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 19. A modified version of the stereological optical disector method [M. J. West, L. Slomianka, H. J. Gundersen, *Anat. Rec.* **231**, 482 (1991)] was performed on peroxidase-stained tissue on coded slides. For every 20th section through the principal sulcus, the number of labeled cells in both banks of the sulcus was determined using an Olympus BX-60 OptiPlex computer. Labeled cells were counted excluding those in the outermost focal plane to avoid counting cell caps. The total volume of the principal sulcus area was estimated with Stereoinvestigator (MicroBrightField). The data were expressed as number of BrdU-labeled cells/mm³. Immunofluorescent tissue was viewed with an Olympus BX-60 fluorescent microscope and with a confocal laser scanning microscope (Zeiss 510 LSM) for verification of double labeling. Z-sectioning was performed at 1- μ m intervals, and optical stacks of three to six images were produced for figures.
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Yeast Gene for a Tyr-DNA Phosphodiesterase that Repairs Topoisomerase I Complexes

Jeffrey J. Pouliot, Kevin C. Yao, Carol A. Robertson, Howard A. Nash*

Covalent intermediates between topoisomerase I and DNA can become dead-end complexes that lead to cell death. Here, the isolation of the gene for an enzyme that can hydrolyze the bond between this protein and DNA is described. Enzyme-defective mutants of yeast are hypersensitive to treatments that increase the amount of covalent complexes, indicative of enzyme involvement in repair. The gene is conserved in eukaryotes and identifies a family of enzymes that has not been previously recognized. The presence of this gene in humans may have implications for the effectiveness of topoisomerase I poisons, such as the camptothecins, in chemotherapy.

Topoisomerases are cellular enzymes that are crucial for replication and readout of the genome; they work by breaking the DNA back-

bone, allowing or encouraging topological change, and resealing the break (1). The enzymes are efficient because DNA breakage is accompanied by covalent union between protein and DNA to create an intermediate that is resolved during the resealing step. This mechanism, although elegant, also makes topoisomerases potentially dangerous. If the resealing step fails, a normally transient break

Laboratory of Molecular Biology, National Institute of Mental Health, Building 36, Room 1B08, Bethesda, MD 20892-4034, USA.

*To whom correspondence should be addressed. E-mail: nash@codon.nih.gov

REPORTS

in DNA becomes a long-lived disruption, one with a topoisomerase protein covalently joined to it. Unless a way is found to restore the

continuity of DNA, the cell will die.

In virtually all topoisomerases, the heart of the covalent complex is a phosphodiester between a specific tyrosine residue of the enzyme and one end of the break (the 3' end for eukaryotic topoisomerase I and the 5' end for topoisomerases II and III). The high-energy nature of this bond normally ensures the resealing step. But failure of resealing is markedly increased by several drugs, such as camptothecin (CPT), a promising anticancer agent that specifically targets eukaryotic topoisomerase I (2). Protein-linked breaks also accumulate when topoisomerases act on DNA containing structural lesions such as thymine dimers, abasic sites, and mismatched base pairs (3). To the extent that such lesions arise during the normal life of a cell, topoisomerase-associated damage may be unavoidable.

Repair of topoisomerase covalent complexes is of obvious value to the cell, but the subject remains largely unexplored. A plausible pathway invokes hydrolysis of the bond joining the topoisomerase to DNA; release of the topoisomerase would then permit the cleaved DNA to undergo conventional modes of break repair (4). Although no such hydrolysis has been reported for covalent complex-

es of topoisomerases II or III, we described (5) an activity that specifically hydrolyzes the type of bond found in complexes between DNA and topoisomerase I (Fig. 1A). The specificity of this activity and its conservation from yeast to man suggested that it might be part of a repair pathway. But without specific inhibitors or mutants, no assessment of its function could be made. We now report the identification of the gene encoding this enzyme and the demonstration of its importance for topoisomerase metabolism.

