Repair of topoisomerase I covalent complexes in the absence of the tyrosyl-DNA phosphodiesterase Tdp1

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Accidental or drug-induced interruption of the breakage and reunion cycle of eukaryotic topoisomerase I (Top1) yields complexes in which the active site tyrosine of the enzyme is covalently linked to the 3′ end of broken DNA. The enzyme tyrosyl-DNA phosphodiesterase (Tdp1) hydrolyzes this protein-DNA link and thus functions in the repair of covalent complexes, but genetic studies in yeast show that alternative pathways of repair exist. Here, we have evaluated candidate genes for enzymes that might act in parallel to Tdp1 so as to generate free ends of DNA. Despite finding that the yeast Apn1 protein has a Tdp1-like biochemical activity, genetic inactivation of all known yeast apurinic endonucleases does not increase the sensitivity of a *tdp1* mutant to direct induction of Top1 damage. In contrast, assays of growth in the presence of the Top1 poison camptothecin (CPT) indicate that the structure-specific nucleases dependent on *RAD1* and *MUS81* can contribute independently of *TDP1* to repair, presumably by cutting off a segment of DNA along with the topoisomerase. However, cells in which all three enzymes are genetically inactivated are not as sensitive to the lethal effects of CPT as are cells defective in double-strand break repair. We show that the *MRE11* gene is even more critical than the *RAD52* gene for double-strand break repair of CPT lesions, and comparison of an *mre11* mutant with a *tdp1 rad1 mus81* triple mutant demonstrates that other enzymes complementary to Tdp1 remain to be discovered.

Covalent complexes are intermediates in the catalytic cycle of topoisomerases that form when a tyrosine residue from the active site of the enzyme becomes linked to the backbone of DNA (1). Under normal circumstances, the covalent complex is transient, but a wide variety of circumstances lead to its stabilization, and the resulting long-lived disruption in the continuity of the DNA backbone can have dire consequences for genome stability (2). Because topoisomerases are abundant enzymes, covalent complexes must be common, and, given their potential for stabilization, their repair is likely to be an important part of DNA metabolism. That such repair occurs has long been inferred from the fact that mutants defective in DNA repair display greatly increased sensitivity to topoisomerase poisons (3, 4).

Repair of a topoisomerase lesion presents special problems because the strand break is encumbered with a covalently bound polypeptide, which must be removed to restore the continuity of the chromosome. Our laboratory has been interested in the mechanisms by which eukaryotic cells, exemplified by the budding yeast *Saccharomyces cerevisiae*, deal with covalent complexes of topoisomerase I (Top1). A defining characteristic of eukaryotic Top1 is the linkage of its active site tyrosine to the 3′ end of DNA (1). We have described an enzyme, tyrosyl-DNA phosphodiesterase (Tdp1), that can specifically hydrolyze this linkage (5). We further showed that genetic inactivation of this enzyme resulted in sensitization of yeast to Top1 damage (6, 7). Although this served to prove that Tdp1 was involved in repair of topoisomerase damage, the degree of sensitization was less than that produced by inactivation of the *RAD52* gene. The latter is a vital element for homology-dependent double-strand break repair, a process whose completion should rely on removal of topoisomerase lesions. The more modest deficit in repair of *tdp1* mutant cells vs. *rad52* mutant cells implies that, in addition to Tdp1, yeast has other ways to remove covalently bound Top1. In this article, we evaluate several plausible candidate enzymes for the extent to which each contributes to such repair.

Materials and Methods

Strains. All strains used in this work are derived from auxotrophic versions of *S. cerevisiae* strain S288C (8). In addition to the alleles described in ref. 7, we purchased a KanMX substitution of *MUS81* (Research Genetics, Huntsville, AL) and generated one-step disruptions of *APN1*, *APN2*, *MRE11*, *RAD1*, and *TOP1*. For *apn1* and *top1*, we used, respectively, plasmids in which the gene is disrupted by addition of a *URA3* cassette (9) or carries an 849-bp deletion and a *LEU2* cassette (10). For the other genes, as before (7) we used a PCR protocol to generate a complete deletion with either *LEU2* (for *apn2*) or *MET15* (for *rad1* and *mre11*) as the substituted selectable marker. To facilitate drug accumulation (11, 12), all strains also carried a KanMX substitution of the *ERG6* gene. Mutant combinations were prepared by sequential disruption or sporulation of diploids heterozygous for the desired alleles, followed by selection for appropriate markers and confirmation of the construct by PCR. Strains were grown as before (7) in yeast extract/peptone/dextrose (YPD), yeast extract/peptone/glucose, or complete synthetic medium (Qbiogene, Carlsbad, CA), appropriately supplemented with a carbon source and nutrients (13).

