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Cloning, characterization, and sequence of the yeast DNA topoisomerase I gene

(*Saccharomyces cerevisiae/mak1* mutants)

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ABSTRACT **The structural gene for yeast DNA topoisomerase I (*TOP1*) has been cloned from two yeast genomic plasmid banks. Integration of a plasmid carrying the gene into the chromosome and subsequent genetic mapping shows that *TOP1* is identical to the gene previously called *MAK1*. Seven *top1* (*mak1*) mutants including gene disruptions are viable, demonstrating that DNA topoisomerase I is not essential for viability in yeast. A 3787-base-pair DNA fragment including the gene has been sequenced. The protein predicted from the DNA sequence has 769 amino acids and a molecular weight of 90,020.**

We have recently identified both DNA topoisomerase I and topoisomerase II mutants in the yeast *Saccharomyces cerevisiae* (1, 2). Our aim was to use the mutants to learn more about the *in vivo* roles of eukaryotic DNA topoisomerases. The topoisomerase II mutant is a temperature-sensitive conditional lethal mutant; its phenotypes demonstrate that topoisomerase II is required for termination of DNA replication and segregation of chromosomes at nuclear division (2). On the other hand, the topoisomerase I mutants that were identified have <1% of the normal level of topoisomerase activity, but they grow normally and have no obvious defects. These mutants were mapped to the *MAK1* gene (1), previously identified as required for the maintenance of a double-stranded linear, killer RNA found in many yeast strains (3). Because of these surprising results we decided to clone the topoisomerase I gene, to determine whether it corresponds to the *MAK1* gene and to study the effects of chromsomal disruptions of the gene. This paper presents those studies as well as the complete nucleotide sequence of the gene.

MATERIALS AND METHODS

***Escherichia coli* Strains and Media.** *E. coli* K-12 strain JA221 *trpE5 leuB6 lacY recA thi hsdR* was used for plasmid propagation. It was grown in L broth (4) with 50 μg of ampicillin per ml. Strain JA194 *trpC leuB* grown on M9 medium (4) supplemented with 5 μg of tryptophan per ml and 50 μg of ampicillin per ml was used to screen for *LEU2* plasmids; the yeast *LEU2* gene complements the *E. coli leuB* mutation. Plasmids were prepared by an alkaline lysis protocol (4).

Yeast Strains and Media. The following *S. cerevisiae* strains were used: CT552: *MATa ura3-52 his3 his7 leu2-3,112 trp1 ade2-101 can1*; SD2a: *MATa ade2 ura3 leu2 his3 trp1 can1 top1-1 (mak1-1) top2-1*; SD2c: *MATα ade2 ura3 his3 his7 leu2 can1 top1-1*; CT553: *MATa leu2 lys11 his1 ura3 trp1 pet17*; the diploid CT554 *MATa/α leu2/leu2 trp1/+ lys11/+ pet17/+ his1/+ ade1/+ ura3/+*. All the above strains were constructed in this laboratory using strains described previously (1, 2). Other yeast strains used include 6287-20B: *MATα kar1-1 leu2* from F. Winston (5); 418D:cir^0 *MATα leu2 his* from J. Broach; and A364A: *MATa ade1 ade2 ura1 his7, lys2 tyr1 gal1* (KIL-o). Three new *top1* alleles created by gene disruptions are described in the text. The *top1-6* allele contains the inserted *LEU2* gene, the *top1-7* allele contains deletion 1447–2295 as well as the *LEU2* insertion, and the *top1-8* allele contains deletion 580–1446 as well as the *LEU2* insertion.

Standard genetic methods and growth media were used (6). Transformation was by the spheroplast method (6). Two yeast genomic libraries were used, one was cloned in YCp50, provided by M. Rose and G. Fink, and the other was cloned in YEp13 (7).

DNA Topoisomerase I Assays. A method was developed by S. DiNardo of this laboratory whereby individual yeast colonies could be assayed directly for topoisomerase I activity without growth in liquid medium. A colony was scraped from a plate, resuspended in 30 μl of SED (1 M sorbitol/25 mM EDTA/50 mM dithiothreitol), and incubated at 37°C for 30 min. Four microliters of zymolyase 60,000 (2.5 mg/ml) (Miles) was added. Cells were incubated at 30°C for 30 min. Spheroplasts were pelleted in a desktop centrifuge, resuspended in 12 μl of yeast lysis buffer (2), and kept on ice for 30 min. After a 10-min centrifugation, 1 μl of the supernatant could be assayed in a 10-μl reaction mixture as described (1).

