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Isolation of Mutants of *Saccharomyces cerevisiae* Requiring DNA Topoisomerase I

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ABSTRACT

Despite evidence that DNA topoisomerase I is required to relieve torsional stress during DNA replication and transcription, yeast strains with a *top1* null mutation are viable and display no gross defects in DNA or RNA synthesis, possibly because other proteins provide overlapping functions. We isolated mutants whose inviability or growth defect is relieved when *TOP1* is expressed [*trf* mutants (topoisomerase one-requiring function)]. The *TRF* genes define at least four complementation groups. *TRF3* is allelic to *TOP2*. *TRF1* is allelic to *HPR1*, previously shown to be homologous to *TOP1* over two short regions. *TRF4* encodes a novel 584-amino acid protein with homology to the N-terminus of *Saccharomyces cerevisiae* topo I. Like *top1* mutants, *trf4* mutants have elevated rDNA recombination and fail to shut off RNA polymerase II transcription in stationary phase. *trf4* null mutants are *cs* for viability, display reduced expression of *GAL1* and *Cell Cycle Box UAS::LacZ* fusions, and are inviable in combination with *trf1* null mutants, indicating that both proteins may share a common function with DNA topoisomerase I. The existence of multiple *TRF* complementation groups suggests that not all biological functions of topo I can be carried out by topo II.

CHROMOSOMES are involved in dynamic cellular processes such as DNA replication, transcription, chromatin assembly and genetic recombination that lead to the formation of local domains of torsional stress (reviewed in WANG and LYNCH 1993). During DNA replication the movement of a large polymerase complex along the helical DNA backbone is presumed to lead to the formation of domains of positive supercoiling ahead of the polymerase provided that the ends of the template DNA are not free to rotate about the helical axis. Chromosomal DNA is generally not free to rotate about its axis due to interaction with chromosomal proteins such as nucleosomes, the presence of convergently oriented transcription forks that confine domains of supercoiling, and, perhaps, because of the association of certain regions of chromosomes with the nuclear matrix (summarized in COZZARELLI and WANG 1990). In *Saccharomyces cerevisiae* DNA topoisomerases I and II (topo I and II) appear to act together during DNA replication as a swivel to prevent the formation of positive supercoils ahead of the DNA replication fork. As initially proposed (CAIRNS 1963), a swivel is an activity that prevents the formation of torsional stress by unwinding the DNA helix simultaneously with the movement of a protein complex along the helical backbone. In *S. cerevisiae* under conditions where both topo I and II are inactivated, DNA replication stops rapidly (BRILL *et al.* 1987), with elongation of new DNA chains continuing for only a few thousand nucleotides (KIM and WANG 1989). Similar results have been obtained for *Schizosaccharomyces pombe* (UEMURA and YANAGIDA 1984). Thus, both topo I and topo II probably act as DNA replication swivels.

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Transcription can also lead to the formation of locally supercoiled domains in DNA. In the twin domain model (LIU and WANG 1987), movement of a transcription complex along the helical backbone generates positive supercoils ahead of the complex and negative supercoils behind the complex. It has been suggested that transcription is a major determinant of supercoiling *in vivo* (GIAEVER and WANG 1988). Cytological studies have shown that topo I from *Drosophila melanogaster* is associated with puffs in polytene chromosomes, which are regions of active transcription (FLEISCHMANN *et al.* 1984; GILMOUR *et al.* 1986). In addition, transcription of ribosomal DNA transfected into animal cells requires an active topo I (ZHANG *et al.* 1988). Furthermore, when the *c-fos* oncogene is transcriptionally induced, the sites of topo I binding to the *c-fos* gene appear to move progressively in a 5′ to 3′ direction along with the transcription fork (STEWART *et al.* 1990). In *S. cerevisiae* topo I and II appear to function together as a swivel for rRNA transcription and, to a lesser extent, for mRNA transcription (BRILL *et al.* 1987). Transcription from a strong promoter can lead to hypernegative supercoiling of plasmids in *top1* mutants (BRILL and STERNGLANZ 1988; GIAEVER and WANG 1988), suggesting that topo I normally removes negative supercoils formed during transcription. These results and others have led to the

suggestion that another major role for topo I is to relieve torsional stress generated during transcription.

Despite the considerable evidence for the involvement of topo I in both DNA replication and transcription, topo I is not essential in either *S. cerevisiae* or *S. pombe.* Null mutations in the gene encoding the only type I DNA topoisomerase activity detectable in crude extracts cause only modest growth defects (Thrash *et al.* 1984, 1985; Uemura and Yanagida 1984). Furthermore, the overall rates of both DNA and RNA synthesis are normal in *top1* mutants (Brill *et al.* 1987), supporting the suggestion that topo II activity can substitute for topo I activity in these crucial processes (Uemura and Yanagida 1984; Goto and Wang 1985; Brill *et al.* 1987). In *S. pombe,* conditional *top2* mutants have been isolated that are inviable at the permissive temperature in combination with a *top1* null mutation (Uemura *et al.* 1987). In *S. cerevisiae, top1* null mutants display a synthetic growth defect in combination with some *top2* alleles (Goto and Wang 1985). These results demonstrate that some of the functions of type I and type II topoisomerases overlap. It remains unclear, however, whether topo I has additional functions distinct from those of topo II. The biological function of a second type I topoisomerase, topo III (Wallis *et al.* 1989; Kim and Wang 1992), remains unclear. Null mutations in *TOP3* result in hyperrecombination and slow growth (Cozzarelli and Wang 1990; Bailis *et al.* 1992), but *top1 top3* double mutants are viable. Association of Top3 with Sgs1, a DNA helicase, has been proposed to result in formation of a eukaryotic gyrase (Gangloff *et al.* 1994).

To clarify the *in vivo* functions of topo I, we have perfomed a genetic screen to identify mutations affecting gene products that perform overlapping or dependent functions (Guarente 1993) with topo I and, thereby, to further elucidate which processes in the cell require topo I. We have identified four complementation groups of mutants with this phenotype. The existence of multiple *TRF* (topoisomerase one-requiring function) complementation groups suggests that topo II cannot substitute for all topo I functions.

MATERIALS AND METHODS

Microbial techniques: Yeast strains (Table 1) were transformed using the lithium acetate method (Ito *et al.* 1983). Putative *TRF* clones were isolated from a plasmid library consisting of random *S. cerevisiae* DNA *Sau*3A fragments inserted into the *Bam*HI site of YCp50 (Rose *et al.* 1987). *Escherichia coli* cells (strain DH5α) were transformed by electroporation (Dower *et al.* 1988). Small scale plasmid DNA preparations were made by the boiling lysis method (Holmes and Quigley 1981) or using Qiagen columns (Qiagen, Chatsworth, CA).

Strain constructions and crosses: CY715: pBS14 (*URA3 TRF4* integrating plasmid) was linearized with *Sna*BI and integrated at the *TRF4* locus in CY445. CY715 was shown to have integrated pBS14 at homologous sequences by Southern blot (data not shown). CY855: Plasmid pCB470 was digested with *Xho*I to release a *trf4-101::HIS3* fragment. This fragment was used to disrupt *TRF4* in the *TRF4/TRF4* diploid, CY858. The resulting diploid was sporulated and several tetrads were dissected. One of the *HIS3*$^+$ cold-sensitive spores was designated CY855. Disruption at the *TRF4* locus in CY855 was confirmed by Southern analysis. CY891: The *trf4-101::HIS3* fragment from pCB470 was used to disrupt *TRF4* at one locus in the diploid CY868. Disruption of *TRF4* in CY891 was confirmed by Southern blot. pSH1 encodes TRF4: To examine linkage of pSH1 sequences to the *trf4-1* mutation, we cloned an internal 2.2-kb *Eco*RI fragment from pSH1 into the *URA3*-marked integrating vector pRS306 to generate pBS14. CY715 (*TRF4::URA3::TRF4*) was crossed to CY725, a *trf4-1* strain containing pBS9 (*TOP1 ADE3 TRP1 2μ*). Of 40 tetrads analyzed, there were four tetrads in which each of the four spores carried plasmid pBS9. All four were parental ditypes for the integrated *URA3* marker and the nonsectoring and *cs* phenotypes of *trf4-1.* In addition there were 10 tetrads that displayed 2:2 segregation of viability. These most likely resulted from sporulation of diploid cells that had lost pBS9 during mitotic growth. For all 10 tetrads the two surviving spores were Ura$^+$, indicating that these 10 tetrads were also parental ditypes for *trf4-1* and the integrated *URA3* marker. This indicates that plasmid pSH1 carries the *TRF4* gene.

Media and growth conditions: Yeast strains were routinely grown in either YEP or synthetic complete media lacking uracil (SC −Ura) or adenine (SC −Ade) (Sherman 1991). Either glucose or galactose was added as a carbon source to a final concentration of 2%. Synthetic complete medium containing galactose is referred to as Gal. The YEP medium with dextrose added is referred to as YPD. To test plasmid complementation of the nonsectoring phenotype of *trf* mutants (see below), SC −Ura with low (0.28×) histidine (0.083 mM) and low (0.25×) adenine (0.075 mM) was used to facilitate visualization of red and white sectors in colonies.