Growth and Testing of Cells. Cell killing by a toxic Top1 (14) was performed essentially as described (7). In brief, cells carrying an expression plasmid containing a mutant Top1 (p*top1*T722A) were grown to midlogarithmic phase with raffinose as the carbon source and induced by addition of galactose. At various times, aliquots were spread on plates containing 2% glucose. Survival was calculated as the number of colony-forming units divided by the number before induction; the average (±SEM) of 3–8 experiments is reported. Cell killing by camptothecin (CPT) was performed as described (6). Briefly, because cells with defective oxidative metabolism are resistant to many drugs (15), including CPT (C. A. Robertson and J.J.P., unpublished observations), cultures were first grown in yeast extract/peptone/glucose broth to suppress growth of ρ^- strains, then resuspended in YPD broth and grown for 2 h before addition of CPT. The drug (Sigma) was diluted to a final concentration of either 5 or 100 μg/ml from a stock (5 mg/ml in DMSO) that was stored at −20°C; where tested, these two concentrations gave equivalent results. At various times, samples were plated to YPD agar, and survival was calculated as above. For tests of growth in the presence of CPT, spots (3 μl) of 10^2-, 10^3-, and 10^4-fold serial dilutions of freshly prepared cultures (grown in YPD) were applied to the surface of YPD plates that were either drug-free or freshly made to contain a particular concentration of CPT. Plates were incubated at 30°C and photographed after 3 or 4 days. Cell killing by

Abbreviations: Tdp1, tyrosyl-DNA phosphodiesterase; Top1, topoisomerase I; AP, apurinic; CPT, camptothecin; PG, phosphoglycolate; YPD, yeast extract/peptone/dextrose.

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bleomycin was assessed by exposure of logarithmic-phase cells (at $OD_{650} = 0.4$) to various concentrations of the drug for 1 h, followed by dilution and spreading on YPD plates. For each bleomycin concentration, survival is calculated relative to a culture grown in parallel without drug.

Substrates and Enzyme Assays. Tyrosyl-DNA substrates were obtained, labeled, and annealed to form duplexes as described (7). Purified Apn1 (a gift of R. A. O. Bennett and B. Demple, Harvard School of Public Health, Boston) was assayed for hydrolysis of the tyrosyl-DNA phosphodiester exactly as described for purified Tdp1 (7), except that $MgCl_2$ was added to a final concentration of 7.5 mM. A 21-mer oligonucleotide that terminated in a 3′-phosphoglycolate (PG) was purchased (R & D Systems, Minneapolis) and labeled at the 5′-end as described (7). Because 3′-PG and 3′-phosphate oligonucleotides have very similar mobilities in denaturing gels (16), Tdp1 reactions were supplemented with 0.1 unit of T4 polynucleotide kinase (New England Biolabs). To accommodate the manufacturer's recommendations for polynucleotide kinase, the standard (12-μl) reaction mixture (7) was modified to contain 70 mM Tris·HCl at pH 7.6, 10 mM $MgCl_2$, and 5 mM DTT. The 3′-phosphatase activity of polynucleotide kinase served to convert the immediate product of Tdp1 hydrolysis into the more slowly migrating 3′-hydroxyl derivative (5).

Results and Discussion

Estimating the Maximal Capacity for Repair of Top1 Lesions. To evaluate the relative contributions of different pathways for the repair of topoisomerase damage, the ideal scale would be defined by the sensitivity of a WT strain versus one that is lacking in all capacity for such repair. In the past, we and others have used a *rad52* strain for this control. Here, we show that, for Top1 damage induced by the chemotherapeutic agent CPT, a strain deleted for *MRE11* is a better standard.

