A Novel Mutation in DNA Topoisomerase I of Yeast Causes DNA Damage and *RAD9*-Dependent Cell Cycle Arrest

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ABSTRACT

DNA topoisomerases, enzymes that alter the superhelicity of DNA, have been implicated in such critical cellular functions as transcription, DNA replication, and recombination. In the yeast *Saccharomyces cerevisiae*, a null mutation in the gene encoding topoisomerase I (*TOP1*) causes elevated levels of mitotic recombination in the ribosomal DNA (rDNA), but has little effect on growth. We have isolated a missense mutation in *TOP1* that causes mitotic hyper-recombination not only in the rDNA, but also at other loci, in addition to causing a number of other unexpected phenotypes. This topoisomerase I mutation (*top1-103*) causes slow growth, constitutive expression of DNA damage-inducible genes, and inviability in the absence of the double-strand break repair system. Overexpression of *top1-103* causes *RAD9*-dependent cell cycle arrest in G_2. We show that the Top1-103 enzyme nicks DNA *in vitro*, suggesting that it damages DNA directly. We propose that Top1-103 mimics the action of wild-type topoisomerase I in the presence of the anti-tumor drug, camptothecin.

TYPE I DNA topoisomerases remove superhelical turns from DNA by making a transient single-stranded break in the DNA backbone and then acting as a swivel point about which the strands may rotate [Figure 1; reviewed in CHAMPOUX (1990)]. A major role of topoisomerases is believed to be reduction of superhelicity that is generated by such processes as transcription and DNA replication (LIU and WANG 1987). In fact, a number of studies have implicated topoisomerases in these important cellular functions [reviewed in YANAGIDA and STERNGLANZ (1990)]. In the yeast *Saccharomyces cerevisiae*, topoisomerase mutants have been shown to build up supercoils on highly transcribed plasmids (BRILL and STERNGLANZ 1988; GIAEVER and WANG 1988), and topoisomerase activity has been shown to be required for DNA replication and transcription (GOTO and WANG 1985).

The yeast *TOP1* gene is a member of the highly conserved group of eukaryotic type I topoisomerases (GOTO and WANG 1985; THRASH *et al.* 1985). Despite the evidence for involvement of topoisomerase I in essential functions such as transcription and DNA replication, *TOP1* is not required for growth, and deletion of this gene results in only a modest growth defect. Deletion of *TOP1* does, however, cause a large increase in mitotic recombination in the tandemly repeated ribosomal DNA arrays, a chromosomal domain that is normally suppressed for recombination (CHRISTMAN, DIETRICH and FINK 1988). Deletion of *TOP1* in combination with a conditional mutation in the *TOP2* gene, which encodes the yeast type II topoisomerase, results in recombinational excision of rDNA circles (KIM and WANG 1989). The association between topoisomerase mutations and increased recombination levels is also observed for a third yeast topoisomerase gene, *TOP3*, which has been shown to elevate mitotic recombination between delta elements, which are the terminal repeats of the retrotransposon Ty (WALLIS *et al.* 1989).

In order to explore the role of topoisomerase I in suppressing recombination, we have isolated new mutant alleles of the yeast *TOP1* gene. Among these mutants, we identified one allele, designated *top1-103*, that has a number of unexpected phenotypes. *top1-103* causes an extremely high level of mitotic rDNA recombination, giving an rDNA recombination rate approximately 250-fold higher than wild type and 10-fold higher than that found in a *top1* deletion strain. *top1-103* also elevates recombination at other loci, unlike previously studied mutations in *TOP1* or *TOP2*. The *top1-103* mutant grows slowly and expresses DNA damage-inducible genes constitutively. The *top1-103* mutant requires an intact double-strand break repair system for viability. In addition, overexpression of *top1-103* causes cell cycle arrest in G_2 that is dependent on the *RAD9* gene product, a sensor of DNA damage. In the absence of *RAD9*, cells overexpressing *top1-103* traverse the cell cycle without repairing DNA damage, resulting in rapid cell death.

We show here that Top1-103 enzyme nicks DNA

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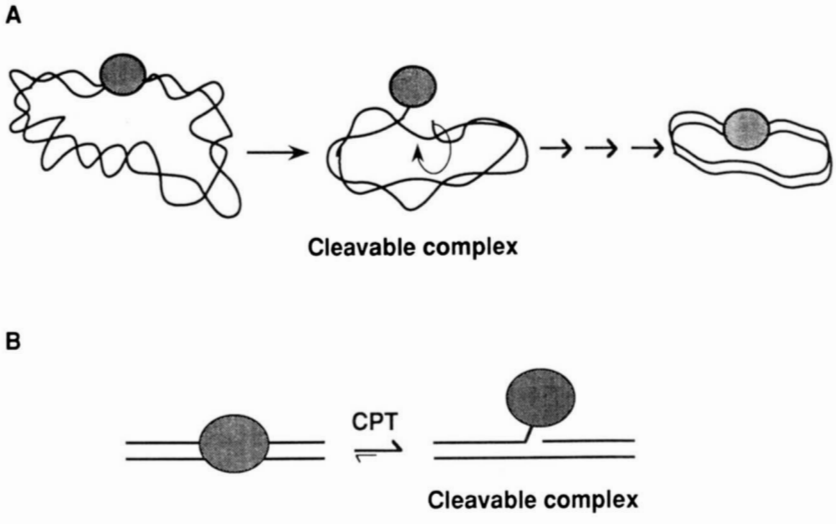

FIGURE 1.—Model of eukaryotic topoisomerase I mechanism. (A) Topoisomerase I binds to supercoiled DNA with high affinity in a noncovalent interaction. The enzyme makes a single-stranded nick in DNA, simultaneously becoming covalently bound to the 3′ end of the broken DNA strand. This topoisomerase-DNA intermediate is called the "cleavable complex." The enzyme provides a swivel about which the broken DNA strand may rotate to relieve superhelical tension. The enzyme then religates the broken DNA strands, releasing itself from the DNA. After multiple rounds of nicking and resealing, the supercoiled DNA is converted to the completely relaxed form. (B) The anti-tumor drug camptothecin (CPT) inhibits the religation step of the reaction, resulting in accumulation of the cleavable complex. (After LIU 1989.)

in vitro, suggesting that it can damage DNA directly. We propose that Top1-103 mimics the action of wild-type topoisomerase I in the presence of the anti-tumor drug, camptothecin. This drug causes topoisomerase I to become trapped during the relaxation reaction as part of a covalent enzyme-DNA complex [Figure 1; reviewed in Liu (1989, 1990)]. The enzyme-bound nicks generated by camptothecin result in hyper-recombination, induction of DNA damage responses, and cell cycle arrest, in close parallel to the effects of *top1-103*.

MATERIALS AND METHODS

Media, strains and genetic techniques: Strains are described in Table 1. YPD, SD, SC and 5-FOA media are as described in GUTHRIE and FINK (1991). "Sectoring plates" are SD supplemented with 75 μM adenine hemisulfate, 75 μM histidine, 400 μM tryptophan and 1.7 mM leucine. The levels of adenine and histidine are lower than those in standard media in order to achieve darker red coloration of *ade2* mutants. Yeast matings, tetrad dissection, lithium acetate transformation and replica-plating are as in GUTHRIE and FINK (1991).

Plasmids: YCp50 is a *CEN-ARS* vector marked with *URA3* (ROSE *et al.* 1987). YCp405 is a *CEN-ARS* vector marked with *LYS2* (MA *et al.* 1987). YEp24 is a 2-μm vector marked with *URA3* (BOTSTEIN *et al.* 1979). YIp5 is an integrative vector carrying *URA3* (STRUHL *et al.* 1979). YIp357r is an integrative vector carrying the *URA3*, amp^r and *lacZ* genes (MYERS *et al.* 1986). pRS303 and pRS305 are integrative vectors carrying *HIS3* and *LEU2*, respectively (SIKORSKY and HIETER 1989).

pCT80ΔTOP is an integrative plasmid used for creating a *top1-7::LEU2* disruption (THRASH *et al.* 1985). pSR16 and pSR18 are integrative plasmids marked with *URA3* for generating *lacZ* fusions to *DIN1* and *DIN3*, respectively (RUBY and SZOSTAK 1985). pR12Sclig2 is an *ARS* plasmid carrying *URA3* and *CDC9* (BARKER and JOHNSTON 1983). YEp13(RAD52) is a 2-μm plasmid carrying *LEU2* and *RAD52* (SCHILD *et al.* 1983). pSM21 is an integrative plasmid for generating the *rad52::TRP1* disruption, consisting of the 2-kb *Bam*HI fragment of *RAD52* cloned into the *Bam*HI site of pBR322 with an insertion of the *Bam*HI-*Bgl*II fragment of *TRP1* cloned in at the *Bgl*II site of *RAD52* (D. SCHILD, personal communication). pGAL::lacZ (pLR1Δ23) is a 2-μm plasmid containing *URA3* and *GAL1*-promoted *lacZ* (WEST, YOCUM and PTASHNE 1984). pWE3 is a *CEN-ARS* plasmid carrying *URA3* and *GAL1*-promoted *TOP1* (ENG *et al.* 1988). pNKY30 is an integrative plasmid for creating a *rad50::URA3* disruption (E. ALANI and N. KLECKNER, personal communication). pRR330 is an integrative plasmid for creating a *rad9::URA3* disruption (SCHIESTL *et al.* 1989).

