p*TOP3-URA3-ADE2* were streaked on YPD next to an unmutagenized *top3*Δ strain. A representative plate is shown with arrowheads marking candidates that were picked as *top3*Δ slow growth suppressors. (B) Mutations in *SGS1* were ruled out by complementation analysis. *top3*Δ slow growth suppressor candidates (*sup*) were crossed to an *sgs1*Δ strain and the diploids were replica plated to 0.03% MMS. Candidates that complement *sgs1*Δ, indicated by arrowheads, were analyzed further.

found that deletion of *RAD51*, *-54*, or *-55* in a *top3*Δ background reduces doubling time to ~155 min. This is greater than the doubling time of the corresponding *rad* mutants in a *TOP3* background, indicating that their suppression of *top3* slow growth is partial. Deletion of *SGS1* in *top3 rad51*, *top3 rad54*, or *top3 rad55* mutants improves the growth rate to that of an *sgs1* mutant, indicating that the slow growth of a *top3 rad* mutant is caused by Sgs1. A catalytically inactive allele of *RAD54*, *rad54-K341A*, also suppresses *top3*Δ slow growth (data not shown). Unlike *rad51*, *rad54*, and *rad55* mutants, the *rad52* strain is slow growing on its own, with a doubling time of 130 min. Deletion of *RAD52* in a *top3*Δ background reduces the doubling time from 260 to ~190 min. Interestingly, in an *sgs1*Δ background, deletion of *RAD52* leads to a synergistic decrease in growth rate and a doubling time of ~170 min. This observation suggests that Sgs1 becomes especially important for normal growth in the absence of Rad52 and vice versa. Removal of *TOP3* does not further reduce the growth rate of an *sgs1 rad52* double mutant. In summary, these results demonstrate that a loss-of-function mutation in a gene affecting homologous recombination can improve the growth rate of a *top3* strain.

In addition to slow growth, *top3* mutants are highly sensitive to the DNA-damaging agents HU and MMS (CHAKRAVERTY *et al.* 2001; Figure 2, B and C). We tested whether homologous recombination contributes to this sensitivity by examining the phenotype of *top3*Δ mutants carrying deletions in *RAD51*, *-52*, or *-55*. To measure HU or MMS sensitivity, the strains were pregrown in liquid YPD medium, sonicated, and plated onto YPD plates containing different concentrations of HU or MMS. Presence of 10 mM HU in the medium severely retards the growth of *top3* mutants, resulting in formation of extremely small colonies after 5 days (Figure 2B). Deletion of *SGS1*, *RAD51*, or *RAD55* in *top3* mutants improves their ability to grow on HU-containing medium. On the other hand, deletion of *RAD52* does not rescue HU sensitivity of *top3* mutants (Figure 3B). Plating of *top3* mutants onto medium containing MMS results in their decreased survival (colony-forming ability) compared to plating onto medium without MMS (Figure 2C). Deletion of *SGS1*, *RAD51*, or *RAD55* in a *top3* background rescues this defect, with *sgs1*Δ being the best suppressor and *rad51*Δ the weakest (Figure 2C). In contrast, deletion of *RAD52*, which leads to significant MMS sensitivity on its own, does not rescue *top3* MMS sensitivity (Figure 2C and Figure 3D).

Previously, WU *et al.* (2001) reported that *RAD51* and *SGS1* are in the same epistasis group for HU and MMS sensitivity. These researchers measured the growth inhibition of *sgs1*, *rad51*, and *sgs1 rad51* mutants during transient exposure to 15 mM HU or 0.002% MMS. In addition, the same strains were plated in 10-fold dilutions and grown for 2–3 days on plates containing the same concentrations of the drugs. Here we explore further the genetic relationship between *SGS1* and the homologous recombination pathway by measuring the sensitivities of *sgs1*, *rad51*, *rad52*, *rad54*, *rad55*, and *sgs1 rad* mutants to different concentrations of HU and MMS using the protocol described in MATERIALS AND METHODS. In our genetic background, we found that *sgs1* and the *rad* mutants can grow on higher concentrations of HU and MMS than those mutants used above, although they are clearly sensitive to these agents. For example, single cell plating experiments show that 30 mM HU retards the

TABLE 2

Non-*sgs1 top3* suppressor mutations

Gene	Amino acid change
rad51	Ala252Thr
rad52	Asp164Asn
rad54	Gly340Ser
	Ser654Leu
	Gly745Ser
rad55	Glu78Lys
	Met175Ile, Glu201Asn
	Gln268Stop
rad57	Gly298Lys

