Yeast Topoisomerase II Mutants Resistant to Anti-Topoisomerase Agents: Identification and Characterization of New Yeast Topoisomerase II Mutants Selected for Resistance to Etoposide

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ABSTRACT

We describe a system that allows us to easily isolate and characterize mutants in yeast topoisomerase II that are resistant to antitumor agents that target this enzyme. The system uses yeast strains that are sensitive to those agents and that carry temperature-sensitive top2 mutations. The temperature-sensitive mutation allows the isolation of recessive drug-resistant mutations. The mutagenized TOP2 gene we have used is under the control of the yeast DED1 promoter; this overexpression of TOP2 is designed to avoid isolating mutants that are drug resistant solely because the mutated topoisomerase II has low enzymatic activity. We describe three mutants that we isolated using this system. Two of the three mutants show resistance to etoposide and amcasrine, while the third mutant is partially resistant to etoposide and fluoroquinolones but not to amcasrine. DNA sequence changes have been identified in all of these mutant TOP2 genes. The mutant with partial resistance to etoposide and fluoroquinolones has an amino acid change at position 738 of TOP2, which is three amino acids from the site homologous to Ser85 of E. coli gyrA, an amino acid which had previously been shown to be an important target for resistance to quinolones in bacteria. In one of the alleles that confers resistance to both etoposide and amcasrine, top2-103, has changes in amino acid 824 and amino acid 1186 of TOP2. Reconstruction of the mutations by oligonucleotide-directed mutagenesis demonstrates that the change at amino acid 824 is responsible for the drug resistance of this allele.

INTRODUCTION

DNA topoisomerases represent a unique target for anticancer chemotherapeutic agents. DNA topoisomerases participate in the full range of DNA metabolic activities in cells including replication, transcription, chromosome segregation, and recombination (reviewed in Refs. 1–3). While it might be expected that inhibitors of DNA topoisomerases are useful anticancer drugs because of their action on enzymes that are essential for the successful completion of DNA metabolic processes, the mechanism of action of antitopoisomerase agents instead depends on conversion of the enzyme into a cellular poison (4–6). This occurs by stabilization of an intermediate in the topoisomerase II reaction cycle termed the cleavage complex. This intermediate includes a covalent attachment of the protein to DNA via a phosphotyrosine linkage following scission of the phosphodiester bond of DNA (reviewed in Ref. 7). This unique mechanism of action predicts that sensitivity to drugs targeting topoisomerase II should parallel enzyme activity, with low activity leading to drug resistance and high activity leading to drug hypersensitivity. These predictions have been experimentally verified using a yeast model system (6, 8).

Many different classes of drugs target eukaryotic topoisomerase II including anthracyclines, epipodophyllotoxins, and aminoacridines. In addition, there are several compounds which do not act by stabilizing cleavage but rather act as classical enzyme inhibitors; these agents include merbarone and ICRF 193 (9, 10).

A different set of agents inhibit prokaryotic type II topoisomerases, which have considerable homology with the eukaryotic enzymes (11). Drugs that stabilize cleavage in prokaryotes are mainly the quinolone antibiotics and their fluorinated derivatives, whereas coumarin antibiotics act as classical inhibitors mainly by inhibiting the ATPase of the enzyme (for a review, see Ref. 12). It should be noted that some fluoroquinolones have recently been described that are potent inhibitors of eukaryotic topoisomerase II (13–16) and that some of the eukaryotic topoisomerase II inhibitors have recently been shown to have weak activity against the prokaryotic enzymes (Gellert and O’Dea, cited in Ref. 12).

The detailed biochemical mechanism of drugs that stabilize cleavage is poorly understood. One way of studying this process is to isolate mutations in the enzyme that result in alterations in drug sensitivity. Various mutations have been identified in bacterial DNA topoisomerase II (gyrase) which confer resistance to quinolones. Ser83 of the gyrA subunit is frequently altered in quinolone-resistant bacterial strains, though other changes, in both gyrus subunits can lead to quinolone resistance (17, 18; reviewed in Ref. 12). Mutation of Ser83 results in an alteration in binding of fluoroquinolones to the gyrase: DNA complex, suggesting that this amino acid may contribute to the drug-binding domain of the enzyme (19).