CB17 consists of the 3.8-kb *Hin*dIII fragment of *TOP1* filled in with Klenow and cloned into the *Sma*I site of YEp24 (M. F. CHRISTMAN, personal communication). CB32 and CB39 are integrative vectors carrying rDNA sequences marked by *URA3* and *HIS3*, respectively (CHRISTMAN, DIETRICH and FINK 1988). CB45 carries *ADE2* on a 6-kb *Bam*HI fragment cloned into the *Bam*HI site of pBR322 and the 6.4-kb *Hin*dIII fragment of rDNA cloned into the *Hin*dIII site. CB51 and CB52 contain the 3.8-kb *Hin*dIII fragment of *TOP1* and of *top1-727*, respectively, cloned into the *Hin*dIII site of YCp405. CB107 is the same as pWE3, but with *top1-727* replacing *TOP1*.

pNL2 contains the 3.2-kb *Hin*dIII-*Nru*I fragment of *TOP1* cloned into the 7.0-kb *Hin*dIII-*Nru*I fragment of YCp50. pNL2-102, pNL2-103 and pNL2-106 are pNL2 containing the mutant alleles *top1-102*, *top1-103* and *top1-106*, respectively, in place of *TOP1*. pNL8 is an integrative vector consisting of the 4.3-kb *Hin*dIII-*Apa*I fragment of pNL2–103 cloned into the 3.5-kb *Hin*dIII-*Apa*I fragment of YIp5. pNL14 is a *CEN-ARS* plasmid consisting of the 2.6-kb *Mlu*I-*Nsi*I fragment of *top1-103* from pNL2-103 cloned into the 12.8-kb *Mlu*I-*Nsi*I fragment of CB51. pNL30 is a *CEN-ARS* plasmid containing the 2.3-kb *Bsu*36I fragment of pNL14 cloned into the 9.1-kb *Bsu*36I fragment of pWE3 to generate *GAL1*-promoted *top1-103*. pNL46 is an integrative plasmid marked with *LEU2* for fusing the *GAL1* promoter to alleles of *TOP1*, consisting of the 2.0-kb *Eag*I-*Eco*RV fragment of pNL30 cloned into the 5.5-kb *Eag*I-*Sma*I fragment of pRS305. pNL48 is an integrative plasmid for marking the rDNA with *LYS2*, consisting of a 2.8-kb *Eco*RI fragment of rDNA cloned into the *Eco*RI site of pUC9 carrying *LYS2*. pNL51 is a *CEN-ARS* plasmid carrying *URA3* and *top1-103,727* made by cloning the 865-bp *Sph*I fragment of CB52 into the 9.3-kb *Sph*I fragment of pNL2-103. pNL52 is a *CEN-ARS* plasmid carrying *URA3* and *GAL1*-promoted *top1-103,727* made by cloning the 865-bp *Sph*I fragment of CB52 into the 10.6-kb *Sph*I fragment of pNL30. pNL53 is an integrative plasmid marked with *HIS3* for fusing the *GAL1* promoter to alleles of *TOP1*, consisting

TABLE 1

Yeast strains

Strain	Genotype	Source
FDY65	*MATα leu2-3 ura3-52 CUP1::URA3 top1-7::LEU2* (pCT80ΔTOP)	CHRISTMAN, DIETRICH and FINK (1988)
CY126	*MATα leu2-3 ura3-52 his4::URA3::his4 top1-7::LEU2* (pCT80ΔTOP)	CHRISTMAN, DIETRICH and FINK (1988)
CY184	*MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rDNA::ADE2* (CB45)	M. F. CHRISTMAN
CY185	CY184 transformed to *top1-7::LEU2* (pCT80ΔTOP)	M. F. CHRISTMAN
NLY107	CY185 carrying pNL2	This work
NLY108	CY185 carrying YCp50	This work
NLY143	CY185 carrying pNL2-103	This work
NLY163	CY185 carrying pNL2-102	This work
NLY164	CY185 carrying pNL2-103	This work
NLY167	CY185 carrying pNL2-106	This work
NLY258	*MATα leu2-3 ura3-52 lys2 rDNA::URA3* (CB32) *top1-7::LEU2*, carrying YCp405	This work
NLY260	*MATα leu2-3 ura3-52 lys2 rDNA::URA3* (CB32) *top1-7::LEU2*, carrying CB51	This work
NLY336	*MATα leu2-3 ura3-52 lys2 rDNA::URA3* (CB32) *top1-7::LEU2*, carrying pNL14	This work
NLY360	*MATα leu2-3 ura3-52 lys2 rDNA::URA3* (CB32), carrying pNL14	This work
NLY363	CY184 carrying pNL2-103	This work
NLY373, 374	CY126 *lys2* carrying YCp405	This work
NLY375	CY126 *lys2* carrying CB51	This work
NLY377, 378	CY126 *lys2* carrying pNL14	This work
NLY379, 380	FDY65 *lys2* carrying YCp405	This work
NLY381, 382	FDY65 *lys2* carrying CB51	This work
NLY383	FDY65 *lys2* carrying pNL14	This work
NLY429	CY185 carrying pWE3	This work
NLY 494	CY185 carrying p*GAL-lacZ*	This work
NLY515	CY184 with *TOP1* replaced by *top1-103*, using pNL8	This work
NLY516	NLY515 switched to *MAT***a**	This work
NLY566	NLY515 carrying pNL2	This work
NLY618	NLY515 carrying CB17	This work
NLY620	CY185 carrying pNL30	This work
NLY638	NLY515 carrying YEp13(*RAD52*)	This work
NLY640	NLY515 carrying pR12Sclig2	This work
NLY642	NLY515 transformed to *rDNA::HIS3*, using CB39	This work
NLY650	*MATα ade2-1 leu2-3,112 trp1-1 lys2 his3 ura3 rad52::TRP1 top1-7::LEU2* (pCT80ΔTOP) *rDNA::ADE* (CB45)	This work
NLY652	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3 ura3 lys2 top1-7::LEU2* (pCT80ΔTOP) *rad52::TRP1* (PSM21) *rDNA::ADE2* (CB45)	This work
NLY678	CY184 transformed to p*GAL1-TOP1* using pNL46	This work
NLY681	NLY515 transformed to p*GAL1-top1-103* using pNL46	This work
NLY730, 731	*MATα ura3 ade2 lys2 trp1 his3 top1-103 rDNA::ADE2* (CB45) *rDNA::LYS2* (pNL48) *rDNA::HIS3* (CB39) carrying YEp24	This work
NLY732, 733	Same as NLY730, 731, but carrying CB17	This work
NLY737	NLY515 transformed to p*GAL-top1-103* (*HIS3*) with pNL53	This work
NLY755	*MAT***a** *leu2 trp1 his3 ade2 ura3 rad50::URA3* (pNKY30) *top1-7::LEU2* (pCT80ΔTOP)	This work
NLY814	CY185 carrying pNL51	This work
NLY816	CY185 carrying pNL52	This work
NLY880	CY185 carrying CB107	This work
NLY884	NLY681 transformed to *rad9::URA3* with pRR330	This work
NLY887	NLY681 transformed to *DIN1::lacZ* (*URA3*) with pSR16	This work
NLY891	*MAT***a** *ura3 ade2 his3 trp1 leu2 DIN1::lacZ* (*URA3*, pSR16) p*GAL-top1-103* (*HIS3*, pNL53) *rDNA::ADE2*	This work
NLY894, 895	NLY681 transformed to *DIN3::lacZ* (*URA3*) with pSR18	This work
NLY896, 897	NLY737 transformed to *DIN3::lacZ* (*URA3*) with pSR18	This work

of the 2.0-kb *Xho*I-*Sac*I fragment of pNL46 cloned into the 4.4-kb *Sac*I-*Xho*I fragment of pRS303.

Isolation of new alleles of *TOP1*: The *TOP1* gene on plasmid pNL2 was mutagenized *in vitro* using hydroxylamine HCl, by the method of NONET *et al.* (1987). The mutagenized plasmid was transformed into *Escherichia coli*, selecting ampicillin resistance, and approximately 10^6 colonies were pooled and grown to make plasmid DNA. An aliquot of the mutagenized plasmid was transformed into *E. coli* strain DB6507, a *pyrF* mutant that is complemented by the wild-type yeast *URA3* gene. By testing approximately 300 colonies for the ability to grow without uracil, the frequency of mutation of the *URA3* gene was determined to be 2.2%, indicating that the mutagenesis procedure was successful.

Mutagenized pNL2 was transformed into the yeast *top1-7::LEU2* strain CY185 (Table 1), selecting for Ura$^+$. Individual transformants were picked to 96-well microtiter dishes and grown overnight in synthetic complete medium lacking uracil at 30°. The cells were collected by centrifugation, digested with Zymolyase (1 mg/ml final concentration), and lysed in the same microtiter dishes. Extracts were tested for topoisomerase I activity by the method of THRASH *et al.* (1984, 1985), except that 4 μl of extract were added to 10 μl of assay buffer. Assays were at 37° for 1 hr, using approximately 200 ng per reaction of YIp357r as the supercoiled substrate plasmid. Reactions were stopped with sodium dodecyl sulfate (SDS) and electrophoresed on an 0.7% agarose gel without ethidium bromide. The gel was stained in ethidium bromide and photographed. All isolates that appeared defective in the initial screen were colony-purified and retested. Plasmid DNA was rescued from each mutant into *E. coli* (GUTHRIE and FINK 1991). The *top1* mutant plasmids were restriction-mapped and retransformed into CY185 to confirm the topoisomerase I activity defect. Of 324 transformants screened for lack of topoisomerase I activity, three *top1* mutants were isolated. An additional 49 mutants were isolated from 14,000 transformants screened by a colony-sectoring assay.

The *top1-103* mutation was shown to reside in the plasmid-borne *TOP1* gene, since transformation with plasmid DNA (pNL2-103) isolated from the *top1-103* mutant (NLY143) conferred on the parent strain all the same phenotypes as the original mutant. In addition, the *top1-103* allele was cloned onto an integrating plasmid (pNL8) and used to replace the wild-type *TOP1* gene in the chromosome. This construct was confirmed by Southern hybridization. The *top1-103* mutation in the chromosome conferred all the same phenotypes as the *top1-103* allele on a plasmid.

Molecular biology techniques: Plasmids were constructed by standard methods (MANIATIS, FRITSCH and SAMBROOK 1982) and are described above. Restriction enzymes were purchased from New England Biolabs. Sequencing was performed on double-stranded DNA (plasmids pNL2, CB51, pNL14, pNL2-103) using the U.S. Biochemical Corp. Sequenase kit. Southern blotting was performed according to MANIATIS, FRITSCH and SAMBROOK (1982), and labeled probes were prepared using the Prime Time random hexamer kit of International Biotechnologies, Inc.

Assays of rDNA recombination rate and frequency: The frequency of loss of the *rDNA::ADE2* marker was measured by picking colonies from medium lacking adenine (to select for *rDNA::ADE2*) and streaking them on medium containing adenine, to allow *ADE2* to be lost from the *rDNA* during growth. Colonies were scraped off the plates containing adenine, serially diluted in sterile water, and plated on medium containing adenine. The resulting colonies were replica-plated to medium lacking adenine to determine the frequency of Ade$^-$ cells. The frequency for each strain was calculated by taking the average of six independent colonies. The frequencies of loss of the *rDNA::URA3*, *rDNA::HIS3* and *rDNA::LYS2* markers were determined in an analogous manner to *rDNA::ADE2*.

The rate of rDNA recombination was determined by growing strains to be tested on SC − adenine medium to select for the *rDNA::ADE2* insertion. Colonies were picked into sterile water, serially diluted, and plated for approximately 200 colonies per plate on YPD (rich) medium. The colonies on YPD plates were replica-plated to SC − adenine to detect half-sectored colonies, which represent recombination events resulting in loss of *ADE2* from the rDNA in the first cell division. The rate of rDNA recombination was calculated as the number of half-sectored colonies divided by the total number of colonies, excluding totally red colonies, which presumably had lost *ADE2* from the rDNA before plating. The average rate for each strain was calculated from measurements on six independent colonies.

