[CANCER RESEARCH 56, 91–99, January 1, 1996]

Functional Expression of Human Topoisomerase IIα in Yeast: Mutations at Amino Acids 450 or 803 of Topoisomerase IIα Result in Enzymes That Can Confer Resistance to Anti-Topoisomerase II Agents[1]

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ABSTRACT

DNA topoisomerase II is the target of a variety of important antitumor agents, including etoposide, adriamycin, and amsacrine. We have constructed a system for analyzing the action of anti-topoisomerase II agents using the yeast *Saccharomyces cerevisiae* and have constructed vectors for expressing human topoisomerase II functionally in yeast. We have demonstrated that temperature-conditional yeast *TOP2* mutants can be complemented by expression of wild-type human topoisomerase IIα. Furthermore, expression of human topoisomerase II in yeast results in a quantitatively unique pattern of sensitivity to amsacrine. We also have constructed mutations in human *TOP2* based on previously identified mutations from a human cell line selected for resistance to teniposide. Our experiments demonstrate that mutation of either arginine 450 or proline 803 of human topoisomerase II can result in an enzyme that has altered sensitivity to anti-topoisomerase II agents, and that a human enzyme carrying both mutations confers a higher level of drug resistance than enzymes carrying either single mutation.

INTRODUCTION

DNA topoisomerases are a major defined target for a large variety of clinically important anticancer agents, including etoposide, adriamycin, and mitoxantrone (1–3). Resistance to antitopoisomerase agents can be mediated by a variety of genetic changes, including alterations in drug uptake (reviewed in Refs. 4 and 5). In addition, resistance to antitopoisomerase agents may arise by changes in the level of expression of DNA topoisomerases or by alterations in the topoisomerase protein itself that renders it insensitive to specific drugs.

Mammalian cells express two isozymes for type II topoisomerases, top2α and top2β. The first identified form, p170, is expressed preferentially in proliferating cells (6–7) and is cell cycle regulated (8). The more recently identified top2β migrates as a M_r 180,000 protein (9) and seems to be expressed at equivalent levels in proliferating and quiescent cells (10). Recent experiments suggest that top2β may be localized preferentially to the nucleolus, suggesting that its function may be related to transcription (11). Identification of the genomic loci of top2α and top2β confirmed that the two proteins are encoded by separate genes (reviewed in Ref. 12).

All type II topoisomerases have regions of significant homology with each other (13–14). The homology extends to prokaryotic type II topoisomerases, including DNA gyrases (15) and topoisomerase IV from *Escherichia coli* (16), as well as the enzymes from T-even bacteriophages (13). There is good evidence that type II topoisomerases from eukaryotes are functionally equivalent. Expression of either *Drosophila* topoisomerase II (17) or human topoisomerase IIα (18) can complement a deficiency of the yeast enzyme.

Several mutations have been identified in human topoisomerase IIα from cell lines that are resistant to anti-topoisomerase II agents. Bugg *et al.* (19) identified a mutation in a human cell line that had been selected for resistance to teniposide. Topoisomerase II purified from this cell line required a higher ATP concentration for complete activity (20). The identified mutation resulted in a change of Arg_{450} to Gln. Bugg *et al.* (19) suggested that the mutation identified was responsible for both the altered enzymatic properties and the teniposide resistance. Subsequently, Danks *et al.* (21) demonstrated by single-strand conformation polymorphism analysis that a second mutation was present in the teniposide-resistant cell lines. The second mutation resulted in a change of amino acid 803 from Pro to Ser. This mutation, located adjacent to the active site tyrosine of topoisomerase II (Tyr_{804}), also would be a candidate change for the observed drug resistance. Because a complete cDNA for *TOP2α* has not been isolated from the teniposide-resistant cell lines, it is not known whether the two mutations are on the same allele.

Several other mutations have been identified in mammalian topoisomerase IIα (22–24). In no case is there clear evidence that the identified mutation is responsible for drug resistance of the cell line. As a model eukaryotic system, yeast has been of great value in dissecting the action of agents that target topoisomerases. Experiments in yeast have been useful in defining the mechanism of cell killing by anti-topoisomerase I (25) and anti-topoisomerase II agents (26–27). Several mutations in yeast topoisomerase II that confer altered sensitivity to anti-topoisomerase II agents have been identified (28). Recently, we have taken advantage of the homology between different eukaryotic topoisomerases to recreate the mutation at Arg_{450} identified by Bugg *et al.* (19) in yeast topoisomerase II. We showed that the equivalent yeast mutation (a change of Lys_{439} to Gln) results in an enzyme that can confer resistance to etoposide and amsacrine (29). This suggests strongly that mutating the equivalent amino acid in human topoisomerase II would result in a drug-resistant enzyme. Because Pro_{803} is not conserved between yeast and human topoisomerase II, we were not able to determine whether that mutation might have an effect on drug sensitivity. We have constructed a system in which human topoisomerase IIα can be expressed functionally in yeast, and the sensitivity of the human enzyme to etoposide, amsacrine, or other topoisomerase II targeting agents can be determined. We demonstrate that introducing either of the mutations identified, Arg450Gln or Pro803Ser, into the VM-1 cell line results in an enzyme that can confer drug resistance in yeast. Interestingly, when both drug resistance mutations are introduced into the h*TOP2* gene, the cells exhibit higher levels of resistance to amsacrine and etoposide than cells carrying either single mutation.

MATERIALS AND METHODS

Yeast Strains. The strains used in these studies are shown in Table 1. All experiments examining drug sensitivity were performed using JN362a and its

Received 7/21/94; accepted 10/31/95.

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[1] This work was supported by NIH Grant CA52814, the Martell Foundation, and the American Lebanese Syrian Associated Charities.

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Table 1 *Yeast strains*

Strain	Genotype
CH326	*MATa ura3–52 his4–539 lys2–801 top2–5*
JCW2	*MATa ura3–52 his4–539 lys2–801 top2–4 top1::HIS4*
JN362a	*MATa ura3–52 leu2 trp1 his7 ade1–2 ISE2*
JN362a t2–4	As JN362a but *top2–4*
JN362a t2–5	As JN362a but *top2–5*
JN394	As JN362a but *rad52::LEU2*
JN394t2–4	As JN394 but *top2–4*
JN394t2–5	As JN394 but *top2–5*

isogenic *rad52*$^-$ and *top2*^{ts} derivatives. The construction of these strains has been described previously (26, 30).

Construction of a Plasmid That Expresses Human Topoisomerase IIα in Yeast. Expression of human topoisomerase IIα in yeast was accomplished by placing the gene under the control of the yeast *TOP1* promoter. The entire yeast *TOP1* gene contained on a 3.8-kb *Hin*dIII-*Hin*dIII fragment was inserted into the unique *Hin*dIII site of yCPlac33 (31). The resultant plasmid was digested with *Mlu*I and *Pst*I, and the 3.4-kb fragment of the 5′ portion of the human *TOP2α* cDNA from pBShTOP2 (32) was inserted into this site. This plasmid (pMJΔ1) contains the yeast *TOP1* promoter, and the initiating ATG of h*TOP2α* but lacks about 2 kb of the 3′-coding region of h*TOP2α*. pMJΔ1 was then digested with *Pst*I and *Sal*I, and a 2.2-kb *Pst*I-*Sal*I fragment carrying the 3′ portion of the of the h*TOP2α* gene was inserted into this site. This plasmid (pMJ1) carries the entire open reading frame of the h*TOP2α* gene under the control of the yeast *TOP1* promoter.