Extracts for determining yeast topoisomerase I activity in *E. coli* were prepared by growing 20-ml cultures, pelleting the cells, washing them, and resuspending them in 0.5 ml of yeast lysis buffer. Lysis was by sonication as described (8). One microliter of extract was assayed in a 10-μl reaction mixture at 30°C for 1 hr. The same extracts could be assayed for *E. coli* topoisomerase I as described (8).

Cloning the *TOP1* Gene. A petite (ρ^-) derivative of strain CT552 was isolated and transformed to Ura$^+$ with the YCp50-derived gene library. The first cloning method involved purifying the transformants and assaying them to find ones that had acquired topoisomerase I activity. To facilitate finding rare transformants with enzymatic activity, they were pooled into groups and assayed by a modification of the method described above for individual colonies. Specifically, 20 colonies were resuspended in 200 μl of SED, the suspension was incubated with 30 μl of zymolyase, and it was resuspended in 60 μl of yeast lysis buffer. Reconstruction experiments showed that if only 1 of the 20 colonies had topoisomerase I activity, it could be detected by this method. When a group of transformants with activity was found, the individual members of the group were reassayed to find the one with activity.

The second cloning method involved screening for transformants that had become Mak1$^+$ by using a procedure

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Abbreviation: kb, kilobase(s).

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described previously for introducing the killer RNA into the cells (6). The same Ura$^+$ transformants generated by the first cloning method were syringed from the top agar of the transformation plates. They were mated with strain 6287-20B on YEPD plates (6) for 60 hr at 30°C. After mating, the cells were diluted into SD medium (6) and spread onto plates containing 0.1% dextrose, 3% glycerol, 0.7% Bacto yeast nitrogen base without amino acids, 20 μg of adenine sulfate per ml, 20 μg of histidine per ml, 30 μg of leucine per ml, and 60 μg of canavanine sulfate per ml. These plates select for transformants of CT552 that have received the cytoplasm of strain 6287-20B and, hence, its killer RNA. After 3 days of incubation at 30°C, colonies were printed onto killer indicator plates (6) containing a lawn of sensitive cells, strain A364A. Colonies that could kill the sensitive cells were considered Mak1$^+$ candidates; they were assayed for topoisomerase I activity.

The third cloning method involved transforming strain SD2a, a *top1-1 top2-1* temperature-sensitive double mutant, with the YEp13-derived gene library. Leu$^+$ transformants were selected at 25°C, syringed out of the top agar, and replated on plates without leucine. Rapidly growing colonies were selected and assayed for topoisomerase I.

DNA Sequencing. The basic procedure has been described in a recent review article (9). The 3.8-kilobase (kb) *Hin*dIII fragment from pCT80 was sonicated to generate a library of random fragments. These were size-fractionated, the ends were repaired, and the fragments were subcloned into bacteriophage M13mp8. DNA sequencing by the dideoxynucleotide chain-termination method (10) was carried out using buffer gradient gels and deoxyadenosine 5′-[[α-^{35}S]thio]triphosphate (11). The sequence was compiled with the aid of computer programs DBAUTO and DBUTIL (12). On average, each nucleotide was sequenced 6.8 times; both strands were determined for 84% of the sequence.

RESULTS

Cloning of the DNA Topoisomerase I Gene. The gene was cloned by three independent methods. The first method involved transforming a *mak1-1* mutant, which has no detectable topoisomerase I activity (1), with a yeast gene library cloned in the plasmid vector YCp50. Ura$^+$ transformants were selected and screened by direct enzymatic assay to find ones that had acquired topoisomerase I activity. Transformants were first pooled into groups and assayed. Individual transformants from pools that had activity were then reassayed. By using this approach, 1140 transformants were screened and three with enzymatic activity were identified.

The second method involved using the same Ura$^+$ transformants as described above but this time screening them to find ones that had become Mak$^+$; i.e., ones that were now able to maintain the killer RNA. The transformants were mated with a *kar1-1* strain (5) in order to introduce the killer RNA, and then screened for the ability to maintain the RNA. Three independent transformants with this property were identified; enzymatic assays showed that all three had acquired topoisomerase I activity.