Topo II assays: Five ml overnight cultures were used to inoculate 50 ml cultures that were grown to an A_{600} of 0.5–0.6. Cells were pelleted, washed and resuspended in 0.3 ml of ice-cold breaking buffer by vortexing (breaking buffer: 100 mM Tris hydrochloride pH 8.0, 200 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol, 1 mM PMSF and 0.83 μg/mL each of chymostatin, leupeptin, antipain and pepstatin). Cells were lysed by vortexing with 0.3 ml of acid-washed glass beads. Samples were centrifuged and supernatants were removed to fresh tubes. When comparing different mutants, the amount of protein in each extract was determined by Bio-Rad microassay, and volumes were adjusted with breaking buffer so that the total amount of protein used in each reaction (0.68 mg) was the same. The assay mixture contained 2 μl of kinetoplast DNA (kDNA final concentration was 0.06–0.1 mg/mL or 1.1–1.9 mM) and the assay was performed in a total volume of 18 μl. The reaction buffer was as described (Worland and Wang 1989). Reactions were incubated at 30° for 45 min after which 4 μl of stop mix (Worland and Wang 1989) and 2.5 μl of 10× agarose tracking dye was added. The stopped reactions were electrophoresed on 1% TAE agarose gels (20 cm length) containing ethidium bromide until the tracking dye reached the bottom of the gel. Gels were transferred to nylon membranes and hybridized to radiolabeled kDNA to visualize reaction products. The small amount of nicked circular DNA seen in the wild-type assay was present in the substrate DNA before the addition of cell extract (−E lane of Figure 2A). We have established that the decatenation reaction is dependent on both time (from 0 to 120 min) and amount of extract added (from 0.17 to 2.7 μg), and have

TABLE 1

Yeast strains

Strain	Genotype	Source
CY151	*MAT***a** *top1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::URA3*	This laboratory
CY155	*MATα top1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::URA3*	This laboratory
CY184	*MATα ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	Zhu *et al.* (1995)
CY185	*MATα top1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	This laboratory
CY187	*MATα top1-7::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2* pCB11	This study
CY431	*MAT***a** *top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15 + pBS3*	This study
CY445	*MAT***a** *top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15 +pBS3*	This study
CY446	*MAT***a** *trf3-2 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15 leu2-3,112* pBS3	This study
CY447	*MAT***a** *trf2-1 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15* pBS3	This study
CY527	*MATα hpr1-2:HIS3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	Zhu *et al.* (1995)
CY715	*MATα TRF4::URA3::trf4 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15, leu2-3,112*	This study
CY725	*MAT***a** *trf4-1 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15* pBS9	This study
CY726	*MAT***a** *top1-7::LEU2 trf4-1 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15*	This study
CY738	*MAT***a** *trf4-1 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15, leu2-3,112* pBS3	This study
CY739	*MAT***a** *trf3-1 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15, leu2-3,112* pBS3	This study
CY740	*MAT***a** *trf3-3 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15, leu2-3,112* pBS3	This study
CY741	*MAT***a** *trf3-4 top1-7::LEU2 ade2-1 ade3::hisG ura3-1 trp1-1 his3-11,15, leu2-3,112* pBS3	This study
CY855	*MAT***a** *trf4-101::HIS3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	This study
CY857	*MATα τrf4-101::HIS3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::URA3*	This study
CY858	*MAT***a** *MATα ade2-1 ade2-1 ura3-1 ura3-1 trp1-1 trp1-1 his3-11,15 his3-11,15 leu2-3,112 leu2-3,112 rDNA::URA3 rDNA::ADE2*	This study
CY868	*MAT***a** *MATα top1-7::LEU2 top1-7::LEU2 ade2-1 ade2-1 ura3-1 ura3-1 trp1-1 trp1-1 his3-11,15 his3-11,15 leu2-3,112 leu2-3,112 rDNA::URA3 rDNA::ADE2*	This study
CY891	*MAT***a** *MATα top1-7::LEU2 top1-7::LEU2 trf4-101::HIS3 TRF4 ade2-1 ade2-1 ura3-1 ura3-1 trp1-1 trp1-1 his3-11,15 his3-11,15 leu2-3,112 leu2-3,112 rDNA::URA3 rDNA::ADE2*	This study
YZY3	*MAT***a** *hpr1-103::LEU2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	Zhu *et al.* (1995)
YZY11	*MAT***a** *lys2::URA3::lys2 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	This study
SY6	*MAT***a** *lys2::URA3::lys2 trf4-101::HIS3 ade2-1 ura3-1 trp1-1 his3-11,15 leu2-3,112 rDNA::ADE2*	This study
RS191	*MAT***a** *top2-1(ts) ade2 ura3-1 his3-11,15 trp1 leu2-3,112*	Rolf Sternglanz
P65	*MATα met4 gal2*	Robert Mortimer

chosen assay conditions (30 min with 0.68 mg extract added) in the linear range for these two variables.

EMS mutagenesis: The strain to be mutagenized was grown overnight to saturation in YPD or SC −Ura medium. Cells were washed twice in sterile water and resuspended in 50 mM potassium phosphate pH 7.0. EMS was added to a final concentration of 3.25%, the tubes were vortexed for 20 sec and then incubated at 30° for 1 hr without shaking. Cells were then diluted in 5% sodium thiosulfate, further diluted in water and plated. Survival was determined by comparison to a control lacking EMS.

DNA sequencing: Genomic DNA containing *TRF4* (2.7 kb) was sequenced by the dideoxy method (Sanger *et al.* 1977) using the Sequenase kit (U.S. Biochemicals, Cleveland) and α-^{35}S-dATP. Sequential exonuclease III-generated, nested deletions (Sambrook *et al.* 1989) were made from each end of a 4-kb genomic insert in pBS12 to sequence both strands with one primer. The remaining *TRF4* sequence on the 5′ side of the EcoRI site (nucleotide 557 in Figure 3) was obtained using synthetic primers and plasmids pCB432 and pBS15 as templates. *trf4-1* was sequenced after using PCR to amplify *trf4-1* from genomic DNA isolated from strain CY738. Double-stranded PCR fragments were gel purified, extracted with the Geneclean kit and directly sequenced with primers complementary to *TRF4*. For PCR sequencing, DNA fragments and oligos were heat denatured and quick-frozen in a dry ice/ethanol bath, followed immediately by the extension step of the sequencing reaction.

rDNA recombination frequency assay: Strains CY184, CY185 and CY855 were grown on SC −Ade plates; three colonies from each strain were then streaked on a YPD plate and incubated at 24° until exponential growth ceased (2 days for CY184, 3 days for CY185 and CY855). Six different colonies from each YPD plate were then separately diluted in sterile water and plated on YPD plates. After 2 days the plates were replica-printed to SC −Ade plates. The recombination frequency was calculated by dividing the number of Ade$^-$ colonies by the total number of colonies growing on YPD plates.

***LYS2* recombination frequency assay:** After digestion with *Hpa*I, plasmid pYZ44, which contains a *lys2* allele truncated at both the 5′ and 3′ ends, was integrated into strains CY184 and CY855 at the *LYS2* locus. This creates two truncated copies of *lys2* at the *LYS2* locus separated by *URA3* and vector sequences and makes the strains Lys$^-$. A recombination event between the truncated copies of *lys2* would lead to the recreation of a wild-type *LYS2* gene and a Lys$^+$ phenotype. Two purified transformants of the new Lys$^-$, Ura$^+$ strains were grown on YPD plates for 2 days at 24°. Six different colonies of each strain from each YPD plate were then diluted in sterile water and plated on YPD and SC −Lys plates. Recombination

frequencies were calculated by dividing the number of colonies growing on SC −Lys plates by the total number of colony forming units on YPD plates.

RNA isolation and analysis: Cultures were grown in YPD to early log phase (for primer extension analysis) or to ≥15 hr beyond stationary phase (for transcription shut-off experiment). Entry into stationary phase was defined as the time when the OD600 did not continue to increase. Total yeast RNA was isolated using standard methods (KOHRER and DOMDEY 1991). For Northern blots, 15 μg of total RNA was electrophoresed in a 1.5% agarose/6% formaldehyde gel, transferred to a nitrocellulose membrane and hybridized with α-^{32}P-dATP-labeled probes. The *ACT1* probe [1.0-kb *Xho*I-*Hin*DIII fragment from pRB149 (NG and ABELSON 1980)] was labeled with random hexamers (FEINBERG and VOGELSTEIN 1983). For primer extension analysis of *TRF4* in strains CY855 and CY184, 10 μg of total RNA was annealed to a γ-^{32}P-dATP-labeled oligo (5×10^5 cpm) with the sequence 5′-CTCTTTTTCACTTTCCCA-3′. The 3′ end of this sequence is at +57 nucleotides (Figure 3) relative to the translation start site.

***β*-galactosidase activity assay:** Plasmids pRY121Δ10 and pBA161 (PETERSON and HERSKOWITZ 1992) were transformed into the following strains: CY184 (wild type), CY185 *(top1-7::LEU2)*, and CY855 *(trf4-101::HIS3)*, as well as the strains CY726 *(trf4-1 top1-7::LEU2)* containing either pCB368 (*TOP1 TRP1 CEN*) or pCB431 (*TRF4 TRP1 CEN*). Transformed strains were grown in SC −Ura or SC −Ura,Trp medium containing 2% galactose (for pRY121Δ10) or 2% glucose (for pBA161). Cultures were harvested by centrifugation in early log phase. Crude extracts were prepared using glass beads (KOLODZIEJ and YOUNG 1991); protein concentration of the crude extract was measured by the BioRad microassay. Ten micrograms of extract were used in liquid β-galactosidase assays at 28° for 20 min.