Construction of Drug-resistant Alleles of Human Topoisomerase II in pMJ1. h*TOP2α* containing the mutation of Arg_{450} to Gln was constructed by PCR. Two pairs of PCR primers were used. The first pair was primer A (universal primer), which corresponds to the sequences located 5′ of h*TOP2α* in pBShTOP2, and primer B (TCAGTGGAGTTTTGGCCCCCTGCATCATTG), which corresponds to the antisense strand of the h*TOP2α* cDNA and also contains a nucleotide change corresponding to the Arg_{450}-to-Gln mutation (bold letter). The second pair of primers are primer C (ATGCAGGGGGCCAAAACTCCACTGAGTGTA), which corresponds to the sense strand of the gene and also contains the nucleotide change that alters Arg_{450} to Gln (bold letter) and primer D (CAAAGAGCTGAGCATTGTA), which corresponds to the antisense strand of the gene and contains a *Bpu*1102 I restriction endonuclease site (underlined).

A 1.3-kb fragment was synthesized via PCR using primers A and B, and a 1.1-kb fragment was synthesized using primers C and D. These fragments were gel purified and used as templates for secondary PCR. The two fragments were allowed to anneal and polymerize for five cycles without primers and for 25 cycles with primers A and D. A 2.4-kb fragment containing a unique *Mlu*I site and a unique *Bpu*1102 I site was obtained.

pBShTOP2α was digested with *Bst*BI, filled in with the Klenow fragment of *E. coli* DNA polymerase I, and digested further with *Eco*RV. The resulting DNA was ligated to give rise to a truncated form of pBShTOP2α (6.6 kb) in which a second *Bpu*1102 I site in the 3′ untranslated region was deleted. The truncated plasmid as well as the 2.4-kb PCR fragment were digested with *Mlu*I and *Bpu*1102 I. The plasmid and PCR fragment were gel purified and then ligated. The ligation mixture was transformed into *E. coli* DH5α-competent cells; and plasmids carrying the Arg450Gln mutation (designated pBShTOP2-R*Q) were confirmed by DNA sequencing. pMJ1 and pBShTOP2-R*Q were then digested with *Mlu*I and *Pst*I. The 8.3-kb fragment from pMJ1 and the *Mlu*I-*Pst*II fragment from pBShTOP2-R*Q were gel purified and ligated subsequently. The resulting plasmid was transformed into *E. coli* DH5α-competent cells, and plasmids that had a restriction pattern identical to pMJ1 were identified. The presence of the Arg450Gln encoding mutation was confirmed by DNA sequencing. The plasmid carrying the mutation was termed pMJ1-R*Q.

h*TOP2α* containing a mutation that changes Pro_{803} to Ser was constructed by PCR using primers A and E (CAAAGAGCTGAGCATTGTAAAGATGTATCGTGAACTAGCAGAATC) Primer E corresponds to the antisense strand of the gene and contains both a mutation that changes Pro_{803} to Ser (bold letter) and a unique *Bpu*1102 I restriction site (underlined). After a PCR reaction, a 2.4-kb fragment containing *Mlu*I and *Bpu*1102 I sites was obtained. This fragment and the 6.6-kb truncated pBShTOP2α were digested with *Mlu*I and *Bpu*1102 I to generate pBShTOP2-P*S. The mutation in pMJ1, pMJ1-P*S, was constructed using the same method as that used for pMJ1-R*Q.

A version of pMJ1 carrying both the Arg_{450}-to-Gln mutation and the Pro_{803}-to-Ser mutation (pMJ1-P*S/R*Q) was constructed by ligating the 3.8-kb *Kpn*I fragment of pMJ1-P*S with the 8.4-kb *Kpn*I fragment of pMJ1-R*Q. The presence of both mutations was verified by DNA sequencing.

Topoisomerase Assays. Yeast cell extracts for topoisomerase assays were prepared by glass bead lysis as described previously (30). Briefly, cells were lysed by vortexing 1 volume cells with 4 volumes acid-washed glass beads and 3 volumes extraction buffer [20 mM $KHPO_4$ (pH 7.0), 10 mM $MgCl_2$, 1 mM EDTA, 0.3 M $(NH_4)_2SO_4$ 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 5% (v/v) glycerol]. Cells were vortexed for 1 min, followed by 3 min on ice. After 7–10 cycles of vortexing and ice incubation, cell extracts were centrifuged for 1 h at 12,000 × *g*, and the supernatant was used for topoisomerase assays. The protein concentration of the supernatant was determined using the Bradford assay (33) with BSA as a standard.

Topoisomerase I (25) and II (30) activity was determined as described previously. The substrate for topoisomerase I activity was the plasmid pUC18. Topoisomerase II activity was determined by decatenation of kinetoplast DNA. The kinetoplast DNA from *Crithidia fasciculata* was prepared by the method of Morel *et al.* (34).

Determination of Drug Sensitivity. Drug sensitivity was determined as described previously (27–28). Briefly, cells were grown to midlog phase in liquid YPDA (yeast extract/peptone/dextrose/adenine) medium (35). Cells were diluted to 2 × 10^6 cells/ml with fresh YPDA, then appropriate volumes of drug or solvent were added. Cells were incubated with shaking, and at various times, samples of cells were removed, diluted, and plated to YPDA plates solidified with 16 g/liter agar. Plates were incubated at 30°C, and the number of cells giving rise to colonies was counted. All results are expressed relative to the viable titer at the time of drug addition (denoted t = 0).

Immunoblot of Topoisomerase IIα Protein. Crude yeast extracts were prepared using the same procedure as that used to assay topoisomerase II activity. Fifty micrograms of each extract were loaded onto a 7.5% SDS-PAGE Laemmli protein gel (36). The proteins were electrophoresed for 1.5 h at 120 V, then transferred to nitrocellulose.

The transferred proteins were blocked overnight in 50 mM Tris (pH 8.0), 0.15 M NaCl, 5% nonfat dried milk, 0.1% Tween 20, and 3% BSA. Antihuman topoisomerase II antibody (a gift from Dr. Mary Danks, St. Jude Children's Research Hospital) was diluted 1:400 in blocking buffer and allowed to incubate with the membrane for 1 h at room temperature. Visualization of the Western reaction was performed using a bromo-chloro-indolyl phosphate-nitroblue tetrazolium reaction (37).

RESULTS

Expression of Human Topoisomerase IIα in Yeast. We constructed the vector pMJ1 shown in Fig. 1 to express the h*TOP2α* gene in yeast. The vector includes the entire coding region of the h*TOP2α* gene; the expression of the h*TOP2* gene is driven by the promoter of the yeast *TOP1* gene. The vector also includes the yeast *URA3* gene, for selection of the plasmid in yeast, a yeast origin of replication, a yeast centromere, and the ampicillin resistance gene and pUC18 origin of replication for replication and maintenance of the plasmid in *E. coli*. Bacterial cells that carry the intact plasmid grow very slowly, whereas cells that carry truncated copies of human topoisomerase II (*e.g.*, plasmid pMJΔ1) grow normally. Because the yeast topoisomerase I promoter functions in *E. coli*, the slow growth is probably due to the deleterious effect of the expression of human topoisomerase II in *E. coli*. This also has been observed with other eukaryotic topoisomerases.