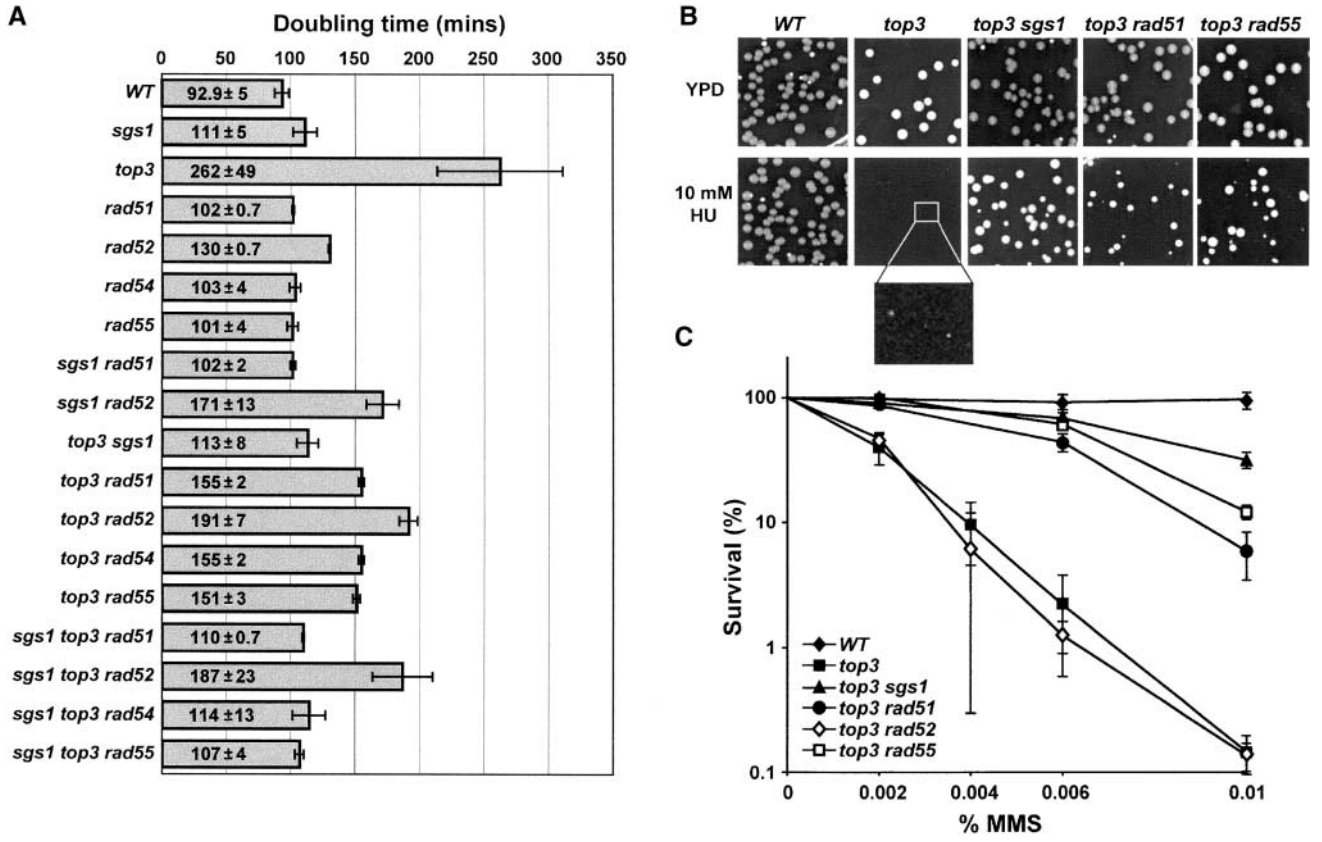

FIGURE 2.—Disruption of homologous recombination genes rescues *top3* defects. All experiments shown were performed with strains containing deletions of the indicated genes. (A) Deletion of *SGS1*, *RAD51*, *RAD52*, *RAD54*, or *RAD55* improves the growth rate of a *top3* mutant. Doubling times of the indicated strains in rich medium are shown. (B) Deletion of *SGS1*, *RAD51*, or *RAD55* rescues HU sensitivity of a *top3* mutant. All plates were scanned 5 days after plating, which allowed the *top3* colonies on YPD to attain the same size as the other strains. The very small *top3* colonies on 10 mM HU are visible on the enlarged section of the plate, which is shown with enhanced contrast for clarity. (C) Deletion of *SGS1*, *RAD51*, or *RAD55*, but not *RAD52*, rescues MMS sensitivity of the *top3* mutant. Survival curves of the indicated mutants are shown. As described in MATERIALS AND METHODS, log-phase cells growing in rich liquid medium were harvested, sonicated, and plated to the indicated concentrations of MMS (abscissa). After 5 days, survival was measured by counting colonies. The number of colonies on plates containing no MMS was taken as 100%. Error bars represent the standard deviation.

growth of *sgs1*, *rad51*, *rad54*, and *rad55* single mutants but not that of an isogenic wild-type strain (Figure 3A; data not shown). Interestingly, *sgs1 rad51*, *sgs1 rad54*, and *sgs1 rad55* double mutants grow even more slowly on 30 mM HU, forming smaller colonies after 5 days of growth than those of either the *sgs1* or the *rad* mutants alone (Figure 3A; data not shown). The *rad52*Δ strain is more sensitive to HU than the *rad51*Δ, *rad54*Δ, or *rad55*Δ strains, and *rad52*Δ HU sensitivity is also exacerbated by deletion of *SGS1* (Figure 3B). These data indicate that *SGS1* and the *RAD* genes participate in different pathways that repair HU-induced damage.

Whereas addition of HU to the medium retards the growth of *sgs1* and *rad* mutants, addition of MMS to the medium results in decreased survival (colony-forming ability) of these strains compared to an isogenic wild-type strain (Figure 3, C and D). Deletion of *SGS1* in a *rad51*, *rad52*, *rad54*, or *rad55* background even further reduces survival in the presence of MMS (Figure 3, C and D, and data not shown). This result suggests that, similar to their roles in HU resistance, *SGS1* and the *RAD* genes participate in different pathways that repair MMS-induced DNA damage.

Frequency and mechanisms of recombination at the *SUP4* locus in *top3*Δ mutants: The *SUP4*-**o** gene in *S. cerevisiae* encodes a tyrosine tRNA ochre suppressor surrounded by five δ sequences derived from long terminal repeats of the yeast Ty transposon (ROTHSTEIN *et al.* 1987; Figure 4A). These δ repeats occur in both direct and inverted orientation and are from 71 to 97% homologous to each other. The *SUP4* region may be difficult to replicate as it contains natural replication pause sites (*e.g.*, three tRNA genes) as well as the δ repeats. Such elements may promote formation of recombinogenic lesions (*i.e.*, chromosomal structures that lead to increased genetic recombination) during DNA replication. In wild-type cells, these lesions occur at low frequencies and are normally repaired by rearrangement-free means, such as gene conversion (GC) using the sister chromatid as a template (ROTHSTEIN *et al.* 1987; MCDONALD and ROTHSTEIN

1994). If the lesions are repaired by GC between misaligned sister chromatids or by single-strand annealing (SSA), this can lead to a deletion of the *SUP4*-**o** gene (Rothstein *et al.* 1987). Insertion of a *URA3* marker adjacent to *SUP4*-**o** simplifies the detection of such deletions as they result in the simultaneous loss of both genes. In wild-type cells, seven distinct deletion classes arise and they can be distinguished by genomic blotting or PCR (Rothstein *et al.* 1987; Figure 4B; materials and methods). Five classes that are either unassociated (classes I and II) or associated (classes III, IV, and VII) with crossing over (CO) arise via GC. The other two deletion classes (V and VI) are thought to arise primarily by direct repeat recombination via SSA between δ sequences 3 and 5, although other mechanisms for their formation (*e.g.*, unequal sister chromatid exchange of break-induced replication, or BIR) are also possible (Rothstein *et al.* 1987). For simplicity, we refer to classes V and VI as the SSA classes.

We analyzed the distribution of deletion classes and the overall deletion frequency in *top3*, *sgs1*, *rad*, and relevant double mutants to understand the role of these proteins in GC and SSA and to explore the reasons for *top3* rescue by *rad* mutations. Originally, *TOP3* was identified as a mutation that leads to increased *SUP4*-**o** deletion formation (Wallis *et al.* 1989). *SUP4*-**o** marker loss is increased 90-fold in *top3*Δ mutants compared to wild-type cells (Figure 4B). Mutation of *SGS1* also leads to hyper-recombination at *SUP4*-**o**, with a 16-fold increase in deletion formation (Figure 4B). In a *top3* background, mutation of *SGS1* reduces *SUP4*-**o** recombination from a 90-fold increase to a 35-fold increase, supporting the notion that Sgs1 functions upstream of Top3. Table 3 lists the distribution of deletion classes in various mutant backgrounds. Both GC and SSA classes are seen in *top3* and *sgs1* mutants, suggesting that neither protein is essential for either GC or SSA. In *top3* mutants, however, among the GC classes, the proportion of CO classes III and IV is increased and the proportion of non-CO classes I and II is decreased relative to wild type (Table 3).