CPT is known to specifically block the religation step of the enzyme cycle (2). An *mre11* strain not only is very sensitive to the killing action of this drug, it is significantly more so than a *rad52* strain (Fig. 1*A*). Hypersensitivity of the *mre11* mutant to CPT can also be seen by comparing growth of *mre11* and *rad52* cultures on plates containing different concentrations of the drug (Fig. 1*C*). Although we chose to examine *MRE11* because the gene was known to be involved in double-strand break repair (17), the difference with *RAD52* was surprising and prompted us to perform two additional control experiments. First, we determined that no death of an *mre11* strain occurred when the cells were exposed just to the solvent used to dissolve the drug (data not shown); this result shows that it is CPT and not a toxic derivative of DMSO (18) that kills *mre11* cells. Second, we tested the CPT sensitivity of a *top1*-deleted derivative of the *mre11* strain; again, no cell death was seen (Fig. 1*A*). This observation eliminates the hypothesis that, in addition to Top1, CPT has a second target that generates potentially lethal lesions, and hypersensitivity is observed because repair of these lesions depends strongly on Mre11 but not Rad52. Before concluding that the two repair genes contribute differently to repair of Top1 damage, it should be noted that the *mre11* mutation we have used is a complete gene deletion (see *Materials and Methods*), whereas the *rad52* mutation is a simple gene disruption, achieved by insertion of a marker cassette (19). However, the possibility that the differential sensitivity to CPT merely reflects residual activity in the *rad52* allele is refuted by two observations: (*i*) in other assays for DNA repair, the *rad52* disruption allele is as defective as a complete deletion of the *RAD52* gene (data not shown); and (*ii*) the *rad52* disruption is more sensitive to some kinds of damage than the *mre11* knockout. The latter case is exemplified by the sensitivity of the two strains to induction of a mutant Top1, one that accumulates covalent complexes because it is intrinsically defective in ligation (14); here, the *rad52* disruption is more readily killed than the *mre11* deletion (Fig. 1*B*). Although differences in the way *RAD52* and *MRE11* contribute to other kinds of DNA repair are documented (17, 20), we do not know why one gene is more important for repair of toxic topoisomerase damage and the other for CPT damage. We do not even know whether all of the effects of an *mre11* mutation reflect the contribution of the Mre11–Rad50–Xrs2 complex (20) or whether the *MRE11* gene can sometimes make a separate contribution. [The latter possibility is suggested by the finding that *rad50* and *rad52* mutants have an identical sensitivity to CPT, albeit in assays different from the ones used here (21, 43).] Despite these uncertainties, the distinction between the effect of *mre11* and *rad52* for the repair of the two kinds of topoisomerase damage supports our previous inference (7) that the corresponding lesions differ in structure and/or in kinetics of production.

Compared with the degree of sensitization caused by *rad52*

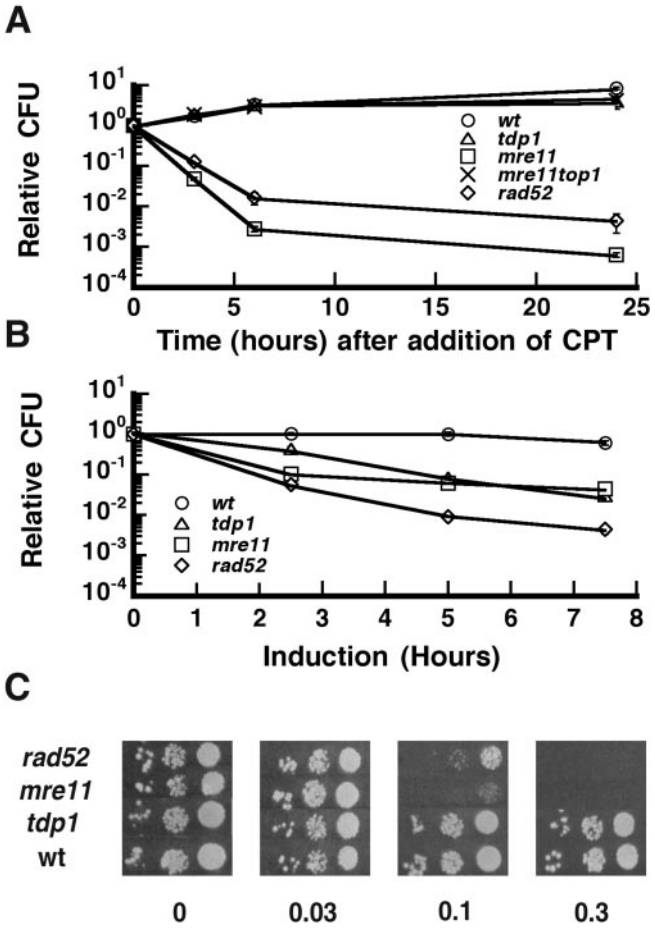

Fig. 1. Sensitivity to topoisomerase damage of WT yeast and mutants defective in global repair. (*A*) Cell death after exposure to CPT. Colony-forming ability of strains of the indicated genotype was assessed just before and after addition of CPT (5 μg/ml) to a midlogarithmic culture. CFU, colony-forming units; wt, wild type. (*B*) Cell death after induction of a toxic topoisomerase. Strains of the indicated genotype each contained a plasmid bearing a mutant form of Top1 downstream of a galactose-inducible promoter. Colony-forming ability was assessed just before and for several hours after addition of galactose to a midlogarithmic culture. (*C*) Growth in the presence of CPT. Spots of serial dilutions of saturated cultures of the indicated strains were applied to plates containing the indicated concentration (μg/ml) of CPT. Plates were incubated at 30°C for 4 days and photographed. In all panels, a *tdp1* deletion strain is included for comparison; in addition to the indicated genotype, all strains contained a null mutation of the *ERG6* gene.