Mutant screens: *pGAL1::top1:* We examined a total of 64,000 mutagenized yeast colonies. Colonies that grew well on galactose plates at 36° but not on glucose plates at 36° were identified. Mutant candidates were examined to determine whether the galactose-dependent complementation of their growth defect at 36° required *TOP1* expression, because the same phenotype (ability to grow on galactose but not glucose) could also be displayed by mutants defective in glucose uptake. To test this, candidate *trf* mutants were grown nonselectively for pWE3 (*pGAL1::TOP1 URA3*) in galactose at 24° and the cells were then plated for single colonies on galactose at 24°. All mutant candidates were able to segregate away pWE3, indicating that the *trf* mutants among the candidates do not require *TOP1* expression for viability at 24°, although they do require *TOP1* expression for a normal growth rate. We define this phenotype as a synthetic growth defect with *top1-7::LEU2*. Ura$^-$ colonies were subsequently streaked onto glucose medium and galactose medium at 36°. We expected that the *trf* mutant colonies, having lost plasmid pWE3 (*pGAL1::TOP1*), would not grow well on the galactose medium compared to the original mutant candidate with pWE3. The majority of the mutants identified initially were not dependent on pWE3 (*pGAL1::TOP1*) for growth on galactose at 36° and most likely were glucose uptake mutants.

Colony sectoring screen: Mutagenized colonies of strain CY431 were spread at a density of ~300 per plate on YPD plates and examined for sectoring after 5 days at 30°. Candidate mutants that formed solid red colonies without white sectors were restreaked on YPD plates at 17°, 30° and 36° to determine whether nonsectoring was reproducibly observed at 30° and whether nonsectoring or viability was conditional.

Plasmid constructions: pCB69: The 3.8-kb *Hin*dIII fragment containing *TOP1* from pCT80 (ROLF STERNGLANZ) was isolated, made blunt ended by filling in the 5~ overhang with Klenow fragment, and ligated to YCp407 (MA *et al.* 1987) digested with *Sal*I and made blunt using Klenow fragment. pBS3: The 3.7-kb fragment containing *ADE3* from pPB55 (David Pellman) was isolated and ligated into the large *Bam*HI/*Nhe*I fragment (11.2 kb) of YEp24. pYZ3: The 6.8-kb *Sal*I/*Sma*I fragment containing *TOP2* from pBB6 (ROLF STERNGLANZ) was isolated, made blunt ended by filling in the SalI overhang with Klenow fragment and ligated into pRS316 (SIKORSKI and HIETER 1989) digested with SMAI. pYZ8: The 6.4-kb *Bam*HI fragment containing *TOP2* from pYZ3 was isolated and ligated into YEp24 digested with *Bam*HI. pCB62: A 15-kb *URA3 CEN ARS* plasmid that carries the *HPR1* gene on a 7-kb genomic insert isolated from a *CEN ARS* library (ROSE *et al.* 1987). pCB75: A derivative of pCB62 made by deleting the 4.4-kb *Bam*HI fragment from pCB62. The *HPR1* gene can be isolated from pCB75 on a 3.7-kb *Bam*HI/*Sal*I fragment. pBS6: A 3.6-kb *Bam*HI-*Nhe*I fragment from pPB55 containing ADE3 was ligated into the 5.9-kb *Bam*HI-*Nhe*I fragment of YEp427 (MA *et al.* 1987). pBS8: A 0.85-kb *Bam*HI-*Bgl*II fragment containing *TRP1* was ligated into the *Bam*HI site of pBS6. pCB36: A 2.36-kb *Hin*dIII fragment of *TOP1* gene was cloned into *Hin*dIII sites of YCp50. pBS9: A 14.1-kb plasmid made by ligating a 3.8-kb *Bam*HI/*Xho*I fragment carrying *TOP1* (from pYZ2) to a 10.3-kb *Bam*HI/*Sal*I fragment of pBS8 that contains *2μ*, *ADE3* and *TRP1*. pBS12: A 4.1-kb *Eco*RI genomic fragment from pSH1 containing the *TRF4* open reading frame (ORF) was ligated into the *Eco*RI site of pRS314. pBS15: A 2.2-kb *Eco*RI genomic fragment from pSH1 containing 0.25-kb of the *TRF4* ORF was ligated into the *Eco*RI site of pRS314. pBS14: An internal 2.2-kb *Eco*RI fragment from pSH1 was cloned into the *Eco*RI site of pRS306 (*URA3* integrating vector) (SIKORSKI and HIETER 1989). Digestion with *Sna*B1 was used to direct integration to the *TRF4* locus. pSH1: A plasmid carrying *TRF4* on a 7.8-kb genomic insert isolated from a *CEN ARS* library. pYZ2: A 3.8-kb *Hin*dIII fragment carrying *TOP1* was isolated from pCB36 and cloned into the *Hin*dIII site of pRS316. pYZ36: The entire *HPR1* ORF made as a PCR product (with *Bam*HI ends) and ligated into the *Bam*HI site in pRD56 (RAY DESHAIES). pYZ36-1: pYZ36-1 was made by removing the *Sph*I-*Nru*I *HPR1* PCR product of pYZ36 and replacing it with a genomic (non-PCR derived) *Sph*I-*Nru*I fragment from pYZ1 (ZHU *et al.* 1995). pYZ36: A 2.3-kb *Bam*HI fragment of *HPR1* gene PCR product was cloned into *Bam*HI sites of pRD56. pYZ44: A 2.5-kb *Bgl*II-*Xho*I fragment from pDP4 (GERRY FINK) containing a *lys2* allele truncated at both the 5′ and 3′ ends was ligated into the 5.2-kb *Bam*HI-*Sal*I fragment of YIp5. pCB470: A 1.2-kb *Bam*HI fragment containing an internal segment of *TRF4* was removed from pCB432 and replaced by the 1.8-kb *Bam*HI *HIS3* fragment from pCB65. This plasmid was used to disrupt *TRF4* (*trf4-101::HIS3*) after digestion with *Xho*I. pCB494: pCB431 was digested with *Nco*I, filled in with Klenow fragment to make blunt ends and religated to create a frameshift mutation in the *TRF4* ORF (*trf4-nco163*). pCB431: A 4.5-kb *Xho*I-*Bgl*II fragment of pSH1 containing *TRF4* was cloned into the *Xho*I and *Bam*HI sites of pRS314 (SIKORSKI and HIETER 1989). pCB432: A 3.7-kb *Sna*BI-*Hin*dIII fragment of pSH1 containing *TRF4* was cloned into the *Sma*I and *Hin*dIII sites of pRS316 (SIKORSKI and HIETER 1989).

RESULTS

Isolation of *trf* mutants using *pGAL1::fTOP1:* A strain that carries a deletion in the chromosomal *TOP1* gene (CY187, *top1-7::LEU2*) and a plasmid with a galac-

TABLE 2

Plasmids

Plasmid	Genotype	Source
pBA161	*CCB::lacZ URA3 2μ*	PETERSON and HERSKOWITZ (1992)
pBB6	*TOP2 URA3 2μ*	ROLF STERNGLANZ
pBS1	*hpr1-102::HIS3 URA3*	ZHU *et al.* (1995)
pBS3	*TOP1 ADE3 URA3 2μ*	This study
pBS6	*ADE3 2μ*	This study
pBS8	*ADE3 TRP1 2μ*	This study
pBS9	*TOP1 TRP1 ADE3 2μ*	This study
pBS12	*3' ORF of TRF4 URA3 CEN*	This study
pBS14	*TRF4 URA3*	This study
pBS15	*5'* ORF *of TRF4 URA3 CEN*	This study
pCB36	*TOP1 URA CEN*	This study
pCB51	*TOP1 LYS2 CEN*	This study
pCB62	*HPR1 URA3 CEN*	This study
pCB65	*HIS3 CEN*	This study
pCB69	*TOP1 HIS3 CEN*	This study
pCB368	*TOP1 TRP1 CEN*	This study
pCB431	*TRF4 TRP1 CEN*	This study
pCB432	*TRF4 URA3 CEN*	This study
pCB470	*trf4::HIS3 URA3 CEN*	This study
pCB494	*trf4-Nco163 TRP1 CEN*	This study
pCT80	*TOP1 Ap^r*	THRASH *et al.* (1985)
pPB55	*ADE3*	DAVID PELLMAN
pRB149	*ACT1 URA3*	NG and ABELSON (1980)
pRD56	*URA3 CEN pGAL1, 10-GST*	I. HERSKOWITZ
pRS314	*TRP1 CEN*	SIKORSKI and HEITER (1989)
pRS316	*URA3 CEN*	SIKORSKI and HEITER (1989)
pRY121Δ10	*GAL1::lacZ URA3 2μ*	PETERSON and HERSKOWITZ (1992)
pSH1	*TRF4 URA3 CEN*	This study
pWE3	*pGAL1::TOP1 URA3 CEN*	ROLF STERNGLANZ
pWJ459	*TOP3 URA3 2μ*	ROD ROTHSTEIN
pYZ1	*HPR1 URA3 CEN*	ZHU *et al.* (1995)
pYZ2	*TOP1 URA3 CEN*	This study
pYZ3	*TOP2 URA3 CEN*	This study
pYZ8	*TOP2 URA3 2μ*	This study
pYZ36	*pGAL GST::HPR1 URA3 CEN*	This study
pYZ36-1	*GAL GST::HPR1*	This study
pYZ44	*lys2Δ URA3*	ZHU *et al.* (1995)
YEp24	*URA3 2μ*	G. FINK
YEp427	*TRP1 2μ*	MA *et al.* (1987)
YCp5	*URA3 Ap^r*	G. FINK
YCp50	*URA3 CEN*	G. FINK
YCp407	*HIS3 CEN*	G. FINK

tose-inducible *TOP1* gene (pWE3) was mutagenized with EMS to 50% survival. The mutagenized culture was plated on galactose medium at 24°. On galactose plates CY187 shows a 10-fold elevation of topo I activity over the level in a wild-type strain (data not shown). Colonies were replica printed first to glucose and then to galactose medium, and mutant candidates were identified based on their inability to grow well on glucose (where *TOP1* is not expressed, see MATERIALS AND METHODS) and their ability to grow well on galactose (where *TOP1* is expressed). Characterization of mutant 8-1, isolated in this screen, is presented here.