Next, we determined the topoisomerase II activity of the human enzyme expressed in yeast by decatenation of kinetoplast DNA. Whole-cell extracts were prepared from the logarithmically growing yeast strain JN394t2–5, with the cells either carrying no plasmid or plasmid pMJ1. Topoisomerase II activity was determined at 37°C; at this temperature, the top2–5 protein is inactive

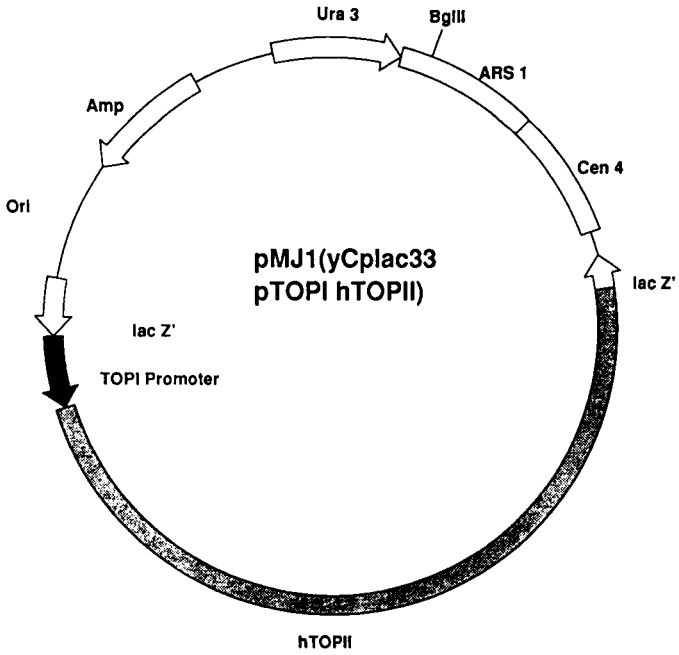

Fig. 1. Map of the topoisomerase II expression plasmid pMJ1. The construction of the plasmid is described in detail in "Materials and Methods."

(30); therefore, the only topoisomerase II activity will be from the htop2 protein. The results of this experiment are shown in Fig. 2. In *Lane 7*, the topoisomerase II activity from the JN394t2–5 extract is shown. No decatenated product is observed; all of the substrate DNA remains in the well. By contrast, 4 μg extract from cells carrying pMJ1 results in complete decatenation of 0.2 μg kinetoplast DNA (*Lane 6*). Titration experiments demonstrated that extracts of CH326 cells have about 1 unit topoisomerase II activity/μg protein; this is similar to the activity obtained from the *TOP2* strain carrying yeast *TOP2* under the control of its own promoter (data not shown). To ensure the integrity of the extracts, we also determined topoisomerase I activity of both extracts. *Lanes 2 and 3* show Mg^{2+}-independent relaxation activity determined at 37°C. Both the JN394t2–5 extract and the JN394t2–5/pMJ1 extract can carry out Mg^{2+}-independent relaxation efficiently.

Expression of Human Topoisomerase IIα Complements a Deficiency of Yeast Topoisomerase II. The yeast *TOP2* gene is essential for viability (38–40). We transformed pMJ1 into strains CH326 (38) and JN362at2–4 to determine whether the h*TOP2* gene could complement the essential function of the enzyme. The results with CH326 are shown in Fig. 3. Independently transformed colonies carrying no plasmid, pMJΔ1, or pMJ1 were grown at 25°C, and replica plated to YPDA at 36°C. Cells that carried no plasmid or that were transformed with a truncated copy of h*TOP2* were unable to grow at 36°C, whereas cells carrying the intact human gene are able to grow at this temperature.

This result suggests that human topoisomerase II is able to support the essential function of yeast topoisomerase II. To exclude the possibility that the complementation was specific for the *top2–5* allele, we also transformed pMJ1 into JN362at2–4, which carries a different topoisomerase II allele. pMJ1 was also able to complement the growth defect at 36°C of the *top2–4* allele (data not shown; see also Fig. 4). Therefore, human topoisomerase II can replace yeast topoisomerase II functionally. This result is in accord with previous results showing that either *Drosophila* topoisomerase II or human topoisomerase II overexpressed from the yeast *GAL1* promoter is able to replace the yeast enzyme functionally (17–18).

Expression of Human Topoisomerase II Supports Growth of Yeast Strains Lacking Both Topoisomerase I and Topoisomerase II. Although yeast topoisomerase II is essential for growth, yeast topoisomerase I is not essential (41–42). However, topoisomerase I probably carries out essential functions in the cell. A deficiency of *TOP1* can be compensated for by the action of topoisomerase II. Consequently, cells carrying both *top1* and $top2^{ts}$ mutations grow considerably more slowly than either single mutant. At the nonpermissive temperature for *top2*, yeast cells carrying both *top1* and $top2^{ts}$ mutations are defective in DNA replication and transcription, as well as the defect in chromosome segregation caused by *top2* single mutations (42–43). Therefore, we examined whether expression of human topoisomerase II in yeast could complement a lack of both *TOP1* and *TOP2*. We transformed pMJ1 into strain JCW2, which carries a deletion of the yeast *TOP1* gene and the *top2–4* mutation. The cells carrying the plasmid were grown at 25°C, and replica plated to 36°C. The results are shown in Fig. 4. As was the case with the $top2^{ts}$ single mutant, expression of human topoisomerase II can complement a deficiency of both enzymes. This result suggests that human topoisomerase II can carry out all of the *in vivo* functions of the yeast

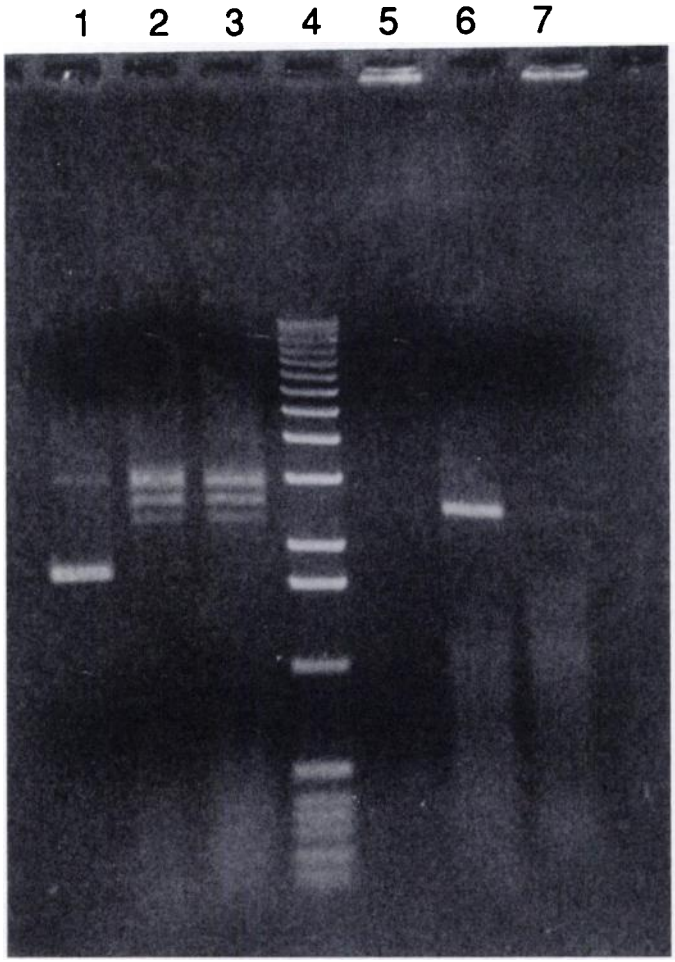

Fig. 2. Topoisomerase activity of yeast cells expressing human topoisomerase IIα. Topoisomerase II activity was measured in cell extracts prepared from strain JN394t2–5. Extracts were prepared from cells carrying pMJ1 and from JN394t2–5 cells with no plasmid. Cells were grown at 25°C, and topoisomerase activity was assayed at 37°C, the nonpermissive temperature of the *top2–5* allele. *Lane 1*, supercoiled pUC18 DNA; *Lane 2*, 200 ng pUC18 treated with extracts from JN394t2–5 cells carrying pMJ1; *Lane 3*, pUC18 treated with extracts from JN394t2–5 cells; *Lane 4*, molecular weight markers; *Lane 5*, 200 ng kinetoplast DNA from *C. fasciculata*; *Lane 6*, kinetoplast DNA treated with extracts from JN394t2–5 cells carrying pMJ1; *Lane 7*, extracts from JN394t2–5 cells. The reaction buffer used with pUC18 contains EDTA and, therefore, is specific for the topoisomerase I activity of the extracts.