Frequency of recombination at *HIS4::URA3::HIS4* and *CUP1::URA3*: Strains to be tested were streaked for single colonies on medium lacking uracil to select for the *URA3* insertion. Colonies were picked to liquid medium containing uracil (to allow *URA3* to be lost during growth) in microtiter dishes. Strains were grown to saturation and serially diluted in sterile water. Dilutions were plated on medium containing 5-fluoro-orotic acid (5-FOA) to determine the number of Ura$^-$ cells [5-FOA selects against Ura$^+$ cells (BOEKE *et al.* 1987)] and on medium without 5-FOA to determine the total number of cells. The frequency of recombination for each strain was calculated by taking the average of six independent colonies.

Determination of synthetic lethality of *top1-103* with DNA repair mutants: We assayed for a genetic interaction between *top1-103* and a number of DNA repair mutants by crossing a *top1-103* haploid strain by a haploid strain of the opposite mating type carrying a marked deletion of the DNA repair gene to be tested, which we will refer to as *RADX*. The diploid generated by this cross was therefore heterozygous both at the *TOP1* locus and at *RADX*. When this diploid was sporulated, the alleles of *TOP1* and *RADX*, which are unlinked genes in all cases tested, should have assorted independently, generating equal numbers of all four possible combinations of *TOP1* and *RADX* alleles. If *top1-103* were lethal in combination with a deletion of *RADX*, however, the double-mutant spore clones would not grow, and this combination of alleles would not be recovered. We scored ascospore phenotypes by printing tetrad dissection plates to selective media in order to assay for the presence of the the marked deletion alleles.

β-Galactosidase assays: The *DIN1::lacZ* (pSR16) and *DIN3::lacZ* (pSR18) fusions (see above) were integrated at *DIN1* and *DIN3*, respectively, by transforming strains carrying a p*GAL-top1-103* plasmid (pNL46), and selecting for Ura$^+$ transformants. Single colonies were grown overnight at 30° in 10 ml minimal synthetic raffinose medium to select for the *DIN::lacZ* fusion. The cultures were split in two, and galactose was added to one half to 2% final concentration, in order to induce p*GAL-top1-103*. Glucose was added to the other half to 2% final concentration as a negative control. Cells were grown for 24 hr, collected by centrifugation, resuspended in 0.4 ml breaking buffer [0.1 M Tris, pH 8.0, 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM AEBSF protease inhibitor (CalBiochem)], and broken by vortexing with 300 mg acid-washed glass beads for 2 min at full speed. Cell debris and glass beads were removed by centrifugation. Extracts were assayed as in MILLER (1972). β-Galactosidase units [nanomoles *o*-nitrophenyl β-D-galactopyranoside (ONPG) cleaved per min per mg protein] were calculated using the following formula: (A_{420})(assay volume)/

(0.0045)(protein concentration)(assay time)(extract volume), and protein concentration was determined using the Bio-Rad dye reagent. Isogenic strains not carrying any *lacZ* fusion were assayed in parallel to determine the baseline units for each strain, and these baseline values were subtracted from the values of strains with the *DIN::lacZ* fusion.

Quantitation of cell cycle stages: Strains were grown overnight in synthetic raffinose medium lacking leucine (NLY681 and NLY884) or uracil (NLY429, NLY494, NLY620) to an OD_{600} of about 0.1. Galactose was added to a final concentration of 2%, and cultures were grown for 48 hr (NLY429, NLY494 and NLY620) or 29 hr (NLY681 and NLY884). An aliquot of each culture was sonicated to disperse aggregated cells, and 3 μl of each culture was observed using a hemacytometer. At least 300 cells of each culture were scored as unbudded, small-budded (bud diameter less than 1/2 that of mother), or large-budded (bud diameter greater than or equal to 1/2 that of mother).

Purification of topoisomerase I from yeast: Topoisomerase I was purified from cells overexpressing *TOP1* (NLY429) or *top1-103* (NLY620) from the *GAL1* promoter on a *CEN*, *URA3* vector (pWE3 and pNL30, respectively), and a mock purification was done from the isogenic strain without any *TOP1* construct (NLY108). The method of purification will be described in greater detail elsewhere (A. KNAB and M.-A. BJORNSTI, unpublished results). Cells were grown overnight at 30° in synthetic medium lacking uracil, diluted 1:100 into 300 ml synthetic raffinose medium lacking uracil, and grown to an OD_{600} of approximately 1.0. Galactose was added to 2% final concentration and the cells were grown for approximately 6 hr. Cells were collected by centrifugation, washed in sterile water, resuspended in 2 ml of TEEG (Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol) per gram wet weight, supplemented with 0.3 M KCl and standard protease inhibitors, and stored frozen at −70°. Cells were broken by vortexing with acid-washed glass beads for 15 min on a fixed-head vortexer. Cell breakage was monitored by determining the protein concentration of the extracts. Cell debris and glass beads were removed by centrifugation.

Cell lysates were fractionated by ammonium sulfate precipitations, collecting the material that was soluble at 30%, but insoluble at 70% saturation and resuspending it in TEEG buffer with protease inhibitors but without salt. The conductivity was adjusted to that of TEEG + 0.2 M KCl. This material was loaded on a 2-ml bed volume phosphocellulose (Whatman) column that had been equilibrated with TEEG + 0.2 M KCl in a Bio-Rad 10-ml capacity Poly Prep column. The column was washed with five volumes TEEG + 0.2 M KCl, and fractions were eluted in two column volume steps using TEEG containing increasing concentrations of KCl (0.4, 0.6, 0.8 and 1.0 M KCl). Topoisomerase I activity eluted at 0.6 M KCl.

Column fractions were stored at −20° in topoisomerase I storage buffer (0.3 M KCl, Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 50% glycerol). The protein concentration of each fraction was determined by Bio-Rad assay, and the quantity of topoisomerase I enzyme in a given fraction was determined by electrophoresis on a polyacrylamide gel and visualization of the proteins by silver staining. The identity of topoisomerase I, a 90-kD protein, was confirmed by Western blotting of a gel run in parallel to the silver stained gel, using polyclonal anti-yeast topoisomerase I antiserum (M.-A. Bjornsti, unpublished).

Nicking assays: The ability of wild-type and mutant topoisomerase I to nick a supercoiled plasmid substrate was determined as in HSIANG *et al.* (1985), except that the reaction buffer was 40 mM Tris, pH 7.6, 50 mM KCl, 0.5 mM DTT, 15 mM EDTA, and 30 μg/ml acetylated bovine serum albumin (BSA). Reactions were performed in 34 μl total volume, containing 4 ng negatively supercoiled pUC118 DNA and 6 μl diluted partially purified topoisomerase I. Camptothecin (Sigma) in dimethylsulfoxide (DMSO) was added to reactions to a final concentration of 0.11 mg/ml or DMSO alone was added to 11% final concentration as a control. Reactions were at 37° for 10 min. Reactions were stopped by adding SDS to 0.5% final concentration, followed by digestion with proteinase K (0.15 mg/ml final concentration) for one hour at 37°, except as noted. Reaction products were electrophoresed on 0.9% agarose gels containing 50 ng/ml ethidium bromide in order to separate supercoiled, relaxed and nicked topoisomers. The gels were blotted to Biotrans nylon membranes (ICN) and visualized by hybridization to hexamer-labeled pUC118, followed by autoradiography. Quantitation of radioactivity was performed using a Fujix BAS-2000 phosphorimager.

RESULTS

Isolation of new alleles of yeast *TOP1*: We obtained new *top1* mutations by randomly mutagenizing the cloned *TOP1* gene on a centromere-based plasmid (pNL2) and transforming the library of mutagenized plasmid DNA into a strain (CY185) carrying the *top1-7::LEU2* deletion allele. The transformants were screened for a lack of topoisomerase I activity by growing random transformants in liquid culture in microtiter dishes, making crude extracts from each of the cultures, and testing them for activity in an *in vitro* assay (THRASH *et al.* 1984, 1985). In this assay, the substrate, negatively supercoiled plasmid DNA, and the product, relaxed covalently closed circles, are easily distinguished because of their different mobilities on an agarose gel. Extracts from a strain expressing wild-type *TOP1* (NLY107) fully convert the supercoiled substrate plasmid to the relaxed form, whereas extracts from the isogenic *top1-7::LEU2* deletion strain (NLY108) fail to convert the substrate plasmid to the relaxed form (Figure 2). Transformants receiving a plasmid bearing a mutant allele of *TOP1* would, like *top1-7::LEU2*, be expected to lack topoisomerase I activity. Topoisomerase I assays on several new *top1* mutants are shown in Figure 2.

We tested the new *top1* mutants for their ribosomal DNA recombination levels. CHRISTMAN, DIETRICH and FINK (1988) showed that yeast strains with a null (deletion) allele of *TOP1* have elevated mitotic recombination in the rDNA arrays. The majority of new *top1* mutants also had elevated rDNA recombination levels that were indistinguishable from a strain carrying the *top1-7::LEU2* deletion (NLY108). One of the new *top1* mutants (NLY164), however, had an unusually high frequency of rDNA recombination compared to the *top1-7::LEU2* deletion strain, as judged by the colony sectoring assay (Figure 3). In this assay, the number of red sectors in a colony reflects the level of rDNA recombination. This unusual mutant, designated *top1-103*, was chosen for further study.

***top1-103* causes hyper-recombination in the rDNA arrays and at other loci:** Quantitation of the rDNA

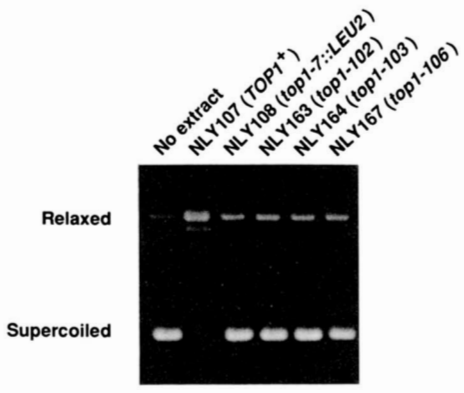

FIGURE 2.—Topoisomerase I assays of extracts from wild-type and *top1* mutant strains. Extracts from isogenic wild-type and *top1* mutant strains were incubated with a supercoiled plasmid substrate (pUC118) for 1 hr at 37° in topoisomerase I assay buffer (THRASH *et al.* 1984, 1985), and the reaction products were separated by electrophoresis on an 0.7% agarose gel. The DNA was visualized by staining in ethidium bromide. See Table 1 for complete genotypes.

recombination effect using the *rDNA::ADE2* marker showed that a *top1-7::LEU2* strain bearing *top1-103* on a plasmid (NLY164) had an rDNA recombination frequency 108-fold higher than the same strain carrying wild-type *TOP1* (NLY107). This strain carrying the vector alone (NLY108) had an rDNA recombination frequency 19-fold above wild type (Table 2A). Replacement of the wild-type *TOP1* gene (CY184) with *top1-103* (NLY642) by integrative transformation also resulted in a large (164-fold) stimulation of rDNA recombination frequency, in contrast to replacement of *TOP1* with *top1-7::LEU2* (CY185), which elevated rDNA recombination 23-fold (Table 2A). The stimulation of rDNA recombination by *top1-103* did not depend on using the *rDNA::ADE2* marker, as *top1-103* (NLY336) also increased the frequency of loss of an *rDNA::URA3* insertion to levels 338-fold higher than wild-type (NLY260; Table 2B), and of *HIS3* and *LYS2* insertions (NLY730, NLY731) in the rDNA arrays (Table 2C).