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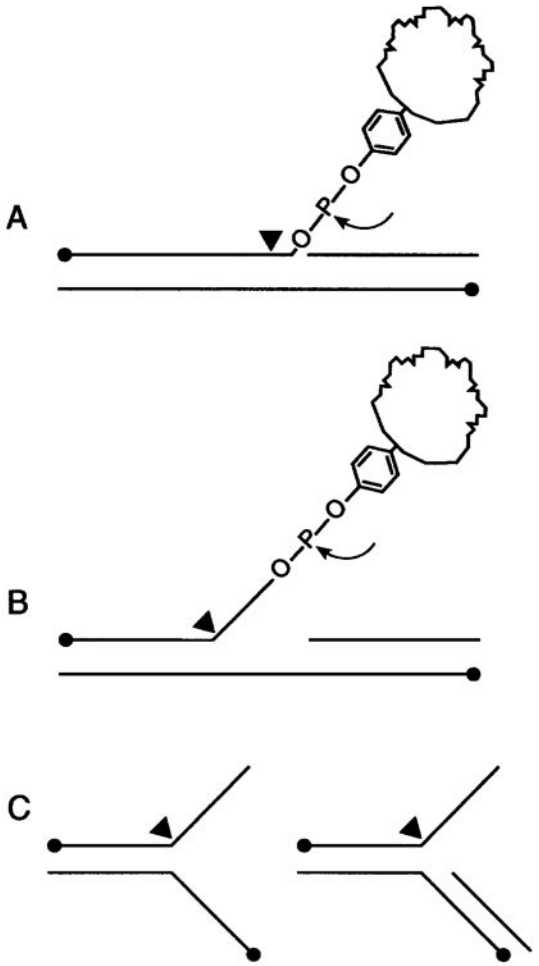

Fig. 2. Hypothetical avenues for repair of covalent complexes. (*A*) A duplex DNA (with 5′ ends marked by filled circles) bearing a covalent complex of Top1 (whose active site tyrosine emerges from an irregular proteinaceous blob). The protein moiety is shown as being removed either by hydrolysis of the tyrosyl-DNA phosphodiester (curved arrows) or by endonuclease action at phosphodiesters 5′ to this bond (arrowheads). (*B*) The DNA portion of the covalent complex has undergone conversion to a more open structure. This might happen after collision with a replication fork or an elongating transcription complex (2) or after the action of a helicase or an exonuclease. Sites of potential removal of the covalent complex are indicated as in *A*. (*C*) Branched DNA substrates for structure-specific nucleases. (*Left*) The preferred site of cleavage by Rad1/Rad10 on a "simple Y flap." (*Right*) The same for Mus81/Mms4 on a "duplex flap." The relationship between these artificial constructs and structures shown in *A* and *B* is suggestive but not proven.

and *mre11*, the effect of *tdp1* on lesion removal (Fig. 1 *A* and *B*) varies from minimal (for CPT damage) to moderate (for toxic topoisomerase damage). This finding implies that, for either kind of damage, specific hydrolysis of the tyrosine-DNA phosphodiester by Tdp1 must not be the only way to effect repair. *A priori*, DNA can be freed from covalently joined peptide in only one of two ways (Fig. 2 *A* and *B*). Either enzymes like Tdp1 can hydrolyze the tyrosyl-DNA bond or enzymes with nuclease activity can recognize some feature of the blocked 3′ end and cut off a segment of DNA together with the peptide residues. Concerning the latter mechanism, one should recall that the Mre11 protein contains a domain that can act as a nuclease at a 3′ end or 3′ branch (22). However, we question whether this nuclease function is critical for topoisomerase repair because we found that a deletion of the *SAE2* gene (23, 24) has only a small effect on sensitivity to CPT, either by itself or in combination with a *tdp1* mutation (data not shown). In a wide variety of tests, *sae2*-null mutants behave as if they were specifically depressed in Mre11 nuclease activity (25). In fact, a mutation that directly inactivates this domain (26) has little or no effect on CPT sensitivity (J. Nitiss, personal communication). Although it would be best to study this mutation in combination with a *tdp1* mutation (and mutations that inactivate some of the genes described below), we favor the idea that it is only one or more of the other demonstrated functions of Mre11 (20, 27) that contribute to Top1 repair. Accordingly, in the remainder of this paper we have proceeded to examine other genes known to encode enzymes that can act at or near the 3′ end of DNA for their contribution to repair of DNA encumbered with a Top1 covalent complex.