Bacteriophage T4 encodes a type II topoisomerase that more closely resembles the eukaryotic than the prokaryotic type II enzymes (20). Like eukaryotic topoisomerase II, the T4 enzyme is very sensitive to agents such as ellipticines and mAMSA (21) but is less sensitive to quinolones such as oxolinic and nalidixic acid (21). Mutants have been isolated that are resistant to mAMSA, with mutations that map to both the gene 39 and gene 52 subunits having been described. Interestingly, the gene 39 mutant appears to be hypersensitive to quinolones (22). The gene 39 subunit includes most of the enzyme that is homologous to the E. coli gyrB subunit and includes the ATPase domains of the enzyme (23). The DNA sequence changes responsible for drug resistance in the mutants has not yet been reported.

Several mutations have recently been reported in eukaryotic DNA topoisomerase II that may play a role in drug resistance. A mutation in topoisomerase II has been identified in an etoposide-resistant Chinese hamster ovary cell line that changes Arg393 to Gln (24). It is of particular interest that transfection of a wild-type Drosophila topoisomerase II gene under the control of the mouse mammary tumor virus promoter restores etoposide sensitivity, indicating that the mutation is recessive, at least to high levels of expression of a drug.
sensitive topoisomerase II (25). Since the mutant enzyme has relatively normal (but drug refractory) enzyme activity in vitro, this change is likely to be the cause of the observed drug resistance.

Zwelling and colleagues have reported a mutation in human topoisomerase II (p170) that changes Arg449 to lysine in a cell line resistant to amsacrine (26, 27). The same mutational change has also been identified from the same cell line by Lee et al. (28). However, reconstruction of the amino acid change in yeast topoisomerase II failed to result in a drug-resistant enzyme (28). Hence, the importance of this amino acid change in drug resistance is unclear.

Bugg et al. (29) and Danks et al. (30) have identified two mutations in a human leukemia cell line that is tenopside resistant. The cell line carries a topoisomerase II with an altered requirement for ATP (31). One of the identified mutations changes Arg449 to glutamine in topoisomerase IIa (p170). More recently, Danks et al. (30) have also found that amino acid 804 is changed from proline to serine, though it is not known whether the mutation is on the same allele of the TOP2α gene as the Arg449 mutation. We have recently reconstructed the Arg449 mutation in yeast topoisomerase II; in this case, the mutated enzyme confers resistance to etoposide and mAMSA, demonstrating the importance of that specific change.4

In most of the mutations identified from mammalian cell lines, it has been difficult to rigorously demonstrate what role the identified mutation plays in drug resistance. The mechanism of cell killing predicts that drug resistance should be recessive, although the mutant topoisomerase II described by Bugg et al. (29) and a separate mutation that confers mitoxantrone resistance in CHO cells (32) may confer dominant resistance. In addition, it is very difficult to obtain a complete spectrum of all of the mutational changes that can lead to drug resistance using mammalian cells.

This paper describes a genetic and biochemical system for isolating drug resistant topoisomerase II mutants in yeast. We describe the characteristics of three mutant alleles identified using this system. The identified mutations are all recessive to wild type. One of the three mutants has wild-type levels of topoisomerase II activity, while the other two have reduced enzyme activity. The sequenced mutations map to several locations in the TOP2 gene, although none of the mutations described here map to the area around amino acids 400–500. We demonstrate that mutation of Pro874, which is near the active site tyrosine (Ty17816 in yeast), and is also very close to the previously described top2-4 mutation (33, 34), is responsible for the observed drug resistance in one of the mutants.

**MATERIALS AND METHODS**

**Yeast Growth and Transformation.** Yeast cells were typically grown in rich medium YPDA or, to select for plasmids carrying URA3 as a marker, in synthetic complete medium lacking uracil, SC-URA (35). Yeast transformation was carried out using the modified lithium acetate protocol of Schiestl and Gietz (36).