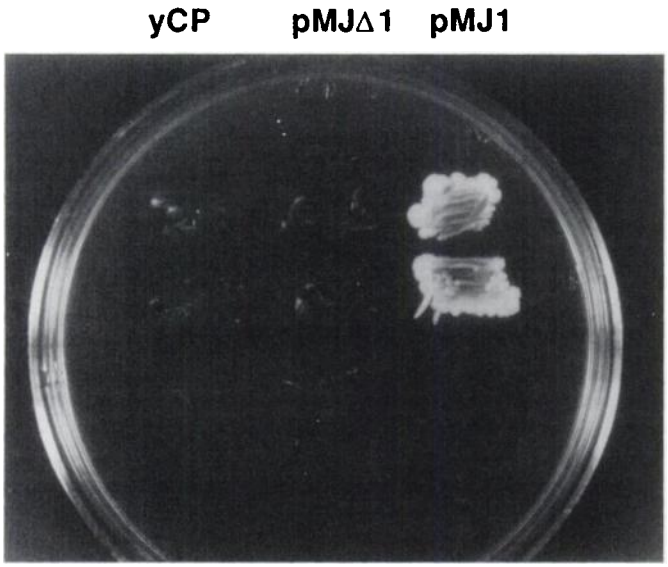

Fig. 3. Complementation of *top2-5* by pMJ1. CH326 cells were transformed with yCP50 (*yCP*), pMJΔ1, or pMJ1. The cells were grown on YPDA plates at 25°C, and replica plated to YPDA plates that were incubated at 36°C. Two independent isolates of each transformant are shown.

enzyme, including the requirement of a swivel for DNA replication and for transcription. It should be noted that the growth of JCW2 cells carrying pMJ1 at the nonpermissive temperature is considerably poorer than JCW2 cells transformed with a single-copy vector that expresses yeast topoisomerase II (Fig. 4; *yTOP2*$^+$). The poor growth may be due to somewhat incomplete complementation by human topoisomerase II of a deficiency of both yeast topoisomerases. Alternately, the level of human topoisomerase II expressed from pMJ1 may not be optimal for growth of the *top1-top2*^{ts} strain.

Expression of Human Topoisomerase II Restores Drug Sensitivity to Yeast Cells Carrying Topoisomerase II Mutations. The human topoisomerase II expression vector was transformed into strain JN394t2–4 (26). This strain carries the *ISE2* mutation that allows drug permeability (25), a mutation in *rad52* to increase drug sensitivity, and a temperature-sensitive mutation, *top2–4*, in the yeast *TOP2* gene. The *top2–4* mutation results in a protein that is not enzymatically active at 34°C, nor does it display any drug-stabilized cleavage at this temperature (30). Hence, the top2–4 protein will not contribute to drug sensitivity at this temperature. JN394t2–4 strains carrying pMJ1 are sensitive to etoposide at 34°C (Fig. 5*A*). The minimum lethal concentration to etoposide is approximately 50 μg/ml, with higher drug concentrations causing progressively greater cell killing. A drug concentration of 200 μg/ml results in about 1% survival after 24 h drug exposure. The sensitivity of yeast cells expressing their own topoisomerase II is comparable; the minimum lethal concentration is also about 50 μg/ml, and cell survival after 24 h exposure to 200 μg/ml etoposide is about 0.5% (Fig. 5*B*).

The sensitivity of yeast cells expressing human topoisomerase IIα to amsacrine is particularly striking. The minimum lethal concentration of yeast cells expressing the human enzyme to amsacrine is less than 2 μg/ml (Fig. 6*A*). Exposure of these cells to 50 μg/ml amsacrine reduces the viable cells to 0.001%. By contrast, cells expressing wild-type yeast topoisomerase II have a minimum lethal concentration to amsacrine of 5–10 μg/ml (Fig. 6*B*). We compared the survival at 24 h between cells expressing yeast and human topoisomerase II using Student's *t* test. At all drug concentrations greater than 2 μg/ml, the difference in sensitivity is highly significant; *e.g.*, at 50 μg/ml, the calculated *P* value is <0.01. Thus, yeast cells expressing human topoisomerase IIα are hypersensitive to amsacrine compared with yeast cells expressing the yeast enzyme. Because the sensitivity of yeast cells to etoposide is comparable whether the cells express h*TOP2* (from pMJ1) or yeast *TOP2*, the amsacrine hypersensitivity may reflect a difference between the intrinsic sensitivities of the yeast and human enzymes.

Reconstruction of Human Topoisomerase IIα Mutations: Arg450Gln and Pro803Ser Both Result in Drug-resistant Topoisomerases. We have demonstrated previously that changing the amino acid homologous to Arg_{450} in yeast results in an enzyme that can confer resistance to both etoposide and amsacrine. To demonstrate directly that mutation of Arg_{450} can lead to a drug-resistant topoisomerase II, we constructed a form of pMJ1 with the Arg450Gln mutation identified previously by Bugg *et al.* (19). The plasmid pMJ1Arg450Gln was introduced into JN394t2–4 cells, and sensitivity to etoposide and amsacrine at 34°C was determined. The results obtained with etoposide are shown in Fig. 7*A*. Even at etoposide concentrations of 200 μg/ml, JN394t2–4 cells are able to grow, although at a reduced rate. Drug concentrations of 100 μg/ml or less have negligible effects on cell growth. By contrast, 50 μg/ml etoposide are sufficient to cause cell killing with wild-type human topoisomerase IIα (Fig. 5*A*). At all concentrations greater than 50 μg/ml etoposide, there was a significantly greater sensitivity to etoposide in cells carrying pMJ1 with the Arg450Gln mutation. Similar results were obtained with the related epipodophyllotoxin teniposide. Teniposide results in a minimum lethal concentration of less than 50 μg/ml in JN394t2–4 cells that carry wild-type pMJ1. By contrast, the minimum lethal concentration to teniposide in the same strain carrying pMJ1Arg450Gln is greater than 200 μg/ml (data not shown). We conclude that the Arg450Gln mutation in human topoisomerase IIα results in resistance to epipodophyllotoxins.

Cells carrying pMJ1Arg450Gln also have enhanced resistance to amsacrine. The minimum lethal concentration increases to about 5 μg/ml from less than 2 μg/ml for wild-type pMJ1 (Fig. 7*B*, compare with Fig. 6*A*). This result demonstrates further that the Arg450Gln mutation results in a drug-resistant topoisomerase II.

As described above, the human cell lines that carried the Arg450Gln mutation in h*TOP2α* also carried an additional mutation

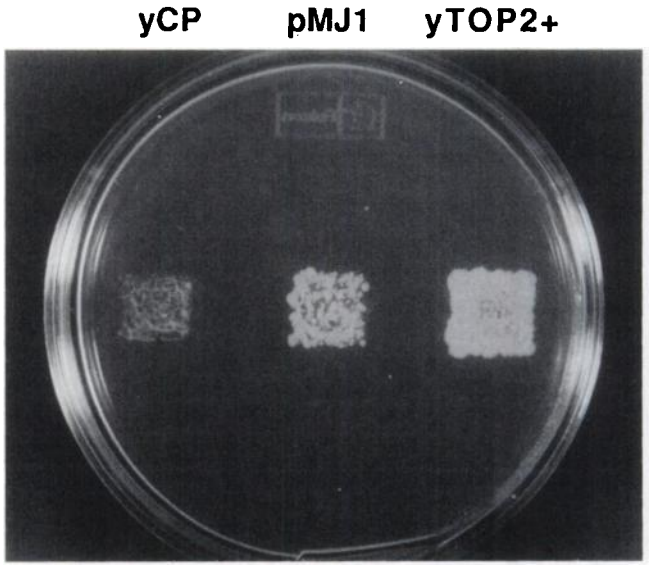

Fig. 4. Complementation of top2–4 Δtop1 strains by human topoisomerase IIα. Yeast strain JCW2 (relevant genotype *Δtop1-top2–4*) was transformed with yCP50, pMJ1, or yCP50TOP2 (indicated as *yTOP2*$^+$). Cells were replica plated to YPDA medium, and the plates were incubated at 36°C. yCP50 carries no *TOP2* gene, and so JCW2 cells are completely unable to grow at the nonpermissive temperature. JCW2 cells carrying either pMJ1 or yCP50TOP2 are able to grow at 36°C.