Apurinic (AP) Endonucleases. These enzymes are defined by their capacity to incise the phosphodiester backbone to the 5′ side of an abasic site (28). However, the catalytic activity of such enzymes is typically broad and often includes hydrolysis of residues from the 3′ terminus of DNA (29).

The principal AP endonuclease of *S. cerevisiae* is Apn1 (30). We obtained a sample of purified enzyme and incubated it with DNA substrates that model the covalent complex (Fig. 3*A*). The purified enzyme removed tyrosine efficiently from the 3′ end of a synthetic oligonucleotide in the context of a nicked duplex or tailed duplex substrate. However, it acted weakly on a tyrosyl phosphodiester at the end of a blunt duplex substrate and had no detectable activity when presented with a single strand. The substrate preference of Apn1 is therefore complementary to that of Tdp1, which disfavors cleaving tyrosyl-DNA phosphodiester bonds presented in the middle of a duplex (7). Experiments with crude yeast extracts argue against the possibility that the activity shown in Fig. 3*A* is an artifact of enzyme purification. When prepared from WT cells, such extracts have a substantial capacity to remove a 3′-tyrosine located at a nick in a duplex. This activity is unaffected by a *tdp1* mutation, as expected from the behavior of purified Tdp1 (7), but is greatly depressed in extracts of *apn1* cells. Indeed, extracts of an *apn1 tdp1* double mutant have no detectable capacity to hydrolyze a tyrosyl-DNA phosphodiester when presented in any of the contexts used in Fig. 3*A* (data not shown).

Does Apn1 contribute significantly to *in vivo* repair of topoisomerase damage? This issue calls for a genetic analysis, but there exists a potential complication. In addition to Apn1, *S. cerevisiae* contains a second enzyme with AP endonuclease activity, Apn2. Although Apn1 accounts for >90% of the AP endonuclease activity in yeast (31), the sensitivity of an *apn1 apn2* double mutant to agents that induce depurination is substantially greater than that of either single mutant (32, 33). This observation suggests that, despite differences in their abundance and/or catalytic efficiencies, the two enzymes function somewhat redundantly. We therefore evaluated their joint contribution to survival of topoisomerase damage.

Fig. 3*B* shows that the *apn1 apn2* double mutant has a modestly increased sensitivity to induction of the toxic topoisomerase. [Studies with *apn1* and *apn2* single mutants (not shown) indicate that neither gene alone accounts for this sensitivity.] However, an *apn1 apn2 tdp1* triple mutant was scarcely more sensitive than a *tdp1* single mutant (Fig. 3*B*); *tdp1* is thus epistatic over *apn1 apn2*. This finding argues against the interpretation that tyrosyl-DNA phosphodiesterase activity of AP endonucleases (as demonstrated in Fig. 3*A* for Apn1) contributes to repair of toxic Top1 damage. If it did, one would expect additivity rather than epistasis between *tdp1* and *apn1 apn2* mutations (34). Thus, we infer that the esterase activity shown in Fig. 3*A* is incidental, and it is some other activity of the AP endonucleases that works in the same pathway as does Tdp1. This is precisely the conclusion drawn by Vance and Wilson (35), who studied growth of yeast