**Yeast Strains and Plasmids.** The yeast strains used in this study are derivatives of IN362a (8). The genotype of this strain is MATα ara3-52 leu2 trp1 his7 ade2 ile2. The isogenic rad52::LEU2 derivative of IN362a, IN394, the top2-4 rad52 double mutant derivative (IN394-2-4), and the top2-1 rad52 double mutant derivative (IN394-2-1) have also been described previously (6, 9). The mutated plasmid carrying yeast topoisomerase II was pDED1TOP2, which carries the yeast top2 gene under the control of the yeast DED1 promoter (8). Reconstruction of mutated alleles of TOP2 was performed using the plasmid pM12. This plasmid was constructed by first eliminating the Asp1718 site of pRS306 (37) by digestion with Asp1718 fill-in synthesis with Klengow fragment, and religation. Then, the 4.1-kilobase BglII-BglII fragment from pY1 (38) which carries most of the yeast TOP2 gene (but

4 P. Vilalta and J. McMahon, unpublished data.
RESULTS

Selection of Etoposide-resistant Mutants. The overall scheme for obtaining yeast mutants that are resistant to etoposide is shown in Fig. 1. The three critical aspects of the screen are the use of the plasmid pDED1TOP2 in order to overexpress yeast topoisomerase II, using yeast strains that are drug permeable and screening for resistance in a strain that carries the temperature sensitive top2-4 allele on the chromosome. A predicted characteristic of mutations in topoisomerase II that lead to drug resistance is that the drug-resistant phenotype will be recessive to a drug-sensitive allele. In order to isolate recessive mutations, we have taken advantage of temperature-sensitive alleles of topoisomerase II. We have used the top2-4 allele since it has no detectable enzyme activity at the nonpermissive temperature (44). Though strains carrying rad52− mutations are much more sensitive to etoposide than isogenic RAD + strains, we carried out the initial selection in RAD + strains in order to be able to isolate top2 mutations that are inviable in combination with rad52− mutations. In later screens, the rad52− strain JN394t2-4 was used since we never obtained any top2 alleles that were inviable in combination with rad52− mutations (data not shown).

The library that was used to screen for the mutants was constructed by hydroxylamine mutagenesis of the plasmid pDED1TOP2 and consisted of approximately 6,000 independent plasmids. The efficiency of mutagenesis was assessed by transforming the mutagenized plasmid library into JN362at2-4 and selecting for URA + transformants at 25°C. About 500 of the transformants were replica-plated to YPDA plates at 35°C; approximately 5% of the transformants were unable to grow at the restrictive temperature. To verify that the plasmids that failed to complement the top2 mutation carried point mutations rather than rearrangements or large deletions, plasmids were isolated from the colonies that failed to grow at the restrictive temperature. The plasmids were transformed into E. coli, plasmid DNA was isolated, and restriction analysis was performed. All of the plasmids had the same restriction pattern as pDED1TOP2, excluding gross rearrangements or large deletions (data not shown).

The mutagenized pool of pDED1TOP2 was transformed into JN362at2-4. A total of about 12,000 individual yeast transformants were pooled, and a portion of the pool was suspended in SC-URA
medium at a concentration of $2 \times 10^6$ cells/ml. Etoposide was added to a final concentration of 100 µg/ml, and the cells were incubated with shaking for 48 h at 36°C. After 48 h, the cells were diluted to $2 \times 10^5$ cells/ml in SC-URA, and fresh etoposide was added to a final concentration of 100 µg/ml. After an additional 48 h, the cells were plated to SC-URA plates. The colonies that grew on SC-URA plates were replica-plated to SC-URA plates containing 100 µg/ml etoposide. After an additional 48 h, the cells were diluted to a final concentration of 100 µg/ml, and the cells were incubated.

Plasmids were isolated from several of the resistant colonies, transformed into E. coli, and retransformed into JN394-2-4. One of four plasmids gave rise to transformants that had the same sensitivity to etoposide as unmutagenized pDED1TOP2. Therefore, the resistance to etoposide in the original strain that carried that plasmid was probably unrelated to the mutagenized plasmid. The other three plasmids conferred altered sensitivity to etoposide. The phenotypes conferred by these plasmids are described below.