The third method was designed to complement a phenotype of a *mak1-1 top2-1* temperature-sensitive double mutant. This mutant grows slowly and forms very small colonies at the permissive temperature, 25°C (properties of this mutant will be published elsewhere). A yeast gene library cloned in the vector YEp13 (7) was used to transform the double mutant. Leu$^+$ transformants were selected and screened to find ones that grew at a normal rate as judged by colony size. Five such transformants were identified and all had acquired topoisomerase I activity.

Plasmid DNA was isolated from all the transformants with topoisomerase I activity, the DNA was passaged through *E. coli*, and then used to retransform a yeast *mak1-1* strain to Ura$^+$ or Leu$^+$. In all cases, the yeast transformants regained topoisomerase I activity. Restriction enzyme analysis of the plasmid DNA from these transformants showed that eight different plasmids had been isolated—three from the YCp50 bank and five from the YEp13 bank. All eight plasmids showed common restriction fragments and thus came from the same region of the yeast genome.

To determine whether we had cloned the structural gene for yeast topoisomerase I, as opposed to a regulatory gene, *E. coli* strains containing the plasmids described above were assayed for Mg^{2+}-independent topoisomerase activity characteristic of the yeast enzyme. A similar method was used by Goto and Wang to demonstrate that they had cloned the yeast DNA topoisomerase II structural gene (13). Fig. 1 shows that *E. coli* strains carrying these plasmids all show yeast topoisomerase I activity (lanes 1–6), while *E. coli* strains with no plasmid (lane 8) or with a YCp50-derived plasmid containing an unrelated insert (lane 7) do not exhibit such activity. If a strain containing one of the yeast topoisomerase I plasmids is assayed in the presence of Mg^{2+}, both the endogenous *E. coli* topoisomerase I and the yeast enzyme are active (lane 10). Under the conditions used in these assays, the two enzymes give different DNA topoisomer distributions and can thus be distinguished (compare lanes 9–11). Some of the plasmids were also used to transform *E. coli* strain DM800, which has a deletion of the *E. coli* topoisomerase I gene (8). These *E. coli* transformants also exhibited yeast DNA topoisomerase I activity (data not shown). The results demonstrate that we have cloned the yeast DNA topoisomerase I structural gene.

The Structural Gene Maps at *MAK1*, Renamed *TOP1*. Since one of the methods used to obtain a clone with the DNA topoisomerase I structural gene involved screening for a plasmid that restored the Mak$^+$ phenotype, it seemed likely that *MAK1* was actually the structural gene for topoisomerase I. To prove this, one of the YEp13-derived plasmids described above, pRSa4-8, was integrated into the chromosome and the site of integration was genetically mapped. Such integrants arise from homologous recombination between yeast sequences on the plasmid and the corresponding sequences on the chromosomes (14), in this case at either *LEU2* or at the topoisomerase I gene. If *MAK1* is the topoisomerase I structural gene, integration of the plasmid at

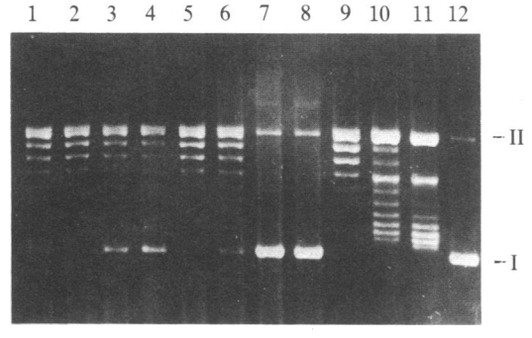

FIG. 1. DNA topoisomerase I assays of *E. coli* strains carrying *TOP1* plasmids. Lanes 1–9, assays carried out in the absence of Mg^{2+}, in a buffer specific for yeast topoisomerase (1). Lanes 1–6, *E. coli* transformants carrying the six plasmids identified by cloning methods 1 and 2. Lane 7, an *E. coli* transformant carrying a YCp50-derived plasmid with an unrelated insert that does not restore topoisomerase I activity in yeast. Lane 8, untransformed *E. coli* strain. Lane 9, an assay of partially purified yeast DNA topoisomerase I. Lanes 10 and 11, same extracts as in lanes 1 and 7 but assayed in the presence of Mg^{2+} in a buffer designed for *E. coli* topoisomerase I (8). Lane 12, no extract added. I and II show the positions of supercoiled and nicked pBR322 DNA, respectively.