Consistent with the elevated frequency of rDNA recombination in *top1-103*, the rate of rDNA recombination was also significantly elevated in the *top1-103* mutant. Whereas the frequency of rDNA recombination events is determined by measuring the fraction of cells in a colony that have lost the rDNA marker, the rate of rDNA recombination is expressed as loss of the marker per cell per generation (see MATERIALS AND METHODS). *top1-103* (NLY515) lost *ADE2* from the rDNA at a rate 250 times greater than wild-type (CY184), whereas the *top1-7::LEU2* strain (CY185) had a rate of loss of *ADE2* that was 25 times greater than wild type (Table 3). As the effects of *top1-103* on the frequency and on the rate of recombination are consistent, only the frequency was measured in subsequent experiments.

The rDNA hyper-recombination phenotype of *top1-103* is semidominant. *top1-103* elevates rDNA recombination levels even in a strain expressing wild-type *TOP1*, although the frequency of rDNA recombination is not as high as in a strain expressing *top1-103* alone. *top1-103* on a low-copy plasmid (pNL14) elevated rDNA recombination to 48-fold above the wild-type level when transformed into a *TOP1* strain (NLY360), but to 338-fold above wild type when transformed into a *top1-7::LEU2* strain (NLY336, Table 2B). Increasing the dosage of *TOP1* in a *top1-103* mutant (NLY730 and 731) by transformation with a high-copy *TOP1* plasmid (CB17) resulted in a greater reduction of rDNA recombination frequency (28–40-fold) than when a single copy of *TOP1* was present (NLY732 and 733; Table 2C; also, compare Figure 3, C and D). In contrast to the reduction in rDNA recombination levels observed when the *top1-103* mutant was transformed with high copy *TOP1* (CB17), introducing the gene for DNA ligase (*CDC9*, NLY640) or the DNA repair gene *RAD52* (NLY638) on high-copy plasmids had no effect on the rDNA recombination level of a *top1-103* mutant (data not shown).

Deletion of *TOP1* elevates mitotic recombination in the rDNA but has little or no effect on recombination outside the rDNA arrays (CHRISTMAN, DIETRICH and FINK 1988). We tested *top1-103* for an effect on recombination at two other loci, by measuring the frequency of loss of the *URA3* gene from a tandem duplication of *HIS4* or from the *CUP1* locus, a tandem array of approximately 10 copies of the metallothionein gene (FOGEL and WELCH 1982). Surprisingly, the *top1-103* allele increases the recombination frequency at *HIS4* approximately 24-fold (NLY377 and NLY378) over the isogenic wild-type strain (NLY375), whereas the *top1-7::LEU2* deletion (NLY373 and NLY374) has only a twofold effect (Table 4A). *top1-103* also stimulates recombination at the *CUP1* locus (Table 4B). A *top1-103* mutant (NLY383) loses the *CUP1::URA3* marker 11 times more frequently than wild type (NLY381 and NLY382), whereas the *top1-7::LEU2* deletion (NLY379 and NLY380) has little or no effect on recombination at *CUP1*.

***top1-103* strains have a growth defect:** Although a deletion of *TOP1* causes only a slight growth defect, the *top1-103* mutation causes a severe growth defect that is readily observed when *top1-103* is streaked for single colonies on solid medium (Figure 4). A *TOP1* strain (NLY107) forms large colonies on solid medium and an isogenic *top1-7::LEU2* mutant (NLY108) forms only slightly smaller colonies, whereas the isogenic *top1-103* mutant (NLY164) grows very slowly, forming extremely small colonies. The slow growth of *top1-103* is recessive to wild type. A single copy of *TOP1* on a centromere vector (NLY566) restores wild-type

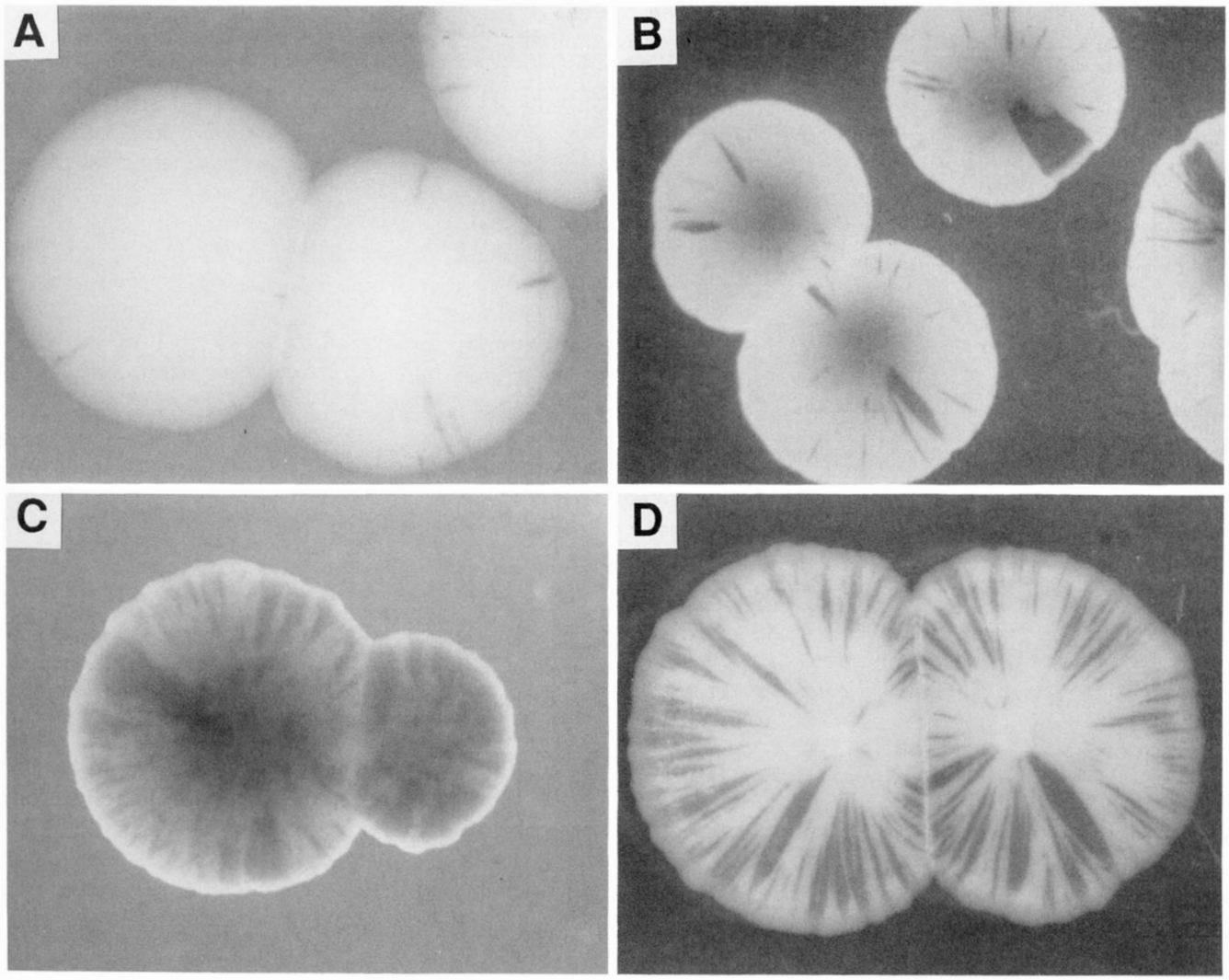

FIGURE 3.—A colony sectoring assay for rDNA recombination. rDNA recombination levels in isogenic wild-type and *top1* mutant strains were measured using a colony sectoring assay. In this assay, the *ADE2* gene is integrated into the rDNA array of an *ade2* mutant strain. Wild-type yeast cells form white colonies, whereas *ade2* mutants form red colonies. Each red sector results from an rDNA recombination event in which the *ADE2* insertion was lost. The number of red sectors in a colony reflects the frequency of rDNA recombination events. The colonies shown here were grown on "sectoring medium" (see MATERIALS AND METHODS). (A) NLY107 (*TOP1*). (B) NLY108 (*top1-7::LEU2*). (C) NLY164 (*top1-103*). (D) NLY618 [*top1-103* carrying CB17 (high copy *TOP1*)]. See Table 1 for complete genotypes.

growth to the *top1-103* mutant as well as *TOP1* on a high-copy plasmid (NLY618; Figure 4).

***top1-103* mutants require an intact double-strand break repair system:** *top1-103* is inviable in combination with a deletion allele of the double-strand break repair gene *RAD52*. When a *top1-103 RAD52* strain (NLY516) is crossed by a *top1-7::LEU2 rad52::TRP1* strain (NLY650), a pattern of spore lethality is observed. Out of 29 tetrads, no viable *top1-103 rad52::TRP1* (Leu^- Trp^+) spores were recovered, whereas all other combinations of Leu and Trp phenotypes were recovered. Since *TOP1* and *RAD52* are unlinked, one would expect one quarter of the spores to be Leu^- Trp^+. Further evidence for the synthetic lethality of *top1-103* and *rad52::TRP1* comes from transformation experiments in which a *rad52::TRP1 top1-7::LEU2* strain (NLY652) was transformed with several micrograms (μg) of either a *top1-103* bearing plasmid (pNL14) or the vector alone (YCp405). Although the vector control gave several hundred transformants, no *top1-103* transformants were recovered. Likewise, transformation of a *top1-103* strain (NLY515) with 1.2 μg of *rad52::LEU2* disrupting plasmid pSM21 was unsuccessful, whereas the same amount of this plasmid gave 14 Leu^+ transformants of the isogenic wild-type strain (CY184).

top1-103 is also lethal in combination with a deletion allele of *RAD50* (*rad50::URA3*), another gene in the double-strand break repair pathway. We crossed a *top1-103 RAD50* strain (NLY515) by a *top1-7::LEU2 rad50::URA3* strain (NLY755) and sporulated the diploid. Out of 17 tetrads examined, no Leu^- Ura^+ (*top1-103 rad50::URA3*) spores were recovered. The other combinations of markers were recovered at the expected frequencies.