**Characteristics of Drug Resistance.** Fig. 2 shows the sensitivity of JN394-2-4 carrying pDED1TOP2 to mAMSA and etoposide. The drug sensitivity was determined at 35°C. For mAMSA, 1 µg/ml is somewhat growth inhibitory, and 2 µg/ml or greater results in cell killing. Thus, the MLC (the concentration required to effect a reduction in viability) is about 2 µg/ml. This level of sensitivity is very similar to what we reported previously for 30°C (8). Higher concentrations of mAMSA result in greater cell killing; at 50 µg/ml, viability is less than 1% after 8 h and about 0.01% after 24 h. Similarly, 10 µg/ml etoposide is the MLC concentration, with lower drug concentrations being partially growth inhibitory and higher concentrations producing greater cell killing (Fig. 2B).

Fig. 3 shows a detailed time course for cell killing in JN394-2-4 with the pDED1TOP2 plasmid carrying the top2-102 allele. The strain with this plasmid has an MLC of about 100 µg/ml mAMSA and greater than 100 µg/ml etoposide as compared to an MLC of 2 µg/ml for mAMSA or 10 µg/ml for etoposide with unmutagenized pDED1TOP2. This plasmid confers the highest drug resistance of the three mutant plasmids described here.

The MLC of the three alleles to etoposide and mAMSA are summarized in Table 1. The top2-101 allele does not have altered sensitivity to mAMSA but is partially resistant to etoposide with an MLC of 50 µg/ml versus 10 µg/ml for wild-type TOP2. The top2-103 allele has an MLC to etoposide similar to what we observed with the top2-101 allele, but unlike top2-101, it is also partially resistant to amsacrine, having a MLC of 20 µg/ml for this drug.

It has been demonstrated that the principal target of the fluoroquinolone CP-115,953 in yeast is DNA topoisomerase II (16). JN394-2-4 carrying pDED1TOP2 has an MLC of 5 µg/ml with this drug (Table 1). We also examined the sensitivity of JN394-2-4 carrying the mutant plasmids to this agent. As is the case with mAMSA and etoposide, the top2-102 allele also has a high level of resistance to this agent with an MLC of greater than 50 µM. Interestingly, the top2-101 allele has an MLC of 50 µg/ml; hence, it is more resistant to CP-115,953 than it is to either etoposide or mAMSA. By contrast, the top2-103 allele has much less resistance to CP-115,953 than top2-101 with a MLC of 10 µg/ml. Hence, all three mutant plasmids confer a unique spectrum of drug sensitivity to JN394-2-4.

**Resistance to Anti-Topoisomerase II Drugs Is Recessive.** The proposed mechanism of action of antitopoisomerase II agents predicts that a drug-sensitive topoisomerase will always render cells sensitive to antitopoisomerase II agents, regardless of the level of drug-resistant topoisomerase II in the cell. We tested this with the three alleles described above by transforming the plasmids carrying top2-101, top2-102, or top2-103 into JN394, which carries a wild-type chromosomal allele of topoisomerase II. The drug sensitivity of JN394 carrying any of the three alleles on pDED1TOP2 is almost the same as JN394 with unmutagenized pDED1TOP2. In particular, the JN394

Table 1 Resistance of mutants to antitopoisomerase II agents

<table>
<thead>
<tr>
<th>Allele</th>
<th>Etoposide (µg/ml)</th>
<th>mAMSA (µg/ml)</th>
<th>Fold resistance</th>
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<tr>
<td>TOP2*</td>
<td>10</td>
<td>2</td>
<td>5</td>
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<tr>
<td>2-101</td>
<td>50</td>
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<td>2-102</td>
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* FO, fluoroquinolones.
cells with this plasmid are more drug sensitive than cells without any plasmid (data not shown). These results demonstrate that expression of a highly drug-resistant topoisomerase II in JN394 confers drug hypersensitivity. All of the drug-resistant alleles are recessive, even though the level of drug-resistant enzyme is considerably higher than the wild-type, drug-sensitive enzyme. The greater than wild-type sensitivity may be due to the formation of heterodimers between the mutant and wild-type enzymes since almost all of the wild-type enzyme is probably present as a heterodimer with a mutant subunit. The mutant:wild-type heterodimer holoenzymes would necessarily be drug sensitive, and since the level of drug sensitive holoenzyme is increased, the cells are drug hypersensitive. Since the level of drug-sensitive holoenzyme is less than the level when wild-type pDED1TOP2 is present, the cells carrying any of the mutant plasmids are not as sensitive as cells carrying wild-type pDED1TOP2.