Isolation of *trf* mutants using the *ade2 ade3* colony color sectoring assay: A second method we used to isolate mutants in topoisomerase one-requiring functions involves a parent strain that is mutant in the gene of interest (*top1-7::LEU2* in this case) and at both the *ADE2* and *ADE3* loci and carries a 2μ plasmid containing the wild-type *TOP1* and *ADE3* genes (KOSHLAND *et al.* 1985; KRANZ and HOLM 1990; BENDER and PRINGLE 1991;). When the parent strain (CY431) was plated on YPD and single colonies were examined, the majority of colonies were found to contain multiple white sectors. After mutagenesis, colonies that failed to give rise to white sectors were identified. One explanation for this phenotype is that these colonies harbor a mutation

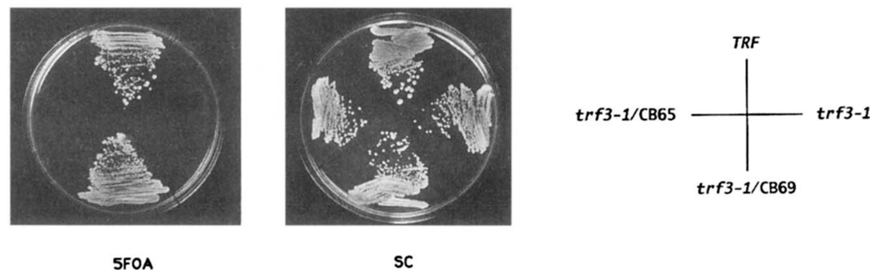

FIGURE 1.—Inviability of a *trf3-1 top1* double mutant. A *trf3-1 top1* double mutant (CY739) carrying a *TOP1* plasmid and its *TRF*$^{+}$ parent strain (CY431) were assayed for the frequency of loss of plasmid pBS3 (*TOP1 ADE3 URA3*). The mutant (*trf3-1*) is unable to lose the *TOP1 ADE3 URA3* plasmid and give rise to *ura3*$^{-}$ segregants that can grow on 5-FOA plates, whereas the parent (*TRF*) readily gives rise to 5-FOA-resistant segregants. The bottom and left quadrants of the plates show that introduction of another plasmid that provides *TOP1* function (pCB69), but not a control vector (pCB65), allows the original mutant to yield 5-FOA-resistant segregants.

that causes inviability in combination with the *top1-7::LEU2* mutation and, therefore, does not allow growth without the *TOP1* plasmid. We mutagenized a *top1-7::LEU2* strain (CY431, Table 1) that carries a *TOP1 ADE3 URA3* 2μ plasmid (pB3, Table 2) with EMS to between 10 and 20% survival and examined 130,000 colonies on YPD plates at 30°. One hundred thirty mutants that failed to sector were identified.

To determine whether nonsectoring was, in fact, a consequence of the inviability or growth defect of a *trf* mutation in combination with *top1-7::LEU2,* candidate mutants were transformed separately with pCB69 (*TOP1 HIS3 CEN*) and pCB65 (*HIS3 CEN* vector) plasmids. Since pCB69 provides *TOP1* function, *trf* nonsectoring mutants were expected to regain their ability to sector while candidates transformed with pCB65 were not. Of 130 candidates examined, six regained their ability to sector after being transformed with pCB69 but not with pCB65.

Each of the candidates that showed sectoring on YPD plates after transformation with pCB69 (*TOP1*$^{+}$) but not pCB65 (vector) also displayed a much higher frequency of 5-fluoroorotic acid (5-FOA)-resistant segregants after transformation with pCB69 than with pCB65. Figure 1 shows the frequency of 5-FOA-resistant segregants observed for mutant 101 (*trf3-1*) with and without a second plasmid providing *TOP1* function. Characterization of the six *trf* mutations isolated by colony sectoring is reported here.

Segregation and complementation of *trf* alleles: Mutant 8-1 was back crossed to a strain isogenic to the parent strain but of the opposite mating type (CY151). Mutant 8-1 displayed a clear 2:2 segregation for very slow growth on YPD at 24° in all 10 tetrads with four viable spores (of the 20 tetrads dissected) demonstrating that the 8-1 mutant phenotype was due to a single nuclear mutation. Each of the six mutants from the nonsectoring screen were also backcrossed (to wild-type CY445). All six showed 2:2 segregation of the *trf* mutant phenotype in at least seven four-spored tetrads. Mutant 27, which was temperature sensitive *(ts)* for viability at 36° even in a *TOP1*$^{+}$ background, showed cosegregation of nonsectoring, low frequency 5-FOA-resistance and the secondary phenotype, *ts.* Thus, mutant 27 carries a *trf* allele that is topo I-requring at 30° but is inviable at 36° even in a *TOP1*$^{+}$ background. Similarly, mutant 130 is a *trf* allele that is topoisomerase one-requiring at 30° but is also cold-sensitive (*cs*) for viability even in a *TOP1*$^{+}$ background. The *cs* phenotype of mutant 130 cosegregates with nonsectoring and low frequency 5-FOA-resistance. *trf* mutants 19 and 101 also had cosegregating *ts* phenotypes in a *TOP1*$^{+}$ background.

The *trf* allele in 8-1 was recessive as judged by the ability of diploids resulting from a backcross to form healthy colonies on glucose-containing medium. This complementation group was designated *TRF1* and the mutant allele *trf1-1.* The six nonsectoring *trf* mutations also were found to be recessive for all phenotypes. The results of pairwise matings of *trf* mutations isolated using the *ADE3* sectoring screen are summarized in Table 3. This analysis showed that the six nonsectoring *trf* mutants could be assigned to three complementation groups. One group contains four members and two other groups contain one member each. Subsequent analysis (see below) demonstrated that all six of the *trf* mutations isolated using the colony sectoring screen were in separate genes from the complementation group isolated using the *pGAL1::TOP1* screen. Thus,

TABLE 3

***TRF* complementation groups**

	Allele (mutant no.)	Phenotype in *TOP1*	Allelic with
TRF1	*trf1-1* (8-1)	Slow growth	*HPR1*
TRF2	*trf2-1* (27)	*ts*	
TRF3	*trf3-1* (101)	*ts*	*TOP2*
	trf3-2 (19)	*ts*	
	trf3-3 (114)		
	trf3-4 (153)		
TRF4	*trf4-1* (130)	*cs*	