Deletion of *RAD51*, *-54*, *-55*, or *-57* leads to a 2- to 7-fold overall increase in *SUP4* recombination. However, in these mutants, the GC classes I–IV and VII are almost entirely abolished and virtually all deletions belong to the SSA classes V and VI, due to hyper-recombination between δ sequences 3 and 5 (Figure 4B; Table 3). These results support previous reports that mutations in *RAD51* epistasis group genes lead to a severe reduction of GC but cause an increase in recombination between direct repeats (McDonald and Rothstein 1994;

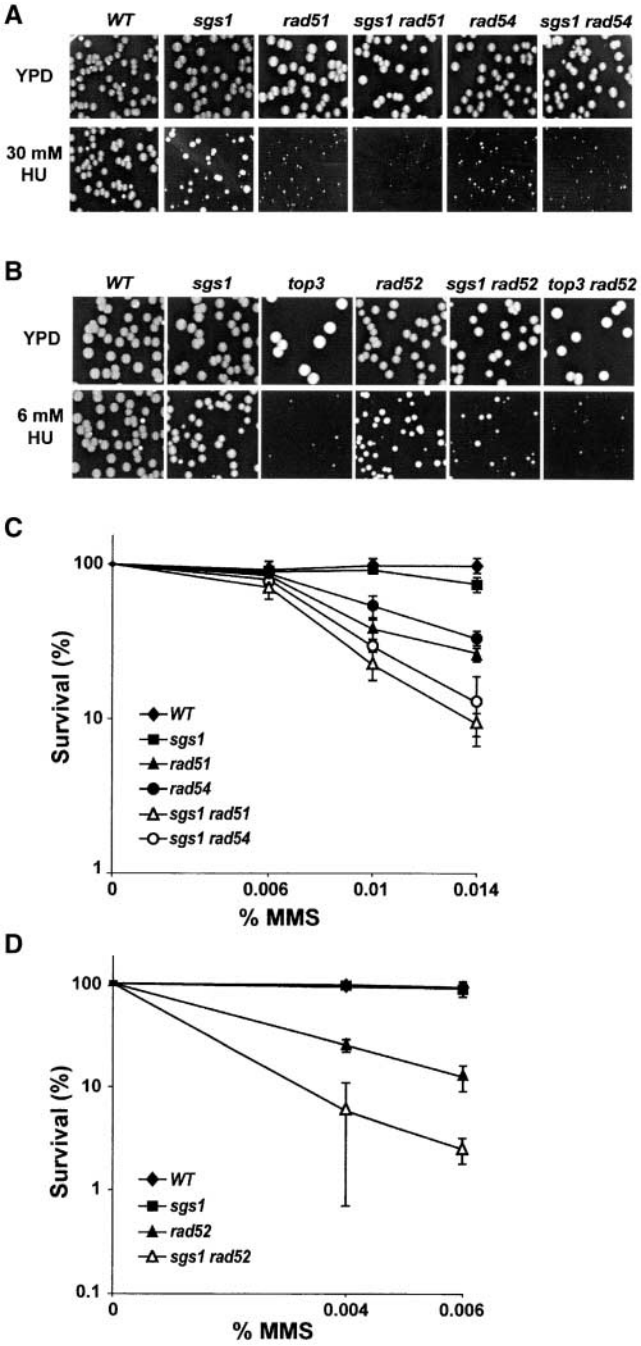

Figure 3.—Deletion of *SGS1* results in additional sensitivity to HU and MMS in strains carrying deletions of homologous recombination genes. Strains were grown in YPD, harvested in the exponential phase of growth, sonicated, and plated to indicated concentrations of HU or MMS. The data for the *rad52* strain are shown separately (B and D) because the *rad52* mutant is significantly more HU and MMS sensitive than *sgs1* or the other *rad* mutants. (A) Deletion of *SGS1* increases HU sensitivity in a *rad51* or *rad54* background. After 5 days of growth on 30 mM HU, *rad51 sgs1* and *rad54 sgs1* colonies are smaller than *rad51* and *rad54* colonies, respectively. (B) Deletion of *SGS1* increases HU sensitivity in a *rad52*Δ background. After 5 days of growth on 6 mM HU, *rad52 sgs1* colonies are smaller than *rad52* colonies. B also shows that deletion of *RAD52* does not rescue HU sensitivity of the *top3* mutant. (C) Deletion of *SGS1* increases MMS sensitivity in a *rad51* or *rad54* background. Survival was measured after 5 days of growth on solid medium containing indicated concentrations of MMS. (D) Deletion of *SGS1* increases MMS sensitivity in a *rad52* background.

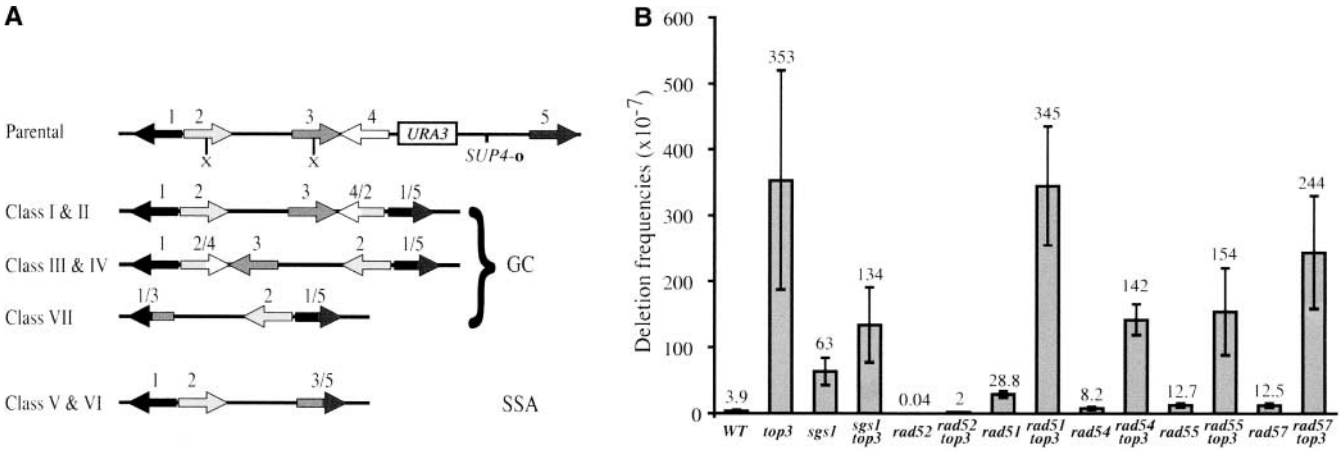

FIGURE 4.—Effects of inactivating *RAD51*, *-52*, *-54*, *-55*, or *-57* on *top3*Δ hyper-recombination at *SUP4*-**o**. (A) Map of the *SUP4* region in wild-type cells (parental) and different deletion classes that arise as a result of either GC or SSA. X indicates an *Xho*I site. Classes I and II are distinguished by the presence of an *Xho*I site in the recombinant 4/2 δ in class II. Classes III and IV are distinguished by the presence of an *Xho*I site in the recombinant 2/4 δ in class IV. Similarly, an *Xho*I site in the recombinant 3/5 δ in class VI is absent in class V (ROTHSTEIN *et al.* 1987). (B) Frequency of deletion formation ($\times 10^{-7}$) in wild-type cells and the indicated mutants as assayed by loss of both *URA3* and the *SUP4* ochre suppressor.