Resistant Does Not Depend on the Chromosomal Topoisomerase II Allele. All of the mutant alleles were analyzed in the presence of the top2-4 allele at the nonpermissive temperature. To exclude the possibility that the top2-4 allele contributes to drug resistance, we also examined drug sensitivity conferred by the three mutant plasmids in JN394rt2-1, which carries the top2-1 allele instead of the top2-4 allele, but is otherwise isogenic. Drug sensitivity of JN394rt2-1 carrying any of the three plasmids at 35°C is essentially the same as in JN394rt2-4 (data not shown). This shows that the resistance conferred by the mutant plasmids does not depend on the specific chromosomal topoisomerase II allele provided that drug sensitivity is assayed at the restrictive temperature for the allele.

Topoisomerase II Activity of the Drug-resistant Alleles. We next examined the topoisomerase II activity in crude cell extracts. Yeast cell extracts were made from strain JN394rt2-4 carrying pDED1top2-101, pDED1top2-102, pDED1top2-103, or unmutagenized pDED1TOP2. Topoisomerase II activity was determined with serial dilutions of the crude extract. The results are shown in Fig. 4. Extracts prepared from cells carrying the top2-102 plasmid had essentially the same level of activity as wild-type, while both top2-101 and top2-103 plasmids result in reduced activity compared to wild-type. The top2-103 activity is about 25% of wild-type, whereas the top2-101 activity is about 10% of wild-type. In all three cases, the DNA topoisomerase II activity is as high or higher than wild-type topoisomerase II when it is expressed from its own promoter. Since each allele confers a level of resistance to at least one drug greater than what is seen with JN394, which has a wild-type level of topoisomerase II activity, the resistance in top2-101 and top2-103 cannot be solely due to reduced enzyme activity. In separate experiments, we observed that the top2-101 activity appears to be rather labile in vitro, with extracts analyzed immediately after preparation showing more enzyme activity than extracts stored on ice for short periods of time (data not shown).

DNA Sequence of the Drug-resistant Alleles. In order to characterize the mutations further, we determined the DNA sequence of the resistant alleles. We sequenced the entire coding region of all three mutant alleles. In all cases, the mutations identified in top2-103a to A or C to T transitions, which would be the predicted change induced by hydroxyxlamine. The top2-101 allele has a change of G15131 to A; this DNA sequence results in a change of Gly738 of the yeast enzyme to Asp. No other mutation was identified in the coding region. This mutation is three amino acids from Ser741, the amino acid position that is homologous to Ser83 of gyrA. Ser83 is the location of several mutations that confer resistance to fluoroquinolones in bacteria (12). Interestingly, this allele confers resistance to the fluoroquinolone CP-115,953 (Table 1). This region is relatively well conserved among eukaryotic type II topoisomerases (Fig. 5). The region around the Ser83 homologue has the consensus sequence Tyr-His-His-Gly-Glu-X-Ser, where the amino acids in the consensus are conserved between yeast, Schizosaccharomyces pombe, Drosophila, and human top1α. The equivalent sequence from prokaryotic gyrA is shown in Fig. 5B. The two prokaryotic enzymes have a proline between the two histidines, while the (nonconserved) amino acid immediately before the serine is absent.

A single mutation was also detected in the top2-102 allele. The mutation changes G2554 to A, resulting in a predicted change of Arg1195 to Lys. This mutation is relatively surprising, given the conservative substitution, and its distance from the active site tyrosine and the ATPase domain of the protein. There is no obvious homology between different type II topoisomerases in this region other than the relatively high number of basic amino acids.