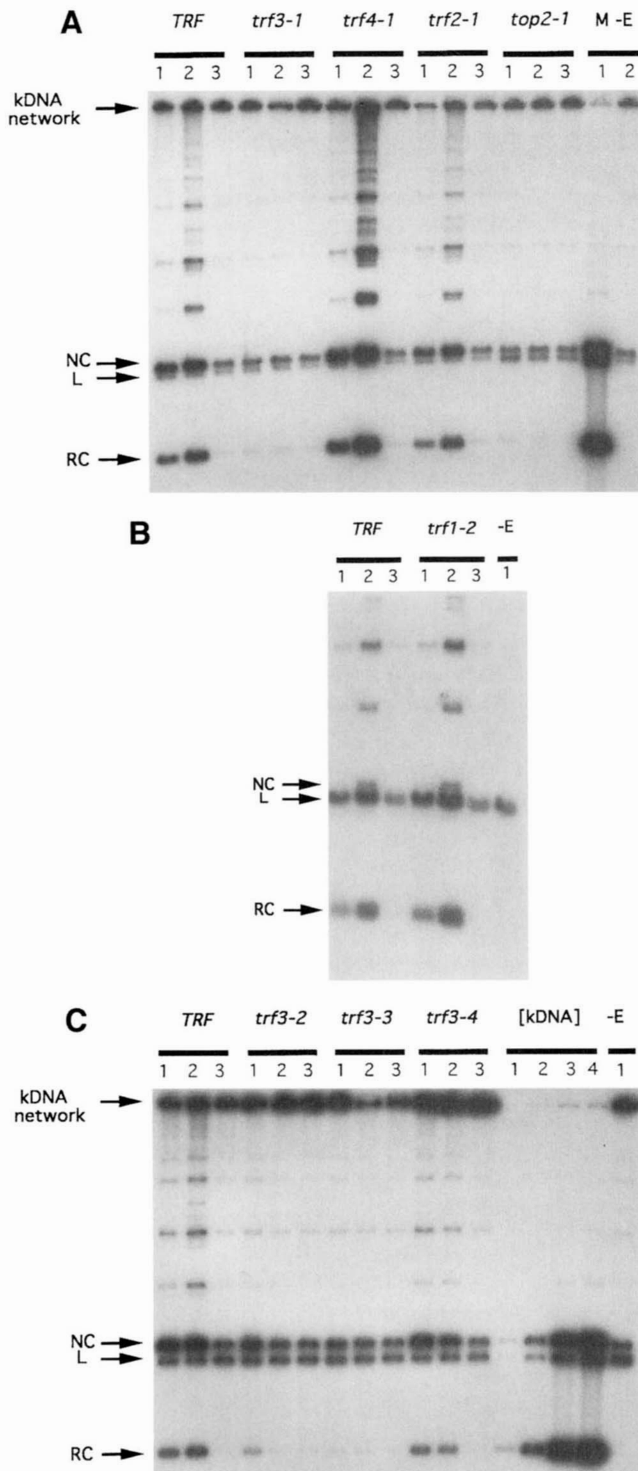

FIGURE 2.—Topo II assays of *trf* mutants. Quantitative assays for decatenation by topo II were performed as described in MATERIALS AND METHODS. All assays were perfomed under conditions that show linear dependency of the appearance of product on both time and amount of extract. kDNA (kinetoplast DNA) network indicates the position of the fully catenated kDNA substrate used in the assay. All other arrows refer to various forms of kDNA monomers that have been released from the kDNA network by the action of topo II. NC, nicked circular monomer; L, linear monomer; RC, relaxed circular monomer. Due to its large size, the kDNA network remains in the well of the 0.8% agarose gels used in the assay. The smaller monomer forms that are released from the networkby the action of topo II are able to migrate into the gel. In each gel M refers to a lane in which markers provided by TopoGen (Columbus, OH) were loaded to determine the migration position of the NC, L and RC forms. A small amount of nicked circular monomer is seen in the -E (no extract) lane. (A) The assay perfomed on the strain marked *TRF* is from CY431, the parent strain used for the nonsectoring mutant screen. The *trf3-1* strain is CY739, *trf4-1* is CY738, *trf2-1* is CY447 and *top2-1* is RS191. For each strain assayed there are three lanes marked 1, 2 and 3. The reactions in lanes marked 1 contained 150 mM KCl final concentration; lane 2, 200 mM KCl; and lane 3, 250 mM KCl. The lane marked -E contains a mock reaction performed with no added extract. (B) The *TRF* strain is CY184, which is the isogenic parent of the *trf1-2* strain (*hpr1-2::HIS3*, CY527) used in the assay. (C) The *TRF* strain is CY431, *trf3-2* is CY446, *trf3-3* is CY740, and *trf3-4* is CY741. The lanes marked kDNA contain various amounts of marker DNA. Lane 1 contains 0.5 nanograms; lane 2, 5 ng; lane 3, 50 ng; and lane 4, 500 ng. The lane marked -E contains no extract.

the *pGAL1::TOP1* screen yielded complementation group *TRF1* while the colony-sectoring screen yielded complementation groups *TRF2*, *TRF3* and *TRF4*.

***TRF3* is allelic to *TOP2*:** The *trf* mutants isolated using the colony-sectoring assay were transformed with plasmids carrying *TOP2* (pYZ3) and *TOP3* (pWJ459) to test for complementation by the two other known *S. cerevisiae* topoisomerase genes. Mutants in the *TRF3* complementation group (19, 101, 114 and 153) were complemented for nonsectoring phenotypes by a *CEN ARS* plasmid carrying *TOP2*. Thus, mutants 19, 101, 114, and 153 are likely to be alleles of *TOP2*. Mutants 27 (*trf2-1*) and 130 (*trf4-1*) showed no complementation of the nonsectoring phenotype or of secondary *ts* and *cs* phenotypes with either of the test plasmids. To further confirm that *trf2-1*, which is *ts* in a $TOP1^+$ background, is not allelic to *TOP2* mutant 27 (*trf2-1*) was crossed to a *met4* strain. *MET4* is 5 cM from the *TOP2* locus (MORTIMER *et al.* 1992). The cross (P65, *met4* × CY447, *trf2-1*) showed that in 26 four-spored tetrads the phenotypes of *ts* and Met^- displayed a ratio of PD:NPD:T of 4:4:18 indicating that *trf2-1* (ts) is not linked to *met4*, and, therefore, not allelic to *TOP2*.

***TRF1* is allelic to *HPR1*, a gene with carboxy-terminal homology to *TOP1*:** The *TRF1* gene was cloned from an *S. cerevisiae CEN ARS* plasmid library (ROSE *et al.* 1987) based on its ability to complement the growth defect of the *trf1-1 top1-7::LEU2* double mutant at 30°. Plasmid pCB62 complements the growth defect of the *trf1-1 top1* mutant. The *CEN ARS* plasmid carrying the putative *TRF1* gene was mapped by hybridization to a set of overlapping contigs that cover 90% of the *S. cerevisiae* genome (LINK and OLSON 1991). This analysis located the putative *TRF1* clone on the right arm of chromosome *IV*. Comparison of DNA sequence and restriction map information confirmed that *TRF1*

is identical to *HPR1* (Aguilera and Klein 1990), a gene that contains a region of C-terminal homology to *TOP1* (25% identity and 49% similarity over 129 amino acids).

We constructed a marked null mutation in *HPR1* (*hpr1-102::HIS3*) by deleting most of the ORF and replacing it with *HIS3* (pBS1, Table 2). Cells containing *hpr1-102::HIS3* are viable but display a much longer doubling time in synthetic complete medium (210 *vs.* 90 min for an isogenic wild-type strain). *hpr1-102::HIS3* mutants also display a synthetic growth defect with *top1-7::LEU2* similar to the *trf1-1* allele.

Topo II activity is not altered in *hpr1* (*trf1*), *trf2* and *trf4* mutants: In the absence of topo I yeast cells depend on topo II activity during DNA synthesis as well as at mitosis. Therefore, *trf* mutants that are not mutated in the *top2* gene itself (*i.e.*, excluding *trf3* alleles) may be defective in gene products necessary for synthesis or activity of topo II. To address this question we assayed topo II activity quantitatively in extracts from each of the *trf* mutants (Figure 2). To distinguish topo II activity from topo I activity (which is provided from plasmid pBS3 in each *trf* mutant), it is necessary to use a decatenation assay (Miller *et al.* 1981). Whereas both topo I and topo II are able to relax supercoiled substrates, only topo II is capable of releasing monomer circles from a catenated network of plasmids called kinetoplast DNA (kDNA). This is because release of a monomer from the catenated network requires passage of one double helix through a double strand break in the adjacent circle.

Topo II activity in wild-type (TRF^+) extracts is readily detected after 30 min (first three lanes of Figure 2A). The release of monomer circles in the reaction is ATP-dependent, as expected, since topo II activity requires ATP (not shown). Each of the non-topo II *trf* mutants (*trf1*, *trf2* and *trf4*) shows at least wild-type levels of topo II activity (Figure 2, A and B). Based on several independent assays, we do not believe that mutant *trf4-1* has higher topo II decatenation activity than the parent strain. In contrast a *bona fide top2* mutant, *top2-1* is clearly defective in decatenation activity (Figure 2A). The topo II enzyme is sensitive to salt and, therefore, we tested the salt dependency of each of the *trf* mutants compared to wild type. In each panel of Figure 2 the three lanes (labeled 1, 2, and 3) have final KCl concentrations of 150, 200 and 250 mM, respectively. None of the mutations in genes other than *TOP2* /*TRF3* show an altered salt dependency of topo II in the decatenation assay. The level of topo II protein is also not altered in any *trf* mutants as determined by Western blot of *trf* mutants containing a *TOP2-HA CEN* plasmid (data not shown).

As expected, most of the *trf3* (*top2*) mutant alleles are defective in topo II activity (Figure 2C). The *trf3* mutants vary in the extent of the defect ranging from allele *trf3-1*, which has undetectable levels of topo II activity (Figure 2A), to allele *trf3-4*, which is only reduced about twofold in activity (Figure 2C). Mutant *trf3-4*, however, does show a greater inhibition by 200 mM salt than the wild-type enzyme and, therefore, may be more defective *in vivo* at physiological salt concentrations. These experiments show that *trf1*, *trf2* and *trf4* mutants are not topo I-requiring merely because they fail to generate normal steady state levels of active topo II.

***TRF4* encodes a novel protein with limited homology to the N-terminus of *TOP1*:** The *TRF4* gene was cloned from an *S. cerevisiae* genomic *CEN ARS* library (Rose *et al.* 1987) by complementation of the secondary *cs* phenotype of *trf4-1*. Plasmid pSH1 complements both the *cs* phenotype of the *trf4-1* mutation and the nonsectoring phenotype of the *trf4-1 top1* double mutant. pSH1 was shown to contain the *TRF4* locus (Materials and Methods). Hybridization to filters containing contigs covering 90% of the yeast genome (Link and Olson 1991) revealed that *TRF4* is <10 kb centromere distal to *SUF1* on the left arm of chromosome *XV*.

Genomic DNA containing *TRF4* (2.6 kb) was sequenced on both strands. Sequence analysis showed that *TRF4* is located adjacent to *MSN1* (Estruch and Carlson 1990) on chromosome *XV*. The sequence contains a novel long ORF of 584 amino acids (Figure 3) with a region of 92 amino acids near the N-terminus that shows 21% identity and 43% similarity to an N-terminal region of *S. cerevisiae TOP1* (Figure 4). Further comparisons of the 92-amino acid putative homology region between *TRF4* and *TOP1* were done using Monte Carlo randomizations. This analysis indicated that the alignment shown in Figure 4 is 4.38 standard deviations above the mean of 100 randomized alignments of the same two sequences from *TRF4* and *TOP1*, indicating that the alignment is likely to be significant. The homology with *TRF4* lies between *TOP1* amino acids 70 and 156. By contrast N-terminal homology between *HPR1* and *TOP1* involves *TOP1* amino acids 5–57. The remainder of the *TRF4* and *TOP1* sequences are unrelated. *TRF4* contains 35% charged amino acids (18% acidic, 17% basic), a characteristic of chromatin-associated HMG proteins (Kruger and Herskowitz 1991). A basic region of the *TRF4* ORF similar to several yeast nuclear localization sequences is marked in Figure 3. *TRF4* shares no overall sequence similarity to other genes in the Genbank database.