AGUILERA 1995; SUGAWARA *et al.* 1995). Loss of Top3 function in a *rad51*, *rad54*, *rad55*, or *rad57* background further stimulates *SUP4*-**o** deletion by 12- to 20-fold, where almost all deletions are in the SSA classes V and VI (Figure 4B; Table 3).

It was previously demonstrated that deletions at *SUP4*-**o** are highly dependent on Rad52, the central *S. cerevisiae* recombination protein (ROTHSTEIN *et al.* 1987). In *rad52* mutants, deletions are >100-fold down for classes I–IV and VII and >20-fold down for classes V and VI (ROTHSTEIN *et al.* 1987; Figure 4B). In a *rad52* background, hyper-recombination at *SUP4*-**o** in *top3* mutants is decreased, but it is still 50-fold higher compared to *rad52 TOP3* cells (2×10^{-7} *vs.* 0.04×10^{-7}) (Figure 4B). In the *top3 rad52* strain, only class V and VI deletions are recovered (Table 3). These results indicate that in the *rad52* background, the absence of Top3 generates chromosomal intermediates that can be processed by Rad52-independent recombination mechanisms.

The MRX complex is important for growth and DNA repair in the absence of Sgs1-Top3: In several processes, such as alternative lengthening of telomeres (ALT) and BIR, genes of the *RAD52* epistasis group have been further subdivided into two groups based on additional epistasis relationships: the *RAD51* group (*RAD51*, *-54*, *-55*, *-57*) and the *RAD50* group (*MRE11*, *RAD50*, *XRS2*, *RDH54*, and *RAD59*; LE *et al.* 1999; Q. CHEN *et al.* 2001; SIGNON *et al.* 2001). For example, BIR and ALT can both occur in *rad51* mutants or in *rad50* mutants but are abolished in *rad51 rad50* double mutants or in a *rad52* mutant.

Since our results indicated that recombinogenic chromosomal intermediates generated in the absence of Top3 can be repaired by Rad51-independent processes, we tested the effect of deleting genes of the *RAD50* group in a *top3* mutant background. Appropriate crosses were performed and tetrads dissected. We found that deletion of *MRE11*, *RAD50*, or *XRS2* (*MRX*) in a *top3*Δ background has a striking deleterious effect on viability and growth (Figure 5A, data not shown). In each case, double mutants, when viable, form only microcolonies. This synthetic defect is most severe for the *top3 mre11* mutants, which are usually inviable. Similar to other *top3* defects, *top3 mrx* mutants are partially rescued by deletion of *SGS1* or *RAD51* (Figure 5A).

We also investigated the genetic interaction between *MRX* and *SGS1*. We found that deletion of *SGS1* exhibits a synergistic slow growth with all three components of the MRX complex, the most severe synthetic defect being with *mre11*Δ. Figure 5B shows the doubling times of the appropriate single and double mutants in liquid YPD. Since deletion of *RAD51* partially rescues *top3* slow growth, we tested whether it would also rescue the *sgs1 rad50* synthetic slow growth. We found that *rad51*Δ indeed reduces the doubling time of an *sgs1 rad50* mutant from 202 to 171 min (Figure 5B). Additionally, *sgs1*, *rad50*, *rad51*, and the double- and triple-mutant combinations were tested for HU, MMS, and IR sensitivity (Figure 5C). We found that *sgs1 rad50* mutants are more sensitive to HU and MMS than either single mutant but exhibit IR sensitivity that is similar to that of the *rad50* mutant. Lastly, deletion of *RAD51* does not rescue these synthetic defects.

Exploring the contribution of Rdh54, Rad59, and Rad1 in the absence of Sgs1-Top3: Rdh54 and Rad59 also belong to the *RAD52* epistasis group (PÂQUES and HABER 1999). *RDH54* encodes a Rad54 homolog thought to be involved in homologous recombination in diploid cells (KLEIN 1997; SHINOHARA *et al.* 2000). *RAD59* encodes a Rad52 homolog whose inactivation results in a strong

TABLE 3

Distribution of *SUP4* deletion classes in different strain backgrounds

Class	Type	Wild type	*top3*	*sgs1*	*sgs1 top3*	*rad52*	*rad52 top3*	*rad51*	*rad51 top3*	*rad54*	*rad54 top3*	*rad55*	*rad55 top3*	*rad57*	*rad57 top3*
I	GC[a]	50	27	36	34	0	0	0	0	0	0	0	5	0	0
II	GC[a]	7	18	21	10	0	0	0	0	0	0	0	0	0	0
III	GC[b]	9	15	12	5	0	0	0	0	0	0	0	0	0	0
IV	GC[b]	9	20	12	10	0	0	0	0	0	0	0	0	0	0
V	SSA[c]	16	18	16	24	20	22	64	68	44	57	53	57	50	55
VI	SSA[c]	7	2	3	17	80	78	36	32	56	43	47	38	50	45
VII	GC[a]	2	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 4A shows the structure of the *SUP4* region and the different deletion classes. For each strain background, the number corresponds to the *percentage* of deletions that belong to a particular class.

[a] Gene conversion unassociated with crossing over.

[b] Gene conversion associated with crossing over.

[c] Single-strand annealing.

decrease in mitotic recombination when combined with mutation of *RAD51* (Bai and Symington 1996). To explore the roles of these proteins in the absence of Top3, *rdh54*Δ and *rad59*Δ were separately combined with a *top3*Δ. We found that, similar to deletion of *RAD50*, *MRE11*, or *XRS2*, deletion of *RDH54* in a *top3* background results in a severe growth defect or lethality, which is rescued by deletion of *SGS1* (Figure 6A). In contrast, deletion of *RAD59* does not significantly alter the size of *top3*Δ colonies (Figure 6B).

The requirement of MRX and Rdh54 functions underscores the importance of Rad51-independent processes for survival in the absence of Top3. The MRX complex is involved in at least two Rad51-independent processes: nonhomologous end joining (NHEJ) and SSA (Ivanov *et al.* 1996; Lewis and Resnick 2000; L. Chen *et al.* 2001). Therefore, we decided to examine genetically the contribution of each of these processes to normal viability and growth in a *top3* background.

NHEJ is a homology-independent error-prone mechanism of DSB repair that ligates two broken DNA ends. This process is dependent on the Ku70 and Ku80 proteins, encoded in yeast by *YKU70* and *YKU80*, DNA ligase 4, and the MRX complex (Lewis and Resnick 2000; L. Chen *et al.* 2001). To address whether NHEJ plays a role in resolving chromosomal intermediates that arise in *top3* mutants, we constructed a *top3*Δ *yku70*Δ double mutant. Deletion of *YKU70* had no detrimental effect in a *top3*Δ background, indicating that NHEJ is likely not involved in the repair of chromosomal structures formed in the absence of Top3 (Figure 6D).