Two mutations were identified in the top2-103 allele. There is a change C474 to T, resulting in a change of Pro474 to Ser (top2-103a) and a change of G3557 to A, changing Gly186 to Glu (top2-103b). The change identified in the top2-103a allele is close to the site of the

Fig. 4. Topoisomerase II activity of mutant alleles. DNA topoisomerase II activity was determined in crude extracts from JN394rt2-4 cells carrying pDED1TOP2 with the wild-type enzyme, top2-101, top2-102, or top2-103. All activity assays were carried out at 37°C; hence, all of the activity is due to the plasmid-born topoisomerase II. Lanes 1–4, activities obtained with top2-103; Lanes 5–8, with top2-102; Lanes 9–12, with top2-101; and Lanes 13–16, with wild-type topoisomerase II. Lane M, molecular weight markers; Lane U, P4 DNA unknotted with purified topoisomerase II; Lane S, the knotted P4 DNA treated without extract. The quantities of protein added were 1 μg (Lanes 1, 5, 9, and 13); 0.5 μg (Lanes 2, 6, 10, and 14); 0.25 μg (Lanes 3, 7, 11, and 12); and 0.125 μg (Lanes 4, 8, 12, and 16).
results in a strain that is highly resistant to both mAMSA and etoposide (Fig. 7A). By contrast, the plasmid carrying the Gly_{1186} to Glu mutation does not alter sensitivity to either agent (Fig. 7B). Hence, the Pro_{824} mutation is clearly responsible for the resistance of the top2-103 allele.

Since the original overexpressing plasmid carrying the top2-103 allele had limited resistance to CP-115,953, we also examined the reconstructed Pro_{824} to Ser allele for sensitivity to this agent. Fig. 8A shows that the Pro_{824} to Ser has a MLC to CP-115,953 of 10 μg/ml versus 5 μg/ml for the isogenic wild-type strain (Fig. 8C). While mutations at this site result in significant resistance to mAMSA and etoposide, the resistance to CP-115,953 is minor.

As described above, the top2-103 allele is quite close to the top2-4 allele, which changes Pro_{823} to Gin. We had demonstrated previously that the top2-4 allele is resistant to etoposide but not to mAMSA (8). In order to determine whether this region is important for interaction with fluoroquinolones, we examined the sensitivity of the top2-4 allele to CP-115,953. Since the top2-4 allele is temperature sensitive, sensitivity to CP-115,953 was determined at 25°C. Interestingly, this allele shows about a 4–5 fold resistance to CP-115,953 (Fig. 8B), a higher level of resistance to the fluoroquinolone than the top2-103a allele. These results suggest that a domain that includes the region from Pro_{821} to Pro_{824} plays important specific roles in the interaction of different classes of antitopoisomerase II drugs with the enzyme.

We also reconstructed the Gly_{1186} to Asp mutation we identified in the top2-101 allele. In this case, we were not able to observe a difference in sensitivity to either etoposide or CP-115,953 (data not shown). Therefore, this mutation, when expressed at a wild-type level, is not sufficient for drug resistance. Since no other mutation was identified in the coding region of the mutant plasmid, the allele may require overexpression to confer a drug-resistant phenotype.

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**top2-4 allele**, which carries a change of Pro_{823} to Gin (34). There is quite good homology between the eukaryotic type II enzymes in this region (Fig. 6). A comparison of amino acids 818–830 of the yeast enzyme with other eukaryotic type II topoisomerases shows that all amino acid substitutions in this region are conservative. There is also very good homology with T4 gene 52, where the major difference is a threonine at position 155 of the T4 subunit instead of the methionine found in all the eukaryotic enzymes. All other differences between T4 gene 52 protein and the eukaryotic enzymes are conservative.

The top2-103a mutation is at the amino terminal residue of one of the homology domains identified by Huang (23) common to all type II topoisomerases. Domain 8 as defined by Huang (23) has the amino acid sequence Pro—X—X—Leu—X—Asn—Gly—X—X—Gly—Ile—X—X—Gly, where X is any amino acid. It is interesting to note that the amino acids immediately to the amino terminal side of domain 8 are well conserved in eukaryotic topoisomerase II but less conserved between gyrA and the eukaryotic enzymes to the carboxyl-terminal side of Pro (Fig. 6B). There is no obvious homology between the different eukaryotic enzymes around the top2-103b allele (see below).