The synthetic lethality of *top1-103* with

TABLE 2

rDNA recombination frequencies in isogenic wild-type, *top1-7::LEU2*, and *top1-103* strains

A. *rDNA::ADE2*

Strain	*TOP1* allele in chromosome	*TOP1* allele on plasmid	Frequency of loss of rDNA marker (%)	Frequency relative to *TOP1*$^+$
NLY107	*top1-7::LEU2*	*TOP1*	0.61	1
NLY108	*top1-7::LEU2*	Vector	11.5	19
NLY164	*top1-7::LEU2*	*top1-103*	66.1	108
NLY363	*TOP1*	*top1-103*	27.2	45
CY184	*TOP1*	—	0.51	1
CY185	*top1-7::LEU2*	—	11.6	23
NLY642	*top1-103*	—	83.6	164

B. *rDNA::URA3*

Strain	*TOP1* allele in chromosome	*TOP1* allele on plasmid	Frequency of loss of rDNA marker (%)	Frequency relative to *TOP1*$^+$
NLY260	*top1-7::LEU2*	*TOP1*	0.24	1
NLY258	*top1-7::LEU2*	Vector	5.0	21
NLY336	*top1-7::LEU2*	*top1-103*	81.0	338
NLY360	*TOP1*	*top1-103*	11.6	48

C. *rDNA::HIS3*, *rDNA::LYS2*

Strain	*TOP1* allele in chromosome	*TOP1* allele on plasmid	Frequency of loss of *HIS3* (%)	Frequency of loss of *LYS2* (%)
NLY730, 731	*top1-103*	Vector (YEp24)	36.2	39.3
NLY732, 733	*top1-103*	*TOP1* (CB17)	0.9	1.0
Ratio of vector to *TOP1*$^+$:			40	28

The frequency of rDNA recombination was determined as described in MATERIALS AND METHODS. Each value is the average of six independent measurements. All strains are isogenic and their complete genotypes appear in Table 1.

TABLE 3

rDNA recombination rates in isogenic wild-type, *top1-7::LEU2* and *top1-103* strains

Strain	*TOP1* allele in chromosome	Rate of loss of *rDNA::ADE2*	Rate relative to *TOP1*$^+$
CY184	*TOP1*	4.8×10^{-4}	1
CY185	*top1-7::LEU2*	1.2×10^{-2}	25
NLY515	*top1-103*	1.2×10^{-1}	250

The rate of rDNA recombination was determined using the *rDNA::ADE2* insertion as described in MATERIALS AND METHODS. Each value is the average of six independent measurements. All strains are isogenic and their complete genotypes appear in Table 1.

rad50::URA3 and *rad52::LEU2* is a specific genetic interaction, as the *top1-103* mutation is viable in combination with mutations in a number of other DNA repair genes, including *RAD1*, *RAD6*, *RAD9*, *RAD10*, the DNA polymerase δ subunit, *CDC2*, and the topoisomerase II gene, *TOP2*.

***top1-103* causes induction of DNA damage-inducible genes:** The synthetic lethality of *top1-103* with *rad50* or *rad52* suggested that *top1-103* causes DNA damage. We investigated this possibility by observing expression of two DNA damage-inducible genes, *DIN1* and *DIN3*, in strains carrying *top1-103* under control of the *GAL1* promoter. *DIN1* and *DIN3* are transcriptionally induced by DNA damaging agents such as UV light or MMS, although the signal directly responsible for induction is not known (RUBY, SZOSTAK and MURRAY 1983; RUBY and SZOSTAK 1985). We assayed the expression of the integrated *DIN::lacZ* fusions by measuring β-galactosidase activity after inducing p*GAL-top1-103* with galactose. We found that 24 hr after galactose induction the level of *DIN1* expression was elevated approximately 70-fold relative to the uninduced control (Table 5). The level of *DIN3::lacZ* expression also increased in response to induction of *top1-103*, reaching levels 5- to 12-fold higher than the uninduced control (Table 5), providing additional evidence that *top1-103* causes DNA damage. In control experiments, we determined that galactose induction of wild-type *TOP1* under control of the *GAL1* promoter had no effect on *DIN1::lacZ* expression.

***top1-103* overexpression causes *RAD9*-dependent cell-cycle arrest:** Overexpression of *top1-103* from the *GAL1* promoter severely inhibits growth of wild-type or *top1-7::LEU2* mutant cells. A strain carrying *top1-103* under control of the *GAL1* promoter on a centromere-based plasmid (pNL30, NLY620) or an integrated plasmid fails to form colonies on galactose

TABLE 4

Recombination frequencies at the *HIS4* and *CUP1* loci in isogenic wild-type, *top1-7::LEU2* and *top1-103* strains

	Strain	*TOPI* allele in chromosome	*TOPI* allele on plasmid	Frequency of loss of *URA3*	Frequency relative to *TOPI*
A.	*HIS4::URA3::HIS4*				
	NLY375	*top1-7::LEU2*	*TOPI*	2.3×10^{-4}	1
	NLY373, 374	*top1-7::LEU2*	Vector	4.9×10^{-4}	2.1
	NLY377, 378	*top1-7::LEU2*	*top1-103*	5.6×10^{-3}	24
B.	*CUP1:URA3*				
	NLY381,382	*top1-7::LEU2*	*TOPI*	3.8×10^{-4}	1
	NLY379, 380	*top1-7::LEU2*	Vector	1.3×10^{-4}	0.34
	NLY383	*top1-7::LEU2*	*top1-103*	4.1×10^{-3}	11

The frequency of recombination was determined as described in MATERIALS AND METHODS. Each value is the average of six independent measurements. All strains are isogenic and their complete genotypes appear in Table 1.

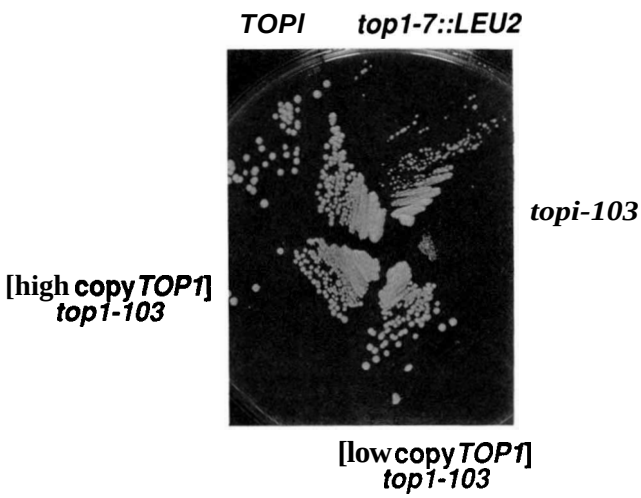

FIGURE 4.—Growth defect of *top1-103* mutant. Isogenic strains were streaked for single colonies on SD medium supplemented with tryptophan, histidine, and leucine. The relevant genotypes are as follows: NLY107 (*TOPI*), NLY108 (*top1-7::LEU2*), NLY164 (*top1-103*). NLY566 [*top1-103* carrying pNL2 (*TOPI* on a single-copy vector)], NLY618 [*top1-103* carrying CB17 (high-copy *TOPI*)]. See Table 1 for complete genotypes.

TABLE 5

Expression of *DIN1::lacZ* and *DIN3::lacZ* fusions in strains expressing *top1-103* from the *GAL1* promoter

Strain	Fusion	β-Galactosidase units: Galactose	β-Galactosidase units: Glucose	Ratio
NLY887	*DIN1::lacZ*	78.1	1.1	70.9
NLY891	*DIN1::lacZ*	45.5	0.68	66.9
NLY894/895	*DIN3::lacZ*	4.98	0.86	5.8
NLY896/897	*DIN3::lacZ*	4.0	0.34	11.8

DIN::lacZ activity was determined 24 hr after induction of p*GAL-top1-103* with galactose, as described in MATERIALS AND METHODS. All values were normalized by subtracting β-galactosidase units of an isogenic strain without any *lacZ* fusion.

medium (Figure 5). In contrast, isogenic control strains carrying plasmids that overexpress *lacZ* (NLY494) or TOPI (NLY429) from the GAL1 promoter form normal colonies on galactose medium (Figure 5). The strain carrying *GAL1*-promoted *top1-103* grows as well as the isogenic control strains on media containing the carbon sources glucose or raffinose (data not shown), which do not induce transcription of the GAL1 promoter.

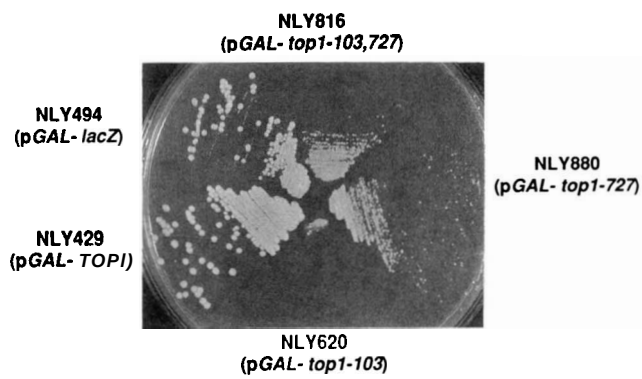

FIGURE 5.—Growth inhibition due to overexpression of *top1-103*. Strain CY185 (*top1-7::LEU2*) was transformed with a series of plasmids bearing the inducible *GAL1* promoter fused to one of the following genes: *lacZ* (NLY494), *TOPI* (NLY429), *top1-103* (NLY620), *top1-727* (NLY880), *top1-103,727* (NLY816). The transformants were streaked on synthetic galactose medium (lacking uracil) in order to induce the *CAL1* promoter, and incubated at 30° for 4 days.

When cells carrying *top1-103* fused to the GAL1 promoter (NLY681) are grown in liquid raffinose medium and then induced with galactose, the number of viable cells rapidly stops increasing and remains constant for greater than 24 hr (Figure 6). The number of viable cells in NLY681 begins to decrease after prolonged incubation in galactose medium, eventually reaching a level some 1000-fold lower than the uninduced control after 100 hr (data not shown). Control strains carrying *GAL1*-promoted *lacZ* (NLY494) or TOPI (NLY429) grow as well after galactose induction as in the uninduced cultures (data not shown).