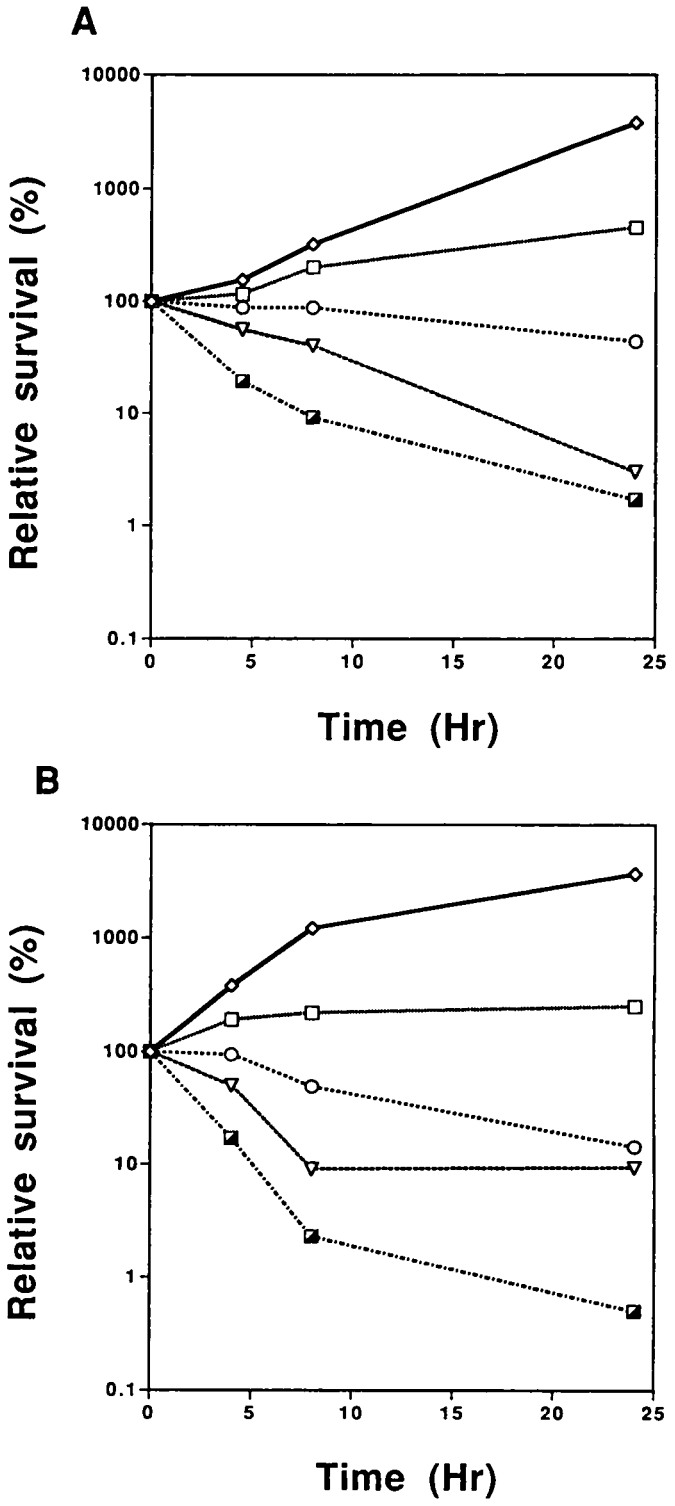

Fig. 5. Sensitivity to etoposide of yeast cells expressing human or yeast topoisomerase II. The sensitivity of JN394t2–4 cells carrying pMJ1 to etoposide was determined at 34°C as described in "Materials and Methods." *A*, sensitivity to various concentrations of etoposide. *B*, sensitivity to etoposide of yeast cells expressing wild-type yeast topoisomerase II. ◇, no drug. □, 20 μg/ml; ○, 50 μg/ml; ▽, 100 μg/ml; and ◪, 200 μg/ml, respectively.

in top2α, which changed Pro_{803} to Ser. Pro_{803} is not conserved in yeast topoisomerase II (29), so we were not able to determine whether this mutation could contribute to drug resistance using yeast topoisomerase II. We again carried out directed mutagenesis and created a version of pMJ1 that carries the Pro803Ser mutation. The plasmid was transformed into JN394t2–4, and drug sensitivity at 34°C was determined as described above. Cells carrying the Pro803Ser mutation have enhanced resistance to etoposide compared with cells expressing wild-type human topoisomerase II (compare Fig. 8*A* with Fig. 5*A*).

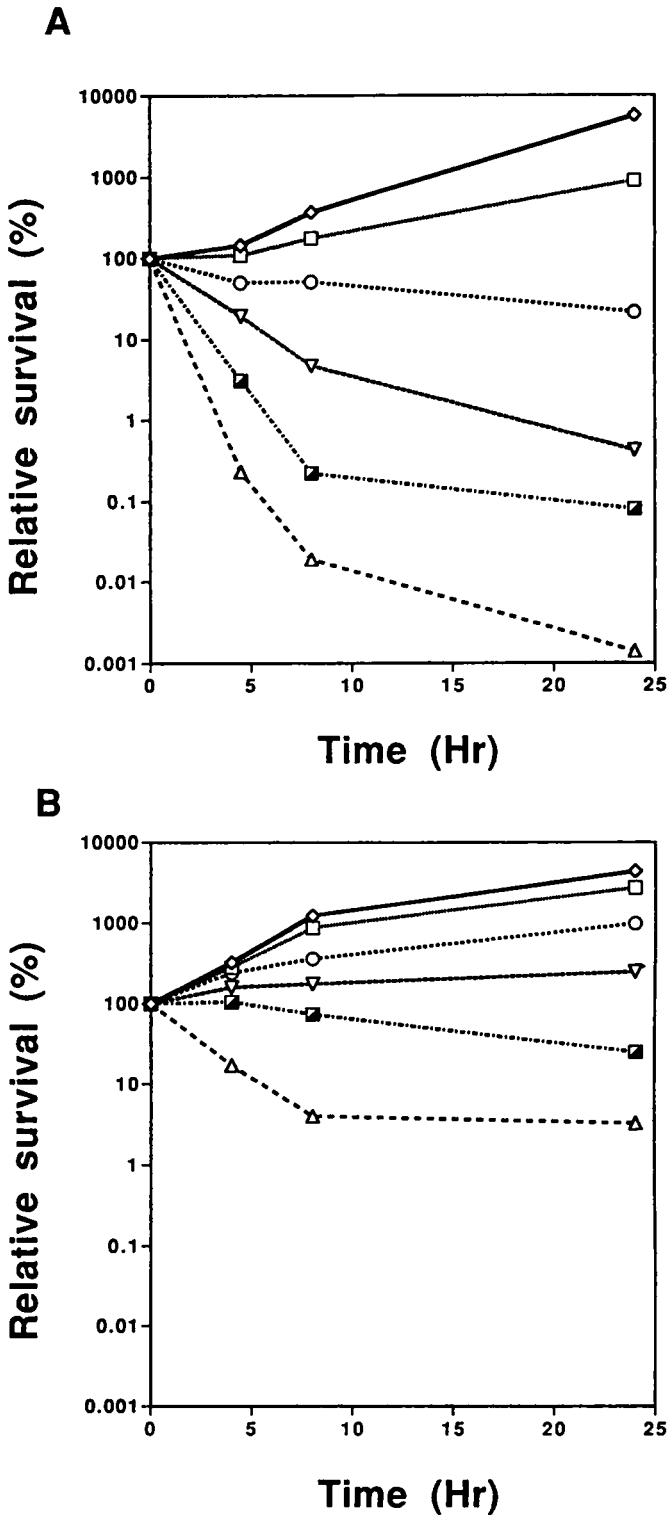

Fig. 6. Sensitivity to amsacrine of yeast cells expressing human or yeast topoisomerase II. The sensitivity of JN394t2–4 cells carrying pMJ1 to amsacrine was determined at 34°C as described in "Materials and Methods." *A*, sensitivity to various concentrations of amsacrine. *B*, sensitivity to amsacrine of the same cells expressing wild-type yeast topoisomerase II. ◇, no drug. □, 0.5 μg/ml; ○, 2 μg/ml; ▽, 5 μg/ml; ◪, 10 μg/ml; and △, 50 μg/ml, respectively.