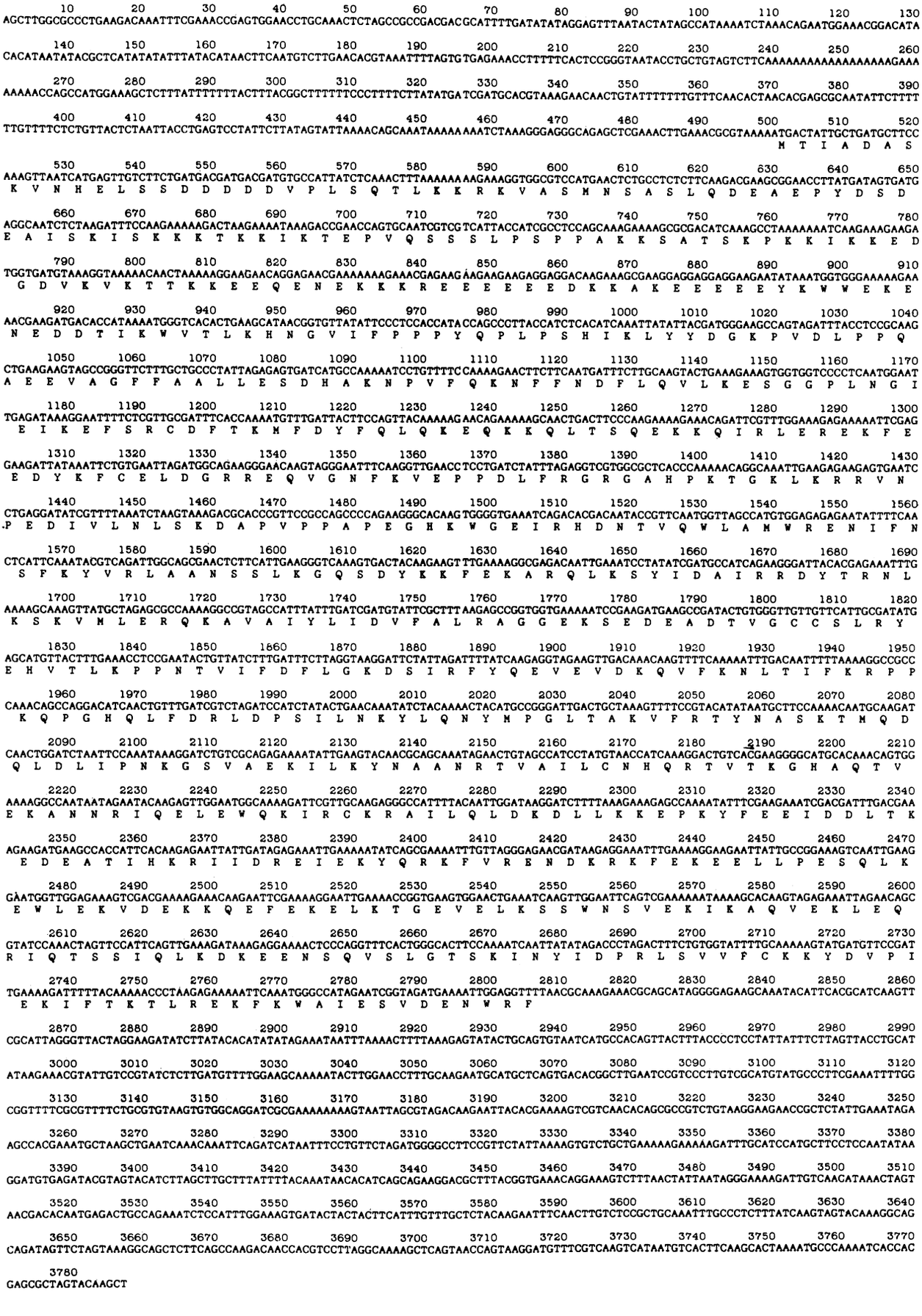

FIG. 2. DNA sequence of the 3787-base-pair *Hin*dIII fragment containing the *TOP1* gene. The deduced amino acid sequence is numbered as indicated. The deduced amino acid sequence of DNA topoisomerase I is given in one-letter code below the DNA sequence.

that locus should cause *LEU2* present in the plasmid to become linked to the centromere and to *PET17* (1, 3).