PCR amplification followed by direct sequencing was used to determine the nature of the *trf4-1* allele. The only mutation found was a +1 frameshift at nucleotide position 1686 (amino acid 445 of 584, Figure 3). Two additional alleles of *trf4* were generated. A plasmid carrying the new allele, *trf4-nco163* (pCB494), a frameshift mutation at the *Nco*I site at nucleotide 838 (amino acid 163, Figure 3), failed to complement both the synthetic lethality of a *trf4-1 top1* double mutant and the *cs* viabil-

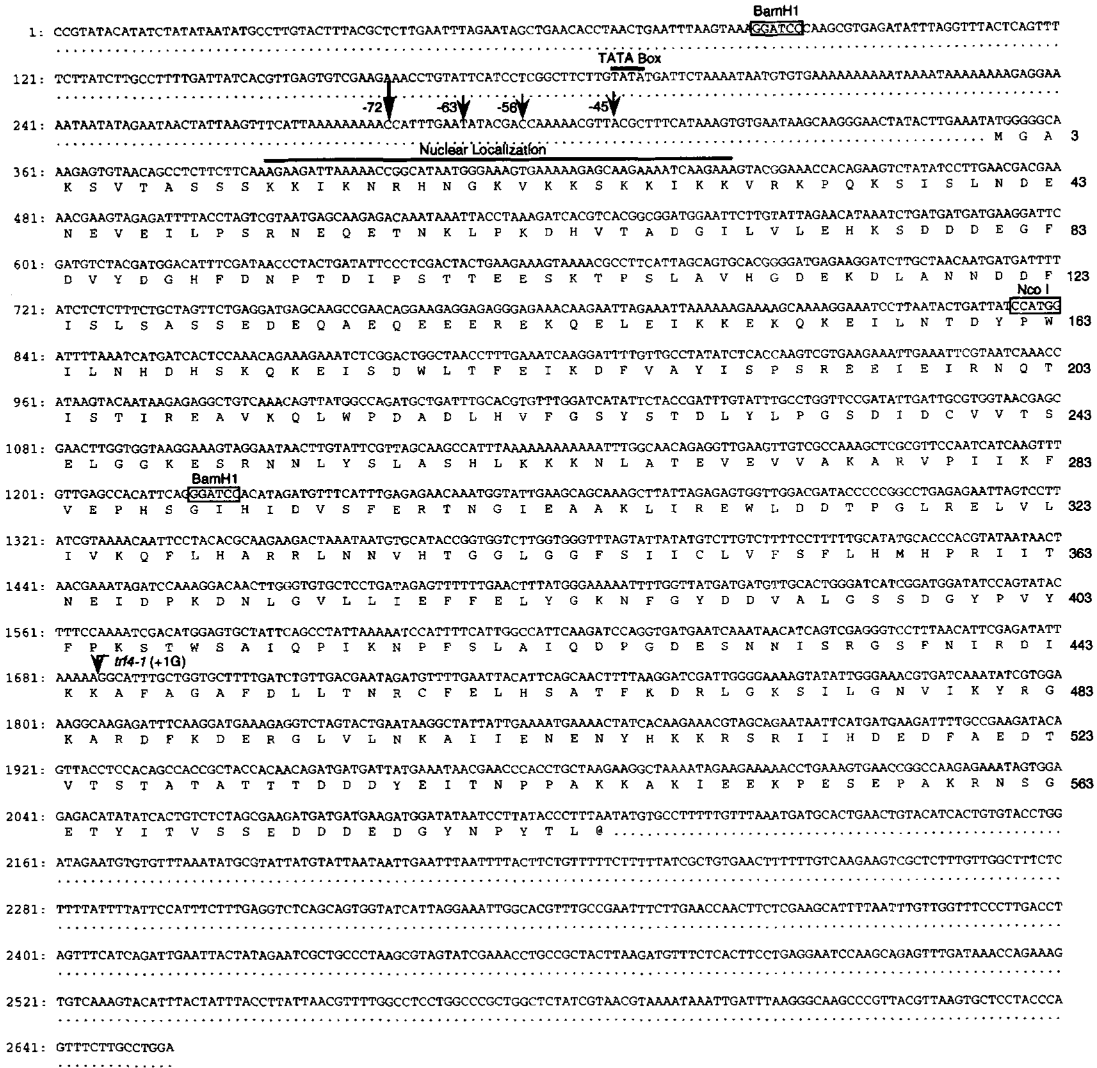

FIGURE 3.—DNA sequence of *TRF4*, position of mutant alleles and transcription start sites. Nucleotide positions are shown on the left side of the sequence and putative amino acid positions on the right side. Arrows indicate the transcriptional start sites (see also Figure 5). The accession number for the DNA sequence is U31355.

ity of a *trf4-1* single mutant (data not shown), indicating that the 584-amino acid ORF is very likely to encode *TRF4*. A deletion allele of *trf4* that removes nucleotides 89–1216 (amino acids 1–288, see *Bam*HI sites in Figure 3), *trf4-101::HIS3*, was constructed and integrated into the genome. The *trf4-101::HIS3* allele also resulted in a *cs* phenotype and was inviable in combination with *top1-7::LEU2* (data not shown).

Northern blot analysis of total RNA using a 1.0-kb probe internal to the *TRF4* ORF shows one major RNA of 2.4 kb, large enough to encode the predicted Trf4

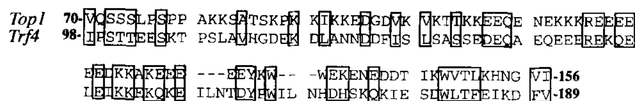

FIGURE 4.—Homology between *Trf4* and *Top1*. Alignment between amino acids 70–156 of *Top1* and 98–189 of *Trf4* proteins.

protein (data not shown). Primer extension analysis showed that there are one major and three minor transcriptional start sites between −72 and −45 nucleotides 5′ to the putative ATG translation initiation codon (Figure 5). A putative TATA box is indicated in Figure 3. This is the only TATA sequence in *TRF4* that is positioned appropriately, between 40 and 120 nucleotides upstream from the sites of transcription initiation (GUARENTE 1992a).

***trf4* and *top1* single mutants have similar phenotypes:** Mutations in *top1* cause mitotic hyperrecombination in the ribosomal DNA (rDNA) (CHRISTMAN *et al.* 1988) and a failure to shut off RNA polymerase II transcription during stationary phase (CHODER 1991). The deletion mutation *trf4-101::HIS3* displays similar phenotypes in a *TOP1*$^{+}$ background.

Recombination in the rDNA was measured by determining the frequency of loss of an *ADE2* marker

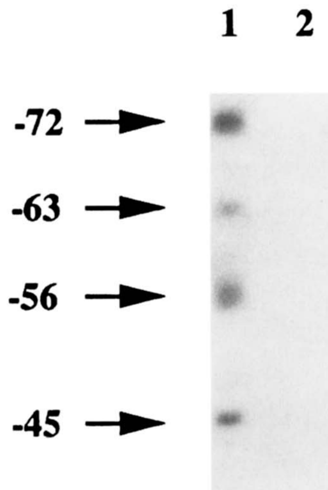

FIGURE 5.—Determination of the mRNA start site of *TRF4* by primer extension analysis. RNA was isolated from isogenic *TRF4* (CY184) and *trf4Δ-101::HIS3* (CY855) strains grown in YPD to mid-log phase. The 3′ end of the primer is at +57 nucleotides relative to the translation start site of *TRF4*. Arrows indicate the products of reverse transcription for RNA isolated from *TRF4* (lane 1) and *trf4-101::HIS3* (lane 2) strains run on an 8% polyacrylamide sequencing gel; their relative sequence position is also included. Sequence position was determined by running a sequencing reaction, using the reverse transcription primer on p*TRF4* (pCB432) next to the primer extension lanes (not shown).

TABLE 4

Mitotic recombination in a *trf4* mutant

Genotype	Frequency of loss of *rDNA::ADE2*	Frequency of reversion to Lys⁺
Wild-type	2.1×10^{-3} (1)	4.2×10^{-4} (1)
trf4-101::HIS3	17.1×10^{-3} (8)	3.8×10^{-4} (0.9)
top1-7::LEU2	58.4×10^{-3} (28)	ND

Isogenic wild-type (CY184), *top1* (CY185) and *trf4* (CY855) strains were streaked on SC −Ade plates to select for the *ADE2* gene in the rDNA array. Three colonies of each strain were then restreaked on to YPD plates to allow nonselective growth. From this plate, six colonies of each strain were separately diluted and plated on YPD; resulting colonies were then printed to SC −Ade and YPD plates. Frequencies of loss of rDNA::*ADE2* were calculated by dividing the number of colonies that failed to grow on the SC −Ade plates by the total number of colonies growing on YPD. For recombination at the *LYS2* locus, pYZ44 was integrated into CY184 and CY855 to yield strains YZY11 and SY6, respectively. This integration creates two truncated *lys2* genes at the *LYS2* locus and makes the strains Lys⁻. Two Lys⁻ colonies of each strain were grown on YPD plates for 2 days at 24°. Six different colonies of each strain from each YPD plate were diluted in water and plated on YPD and SC −Lys plates. Recombination frequencies were calculated by dividing the number of colonies growing on SC −Lys plates by the total number of colonies on YPD plates. ND, not determined.

inserted into the rDNA in isogenic *trf4-101::HIS3* and *TRF4* strains. *trf4* mutants display an eightfold greater frequency of rDNA recombination (Table 4). This increase in recombination frequency is specific to the rDNA because the frequency of recombination at a *lys2* duplication is not affected in *trf4* mutants. Using the same recombination assay, *top1-7::LEU2* mutants also show elevated rDNA recombination but not elevated mitotic recombination at single copy sequences (CHRISTMAN *et al.* 1988).