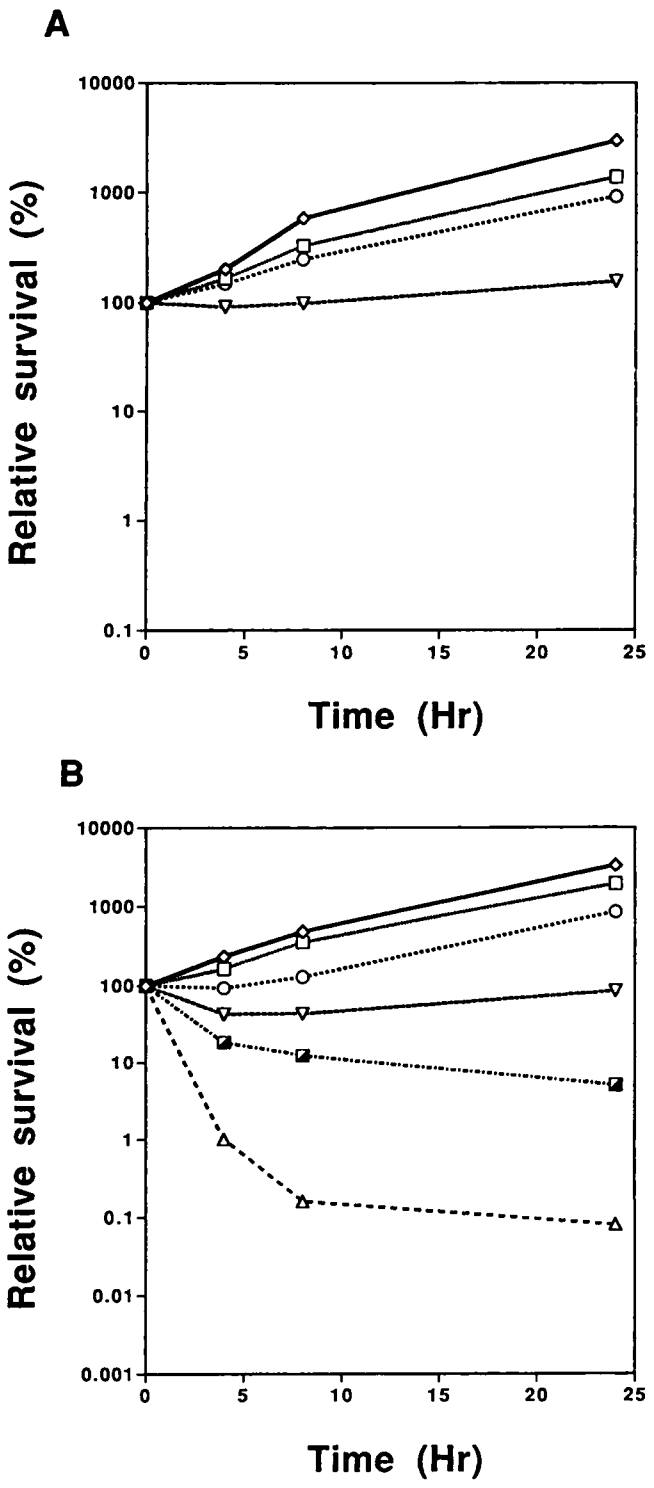

Fig. 7. Sensitivity to etoposide and amsacrine of yeast cells expressing human topoisomerase II carrying the Arg450Gln mutation. Drug sensitivity experiments were carried out with either etoposide or amsacrine under exactly the same conditions as for Figs. 5 and 6. Instead of wild-type human topoisomerase IIα, the yeast cells carry a plasmid that expresses a human topoisomerase IIα cDNA carrying the Arg450Gln mutation. *A*, sensitivity to etoposide. ◇, no drug. □, 50 μg/ml; ○, 100 μg/ml; and ▽, 200 μg/ml etoposide, respectively. *B*, sensitivity to amsacrine. ◇, no drug. □, 0.5 μg/ml; ○, 2 μg/ml; ▽, 5 μg/ml; ◪, 10 μg/ml; and △, 50 μg/ml amsacrine, respectively.

The Pro803Ser mutation also confers slight resistance to amsacrine; the resistance to amsacrine of JN394t2–4 cells carrying the Pro803Ser is similar to that of cells carrying the Arg450Gln mutation. Cells expressing the Pro803Ser mutant have a minimum lethal concentration to amsacrine of 5 μg/ml, compared with <2 μg/ml for wild-type human topoisomerase II. Therefore, the Pro803Ser is also likely to alter the sensitivity of topoisomerase IIα to antitopoisomerase agents.

An Enzyme Carrying Both Arg450Gln and Pro803Ser Mutations Results in a Highly Drug-resistant Topoisomerase II. Although the VM-1 cell line carries both the Arg450Gln and Pro803Ser