Strain 418-D *leu2* was transformed with pRSa4-8 and a stable Leu$^+$ integrant was isolated. It was crossed with strain CT553 *leu2, trp1, pet17*. The resultant diploid was sporulated, and tetrads were dissected and analyzed genetically. The *trp1* marker is used to measure centromere linkage. The results showed that *LEU2* had become centromere linked (*LEU2:TRP1*; 7 parental ditypes, 16 nonparental ditypes, 0 tetratypes) and also was linked to *PET17* (*LEU2:PET17*; 13 parental ditypes, 0 nonparental ditypes, 11 tetratypes). These results showed that the plasmid had integrated at or near *MAK1*.

The same haploid integrant was also crossed with a *mak1-1, leu2* mutant. Diploids were sporulated, dissected, and analyzed. The results for five tetrads showed that Leu2$^+$ cosegregated with topoisomerase I activity and both markers segregated 2:2 in all cases. The simplest interpretation of these results is that the plasmid integrated at *MAK1*, which we suggest be renamed *TOP1*.

DNA Sequence of the Topoisomerase I Gene. Restriction enzyme analysis of the eight different complementing plasmids showed that all of them had a 3.8-kb *Hin*dIII fragment. This fragment was isolated and cloned into the vector pUC12 to yield plasmid pCT80. This plasmid was used to transform *E. coli* and the transformants were found to have acquired Mg^{2+}-independent topoisomerase activity characteristic of the yeast enzyme (as in Fig. 1). These results strongly suggested that the *Hin*dIII fragment contained the *TOP1* gene. The fragment was therefore sequenced using the dideoxynucleotide chain-termination method (9–11).

The 3787-base-pair sequence is shown in Fig. 2. An open reading frame is present, which extends from nucleotide 500 to 2806 and codes for a protein with 769 amino acids and a molecular weight of 90,020. This value agrees well with the published molecular weight of 90,000 for yeast DNA topoisomerase I (15). Results in the next section show that disruptions of this open reading frame eliminate topoisomerase I enzymatic activity in *E. coli* and in yeast. There are two possible transcription start sites at nucleotides 435 and 448. Several possible transcription termination signals are seen beyond the large open reading frame (e.g., at nucleotide 2978) although none agree well with any of the proposed consensus sequences for yeast (16). The sequence TACTAAC, which is found in all yeast introns (17), is not present in this fragment. For these reasons, the amino acid sequence indicated in Fig. 2 is most likely to be the correct sequence for yeast DNA topoisomerase I.

Topoisomerase I Is Not Essential for Viability. A major reason for cloning the gene was to determine whether the enzyme is essential for viability in yeast. To this end, null mutations were introduced into the gene. First a 2.2-kb fragment containing the *LEU2* gene was cloned into the *Sal* I site of pCT80 at nucleotide 2486 of the *Hin*dIII fragment. This insertion within the topoisomerase I coding sequence was sufficient to eliminate yeast topoisomerase I activity in *E. coli* and yeast. Nevertheless, a further gene disruption was performed by deleting an 849-base-pair *Aha* III fragment spanning nucleotides 1447–2295 of the fragment shown in Fig. 2. The resultant plasmid, pCT80ΔTOP, was cut with *Hin*dIII to generate a 5.1-kb fragment with both a deletion and a *LEU2* insertion in the *TOP1* gene. Linear DNA fragments are recombinegenic in yeast and can be used to replace the homologous sequences on the chromosome (18). In this case, the 5.1-kb fragment was used to transform a diploid yeast strain to Leu$^+$. Southern blotting with a *TOP1* probe, pCT80, verified that the disrupted *TOP1* gene had replaced the wild-type gene on one of the two homologous chromosomes but not on the other (Fig. 3). To test whether a disruption of the *TOP1* gene is lethal, this diploid was sporulated and tetrads were dissected. Many fully viable tetrads were found; seven of them were analyzed in detail. As expected, Leu2$^+$ always cosegregated with Top1$^-$ and both markers segregated 2:2. Two complete tetrads were analyzed by Southern blotting. In all cases, the Leu2$^+$ Top1$^-$ haploids had a 5.1-kb *Hin*dIII fragment diagnostic of the disrupted gene, while the Leu2$^-$ Top1$^+$ haploids all had the wild-type 3.8-kb *Hin*dIII fragment (Fig. 3). Haploids with the disrupted gene grow at an almost normal rate (the doubling time is ≈30% longer than for wild type) and have no obvious defect in DNA or RNA synthesis. In a separate experiment, we constructed a null mutation with a deletion in the amino-terminal region of the *TOP1* open reading frame (nucleotides 580–1446), as well as the *LEU2* insertion at nucleotide 2486. Haploid yeast strains carrying this mutation also were viable. Thus, we conclude that DNA topoisomerase I is not essential for viability in yeast.