Cells overexpressing *top1-103* arrest in the G_2 phase of the cell cycle. When strains bearing plasmids that overexpress *top1-103*, *lacZ*, or TOPI were induced with galactose and grown to saturation, 78% of cells overexpressing *top1-103* (NLY620) arrested in the G_2 ("large budded") stage (Table 6). DAPI-staining of

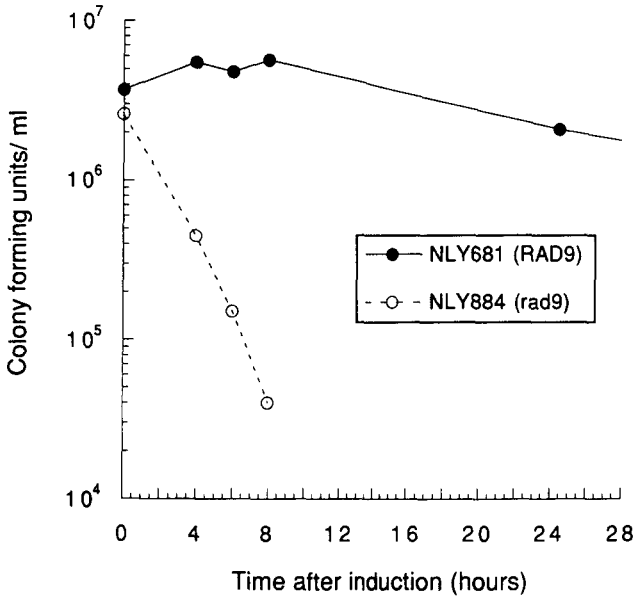

FIGURE 6.—Viability of wild-type and *rad9* mutant strains overexpressing *top1-103*. Isogenic wild-type (NLY681) and *rad9* (NLY884) strains carrying *GAL1*-promoted *top1-103* were grown to early log phase in synthetic raffinose medium lacking leucine. The *GAL1* promoter was induced by addition of galactose to 2% final concentration. At each time point, an aliquot was removed, serially diluted, and plated on SC − leucine (glucose) plates. The number of viable cells per ml was calculated. See Table 1 for complete genotypes.

TABLE 6

Cell cycle arrest of *RAD9* and *rad9* strains overexpressing *top1-103*

Strain	Genotype	Overexpression plasmid	Percent cells		
			Unbudded	Small-budded	Large-budded
NLY429	*RAD9*	*TOP1*	91	6	3
NLY494	*RAD9*	*lacZ*	93	4	3
NLY620	*RAD9*	*top1-103*	16	6	78
NLY681	*RAD9*	*top1-103*	4.5	5.5	90
NLY884	*rad9*	*top1-103*	55.5	17.6	26.9

The percentages of unbudded, small-budded and large-budded cells were determined for each strain as described in MATERIALS AND METHODS. At least 300 cells were counted for each strain. All strains are isogenic and their complete genotypes appear in Table 1.

these cells showed that they contained a single nucleus located at the neck of the bud (data not shown). In contrast, control strains overexpressing *lacZ* (NLY494) or *TOP1* (NLY429), had only 3% cells in G_2. These strains were overwhelmingly (93% and 91%, respectively) in G_1 (unbudded) at this time point (Table 6).

The *RAD9* gene product is required for G_2 arrest and maintenance of viability in cells overexpressing *top1-103*. Whereas wild-type cells overexpressing *top1-103* (NLY681) arrest in the G_2 phase and remain viable for at least 24 hr after induction, the isogenic *rad9::URA3* strain (NLY884) rapidly loses viability

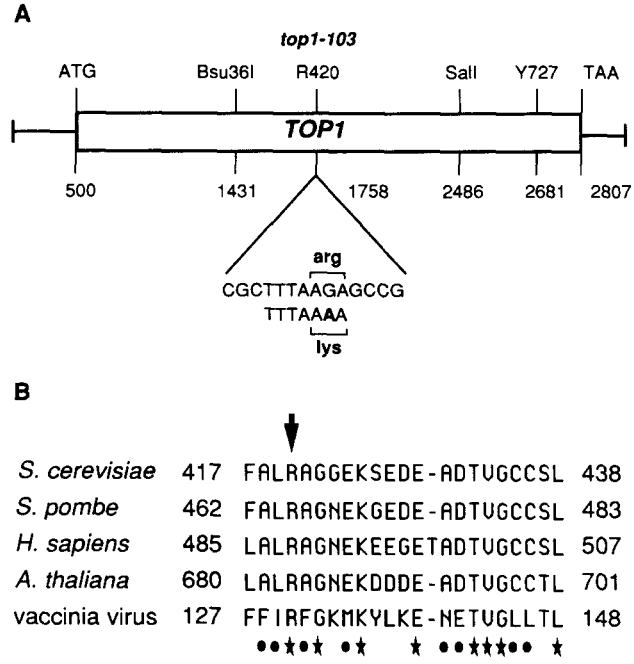

FIGURE 7.—Sequence of the *top1-103* mutation. (A) The coding region of *TOP1* is represented by the open box. The *top1-103* mutation was localized to the 1.1-kb *Bsu*36I-*Sal*I fragment by restriction fragment swapping experiments. The wild-type and *top1-103* mutant genes were sequenced in parallel, revealing a single base change (a G to A transition) at position 1758. This mutation results in an arginine to lysine substitution at amino acid 420. The mutation also generates a new *Dra*I site (TTTAAA) at position 1755, allowing confirmation of the sequence change. The active-site tyrosine (Y727) is indicated. (B) The *top1-103* mutation occurs in a region of high sequence conservation. The amino acid sequence of topoisomerase I from *S. cerevisiae*, *Schizosaccharomyces pombe*, *Homo sapiens*, vaccinia virus (LYNN *et al.* 1989), and *Arabidopsis thaliana* (KIEBER, TISSIER and SIGNER 1992) is shown for a 23-amino acid region identified by MORHAM and SHUMAN (1990) as being important for topoisomerase I activity. The numbers given for each species indicate the position of this region in the amino acid sequence of each protein. Stars indicate residues that are absolutely conserved in all five species, and dots indicate residues that are identical in all species but vaccinia virus, the least conserved of the five. The arginine residue mutated in *top1-103* is indicated by an arrow (After MORHAM and SHUMAN 1990).

(Figure 6). Within eight hours of induction of *top1-103*, the number of viable cells has decreased almost 70-fold. In contrast to the wild-type strain (NLY681) overexpressing *top1-103*, in which 90% of cells were arrested in the large budded stage (G_2) after 29 hr of induction, *rad9::URA3* (NLY884) cells were distributed throughout the cell cycle, with only 27% in G_2 (Table 6).

***top1-103* has an arginine to lysine substitution at a conserved arginine residue:** The *top1-103* mutation was localized by exchanging restriction fragments between the wild-type and mutant genes and determining which fragments conferred the slow growth and rDNA hyper-recombination phenotypes. The *top1-103* mutation was found to lie on a 1.1-kb *Bsu*36I-*Sal*I fragment (Figure 7A). Replacement of the wild-type *TOP1* sequence with this fragment from *top1-103*

resulted in a growth defect and quantitative rDNA recombination frequency that was indistinguishable from the original *top1-103* mutant. Sequencing of this fragment revealed only one base change, a G to A transition at position 1758, resulting in an arginine (AGA) to lysine (AAA) change at amino acid 420. This residue is absolutely conserved in all other eukaryotic type I topoisomerases (LYNN *et al.* 1989; KIEBER, TISSIER and SIGNER 1992; Figure 7B).

***top1-103* phenotypes require the active-site tyrosine:** Eukaryotic type I topoisomerases relax DNA by making a single-stranded nick in the DNA backbone and becoming covalently attached to the 3′ end of the broken strand [Figure 1A; reviewed in CHAMPOUX (1990)]. This covalent attachment occurs through an invariant tyrosine residue, referred to as the "active-site tyrosine." Substitution of phenylalanine for the active-site tyrosine (tyrosine 727 in *S. cerevisiae*; see Figure 7A) renders topoisomerase I catalytically inactive, as it cannot initiate the relaxation reaction (LYNN and WANG 1989; ENG, PANDIT and STERNGLANZ 1989). We examined whether the mutant Top1-103 enzyme requires the active-site tyrosine in order to exert its effects in the cell. If Top1-103 must nick DNA in order to cause hyper-recombination and cell-cycle arrest, then tyrosine 727 would be required for these phenotypes, and the double mutant *top1-103,727* would not be expected to display hyper-recombination or arrest. Alternatively, Top1-103 might not need to be catalytically active to exert its effects in the cell, if it acted, for example, as a dominant inhibitory subunit of an essential protein complex. In this case, Top1-103 would not need to cleave DNA to cause hyper-recombination and cell-cycle arrest, and the *top1-103,727* double mutant would be expected to have the same phenotypes as the *top1-103* single mutant.

As measured by the colony-sectoring assay for rDNA recombination, the *top1-103,727* double mutant (NLY814) has much lower recombination levels than the *top1-103* single mutant (NLY515; data not shown), indicating that Top1-103 must at least be able to initiate the topoisomerization reaction in order to cause hyper-recombination in the rDNA. Quantitation of rDNA recombination showed that strains carrying *top1-727* and *top1-103,727* had comparable recombination frequencies. In addition, *top1-103,727* overexpressed from the inducible *GAL1* promoter permits colony formation on galactose plates (NLY816), unlike the *top1-103* mutation alone (NLY620), which causes growth arrest when overexpressed from the *GAL1* promoter (Figure 5). Overexpressing *top1-103,727* from the *GAL1* promoter (NLY816) does cause some growth inhibition, but this effect is also seen with the *top1-727* single mutant (NLY880 in Figure 5). It is unlikely that the *top1-103,727* phenotypes are less severe than those of *top1-103* because of a difference in protein stability due to the *top1-727* mutation. Purification of Top1-103 and Top1-727 from cells bearing identical overexpression constructs (pNL30 and pNL52) yielded comparable amounts of the two mutant enzymes. No proteolysis of either mutant enzyme was observed. Since the active-site tyrosine is required for *top1-103* phenotypes, the Top1-103 mutant enzyme must at least be able to nick DNA in order to exert its effects in the cell. It is likely, therefore, that the mutant enzyme acts directly to damage DNA.