**Reconstruction of Identified Mutations by in Vitro Mutagenesis.** Since the top2-103 allele consists of two separate mutations, it was of interest to determine which of the two mutations was responsible for the drug resistance. We therefore constructed mutant alleles corresponding to the two single mutations identified in the top2-103 allele. The mutant alleles were constructed in plasmid pMJ2 as described in “Materials and Methods” by oligonucleotide-directed mutagenesis and integrated into JN362a. The transformants were then converted to rad52" using one-step gene replacement.

Transformation of the plasmid carrying the Pro_{824} to Ser mutation
DISCUSSION

We have described a system that allows easy construction of a set of mutations that confer resistance to agents that target eukaryotic topoisomerase II. The use of a temperature-sensitive topoisomerase II allele enables us to screen for drug resistance directly, rather than having to first select against a plasmid carrying a wild-type gene, as would be required for plasmid shuffling. We are able to directly demonstrate that the drug resistance is due to a plasmid-borne mutation by isolating the plasmid and retransforming the plasmid into a yeast strain that had not been exposed to drug selection.

Fig. 7. Drug sensitivity of the reconstructed top2-103 mutations. The two mutations identified in the top2-103 allele were reconstructed using plasmid pMJ2. The mutations were introduced by gene replacement into JN362a, and the strains were then converted to rad52'. A, the sensitivity of the top2-103 allele. □, no drug; ○, 20 µg/ml mAMSA; ▲, 100 µg/ml mAMSA; △, 20 µg/ml etoposide; ▼, 100 µg/ml etoposide. B, the sensitivity of the top2-103b allele. □, no drug; ○, 20 µg/ml mAMSA; ▲, 100 µg/ml mAMSA; △, 20 µg/ml etoposide; ▼, 100 µg/ml etoposide.

Fig. 8. Fluoroquinolone sensitivity of yeast strains carrying the top2-4 and reconstructed top2-103a allele. The sensitivity of the reconstructed top2-103a allele and top2-4 allele to CP-115,953 was determined. A, the sensitivity of top2-103a. □, no drug; ○, 5 µM; ▲, 10 µM; △, 20 µM; ▼, 50 µM CP-115,953. B, the sensitivity of top2-4. □, no drug; ○, 10 µM; ▲, 20 µM; △, 50 µM CP-115,953. C, the results with the isogenic wild-type strain. □, no drug; ○, 5 µM; ▲, 10 µM; △, 50 µM CP-115,953.

It is important to note that the mutants we have isolated may not necessarily reflect mutations that occur in mammalian cell lines selected for resistance to antitopoisomerase agents or in cells from...
patients treated with antitopoisomerase II agents. The major goal of our studies is to identify those portions of the topoisomerase II molecule that are important in sensitivity to inhibitors and thereby construct a model for drug action on the enzyme. Our studies may help to indicate regions of the topoisomerase II molecule that are fruitful to analyze by techniques that can identify changes in short DNA segments, such as single-strand conformational polymorphism analysis (30), but it would not be surprising to find a somewhat different spectrum of mutations in mammalian cells.

The reconstructed top2-103a allele (with a wild-type level of expression) is much more resistant than the allele when it is being overexpressed. This fits well with the expected pattern for drugs that stabilize cleavage. Since the top2-103b allele has no drug-resistant phenotype when reconstructed, we do not think that it contributes to the sensitivity or resistance of the original isolated mutation. We plan to use a similar strategy to the one used for top2-103 to definitely show whether the detected mutant in top2-102 is sufficient for the observed drug resistance.

Since the mutations have been isolated using the plasmid pDED1TOP2, the level of expression of the enzyme may also play a role in the resistance. We would predict that, in most cases, the overexpressed allele would be more sensitive than the same allele under the control of the TOP2 promoter as we observed for the Pro824 mutation in top2-103.