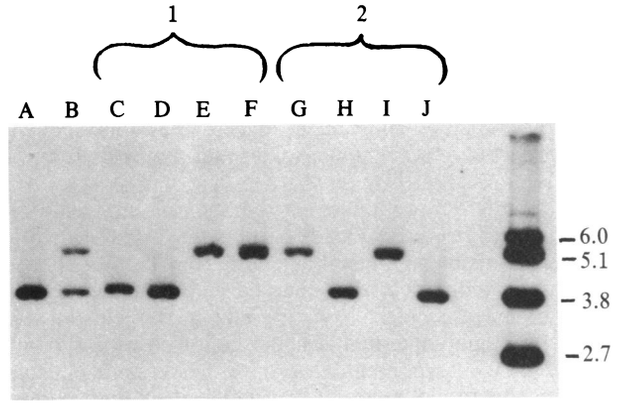

FIG. 3. Southern blotting of strains with a disruption of the *TOP1* gene. Genomic DNA from the following strains was digested with *Hin*dIII, electrophoresed on a 1.0% agarose gel, blotted to nitrocellulose, and probed with pCT80. Lanes: A, the original diploid CT544; B, CT554 transformed with the 5.1-kb DNA fragment with a deletion and insertion in the *TOP1* gene (see text); C–F, tetrad 1 from the transformed diploid; G–J, tetrad 2 from the transformed diploid. The size of known DNA molecules in kb is shown on the right.

DISCUSSION

DNA topoisomerase I is present in large amounts in the nuclei of all eukaryotes. Many workers have speculated that it acts as a swivel during DNA replication and possibly during transcription. It is therefore surprising to find that the enzyme is not essential for viability in yeast. A possible explanation is that DNA topoisomerase II can substitute for topoisomerase I. Recently we have examined *top1 top2-1* double mutants and found that they have defects in DNA replication and transcription not seen for either single mutant (unpublished results). These results suggest that topoisomerase action is necessary for DNA replication fork movement and at least some types of transcription, but that either enzyme can perform the required function. A recent paper suggests that the same situation may exist in the fission yeast, *Schizosaccharomyces pombe*. In that case, topoisomerase I mutants also are viable and topoisomerase I and II double mutants have phenotypes exhibited by neither single mutant (19). It remains to be seen whether topoisomerase II can substitute for topoisomerase I in higher eukaryotes.

We have previously shown that *E. coli* DNA topoisomerase I deletion mutants are viable (8), but in that case compensatory mutations, often in the gyrase genes, are necessary for viability (20). It is clear from the ease with which *top1* mutations are introduced into new strains that no such compensatory mutations are necessary for the viability of *S. cerevisiae top1* mutants.

The DNA sequence shown in Fig. 2 has several interesting features. Upstream of the *TOP1* coding sequence beginning at nucleotide 239, there is a stretch of 27 purines, 26 of which are adenine residues. Studies with synthetic polymers and recombinant DNA molecules suggest that this sequence probably will not form nucleosomes and it may therefore be a regulatory signal (21).

The predicted protein is extremely rich in lysine and glutamic acid residues. This is particularly true for a stretch of amino acids beginning at nucleotide 806. Twenty-six of 30 contiguous amino acids are charged, either acidic or basic, and 2 others are polar. Near the end of this cluster are 5 consecutive glutamic acid residues followed by a tyrosine. This sequence is very similar to the v-*src* tyrosine phosphorylation site found in several proteins including polyoma middle T antigen (22). Recently, it has been reported that several topoisomerases can be phosphorylated by tyrosine protein kinases with concomitant loss of activity (23). It would be of interest to learn whether yeast topoisomerase I can be phosphorylated by tyrosine protein kinases and whether this has any functional significance.

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