A DSB can be processed by SSA if there are regions of homology on both sides of the break (Pâques and Haber 1999). SSA requires the Rad1/Rad10 heterodimer, which functions as an endonuclease and removes single-stranded DNA tails adjacent to the annealed regions (Ivanov and Haber 1995). We previously reported that deletion of *RAD1* reduces the size of *top3* spore colonies (Bailis *et al.* 1992; Figure 6C). We now find that although *top3*Δ *rad1*Δ mutants grow extremely poorly, they form slightly larger colonies than those of *top3*Δ *rad50*Δ mutants. Also, unlike *top3*Δ *rad50*Δ mutants whose synthetic defect is weakly suppressed by deletion of *SGS1*, the size of *top3*Δ *rad1*Δ colonies is almost fully restored to wild type by *sgs1*Δ (compare Figures 5A and 6C). Deletion of *RAD51* also rescues the *top3*Δ *rad1*Δ synthetic defect (data not shown). To investigate whether the MRX complex and Rad1 function in the same processes in the absence of Top3 (*e.g.*, SSA), we created a diploid heterozygous for *top3*Δ, *rad50*Δ, *rad1*Δ, and *sgs1*Δ and dissected 66 tetrads. Assuming 100% spore viability, we expected ~16 *top3*Δ *rad1*Δ *rad50*Δ triple mutants and the same number of quadruple mutants (*top3*Δ *rad50*Δ *rad1*Δ *sgs1*Δ). Although 11 quadruple mutants formed easily discernible microcolonies, no microcolonies were observed for the triple *top3*Δ *rad1*Δ *rad50*Δ mutant. Thus, while simultaneous

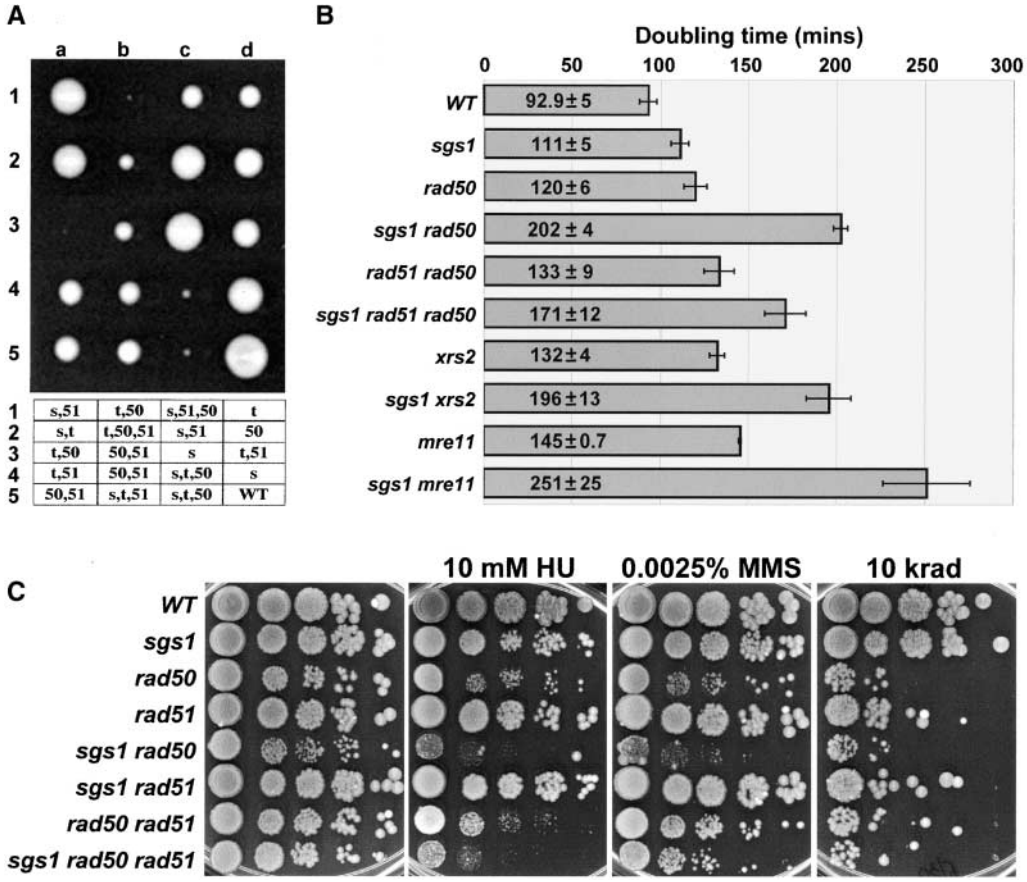

FIGURE 5.—Mutations in *RAD50*, *MRE11*, or *XRS2* have a synergistic deleterious effect in an *sgs1*Δ or *top3*Δ background. (A) Deletion of *RAD50* results in a severe synthetic growth defect or lethality in a *top3* background. Representative tetrads are shown. *top3 rad50* microcolonies are at positions 1b and 3a. The *top3 rad50* defect is somewhat rescued by deletion of *SGS1* (*sgs1 top3 rad50* spore products at positions 4c and 5c) or *RAD51* (*top3 rad50 rad51* spore product at position 2b). t, *top3*Δ; s, *sgs1*Δ; 50, *rad50*Δ; 51, *rad51*Δ. (B) Deletion of *RAD50*, *MRE11*, or *XRS2* results in a synthetic growth defect in an *sgs1*Δ background. Doubling times of the different mutants in YPD are shown. Slow growth of the *sgs1 rad50* mutant is partially rescued by deletion of *RAD51*. (C) Deletion of *RAD50* in an *sgs1*Δ background results in increased sensitivity to HU and MMS but not to gamma irradiation. Deletion of *RAD51* does not rescue HU sensitivity of the *sgs1 rad50* mutant. Similarly, survival of the *sgs1 rad50* mutant on MMS is not improved by *rad51*Δ, although the *sgs1 rad50 rad51* triple mutant forms larger colonies on MMS than the *sgs1 rad50* mutant. Strains were pregrown in liquid YPD medium, sonicated, and spotted in 10-fold serial dilutions onto YPD plates containing indicated concentrations of HU or MMS or exposed to 10 krad of gamma irradiation. Plates were photographed after 5 days.

deletion of *RAD1* and *RAD50* in an otherwise wild-type background does not result in a synthetic growth defect (data not shown), deletion of both *RAD1* and *RAD50* in a *top3*Δ background leads to lethality that is rescued by deletion of *SGS1*. This observation suggests that Rad50 and Rad1 have nonoverlapping functions in the absence of Top3.

Finally, to complete our investigation of the functions of the *RAD52* epsistasis group genes in the absence of Sgs1, we explored the effect of deleting *RAD59* or *RDH54* in an *sgs1*Δ background. Since deletion of *RAD1* shows a synergistic defect with *top3*Δ, we also combined *rad1*Δ with *sgs1*Δ to investigate whether Rad1 has important functions in the absence of Sgs1. Measurements of growth rates in liquid medium showed that none of these mutants are slow growing on their own or retard growth further in the absence of Sgs1 (data not shown). We also tested the effects of deleting *RAD59*, *RDH54*, or *RAD1* on HU and MMS sensitivity in an otherwise wild-type or *sgs1*Δ background. Figure 6, E and F, summarizes these results. The *rad59*Δ strain shows mild sensitivity to HU and intermediate sensitivity to MMS at the concentrations tested. In an *sgs1*Δ background, deletion of *RAD59* results in additional sensitivity to both agents, suggesting that Rad59 is involved in Sgs1-independent and HU- and MMS-resistant pathways. The *rdh54*Δ mutant behaves similarly to the wild-type strain with respect to HU and MMS resistance at the concentrations tested. However, in an *sgs1*Δ background, deletion of *RDH54* results in increased sensitivity to both agents, indicating that Rdh54 function becomes important for HU/MMS resistance in the absence of Sgs1. Similar to *rdh54*Δ, the *rad1*Δ strain does not display HU or MMS sensitivity at the concentrations tested. However, the *sgs1 rad1* mutant is slightly more HU sensitive and significantly more MMS sensitive than the *sgs1* mutant, showing that Rad1 becomes important for resistance to these agents in the absence of Sgs1.