Top1-103 accumulates in the cleavable complex *in vitro*: *top1-103* was isolated in a screen for mutants defective in topoisomerase I activity. Whereas a wild-type cell extract completely relaxed 200 ng of negatively supercoiled substrate DNA after 1 hr at 37°, extract from a *top1-103* mutant was unable to convert a detectable amount of the substrate to the relaxed form (Figure 2). We used a modified version of the standard topoisomerase I assay to show that Top1-103 enzyme does relax supercoiled DNA, although it is very inefficient and tends to accumulate in the nicked enzyme-DNA intermediate (the "cleavable complex"; see Figure 1). In these experiments (Figure 8), we used partially purified fractions of topoisomerase I rather than crude extracts and we increased the enzyme to DNA ratio in order to make the assay more sensitive.

Experiments under a number of different assay conditions and using several independent enzyme preparations led to the same conclusion, that Top1-103 enzyme is defective in converting the cleavable complex to the relaxed covalently closed form. The experiment shown in Figure 8A is typical of the results obtained. During a 10-min incubation at 37° in the absence of camptothecin, Top1-103 enzyme accumulates in the cleavable complex to a much greater degree than wild-type enzyme (compare lanes 6 and 10). The amount of nicked DNA produced by wild-type Top1 (lane 6) is not significantly different from the amount produced when the supercoiled substrate is incubated with enzyme storage buffer (lane 2) or a column fraction of a mock purification from a Δ*top1* strain (lane 4). In contrast, Top1-103 converts a large fraction of the supercoiled substrate to the nicked form (lane 10). It is unlikely that the nicked DNA produced in the Top1-103 reaction in the absence of camptothecin could have been generated by chemical means or by a contaminating copurifying nuclease, because no nicking activity is detected in the parallel column fractions from the Δ*top1* strain or from the strain expressing wild-type Top1. The same amount of topoisomerase I was added to the wild-type and Top1-103 reactions, based on quantitation by silver staining and Western blotting.

Consistent with a defect in converting the cleavable complex to relaxed covalently closed circles, Top1-

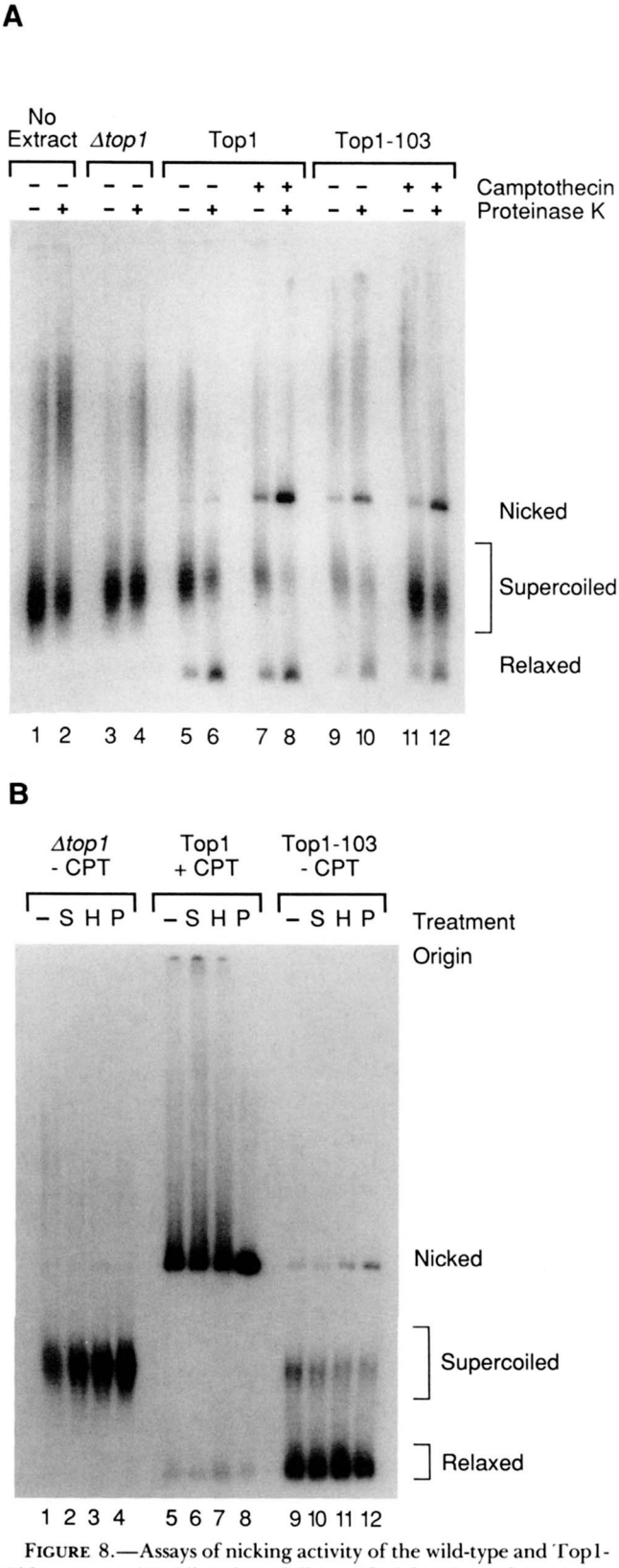

FIGURE 8.—Assays of nicking activity of the wild-type and Top1-103 enzymes. (A) Diluted partially purified fractions of topoisomerase I from strains overexpressing wild-type *TOP1* (NLY429), *top1-103* (NLY620) or no enzyme (*Δtop1*, NLY108) were added to a reaction mix containing 4 ng supercoiled pUC118 DNA. Topoisomerase storage buffer was used as the "No Extract" control. The reaction mixes contained either camptothecin in DMSO at a final concentration of 0.11 mg/ml (lanes 7, 8, 11 and 12) or DMSO alone (all other lanes) at 11% final concentration. Reactions were incubated for 10 minutes at 37°, divided in half, and stopped by adding SDS to 0.5% final concentration. One half of each reaction was digested with 0.15 mg/ml proteinase K (even-numbered lanes) for 1 hr at 37°, and the samples were electrophoresed on an 0.9% agarose gel with 50 ng/ml ethidium bromide. These electrophoresis conditions resolve supercoiled, relaxed, and nicked topoisomers. The DNA was visualized by Southern blotting followed by hybridization with a hexamer-labeled probe. (B) Reactions were performed as in (A), except that camptothecin (0.11 mg/ml) in DMSO was added to the reaction containing wild-type Top1 enzyme (lanes 5–8) whereas DMSO alone was added to the reactions containing *Δtop1* extract (lanes 1–4) or Top1-103 (lanes 9–12). The reactions were stopped by addition of SDS to 0.5% and divided into four parts that were treated as follows: reactions in lanes 1, 5 and 9 ("–") were untreated; NaCl ("S") was added to 0.5 M final concentration to reactions in lanes 2, 6 and 10; reactions in lanes 3, 7 and 11 were heated ("H") to 70° for 15 min; reactions in lanes 4, 8 and 12 were digested with proteinase K ("P") at 0.15 mg/ml for one hour at 37°. The reactions were then electrophoresed, blotted, and hybridized as in (A).

103 enzyme produces less fully relaxed DNA (12.4% of total DNA in lane 10, based on phosphorimager analysis) than wild-type Top1 (32.3% of total DNA in lane 6) in parallel reactions. Experiments designed to follow the conversion of supercoiled DNA to the relaxed form show that Top1-103 enzyme takes longer than 5 min to relax fully a supercoiled substrate DNA, whereas under the same conditions wild-type Top1 relaxes the same substrate in less than 20 sec, at least a 15-fold difference in efficiency (data not shown). These experiments also suggest that Top1-103 is slower to make the initial nick in DNA than is wild-type Top1.

The nicked DNA generated by Top1-103 enzyme is a genuine covalent enzyme-bound species (the cleavable complex), similar to the wild-type Top1 enzyme-DNA complex in the presence of camptothecin. Unlike most nucleases, topoisomerase I remains covalently bound to nicked DNA and does not dissociate until it has religated the broken strand. This covalent enzyme-DNA complex either remains in the wells of an agarose gel or migrates as a broad high molecular weight smear (see Figure 8B, lanes 5, 6 and 7). Thus, the electrophoretic migration of this species as a single band of free nicked DNA is dependent on proteinase K digestion of the Top1 reaction products (Figure 8B, lane 8). The intensity of the band at the position of nicked DNA greatly increases following proteinase K treatment of Top1-103 reaction products (Figure 8A; compare lanes 9 and 10, lanes 11 and 12), showing that Top1-103, like wild-type Top1 in the presence of camptothecin (Figure 8A; compare lanes 7 and 8), produces enzyme-bound nicked DNA. In contrast, the intensity of the band at the position of free nicked DNA does not increase after proteinase K digestion of the reaction products of Top1 in the absence of camptothecin (Figure 8A; lanes 5 and 6). The faint band present at the "nicked " position in the Top1-

103 reaction in the absence of proteinase K digestion (Figure 8A; lane 9) is attributable to contamination of the supercoiled substrate with a small amount of nicked DNA, as it is seen in lanes 1 to 6.

We investigated the resistance of the Top1-103-DNA complex to various treatments that would be expected to dissociate a noncovalent enzyme-DNA complex. After incubating the enzyme with supercoiled DNA for 10 min at 37°, we stopped the reactions with SDS, divided them into aliquots, and treated them with either high salt (0.5 M NaCl for 1 hr), high temperature (70° for 15 min), or proteinase K (1 hr at 37°). A fourth aliquot was left untreated. Figure 8B shows that only proteinase K digestion (lane 12) fully removed Top1-103 from the nicked DNA, allowing it to migrate as a single band. High salt treatment (lane 10) produced no more free nicked DNA than in the untreated control (lane 9), and high temperature treatment (lane 11) produced little more than in the control. Treating the reaction products of wild-type Top1 in the presence of camptothecin with high salt (Figure 8B, lane 6), high temperature (lane 7), or proteinase K (lane 8) gave results that paralleled the results with Top1-103 in the absence of camptothecin. Only proteinase K treatment fully released the nicked DNA to migrate at its characteristic position.

Unlike wild-type Top1, Top1-103 is unaffected by camptothecin (Figure 8A). Top1-103 accumulates in the cleavable complex in the presence (lane 12) or absence (lane 10) of camptothecin. Quantitation of radioactivity shows that 10.6% of total DNA in the Top1-103 reaction without camptothecin (lane 10) is in the nicked form and 11.2% of the total DNA in the Top1-103 reaction with camptothecin (lane 12) is in the nicked form. Camptothecin has no apparent effect on the conversion by Top1-103 of the cleavable complex to relaxed covalently closed circles.