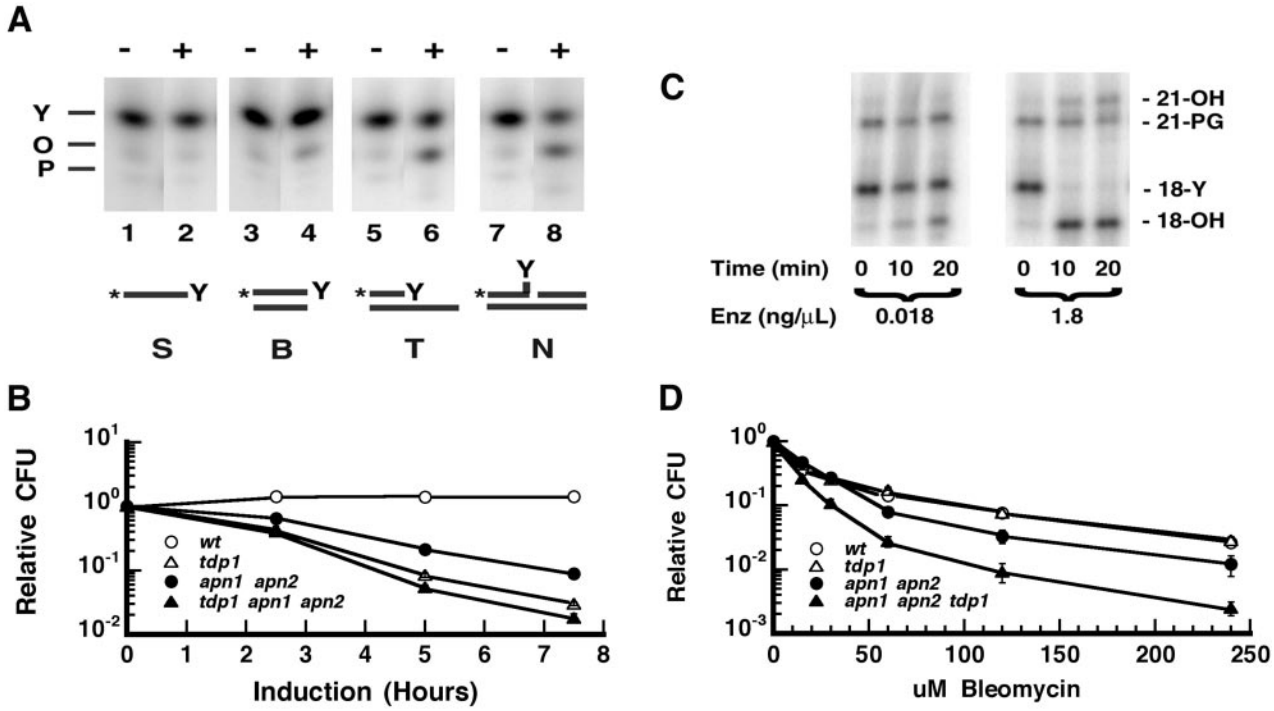

Fig. 3. AP endonucleases and Tdp1. (*A*) Cleavage of the tyrosyl-DNA phosphodiester bond by purified Apn1. An oligonucleotide substrate synthesized to terminate in a 3′-tyrosyl phosphodiester was either used as a single-strand substrate (S) or annealed with conventional oligonucleotides as indicated to produce a blunt duplex (B), a duplex with a 5′-tail (T), or a nicked duplex (N). These were incubated as described in *Materials and Methods* with (+) or without (−) purified Apn1 and electrophoresed in a denaturing gel. The positions of the substrate oligonucleotide (Y) and the products terminated by a 3′-phosphate (P) and 3′-hydroxyl (O) are marked. Under our conditions, hydrolysis is largely limited to the terminal phosphodiester; at lower ionic strengths, we observed some exonucleolytic degradation from the 3′ end, as has been recently described for Apn1 action on conventional oligonucleotides (35). (*B*) Cell death after induction of a toxic topoisomerase. The protocol of Fig. 1*B* was applied to strains of the indicated genotype. (*C*) Relative activity of Tdp1 on 3′-tyrosine and 3′-PG substrates. Yeast Tdp1, purified as described (6) and included at the indicated concentrations, was incubated with a mixture containing roughly equimolar amounts of an 18-mer synthetic tyrosine oligonucleotide and a 21-mer synthetic PG oligonucleotide. Reaction mixtures, supplemented with T4 polynucleotide kinase, were assembled as described in *Materials and Methods*, incubated for the indicated time at 30°C, and electrophoresed on a denaturing polyacrylamide gel. The positions of the substrates (18-Y and 21-PG) and the dephosphorylated products (18-OH and 21-OH) are marked. (*D*) Cell death after exposure to bleomycin. Cultures were incubated with the indicated concentration of bleomycin for 1 h and then diluted and plated. Survival is calculated relative to the number of colonies obtained after 1 h without drug.

in the presence of CPT and inferred that the 3′-phosphatase of the AP endonucleases (working in parallel to the 3′-phosphatase of Tpp1) was the important activity, one that presumably functions subsequent to the action of Tdp1 (5). Our finding that an *apn1 apn2 tdp1* triple mutant is scarcely killed by a 24-h exposure to CPT (data not shown) also implies a lack of additivity and thus provides further support for the idea that AP endonucleases do not function in parallel to Tdp1.