Upon entry of *S. cerevisiae* into stationary phase, there is a decrease in the rate of RNA polymerase II transcription that affects many genes (CHODER 1991). We examined this shut off by monitoring *ACT1* transcription as isogenic *trf4-101::HIS3* and *TRF4* cells entered stationary phase. As in *top1* mutants, *trf4* mutants failed to shut off *ACT1* transcription upon entry into stationary phase as determined by Northern blot analysis (Figure 6). This effect occurs only in stationary phase because *trf4* mutants do not show altered levels of *ACT1* mRNA during logarithmic growth (L lanes in Figure 6). However, while *top1* mutants grow to a higher density in stationary phase than wild-type cells (A600 = 26 for *top1-7::LEU2* *vs.* 10 for wild type), *trf4* mutants do not (A600 = 9 for *trf4–101::HIS3*).

TRF4 mutations decrease utilization of the GAL1 and CCB UAS elements: *TOP1* is thought to be involved in relaxation of transcription-induced torsional stress. Therefore, we examined utilization of an inducible RNA polymerase II gene in a *trf4* mutant background. *GAL1* expression was monitored using a *GAL1::lacZ* plasmid in isogenic strains carrying either *trf4-1*, *trf4-101::HIS3* or wild-type *TRF4* alleles. The *trf4-1* point mutation and the deletion allele *trf4-101::HIS3* showed a threefold reduction in expression of *GAL1::lacZ* when grown in the inducing carbon source galactose compared to isogenic *TRF4* strains (Table 5). The *trf4* deletion allele also caused a threefold reduction in utilization of a *CCB* (cell cycle box) UAS element, whereas the point mutation did not reduce *CCB* UAS function.

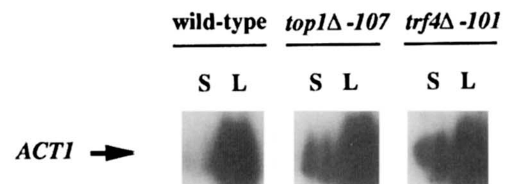

FIGURE 6.—Failure to repress *ACT1* transcription during stationary phase in a *trf4Δ-101* mutant. Strains CY184 (wild-type), CY185 (*top1-7::LEU2*) and CY855 (*trf4-101::HIS3*) were grown in liquid YPD. When the cells reached OD600 = 0.8, half of the culture was spun down and frozen at −20° and the other half was allowed to grow to ≥15 hr past the start of stationary phase before being spun down and frozen. RNA was then isolated; 15 μg of each sample was run on a 1.5% agarose formaldehyde (denaturing) gel then transferred to a nitrocellulose filter. The filter was probed with *ACT1* with resulting bands indicated by an arrow. S, RNA harvested from stationary phase cells; L, RNA harvested from log phase cells.

TABLE 5

UAS::*lacZ* expression in *trf4* mutants

Genotype	β-galactosidase units	
	GAL1::lacZ	*CCB::lacZ*
wild-type	2950	175
top1-7::LEU2	2906	170
trf4-101::HIS3	972	48
trf4-1	1012	176
trf4-1/pTRF4	2850	ND

Strains (CY184, CY185, CY855, CY726/pCB368 and CY726/pCB431) were transformed with plasmids containing *GAL1::lacZ* (pRY121Δ10) or *CCB::lacZ* (pBA161) fusions. The strains were then grown in synthetic media selecting for the plasmid(s) and harvested in mid-log phase of growth. Crude protein extracts were made, protein concentration was determined, and equal amounts of protein were used in a standard β-galactosidase assay at 28°. Unit values are the average of three experiments, one each from the three independent transformants. ND, not determined.

trf4* shows a genetic interaction with *hpr1* (*trf1*), a global positive regulator of transcription, but not with *top2-1(ts)*:** We have shown that *HPR1 (TRF1)* is required for normal induction of *GAL1* transcription and is also required for transcription of many other genes (ZHU *et al.* 1995). Furthermore, Hpr1 is in a 1×10^6 Dalton complex with as yet unidentified associated proteins (ZHU *et al.* 1995). Since *trf4* mutants are also defective for *GAL1 UAS* utilization, we examined genetic interactions between *hpr1* and *trf4* mutations. The following data demonstrate that the *hpr1 trf4* double mutant is inviable. We crossed a *MATa** *hpr1-103::LEU2* strain (YZY3) by a *MATα trf4-101::HIS3* strain (CY857) and dissected 20 tetrads. Of the 80 haploid spores produced from the 20 tetrads, we obtained 19 Leu^+ His^- segregants (20 expected), 18 His^+ Leu^- segregants (20 expected), 19 Leu^- His^- spores (20 expected) and 0 His^+ Leu^+ segregants (20 expected). A similiar cross was performed with a plasmid expressing *HPR1* from the *GAL1* promoter (pYZ36-1) present in the diploid before sporulation. The cross was dissected on galactose plates and, in this case, His^+ Leu^+ segregants were obtained. In each case the His^+ Leu^+ segregants carried the p*GAL1::HPR1* plasmid marker *(URA3)* and were viable on galactose but not on glucose medium (data now shown). This indicates that the *trf4 hpr1 (trf1)* double mutant is inviable.

In contrast, a *trf4-101::HIS3 top2-1(ts)* double mutant shows exactly the same growth defect as a *top2-1(ts)* single mutant at 24°, 27°, 30° and 31° (data not shown). The *top2-1(ts)* mutant shows both hyperrecombination (CHRISTMAN *et al.*, 1988) and greatly reduced topo II activity (Figure 2) at 30°, indicating that partial loss of topo II function is manifest at 30°.

Overexpression of *TOP2* partially suppresses the synthetic lethality of *top1 trf4* but not *top1 trf2* double mutants: Overexpression of topo II from a 2μ plasmid partially suppresses the synthetic growth defect of a *top1-7::HIS3 trf4-1* double mutant (Figure 7). Plasmids expressing 2μ *TOP2* (pYZ8), *TRF4* (pCB432) or vector (YEp24) were introduced into a *top1-7::LEU2 trf4-1* strain, and transformants were purified and incubated at 24° for 2 days. Colonies containing the *TOP2* plasmid grew more rapidly than colonies containing the vector only, but not as rapidly as colonies containing a *TRF4* plasmid. Overexpression of topo II does not result in suppression of the *cs* phenotype of a *trf4-1* single mutant.

As a further test of *TOP2* suppression of the *trf4-1 top1* growth defect, plasmid pYZ8 (2μ *TOP2*) was introduced into a *top1-7::LEU2 trf4-1* or *top1-7::LEU2 trf4-101::HIS3* double mutant containing a *TOP1 LYS2 CEN* plasmid. The transformants were monitored for the ability to lose the *TOP1 LYS2 CEN* plasmid by positive selection for *lys2⁻* using α-amino-adipate (EIBEL and PHILIPPSEN 1983). Whereas introduction of a vector control did not allow the *TOP1 LYS2 CEN* plasmid (pCB51) to be lost, introduction of 2μ *TOP2* (pYZ8) did (data not shown). These data suggest that the inviability of *top1 trf4* double mutants is a consequence of altered DNA topology. However, the lack of a genetic interaction between *trf4* and *top2* mutations indicates that topo II does not normally perform the function that is defective in *trf4 top1*.

***trf4* mutants are hypersensitive to the microtubule drug thiabendazole:** A *trf4* deletion mutant was found to be hypersensitive to killing by the anti-microtubule agent thiabendazole (Figure 8) but only slightly to the DNA synthesis inhibitor hydroxyurea. This suggests that *TRF4* may function at mitosis. To examine this further, we isolated a *ts* allele of *trf4* using hydroxylamine and plasmid shuffle (SIKORSKI and BOEKE 1991) and examined the effect of a spindle assembly checkpoint mutant, *mad1* (LI and MURRAY 1991), and a DNA damage checkpoint mutant, *rad9* (WEINERT and HARTWELL 1988; SCHIESTL *et al.* 1989), on killing at the nonpermissive temperature in a *top1 trf4 (ts)* double mutant. The presence of the *rad9* mutation had no effect on the temperature sensitivity of the *top1 trf4 (ts)* double mutant whereas the *mad1* mutation-enhanced killing at the nonpermissive temperature (data not shown).

DISCUSSION

To further elucidate the functions of topo I in *S. cerevisiae,* we have isolated mutations that result in a requirement for topo I expression for normal growth rate or viability. Using two different genetic screens, we have isolated seven mutants that define four complementation groups that display this phenotype. These genes are designated *TRF1-4* for topoisomerase one-

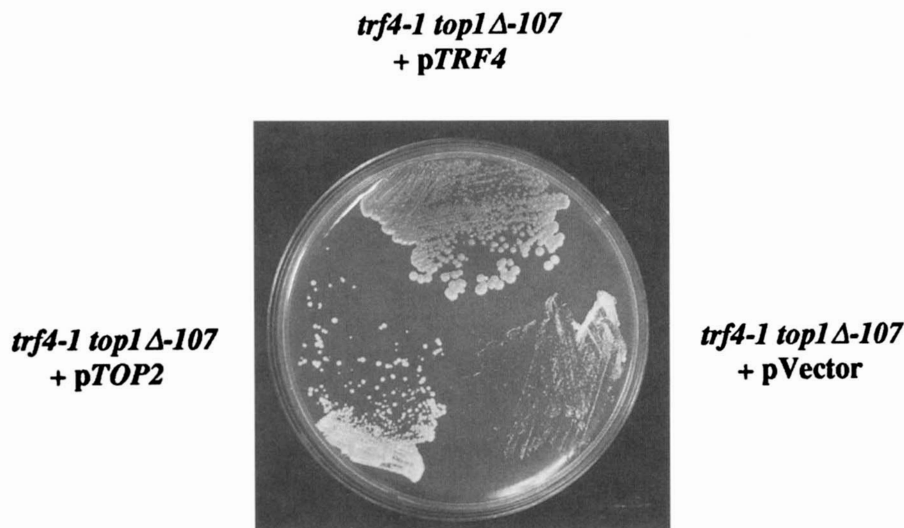

FIGURE 7.—Partial supression of the growth defect of a *trf4-1 top1Δ-107* double mutant at 24° by *TOP2* overexpression. Strain CY726 (*trf4-1 top1-107::LEU2*) was transformed to Ura$^+$ with pCB432 (*TRF4*), pRS316 (vector) or pYZ8 (2μ *TOP2*) and colonies from each transformation were purified on SC −Ura at 30°. A purified transformant of each type was then restreaked on an SC −Ura plate and grown at 24° for 2.5 days.

requiring function. The existence of mutations that are synthetically lethal with *top1* but that do not affect topo II synthesis or activity indicates that not all topo I functions can be carried out by topo II.