DISCUSSION

Mutation of the Rad51 homologous recombination complex rescues *top3* defects: Our results demonstrate that *RAD51*, *RAD54*, *RAD55*, and *RAD57* contribute to slow growth and HU and MMS sensitivity in a *top3* background. Genetic and biochemical studies indicate that, at the molecular level, Rad51 catalyzes the invasion of ssDNA into a homologous duplex and is aided by the

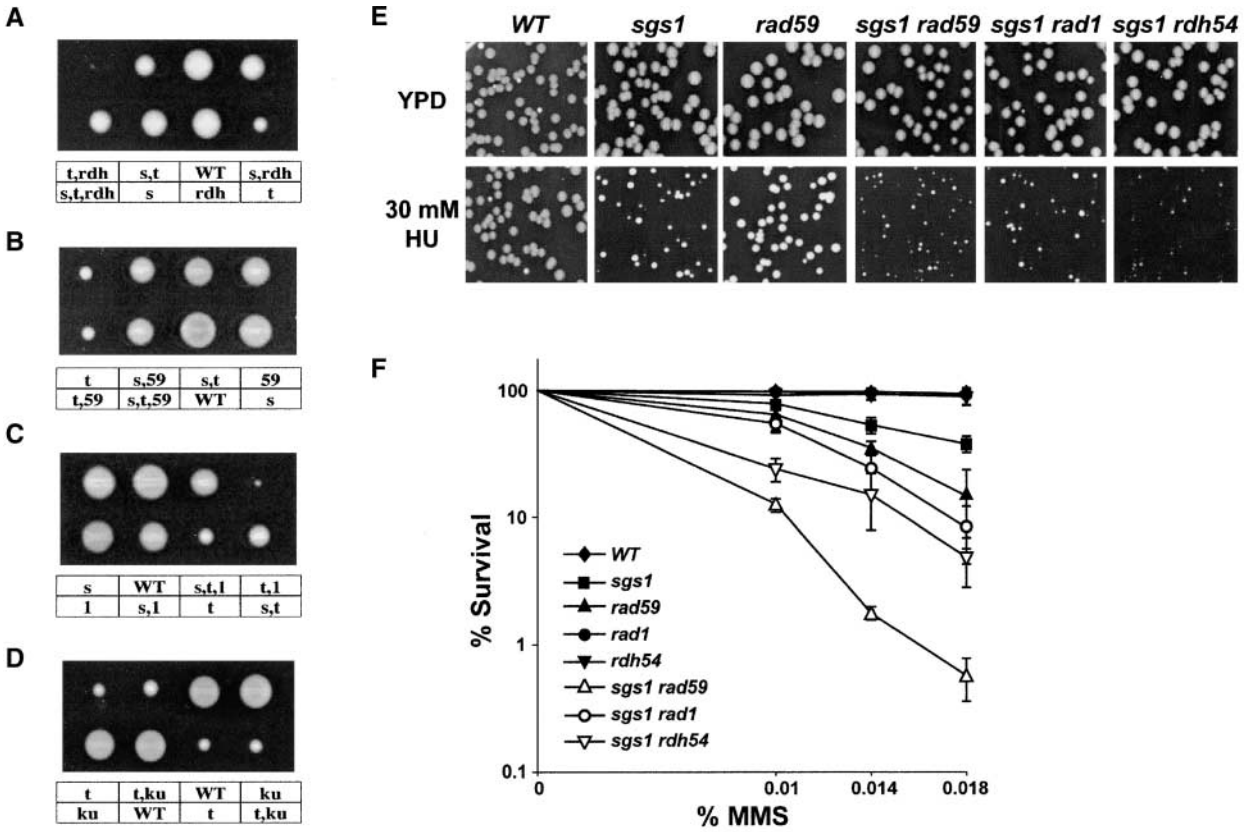

FIGURE 6.—Genetic interactions of *top3* and *sgs1* mutants with deletions of *RAD59*, *RAD1*, *RDH54*, and *YKU70*. (A) Deletion of *TOP3* shows a synergistic growth defect with deletion of *RDH54*, which in turn is suppressed by deletion of *SGS1*. s, *sgs1*Δ; t, *top3*Δ; rdh, *rdh54*Δ. (B) Deletion of *TOP3* does not show a synergistic defect with deletion of *RAD59*. 59, *rad59*Δ. (C) Deletion of *TOP3* shows a synergistic growth defect with deletion of *RAD1*, which in turn is suppressed by deletion of *SGS1*. 1, *rad1*Δ. (D) Deletion of *TOP3* does not show a synergistic defect with deletion of *YKU70*. ku, *yku70*Δ. (E) Deletion of *RAD59*, *RAD1*, or *RDH54* increases HU sensitivity in an *sgs1*Δ background. HU sensitivity of the *rad1* and *rdh54* mutants at the concentration shown is identical to that of the wild-type strain and these controls are not shown. (F) Deletion of *RAD59*, *RAD1*, or *RDH54* increases MMS sensitivity in an *sgs1*Δ background. *rad1* and *rdh54* strains do not exhibit sensitivity at these MMS concentrations and are indistinguishable from wild type in the graph.

DNA annealing protein Rad52 and the Rad55/Rad57 heterodimer (SUNG *et al.* 2000). Rad54, a member of the Swi2/Snf2 family of chromatin-remodeling proteins, presumably makes DNA more accessible for the recombination reaction (SUNG *et al.* 2000). Since mutations in these genes rescue *top3* defects, certain properties or functions of the homologous recombination complex comprised by these proteins must be detrimental in the absence of Top3.

RAD51, *RAD54*, *RAD55*, and *RAD57* belong to the *RAD52* epistasis group. Deletion of *RAD52*, a central *S. cerevisiae* homologous recombination gene, results in a decreased growth rate and resistance to HU and MMS on its own, but *rad52*Δ also partially rescues *top3* slow growth. The *RAD52* epistasis group has been genetically subdivided into two branches: the *RAD51* pathway (*RAD51*, *RAD54*, *RAD55*, and *RAD57*) and the *RAD50* pathway (*MRX*, *RAD59*, and *RDH54*). Processes such as BIR or ALT can occur in the absence of either pathway but not when both pathways or the Rad52 protein are inactivated (LE *et al.* 1999; Q. CHEN *et al.* 2001; SIGNON *et al.* 2001). The two branches also differ in their involvement in several other cellular processes. For instance, *MRX*, unlike the *RAD51* group, has been implicated in SSA, NHEJ, and intra-S-phase checkpoint activation (HABER 1998; D'AMOURS and JACKSON 2001; GRENON *et al.* 2001).