Reconstruction of the top2-101 allele in pM2 does not result in a drug-resistant phenotype. It is possible that this represents a case where resistance requires overexpression of topoisomerase II. We are presently reconstructing the mutant allele in pDED1TOP2 to determine whether that might be the case. In experiments to be reported elsewhere, we demonstrate that Ser414 of yeast topoisomerase II is important for the sensitivity of the enzyme to fluoroquinolones and also the sensitivity to drugs that target eukaryotic topoisomerase II; hence, we believe that the mutation in top2-101 does affect drug sensitivity.

How might overexpression of a mutant top2 allele lead to higher levels of resistance? One possibility is that the mutant enzyme is more sensitive to complete inhibition of cleavage by the drug. In that case, the cells would also be dying due to a lack of topoisomerase II activity rather than just the stabilization of the cleavage complex. This hypothesis will be tested once we have purified the mutant protein.

An important aspect of the mutant alleles is their phenotype in cells carrying a wild-type topoisomerase II. The mechanism of action of drugs that stabilize cleavage is that drug sensitivity should be recessive. Our results for all three alleles support this hypothesis. Interestingly, all three mutant alleles confer hypersensitivity to antitopoisomerase II drugs when expressed in a wild-type strain. This "super-recessive" behavior may be due to the fact that topoisomerase II holoenzyme is a homodimer. If the mutant topoisomerase is overexpressed, there will be two significant populations of holoenzymes, mutant:wild-type heterodimer and mutant:mutant homodimer. If the mutant:wild-type heterodimer is fully drug sensitive and if the level of the wild-type enzyme is not altered by the overexpression of the mutant plasmid, then the net result is a doubling of the number of drug-sensitive holoenzymes. This argument also assumes that the mutant:mutant homodimer makes a negligible contribution to drug sensitivity, which is almost certainly the case, at least for the top2-102 allele. In any case, the experiment strongly suggests that the formation of a heterodimer between a drug-sensitive and a drug-resistant subunit results in a drug-sensitive enzyme.

Our experiments clearly demonstrate that Pro824 is responsible for the drug resistance observed in the top2-103 allele. An interesting aspect of the allele is that it confers resistance to mAMSA and etoposide but that it confers less resistance to fluoroquinolones. It is interesting to compare the effect of this allele with a mutation that is very close to it, the top2-4 allele. Thomas et al. (34) have shown that the top2-4 allele changes Pro241 to Gln. We had reported previously that top2-4 strains are highly resistant to etoposide but do not have significant resistance to mAMSA (8). Along with our result reported here that strains with top2-4 are more resistant to fluoroquinolones than top2-103, the data suggest that the domain defined by these two mutations is quite important in drug action.

These data, along with our previous studies on the top2-5 allele (44), clearly establish an important role for sequences to the carboxy terminal side of the active site tyrosine in drug resistance. It is interesting to note that all three alleles, top2-4, top2-5, and top2-103, involve mutations to or from proline. It is tempting to speculate that alterations in the secondary structure in this region of the protein may lead to the observed alterations in drug sensitivity. It also appears that some mutations that result in drug resistance can occur by subtle alterations in the TOP2 protein structure, e.g., the conservative substitutions found in top2-102 and the mutation reported by Lee et al. (28) and Hinds et al. (27).

Several mutations have been identified in human topoisomerase IIα that lead to drug resistance. Most of the identified mutations are located near one of the consensus ATPase domains of TOP2 (27, 29, 50). Although we do not describe any mutations in that region in this report, we have isolated such mutations by selection with mAMSA. In some cases, the mutations that have been identified in yeast topoisomerase II do not correspond to amino acids that are homologous in the human enzyme. Since we have functionally expressed human topoisomerase IIα in yeast and demonstrated that the expressed enzyme confers drug sensitivity in yeast, we will be able to extend our system to a direct analysis of human topoisomerase II mutations.

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Yeast Topoisomerase II Mutants Resistant to Anti-Topoisomerase Agents: Identification and Characterization of New Yeast Topoisomerase II Mutants Selected for Resistance to Etoposide

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