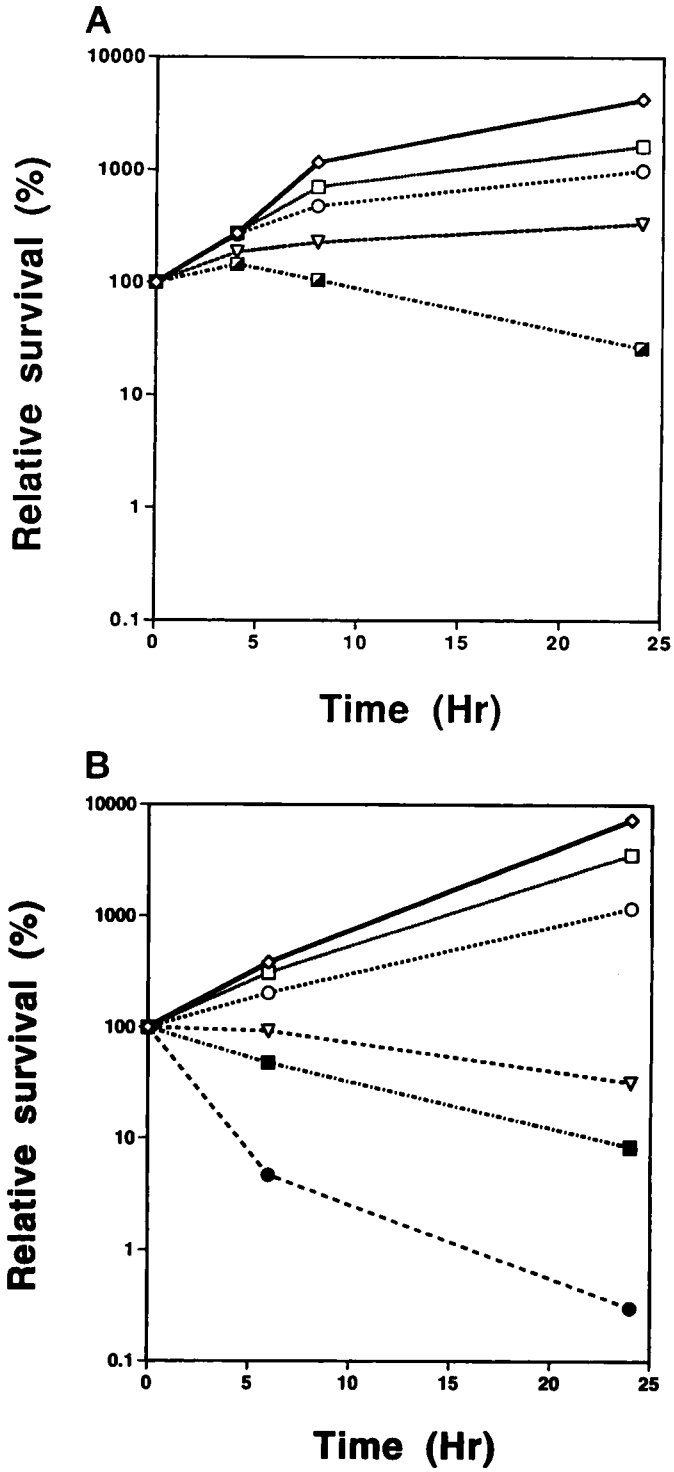

Fig. 8. Sensitivity to etoposide of yeast cells expressing human topoisomerase II carrying a mutation that converts Pro_{803} to Ser. A variant of the human topoisomerase IIα gene carrying the Pro803Ser mutation was constructed. The plasmid carrying the mutation was transformed into JN394t2–4, and etoposide sensitivity was determined at 34°C. *A*, etoposide concentrations are ◇, no drug; □, 20 μg/ml; ○, 50 μg/ml; ▽, 100 μg/ml; and ◪, 200 μg/ml, respectively. *B*, results obtained with amsacrine. ◇, no drug. □, 0.5 μg/ml; ○, 2 μg/ml; ▽, 5 μg/ml; ■, 10 μg/ml; and ●, 50 μg/ml amsacrine, respectively.

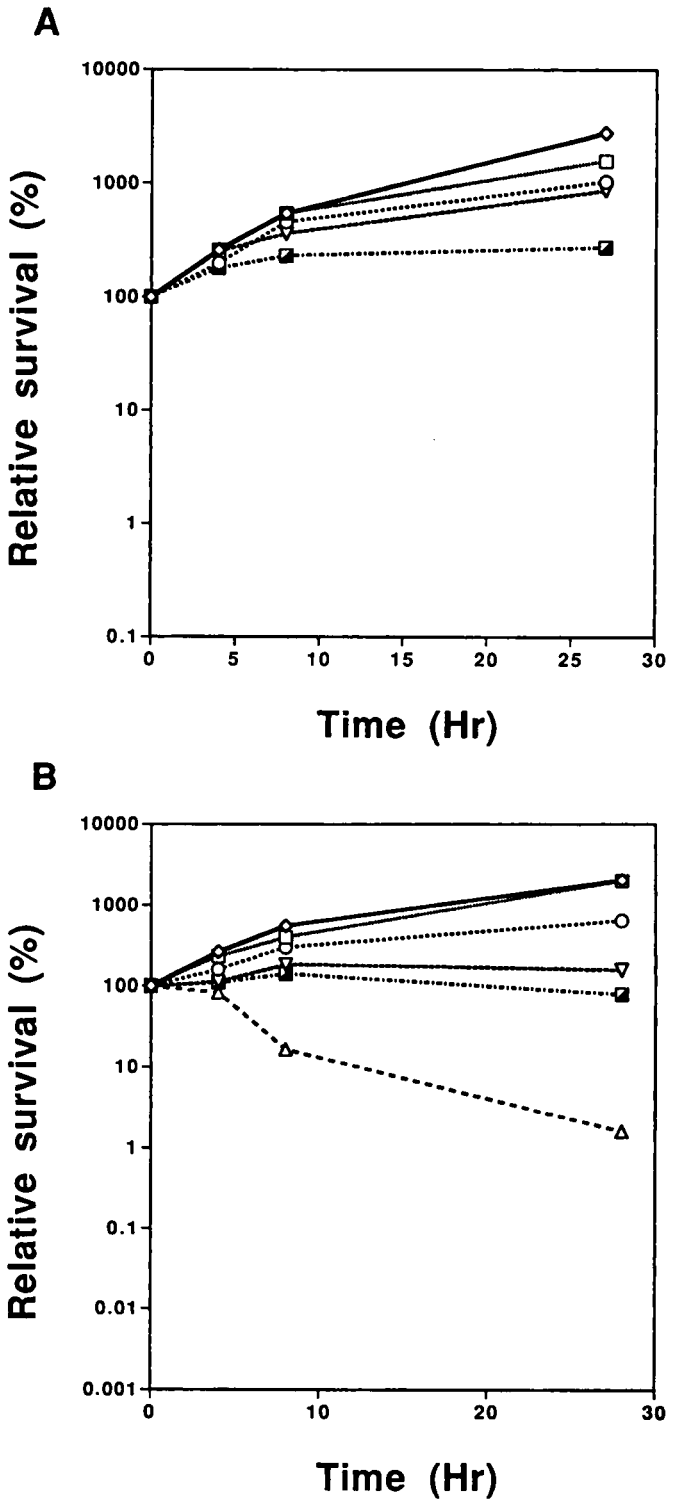

Fig. 9. Sensitivity to etoposide and amsacrine, double mutant. Drug sensitivity experiments were carried out with either etoposide or amsacrine under exactly the same conditions as for Fig. 7, except that the yeast cells carry pMJ1 with both the Arg450Gln mutation and the Pro803Ser mutation. *A*, sensitivity to etoposide. ◇, no drug. □, 20 μg/ml; ○, 50 μg/ml; ▽, 100 μg/ml; and ◪, 200 μg/ml, respectively. *B*, sensitivity to amsacrine. ◇, no drug. □, 0.5 μg/ml; ○, 2 μg/ml; ▽, 5 μg/ml; ◪, 10 μg/ml; and △, 50 μg/ml amsacrine, respectively.

mutations, it is not known whether the mutations are linked, because intact cDNAs that include all of the *TOP2* gene have not been isolated from this cell line. We decided to examine what the effect would be if the two mutations were found in the same polypeptide. The Pro803Ser mutation was introduced into pMJ1Arg450Gln, and the vector was transformed into JN394t2–4. The results of a determination of sensitivity to etoposide are shown in Fig. 9*A*. The double mutant shows high resistance to etoposide, with cell growth occurring at 200 μg/ml etoposide. The observed drug resistance of the double mutant is most striking with amsacrine (Fig. 9*B*). Cells expressing a human topoisomerase with both the Arg450Gln and Pro803Ser mutations have a minimum lethal concentration of amsacrine of between 10 and 50 μg/ml amsacrine. At 50 μg/ml amsacrine, a cytotoxic concentration, viability decreases to about 5%. At that drug concentration, the viability of yeast cells expressing wild-type human topoisomerase IIα is about 0.001% (Fig. 6*A*); with the Arg450Gln mutation alone, it is about 0.1% (Fig. 7*B*); and with the Pro803Ser mutation, it is also about 0.1%. A comparison of the survival of the single mutants with the double mutant indicates again that the differences are highly significant. Therefore, the two mutations together give a significantly greater resistance to amsacrine than either single mutation.

Topoisomerase II Levels and Activity in Yeast Cells Expressing Mutant Topoisomerase II. A possible explanation for the observed drug resistance is that the mutant protein(s) have reduced stability. We examined the level of human topoisomerase II polypeptide by Western blotting using an antibody specific for human topoisomerase II. The results are shown in Fig. 10. The human topoisomerase II antibody recognizes a M_r 170,000 polypeptide in cells carrying pMJ1 (*Lane 1*), whereas no signal is seen in extracts prepared from yeast cells that do not carry pMJ1. The same level of M_r 170,000 polypeptide is seen whether the cells carry pMJ1 with wild type topoisomerase II with either the single-mutant (*Lanes 2* and *3*) or the double-mutant protein (*Lane 5*). There is a slightly higher level of a M_r 160,000 polypeptide in the three mutant samples; however, in other preparations from cells carrying wild type topoisomerase II, a similar level of the M_r 160,000 polypeptide is seen (data not shown). Therefore, the enhanced resistance to anti-topoisomerase II agents cannot be explained by differences in human topoisomerase II protein levels.