DISCUSSION

We have identified a mutant allele of the yeast *TOP1* gene that has phenotypes that are strikingly different from a null allele. This mutation increases mitotic recombination 100–300-fold in the rDNA repeats and 10–25-fold at other loci, whereas a null mutation in *TOP1* results in a 20-fold elevation of mitotic rDNA recombination and no effect outside the rDNA. Other phenotypes of *top1-103*, including slow growth, inviability in the absence of the double-strand break repair system, and constitutive expression of DNA damage-inducible genes, suggest that expression of the mutant enzyme leads to DNA damage. We have shown that Top1-103 enzyme can generate nicked DNA *in vitro*, and is therefore likely to be directly responsible for DNA damage. We propose that Top1-103 enzyme is trapped in the cleavable complex *in vivo*, leading to phenotypes similar to those seen upon treatment of wild-type strains with the anti-tumor drug, camptothecin.

***top1-103* mimics the *in vivo* effects of the anti-tumor drug, camptothecin:** Eukaryotic type I topoisomerases relax DNA by a mechanism that involves a covalent enzyme-DNA intermediate called the "cleavable complex" [Figure 1A; reviewed in CHAMPOUX (1990)]. Although the cleavable complex is ordinarily a transient intermediate, it can be trapped by treatment with denaturing agents such as SDS, alkali, low pH, and by the topoisomerase I-specific drug, camptothecin [Figure 1B; reviewed in LIU (1989, 1990)]. This drug does not alter the ability of topoisomerase I to cleave the DNA substrate, but it impairs either the repositioning of the broken DNA strands for ligation or the religation reaction itself (PORTER and CHAMPOUX 1989; SVESTRUP *et al.* 1991).

The *in vivo* effects of camptothecin have been extensively studied, as this drug and its analogues have shown promise as anti-tumor agents [reviewed in POTMESIL and SILBER (1990)]. Experiments in mammalian tissue culture cells (HSIANG *et al.* 1985) have shown that camptothecin traps topoisomerase I in the cleavable complex *in vivo* as well as *in vitro*. Enzyme-bound nicks generated by topoisomerase I in the presence of camptothecin are expanded to double-stranded breaks by replication fork passage during S phase (HSIANG, LIHOU and LIU 1989; PORTER and CHAMPOUX 1989; D'ARPA, BEARDMORE and LIU 1990; ZHANG, D'ARPA and LIU 1990; DEL BINO, LASSOTA and DARZYNKIEWICZ 1991; RYAN *et al.* 1991). Camptothecin treatment of mammalian cells induces recombination, chromosomal aberrations (LIM *et al.* 1986), and fragmentation of DNA (HORWITZ and HORWITZ 1971). As in mammalian cells, treatment of yeast with sublethal levels of camptothecin causes hyper-recombination, as well as induction of the the DNA damage-inducible gene, *DIN3* (NITISS and WANG 1988; ENG *et al.* 1988). Yeast cells lacking the double-strand break repair gene, *RAD52*, are much more sensitive to killing by camptothecin (NITISS and WANG 1988). Camptothecin treatment also causes G_2 arrest in a variety of mammalian cell lines, presumably as a result of the topoisomerase I-mediated DNA damage (LI, FRASER and BHUYAN 1972; TOBEY 1972; DEL BINO, SKIERSKI and DARZYNKIEWICZ 1990; TSAO, D'ARPA and LIU 1992).

top1-103 has pleiotropic phenotypes that resemble the effects of camptothecin treatment of yeast or mammalian cells. These phenotypes can be understood in light of the model proposed for the *in vivo* effects of camptothecin. If the Top1-103 enzyme has a tendency to become trapped in the cleavable complex, replication fork passage in S phase would lead to double-stranded breaks at sites where the enzyme is bound. These breaks would generate a DNA damage signal and cause cell cycle arrest at the G_2-M

boundary. Breaks occurring in S phase would not be lethal to yeast cells with an intact recombinational DNA repair system, since they could be repaired during G_2 by recombination with the intact sister chromatid.

Consistent with this model, *top1-103* causes mitotic hyper-recombination in the rDNA and at other loci, just as camptothecin treatment elevates recombination. In addition, *top1-103* is inviable in combination with null mutations in either of the DNA-repair genes *RAD50* or *RAD52*, which are required for recombinational repair of double-strand breaks [reviewed in FRIEDBERG (1988)]. Expression of *top1-103* from the *GAL1* promoter causes cell cycle arrest in G_2 and induction of the DNA damage-inducible genes *DIN1* and *DIN3*, providing further evidence of topoisomerase I-mediated DNA damage. Additional support for the model that Top1-103 damages DNA by generating enzyme-bound nicks comes from experiments showing that *top1-103* phenotypes are reversed by mutating the enzyme's active site tyrosine.

***top1-103* overexpression leads to *RAD9*-dependent G_2 arrest:** In both yeast and mammalian cells, DNA damage or incomplete DNA replication leads to arrest in G_2, preventing cells from dividing before their chromosomes have been completely and accurately replicated [WEINERT and HARTWELL (1988) and references therein]. In yeast cells, this arrest is mediated by *RAD9* (WEINERT and HARTWELL 1988). *rad9* mutants fail to arrest properly in response to many types of DNA damage and are thus hypersensitive to DNA-damaging agents.

Overexpression of *top1-103* causes cell-cycle arrest in the G_2 (large-budded) stage (Table 6), suggesting that DNA damage in these cells leads to a cell division block at the *RAD9* checkpoint. Other mutants with a defect in DNA replication, including *cdc9* (DNA ligase), are known to arrest at this same point in the cell cycle (SCHIESTL *et al.* 1989). Deletion of *RAD9* prevents G_2 arrest (Table 6) and maintenance of viability (Figure 6) in cells overexpressing *top1-103*, showing that topoisomerase-induced DNA damage leads to cell-cycle arrest through *RAD9*. Whereas wild-type cells overexpressing *top1-103* arrest uniformly and remain viable for over 24 hr, the isogenic *rad9* mutant continues to traverse the cell cycle and rapidly loses viability.

Treatment of cultured mammalian cells with camptothecin results in G_2 arrest (LI, FRASER and BHUYAN 1972; TOBEY 1972; DEL BINO, SKIERSKI and DARZYNKIEWICZ 1990), analogous to the effect of overexpression of *top1-103*. The mechanism of this arrest is not completely understood, but it has recently been shown to involve inhibition of $p34^{cdc2}$ protein kinase activation (TSAO, D'ARPA and LIU 1992), and is presumably triggered by topoisomerase I-induced DNA damage. The parallels between *top1-103* and camptothecin effects suggest that preventing G_2 arrest in camptothecin-treated tumor cells might increase cell killing. A number of drugs have been identified that interfere with cell-cycle arrest in G_2, including caffeine (SCHLEGEL and PARDEE 1986) and okadaic acid (YAMASHITA *et al.* 1990; PATEL and WHITAKER 1991). Simultaneous treatment of tumors with camptothecin and a drug that blocked arrest might have a synergistic effect, reducing the required dose of camptothecin. This synergy could be important for clinical use of camptothecin, as the drug has toxic side effects (POTMESIL and SILBER 1990). The benefit of using drugs that block G_2 arrest in conjunction with camptothecin would, of course, depend on the relative toxicity of these drugs to normal and tumor cells.

Top1-103 enzyme has altered activity *in vitro*: Top1-103 mutant enzyme differs from wild type in three respects: it is much less efficient at relaxing DNA, it accumulates more covalent enzyme-DNA complex, and it is insensitive to camptothecin. Figure 8 shows that wild-type enzyme converts more of the supercoiled substrate to the relaxed form than does the same amount of Top1-103. Relative to wild type, the equilibrium between free Top1-103 enzyme and the covalent enzyme-DNA complex is shifted toward the enzyme-DNA complex (see Figure 1B). More nicked DNA is produced in reactions containing Top1-103 enzyme than in those containing wild-type enzyme (Figure 8). We have demonstrated that most of the nicked DNA in the Top1-103 reactions is bound to protein, because digestion with proteinase K prior to electrophoresis greatly increases the intensity of the band at the position of free nicked DNA, as compared with a sample that has not been digested (Figure 8).

The amount of nicked DNA found in Top1-103 reactions is unaffected by the presence of camptothecin (Figure 8). Other camptothecin-resistant mutants have been isolated in mammalian cells (TANIZAWA and POMMIER 1992) and yeast (M-A. BJORNSTI, unpublished data). The insensitivity of Top1-103 to camptothecin could be explained in several ways. One explanation is that the drug does not bind to Top1-103 as well as it binds to the wild-type enzyme, and therefore cannot stabilize the enzyme-DNA complex. Another explanation is that camptothecin stabilizes the covalent intermediate by converting the religation reaction, which is normally a very fast step, into a slow step. If the religation reaction is already a slow step for Top1-103, which would explain the accumulation of covalent intermediate even in the absence of camptothecin, then the drug might have little effect.

The *in vitro* activity of Top1-103 enzyme is similar but not identical to that of wild-type enzyme in the presence of camptothecin. Top1-103 enzyme is somewhat defective in the entire reaction pathway, not just the resealing step. However, the critical parameter in

determining the effects of the mutant enzyme in the cell is the relative level of the cleavable complex. Top1–103 clearly accumulates more cleavable complex than wild-type enzyme *in vitro*, and even a small increase in cleavable complex *in vivo* might lead to severe consequences.

The *top1-103* mutation falls in a critical catalytic domain of topoisomerase I: The *top1-103* mutation consists of an arginine to lysine substitution at position 420 of the yeast topoisomerase I enzyme (Figure 7). Although this amino acid change is conservative, substituting one basic residue for another, it alters a residue that is invariant in all cloned eukaryotic topoisomerase I genes (Figure 7B). In addition, arginine 420 falls within a 23 amino acid stretch with very high overall sequence identity (Figure 7B) that was shown by MORHAM and SHUMAN (1990) to be critical for enzyme function in the vaccinia virus topoisomerase I. Four out of the five mutants they isolated that abolished enzyme activity without altering protein structure or stability fell in this region, suggesting an important role in catalysis or DNA binding.

FRIESEN and SADOWSKI (1992) have recently studied the effect on catalysis of an arginine to lysine substitution in the yeast FLP protein, a site-specific recombinase of the integrase family, whose members have a very similar reaction mechanism to topoisomerases. This enzyme class has an invariant tyrosine that makes a covalent phosphotyrosine bond with the 3′ end of the broken DNA strand, as well as invariant histidine and arginine residues. Substitution of lysine for the conserved arginine (RK191) results in a mutant enzyme that can initiate the strand exchange reaction, but becomes trapped in the covalent reaction intermediate, a situation that closely parallels that of Top1-103.

Additional mutagenesis studies of yeast *TOP1* are in progress. These studies should identify other residues that are important for catalysis and for resistance to camptothecin, and should provide insight into the reaction mechanism and structural requirements of topoisomerase I.

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