Despite our results concerning the repair of topoisomerase damage, we have considered whether AP endonucleases and Tdp1 might play complementary roles in another kind of repair. Recently, it has been demonstrated that, like Apn1 and Apn2 (30, 36), purified Tdp1 can hydrolyze a PG residue from the 3′ end of DNA (16). In our hands, compared with its hydrolysis of a 3′-terminal tyrosine, yTdp1 removes PG very inefficiently. For example, when the two kinds of oligonucleotides were mixed, the one terminating in a 3′-PG required at least a 50-fold higher concentration of enzyme to achieve a comparable initial rate (Fig. 3*C*). This finding suggests that Tdp1 may be of limited value in repairing oxidative damage. Indeed, survival of damage induced by bleomycin, an agent that frequently generates breaks that terminate in a 3′-PG (37), is unaffected by genetic inactivation of *TDP1* (Fig. 3*D*). However, when *APN1* and *APN2* have been inactivated, some of the residual repair of bleomycin damage depends on *TDP1* (Fig. 3*D*). We do not know whether this reflects Tdp1 hydrolysis of 3′-PG or whether some of the single-strand breaks introduced by bleomycin (37) trap topoisomerase complexes (38) and thereby sensitize the cell to loss of *TDP1* function. In this context, it would be of interest to see how much of the bleomycin sensitivity of an *apn1 apn2 tpp1* strain (35) reflects phosphatase action downstream of Tdp1. In any case, under conditions where Top1 damage is the unquestioned cause of cell death, our results with AP endonucleases force us to look elsewhere for enzymes that can repair the covalent complex.

***RAD1* and *MUS81*.** Each of these closely related genes encodes a subunit of a nuclease that displays a preference for attacking flapped or branched DNA (Fig. 2*C*). There are subtle differences in biochemical activity that distinguish the two yeast enzymes. The *RAD1* gene product, together with a partner subunit encoded by the *RAD10* gene, has a preference for "simple Y flap" structures, whereas the *MUS81* gene product, together with a partner subunit encoded by the *MMS4* gene, has a preference for cutting "duplex flap" structures (39, 40). In addition to differences between the two yeast enzymes, there are also reported differences in substrate preference between the enzyme from yeast (especially Mus81/Mms4) and the orthologs purified from *Schizosaccharomyces pombe* and *Homo sapiens* (41). Nevertheless, the common feature of these enzymes, action at a distorted 3′-boundary structure, makes *RAD1* and *MUS81* reasonable candidates for genes that might contribute to repair of a DNA with a topoisomerase covalently joined to its 3′ end.

Spot tests of growth on plates doped with CPT constitute a particularly sensitive test for such a contribution. This is because,

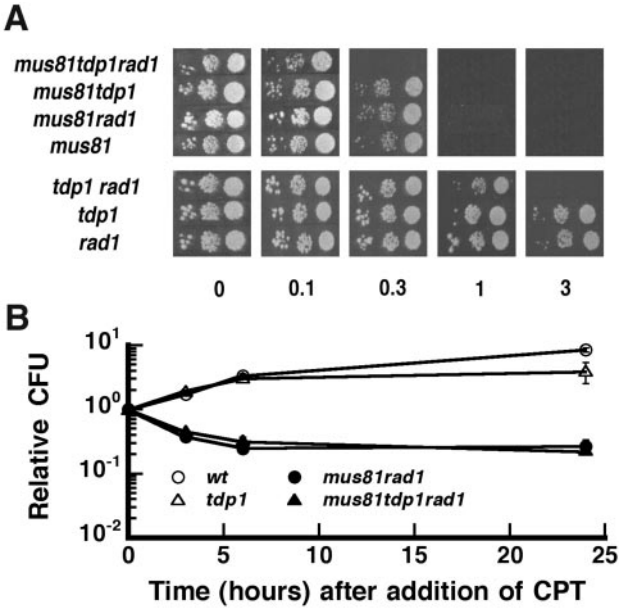

Fig. 4. Sensitivity to topoisomerase damage of yeast mutated in genes for structure-specific nucleases. (*A*) Growth in the presence of CPT. The indicated strains were grown on plates containing various concentrations of CPT as in Fig. 1*C*. (*B*) Cell death after exposure to CPT. Assays on the indicated strains were carried out and analyzed as in Fig. 1*A*. CFU, colony-forming unit.