Here we demonstrate another difference between the *RAD51* and the *RAD50* pathways: their effect in *top3* mutants. In contrast to the Rad51 group proteins, the MRX complex becomes almost essential upon mutation of *TOP3* (Figure 5A). The *mrx* mutants also show marked synthetic growth and HU/MMS resistance defects with mutation of *SGS1* (Figure 5, B and C). Thus, MRX function is important when the Sgs1-Top3 pathway is inactivated and almost essential when Sgs1 acts alone. MRX involvement in SSA and in intra-S-phase checkpoint activation may make it important in both *sgs1* and *top3* backgrounds. Because DNA metabolism defects are characteristic of both *top3* and *sgs1* mutants, checkpoints are particularly important in these mutants for activation of repair and recombination pathways. Presumably,

such defects are more severe in *top3* mutants, so checkpoints become essential. This idea is supported by the observation that mutation of the central *S. cerevisiae* checkpoint genes, *MEC1* and *RAD53*, leads to lethality in a *top3* background and to slow growth in an *sgs1* background (Chakraverty *et al.* 2001; our unpublished results).

Roles of other recombination and repair genes in *top3* mutants: Rdh54 and Rad1, neither of which is required to maintain a normal growth rate in an otherwise wild-type background, become critical for growth when Top3 is inactivated (Figure 6, A and C). Similarly, while neither Rdh54 nor Rad1 is important for repairing HU- or MMS-induced damage in wild-type cells (at least at the drug concentrations we tested), they become important for HU/MMS resistance in the absence of Sgs1 (Figure 6, E and F). The Rad1/Rad10 complex is thought to be involved in recombination processes that require removal of nonhomologous sequences from the ends of recombining DNA molecules (Fishman-Lobell and Haber 1992). *top3* mutants may rely on such recombination processes for improved survival, while *sgs1* mutants may utilize these pathways when confronted with HU- or MMS-induced damage. We have shown previously that genome rearrangement in *top3* mutants is dependent on *RAD1* (Bailis *et al.* 1992). The results presented here suggest that SSA, a *RAD1*-dependent process, provides an alternative to *RAD51*-dependent recombination in *top3* mutants, especially in regions that contain direct repeats, such as *SUP4* and rDNA.

The importance of Rdh54 in *top3* and *sgs1* backgrounds may reveal new functions of this protein in DNA metabolism in haploid cells. Previously, Rdh54 was shown to have diploid-specific roles in mitotic and meiotic recombination (Klein 1997; Shinohara *et al.* 2000). In haploid yeast, Rdh54 has been implicated in "adaptation," a process during which a cell proceeds with mitosis in the presence of an unrepaired DSB after a cell cycle arrest in G2/M (Lee *et al.* 2001). This role of Rdh54 may be critical in *top3* mutants, since "DNA damage" is constantly created by Sgs1 and the cell may need to "adapt" to it to divide. yKu70 has also been implicated in adaptation, yet deletion of *YKU70* does not have a detrimental effect on growth in the absence of Top3 (Lee *et al.* 1998). This difference can be explained by proposing that Rdh54 and yKu70 detect different structures as signals of DNA damage and that yKu70 does not detect DNA intermediates generated by Sgs1. For example, yKu70 may detect DSBs, whereas Rdh54 recognizes other kinds of DNA structures. Alternatively, yKu70 may recognize damage mainly during G1, while Rdh54 is involved in damage recognition during S and/or G2 phases.

The results presented in this report are supported by other observations suggesting that when the Sgs1-Top3 pathway is impaired, Rad51-dependent recombination plays a detrimental role and cells rely on MRX- and Rad1-dependent processes for survival. For example, mutations of *SGS1* or *TOP3* exhibit synergistic growth defects with mutation of another helicase, Srs2 (Gangloff *et al.* 2000; McVey *et al.* 2001). Interestingly, mutation of several *RAD51* epistasis group genes rescues *sgs1 srs2* defects (Gangloff *et al.* 2000; McVey *et al.* 2001). *sgs1 srs2* mutants also show several other genetic interactions similar to *top3* mutants: they are inviable in combination with mutations in *MRX* genes and *RAD1* but show no synthetic defect with a deletion of *YKU70* (McVey *et al.* 2001).

Recombination at the *SUP4* locus: While mutation of *TOP3* or *SGS1* leads to an overall hyper-recombination phenotype at *SUP4*, both GC and SSA events are observed in these mutants in proportions roughly similar to the wild-type strain. This is consistent with previous observations where mutation of *SGS1* did not affect GC rates (Watt *et al.* 1996). Interestingly, at *SUP4*-**o**, the fraction of GCs with crossing over (classes III and IV) is increased in *top3* mutants relative to wild type. This may indicate that the absence of Top3 affects resolution of recombination intermediates, leading to increased formation of crossover events. Alternatively, the recombinogenic structures created by Sgs1 and left unresolved by Top3 may be preferentially channeled into recombination pathways that result in crossover products.

Although mutation of *RAD51*, *-54*, *-55*, or *-57* leads to a slight increase in overall deletion formation at *SUP4*-**o**, no GC events were observed in these mutants. We interpret this to mean that these proteins are required for GC at this locus, and that, in these mutants, recombinogenic lesions are channeled into *RAD51*-independent, rearrangement-prone recombination events, such as SSA, BIR, or NHEJ. However, unless extensive degradation of DNA ends takes place prior to end joining, NHEJ would not result in large deletions and therefore would not be detected in our assay. Deletion of *TOP3* in a *rad51*, *-54*, *-55*, or *-57* background further increases *SUP4*-**o** deletion rates in these mutants 12- to 20-fold. This increase is less severe than the effect that a *top3* mutation has in a Rad^+ strain, where *SUP4* recombination is increased 90-fold. These results suggest that in *top3* mutants, in the absence of the Rad51 pathway, Sgs1 creates fewer recombinogenic intermediates. Such intermediates are consequently processed by *RAD51*-independent mechanisms, leading to an increase in SSA classes and to an abolition of GC classes at *SUP4*.