***TOP2* and *HPR1* are *TRF* Gene:** Double mutants between *top1* and some conditional alleles of *top2* display a synthetic growth defect (UEMURA and YANAGIDA 1984; GOTO and WANG 1985). Therefore, obtaining *top2* mutants among our collection of *trf* mutants serves to validate the screen. DNA sequencing showed that *TRF1* is allelic to *HPR1*, a gene that was identified in a screen for intrachromosomal hyperrecombination mutants and has a region of C-terminal homology to *TOP1* (AGUILERA and KLEIN 1990), indicating that the two proteins may share a functional domain. In previous work we have shown that *HPR1* is required for the transcription of a large number of physiologically unrelated genes and that the transcription defect can be suppressed by a mutation in the HMG1-like gene, *SIN1* (ZHU *et al.* 1995). In addition, *hpr1* mutants are hypersensitive to histone gene dosage imbalance (ZHU *et al.* 1995). This is a surprising result given the hyperrecombination phenotype of *hpr1* mutants (AGUILERA and KLEIN 1990) and may indicate that *hpr1* mutants affect recombination indirectly due to a defect in chromatin structure. Furthermore, this may indicate that *HPR1* and *TOP1* have a common function in maintaining proper chromatin structure (see below).

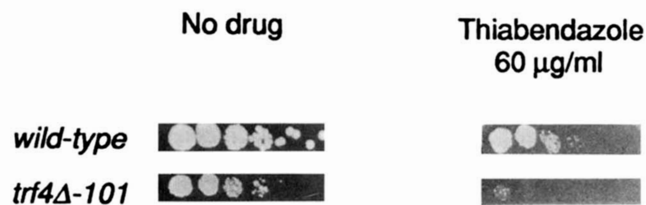

FIGURE 8.—Hypersensitivity of a *trf4* deletion mutant to thiabendazole. Isogenic wild-type (CY184) and *trf4Δ-101::HIS3* (CY855) were grown in liquid YPD. The OD600 of exponentially growing cultures was adjusted to 1.0, and 10-fold serial dilutions were made in sterile water. Five milliliters of each dilution was spotted on YPD agar plates with 0.3% DMSO (no drug) or YPD with 60 mg/ml thiabendazole (dissolved in DMSO) and incubated for 4 days at 24°.

***TRF4* has a region of homology with *TOP1*:** The 584-amino acid predicted ORF of *TRF4* shares a region of sequence similarity to *S. cerevisiae TOP1* (Figure 4). This region is not conserved among type I topoisomerases and, furthermore, is not required for the DNA relaxing activity of *S. cerevisiae* topo I when the enzyme is expressed in *E. coli* (BJORNSTI and WANG 1987). Thus, it is unlikely that *TRF4* encodes a type I topoisomerase.

The two short regions of protein sequence homology between Hpr1 and Top1 (AGUILERA and KLEIN 1990) are also in areas of Top1 that are poorly conserved among human, *S. pombe,* and *S. cerevisiae* type I topoisomerases. Furthermore, the human *TOP1* gene has a deletion of much of the C terminal region of *HPR1* homology. Thus, it is unlikely that *HPR1* encodes a type I topoisomerase and may indicate, instead, that *S. cerevisiae TOP1* has a functional domain that is involved in an as yet unkown function that is shared by *HPR1*. Analysis of the defect in *hpr1 top1* double mutants should allow this function to be defined. The fact that *HPR1* and *TRF4* share homology with *TOP1* in nonconserved regions may indicate that these regions have a distinct function that is shared by all three proteins. This is supported by the fact that *hpr1* (*trf1*) *trf4* double mutants are also inviable.

TRF4 has a very high percentage of charged residues but little overall net charge, as does *TOP1,* a characteristic of high mobility group (HMG), chromatin-associated proteins (KRUGER and HERSKOWITZ 1991). Searches of Genbank and SwissProt databases failed to show overall homology of *TRF4* to other proteins. We have recently isolated and sequenced a high copy number suppressor of the *trf4-1 cs* phenotype, designated

TRF5, that is 57% identical and 74% similar to *TRF4* (S. HEATH-PAGLIUSO and M. CHRISTMAN, unpublished results). *trf4 trf5* double mutants are inviable (I. CASTANO and M. CHRISTMAN, unpublished results) demonstrating that this function is essential.

Inviability of *trf4 hpr1* (*trf1*) double mutants: All pairwise combinations of null mutations in *top1, trf4* and *hpr1 (trf1)* are inviable. This is similar to the family of nuclear pore mutants that show multiple synthetic lethal interactions among mutant alleles of genes encoding different components of the large nuclear pore complex (BELANGER *et al.* 1994). We have recently shown that Hpr1 is globally required for transcription and is present in a large (1×10^6 Dalton) protein complex that is distinct from the SWI/SNF and RNA polymerase II holoenzyme complexes (ZHU *et al.* 1995). *TRF4* and *TOP1* may be members of the Hpr1 complex.

***trf4* and *top1* single mutants have similar phenotypes:** *trf4* single mutants display two phenotypes that are similar to *top1* mutants. These are rDNA hyperrecombination and failure to halt RNA polymerase II transcription upon entry into stationary phase. While the molecular basis for these phenotypes in *top1* mutants is not known, the striking similarity of the phenotypes in *trf4* mutants strongly suggests that *TRF4* has an *in vivo* function that is related to *TOP1*'s function. It is likely that *TRF4* and *TOP1* have a common function either separately or as part of a complex of proteins. The rDNA-specific phenotypes that are common to *trf4* and *top1* could indicate that the shared function involves rDNA metabolism. Topo I is concentrated in the nucleolus in yeast and mammalian cells (MULLER *et al.* 1985; HIRANO *et al.* 1989).

Biological function of *TRF* and *TOP1* gene products: *TRF1 (HPR1), TRF2* and *TRF4* do not encode proteins that are essential for proper topo II transcription, translation, protein stability or activity because *trf* mutants have wild-type levels of topo II activity (Figure 2). Thus, it is more likely that *TRF1 (HPR1), TRF2* and *TRF4* participate in a *TOP1*-specific function that *TOP2* does not normally perform. This would mean that not all *TOP1* functions can be carried out by *TOP2.* Two of the *TRF* genes are involved in transcription, *HPR1* and *TRF4,* indicating that this may be a special function of *TOP1* in which *TOP2* is not normally involved.

Alternatively, the *TRF* gene products could be required for some essential posttranslational aspect of Top2 function *in vivo.* For example, they could encode proteins that modify or associate with Top2 during S-phase or mitosis. This could be the case for *TRF4* since *trf4 top1* but not *trf2 top1* double mutants can be partially suppressed by *TOP2* overexpression. Drosophilia embryos contain at least three functionally distinct pools of Top2 that appear to dissociate from mitotic chromosomes at different times in mitosis (SWEDLOW *et al.* 1993). *TRF2* and *TRF4* might be involved in the generation or maintenance of those distinct pools.

However, no interaction between *TRF4* and *TOP2* is detected using the two-hybrid system (data not shown), and a *trf4-101::HIS3 top2-1(ts)* double mutant does not show any synthetic growth defect at semipermissive temperatures for the *top2-1(ts)* mutant. Thus, we favor a model in which *TRF4* and *TOP1* function in a process that *TOP2* is not normally involved in.

We propose that the *top1 trf* mutants are primarily defective in some aspect of chromatin structure. For example, the absence of topo I activity during DNA replication could result in a greater dependence on *HPR1* and *TRF4* for proper nucleosome reassembly after replication. Genetic evidence for a role of topo I in chromatin structure has not been described previously although topo I activity is known to be required for nucleosome assembly in Xenopus extracts (ALMOUZNI and MECHALI 1988). Consistent with a role in nucleosome reassembly is the fact that *hpr1* mutants are hypersensitive to histone gene dosage imbalance (ZHU *et al.* 1995) and that a transcriptional defect in an *hpr1* mutant can be suppressed by a mutation in an HMG1-like gene (ZHU *et al.* 1995). Furthermore, both *TOP1* and *TRF4* encode proteins with a high percentage of charged residues, a characteristic that may facilitate their direct interaction with chromatin. However, *trf4* mutants are not sensitive to histone gene dosage imbalance (I. CASTANO and M. CHRISTMAN, unpublished results) and, thus, may be defective at a later step in chromatin assembly such as formation of chromatin loops or in subsequent mitotic chromosome condensation. The thiabendazole hypersensitivity of *trf4* mutants and the genetic interaction of *top trf4 (ts)* double mutants with *mad1* is consistent with a common function at mitosis. Further support for a common mitotic function between *TRF4* and *TOP1* comes from the finding that overexpression of *MIH1* (RUSSELL *et al.* 1989), the *S.cerevisiae* homologue of *S. pombe cdc25,* partially suppresses a *top1 trf4 (ts)* double mutant (B. SADOFF, I. CASTANO and M. CHRISTMAN, unpublished observation). Further analysis of *top1 trf* double mutants should yield insight into the role of topo I in chromatin structure.

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