We also measured topoisomerase II activity levels in the same extracts that were used to measure topoisomerase II protein levels. Although it is not possible to make rigorous comparisons of topoisomerase II activity in the crude extracts, we found that approximately 10 μg extract were able to decatenate 100 ng *Crithidia* mitochondrial DNA fully (Fig. 11). We certainly cannot exclude the possibility that there are 2–3-fold differences in topoisomerase II activity between the mutant proteins; indeed, Danks *et al.* (20) observed a difference in topoisomerase II activity between the wild-type and mutant CEM cell lines. In any case, the large differences in drug sensitivity we have

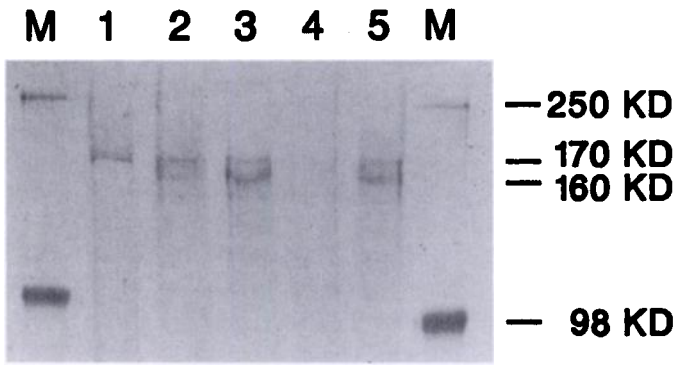

Fig. 10. Topoisomerase polypeptide levels in cells expressing wild-type or mutant human topoisomerase II. Western blot analysis of topoisomerase II protein was carried out using different strain JN394t2–4 human topoisomerase IIα alleles. Fifty μg of protein were loaded in each lane. The plasmids in the strains were pMJ1 (*Lane 1*), pMJ1Arg450Gln (*Lane 2*), pMJ1Pro803Ser (*Lane 3*), no plasmid (*Lane 4*), and pMJ1 Arg450Gln Pro803Ser (*Lane 5*). *M*, molecular weight markers.

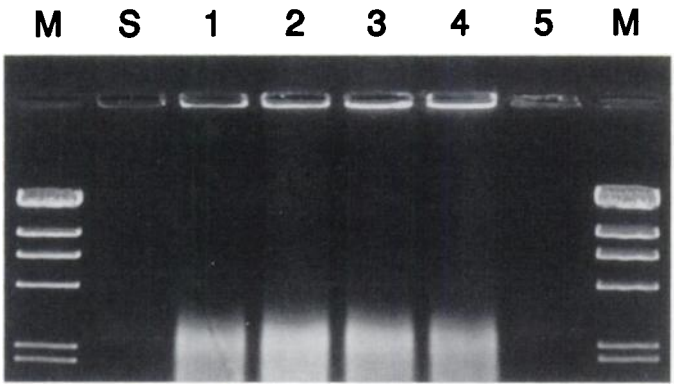

Fig. 11. Topoisomerase II activity of yeast cells expressing wild-type or mutant human topoisomerase IIα alleles. Topoisomerase II activity was assayed in cells carrying either pMJ1 or mutant derivatives. Activity was determined by decatenation of 100 ng *Crithidia* mitochondrial DNA. *Lane 1*, substrate DNA; *Lane 2*, pMJ1; *Lane 3*, pMJ1Arg450Gln; *Lane 4*, pMJ1Pro803Ser; *Lane 5*, pMJ1Arg450Gln and pMJ1Pro803Ser; and *Lane 6*, no plasmid. Topoisomerase II determinations were carried out at 37°C.

observed make it unlikely that the drug resistance is due solely to reduced catalytic activity.

DISCUSSION

We have demonstrated that the expression of human topoisomerase IIα in yeast is able to complement both the essential function of topoisomerase II as well as all other functions for which topoisomerases are required. The complementation of a Δ*top1-top2*^{ts} double-mutant yeast strain by human topoisomerase II is significantly poorer than by the yeast enzyme, which may reflect some specific interactions between topoisomerase II and other DNA metabolic proteins. Alternatively, the relatively poorer complementation may be simply due to less efficient import into the nucleus or a relatively lower stability in yeast. In any case, human topoisomerase IIα can perform all of the biological functions that are performed by yeast topoisomerase II.

A relatively large number of human cell lines have been isolated that show specific resistance to anti-topoisomerase II agents. In many cases, mutations have been identified in the h*TOP2α* gene. However, it has been quite difficult to demonstrate whether the observed mutation is actually responsible for drug resistance. Recently, we have provided strong evidence that the Arg450Gln mutation identified by Bugg *et al.* (19) can be mutated to produce a drug-resistant topoisomerase. We constructed a homologous mutation in yeast topoisomerase II and showed that expression of that mutation resulted in etoposide and amsacrine resistance (29). However, if we had observed no drug resistance in the yeast strain carrying the yeast mutant, we would have been unsure whether the lack of resistance was due to the unimportance of the tested mutation, or whether the differences between the yeast and human enzymes caused the lack of resistance. Expression of human topoisomerase IIα in yeast and an examination of forms of the enzyme that carry specific mutations eliminates the second possibility rigorously.

An understanding of the drug-resistant topoisomerase II in the teniposide-resistant cell lines was complicated by the identification of two mutations in the *TOP2α* gene. Our experiments show that both the Arg450Gln and Pro803Ser mutations probably contribute to the drug resistance of these cell lines. Our experimental evidence is formally analogous to the experiments demonstrating that overexpression of *MDR1* is sufficient to confer drug resistance (44). With *MDR1*, transfection of a plasmid that can confer high-level expression of (wild-type) *MDR1* is sufficient to confer drug resistance to cells that have never been exposed to drugs. In our experiments, a mutant h*TOP2* gene is expressed in yeast under conditions in which the endogenous (yeast) enzyme is inactive. Therefore, the human topoisomerase II must be active; otherwise, the cells are inviable. Furthermore, because we have demonstrated that expression of wild type human topoisomerase II confers drug sensitivity in yeast, a mutated h*TOP2* that is transformed into yeast and results in resistance to anti-topoisomerase II agents must be inherently drug resistant. However, these experiments do not exclude the possibility that other mechanisms of drug resistance are also present in the mammalian cell lines from which the mutations were first identified.

It is interesting to note that the expression of the double-mutant topoisomerase II conferred higher levels of resistance to etoposide and amsacrine than did the expression of either single mutation. It seems that the two mutations are able to act additively to produce a highly drug-resistant enzyme. Because the mutations are in separate domains of the protein (Arg450Gln in the *gyrB* homology domain, Pro803Ser in the *gyrA* homology domain), it will be interesting to determine whether mutations in the same domain can also cooperate to produce higher levels of drug resistance. It also should be emphasized that it has not been determined whether the two mutations actually reside on the same allele in the human VM-1 or VM1–5 cell lines.

The expression of human topoisomerase II should provide a very useful tool for analyzing mutations detected in the *TOP2α* gene from mammalian cell lines selected for resistance to topoisomerase II-targeting drugs. Presently, we are using the expression system to test other mutations that had been identified in human topoisomerase IIα. We are also introducing other mutations into the h*TOP2α* gene to demarcate domains important for drug interactions.

ACKNOWLEDGMENTS

We thank Jim Wang for the gift of the human TOP2 cDNA and for communicating results prior to publication. We also thank Dr. Mary Danks for the antibody to human topoisomerase II and Karin Sykes for comments on this manuscript.

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