Sgs1-Top3 and recombination during DNA replication: We observe that in mutants of the *RAD51* epistasis group, the ability of Sgs1 to cause slow growth and hyper-recombination in the absence of Top3 is diminished. This observation can be explained by a model in which the homologous recombination machinery helps recruit the Sgs1-Top3 complex to its site of action via the Rad51-Sgs1 interaction (Figure 7). In *top3* mutants, Sgs1 molecules create intermediates that are channeled into *RAD51*-dependent (GC) and *RAD51*-independent

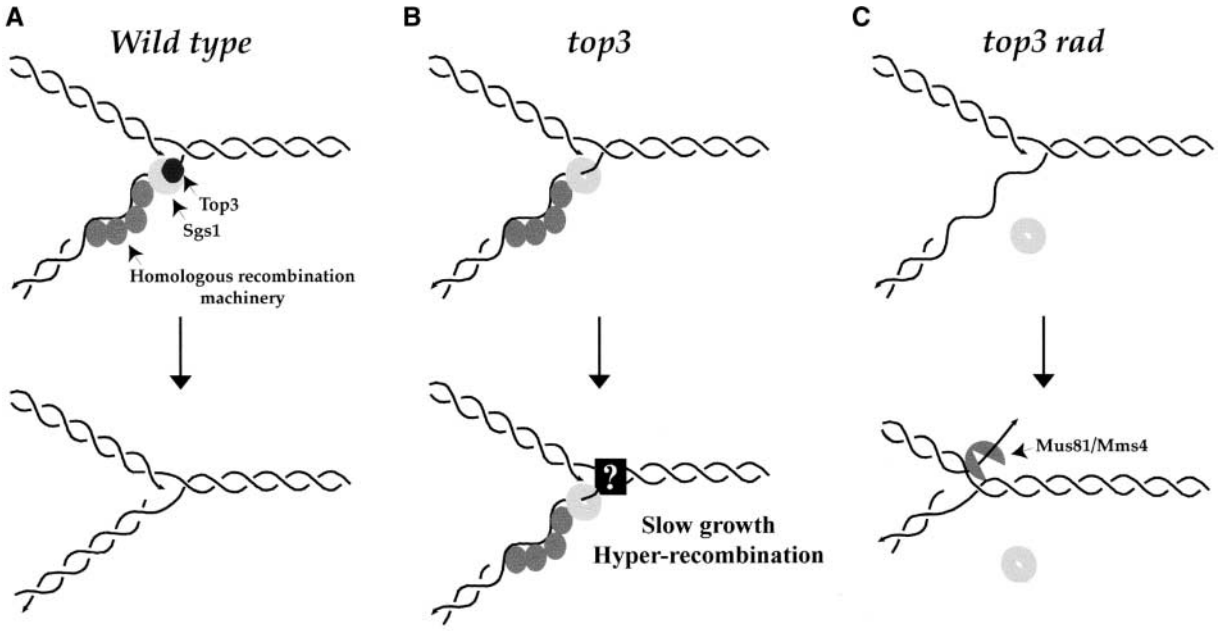

FIGURE 7.—A model to accommodate genetic and physical interactions between Sgs1, Top3, and homologous recombination proteins. As it is not known on which chromosomal structures Sgs1-Top3 acts during DNA replication, a replication fork is shown for the sake of simplicity. Arrowheads indicate the 3′-end of the nascent DNA strand. (A) The homologous recombination complex helps recruit Sgs1/Top3 to sites of replication-induced damage (*e.g.*, regions of single-stranded DNA at the replication fork). The coordinated action of these two protein complexes restores normal DNA replication. (B) In *top3* mutants, Sgs1 creates a chromosomal intermediate that normally requires resolution by Top3. The nature of this intermediate is not known (thus it is depicted as a solid box with a question mark), but the genetic interaction between *SGS1* and *TOP3* suggests that it causes slow growth in *top3* mutants. This intermediate is likely processed by different recombination pathways (consistent with the increase in gene conversion and single-stranded annealing classes of recombinants at regions such as *SUP4*). (C) Removing the homologous recombination complex may effectively reduce Sgs1 concentration at sites like that shown in A or impair its ability to create the harmful intermediate referred to in B, resulting in improved cell growth. Pathways that do not require the homologous recombination complex process the replicative damage in these mutants. In the example depicted here, a single-stranded tail produced by replication fork reversal is removed by the Mus81/Mms4 complex, restoring a normal replication fork (KALIRAMAN *et al.* 2001).

(SSA) recombination pathways, in proportions similar to those in wild-type cells (Table 3). These intermediates are also responsible for the slow growth of *top3* mutants, since inactivation of *SGS1* fully rescues the slow growth phenotype. Mutation of the homologous recombination complex results in decreased localization of Sgs1 to chromosomal sites and/or in decreased ability of Sgs1 to create these detrimental structures. Thus, fewer intermediates that require resolution by Top3 are created and growth is improved. The lesions that Sgs1 does create in these *rad top3* mutants are channeled into *RAD51*-independent recombination pathways, such as SSA.

In an alternative model, Sgs1 creates a substrate for Top3 that, when left unresolved in *top3* mutants, is channeled into various recombination pathways for resolution. The Sgs1-Rad51 physical interaction may help channel this intermediate into the Rad51 recombination pathway. The processing of the substrate by the homologous recombination machinery may create detrimental chromosomal structures that cause slow growth and other defects seen in *top3* mutants, while its alternative processing (*e.g.*, by Rad1-dependent SSA) may be beneficial. Further biochemical and cell biological studies are necessary to distinguish between the two models.

Both scenarios presented above are consistent with the idea that the Sgs1-Rad51 interaction is important for creation of the detrimental intermediate in *top3* mutants. A compelling piece of evidence in favor of this notion comes from studies of the *in vivo* roles of different domains of Sgs1. The C-terminal 200 amino acids that mediate Sgs1 interaction with Rad51 are dispensable for Sgs1 function in an otherwise wild-type background (MULLEN *et al.* 2000; WU *et al.* 2001). However, in a *top3* background, the *sgs1-ΔC200* allele behaves similarly to *sgs1Δ*, rescuing the slow growth caused by mutation of *TOP3* (MULLEN *et al.* 2000). These results suggest that the Sgs1-Rad51 interaction is important for Sgs1 to create chromosomal intermediates that require resolution by Top3.

Other studies have suggested a relationship between Sgs1 and homologous recombination. A model has been proposed in which Sgs1 and Srs2 regulate the processing of recombination intermediates during DNA replication (GANGLOFF *et al.* 2000). This model is supported by the observation that Sgs1 physically interacts with Rad51 via the Sgs1 C terminus (WU *et al.* 2001). To investigate further the relationship between Sgs1 and the homologous recombination complex, we tested the sensi-

tivity of the *sgs1* and *sgs1 rad* mutants to HU and MMS. Here we show that *SGS1* is not in the *RAD52* epistasis group for HU or MMS resistance, suggesting that the Sgs1/Top3 complex and the Rad proteins have independent roles in protecting the genome against damage induced by these agents (Figure 3). However, these results do not contradict the hypothesis that Sgs1/Top3 and the homologous recombination complex interact during DNA replication to promote genome integrity.

The data we present in this report are supported by other observations that homologous recombination plays a detrimental role in cells that lack the activity of either a RecQ homolog or a topoisomerase III. For example, in budding yeast, blocking meiotic recombination rescues the sporulation defect of *top3* homozygous diploids (Gangloff *et al.* 1999). Additionally, as discussed above, *sgs1 srs2* mutants are rescued by inactivating the *RAD51* pathway (Gangloff *et al.* 2000). Moreover, in humans, hyper-recombination between sister chromatids is a major contributor to the genomic instability characteristic of BLM cells, while aberrant recombination intermediates are found in WRN cells (German 1993; Prince *et al.* 2001). Further studies of the Sgs1-Top3 complex and its homologs promise to yield new information about its cellular role, the defects associated with its loss, and its relationship to homologous recombination.

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