in contrast to clonogenic assays in which the drug is present for a fixed interval and repair can follow at leisure, in spot tests the drug is continuously present and lesions must be repaired as fast as they are introduced, or checkpoint mechanisms (42) will arrest the cell cycle and consequently stop growth of the spot. Even with this sensitive assay, a *rad1* mutant cannot be distinguished from a WT strain, being able to grow at the highest drug concentration tested (Fig. 4*A*). This observation is consistent with a previous study that found no effect of a *rad1* mutation on the concentration of CPT needed to suppress growth of yeast in liquid culture (43). However, although a *tdp1* mutant can also grow at 3 μg/ml, when a *rad1* mutation is combined with a *tdp1* mutation, the double mutant is unable to grow (Fig. 4*A*). The synergy between the mutations thus implies that their respective genes contribute in nonoverlapping ways to repair of topoisomerase damage, but the effect is small. The double mutant grows well in the presence of 1 μg/ml CPT, whereas an *mre11* mutant is sensitive under conditions in which many fewer lesions are introduced per unit time, i.e., at 10-fold lower concentrations of the drug (Fig. 1*C*). In comparison with the elimination of *RAD1* function, deletion of the *MUS81* gene has a larger impact. The single mutant fails to grow in the presence of 1 μg/ml CPT (Fig. 4*A*). And, whereas double mutant combinations are not more sensitive, a *mus81 rad1 tdp1* triple mutant fails to grow at 0.3 μg/ml CPT (Fig. 4*A*). The synergy implies that all three genes contribute to different pathways for the repair of CPT damage.

Despite the impressive appearance of the spot tests, it would be wrong to conclude that the combination of mutations in *MUS81*, *RAD1*, and *TDP1* eliminates the vast majority of topoisomerase repair capacity from a yeast cell. With assays that measure survival of clonogenic potential after a fixed time of exposure to a topoisomerase-damaging agent, there is at best a minor effect of inactivating these structure-specific nucleases. For example, when tested in such a clonogenic assay a *mus81 rad1* double mutant is only slightly sensitive to CPT (Fig. 4*B*), an effect that is almost entirely accounted for by the *mus81* mutation alone (data not shown). Moreover, in the survival assay, there is no synergy between a *tdp1* mutation and any combination of *rad1* and *mus81* mutations (Fig. 4*B* and data not shown). Indeed, compared with CPT killing of an *mre11* strain (Fig. 1*A*), the sensitivity of the triple mutant (Fig. 4*B*) is marginal. Similarly, the sensitivity of a *tdp1* strain to induction of a toxic topoisomerase (Fig. 1*B*) is not enhanced by the joint inactivation of *RAD1* and *MUS81* (data not shown).

In summary, the data of Fig. 4*A* indicate that the three enzymes we have evaluated genetically (Tdp1, Rad1, and Mus81) make a substantial contribution to the repair capacity of a yeast cell. While this work was being completed, we learned that T. E. Wilson and his colleagues had come to the same conclusion (21). However, even in cells in which all three genes have been inactivated, assays of cell killing (Fig. 4*B*) reveal that there must be additional pathways of repair that can undo topoisomerase damage, albeit slowly, and thereby restore clonogenic capacity once the generation of lesions is terminated.

Perspective. Taken together with our previous work, the data in this paper lead us to conclude that multiple pathways of repair of topoisomerase damage function in parallel with the Tdp1-dependent pathway. We have shown that some of these alternative pathways depend significantly on the function of *RAD9* (6, 7), a gene that is likely to act high in the cascade of the damage response (42). In contrast, the genes we identify herein as contributing in parallel to *TDP1* (*MUS81* and *RAD1*) are likely to be direct players in the enzymatic removal of covalent complexes from DNA. However, it remains to be determined whether these genes function to remove covalent complexes from simple duplexes or, like Tdp1 (7), from complexes that have been converted into double-strand breaks. It must be emphasized that the list of genetic pathways is still incomplete; the sensitivity to topoisomerase damage of a *mus81 rad1 tdp1* strain is well below that which is achieved by inactivation of global repair genes. Given the multiplicity of potential repair pathways, it will be difficult to assess their relative importance by studying cell growth and clonogenic potential in mutant lines. This is because removal of one set of repair pathways may merely increase the flux of damage through pathways that play only a minor role in a WT cell. Following the kinetics of lesion removal and/or determining the structures generated during processing may be required. Such studies may also serve as guideposts for the evaluation of the way Top1 damage is repaired in metazoans. The enzymatic players we have identified in yeast (Tdp1, Mus81, and Rad1) all have orthologs in higher eukaryotes. Determining the extent to which a particular cell type expresses each of these genes might predict the chemotherapeutic efficacy of Top1 poisons against different types of cancer (44). Such a determination might also explain why, in patients that inherit a defective *TDP1* gene, certain nondividing cells are particularly sensitive to spontaneous DNA damage (45).

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