Crude extracts of the yeast *Saccharomyces cerevisiae* contain readily detectable amounts of tyrosyl-DNA phosphodiesterase (TDP) activity (5). We disrupted (6) four yeast genes—*RAD9*, *RAD17*, *RAD52*, and *TOP1*—that we suspected might encode or control the activity, but none of the disruptions affected activity in extracts (Fig. 1B) (7). To search for previously unknown genes, we assayed extracts from colonies of chemically mutagenized yeast (8); this screen yielded a single strain, KYY337, with very low TDP activity (Fig. 1B). In back crosses to the parental line, the enzyme defect appeared to reflect a single mutation (denoted here as *enz*). That is, when a diploid between the parental line and a defective line was sporulated and haploid colonies were assayed at random (8), about equal numbers were found with normal and with low enzyme activity. The activity of representative colonies after four rounds of back crossing is shown in Fig. 1B.

To assess the role of TDP activity in repair of topoisomerase damage, we compared strains for sensitivity to killing by CPT (9). Despite the marked difference in TDP activity, the parental line and the backcrossed *enz* mutant were both insensitive to CPT (Fig. 2A, bars 1 and 2). We reasoned that, as for other kinds of damage (4), repair of topoisomerase lesions might take place by multiple pathways. If so, a genetic background in which some of these pathways were disabled might reveal a role for TDP activity. Indeed, when combined with a dis-

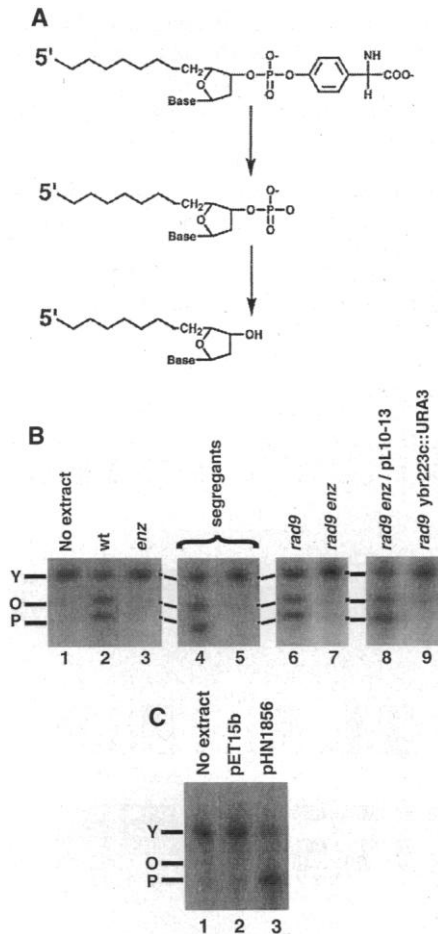


Fig. 1. Molecular genetics of tyrosyl-DNA phosphodiesterase (TDP) activity. (A) Enzymatic transformations. The jagged line represents the single-strand 18-mer oligonucleotide of oHN279Y. TDP activity removes the tyrosine from this chemically synthesized substrate (5) and leaves a 3'-terminal phosphate. In crude extracts, subsequent action by unidentified phosphatases can produce a 3'-terminal hydroxyl. (B) Denaturing gel analysis of TDP activity in yeast strains. Incubations with 5'-radiolabeled oHN279Y were for 12 min as described (5) with buffer (lane 1) or extract (150 μ g/ml) from the following strains: HNY102 and KYY337 (lanes 2 and 3); E17 and E6, two haploid segregants derived from KYY337 after four rounds of back crossing (lanes 4 and 5); HNY243 and HNY244, *rad9::hisG* derivatives of HNY102 and E6 (lanes 6 and 7); HNY244 containing plasmid pL10-13 (lane 8); and HNY383, a derivative of HNY243 with a disruption of the gene for ORF YBR223c (lane 9). The positions of the labeled substrate (Y) and oligonucleotides terminated by phosphate (P) and hydroxyl (O) residues are marked. Total TDP activity is best judged as the ratio $P + O/Y + P + O$. (C) TDP activity in *E. coli*. Radiolabeled oHN279Y was incubated as above with buffer (lane 1) or sonic extracts (10 ng/ml) of strain BL21(DE3) transformed either with plasmid vector (lane 2) or vector plus the coding region of YBR223c (lane 3). wt, wild type.

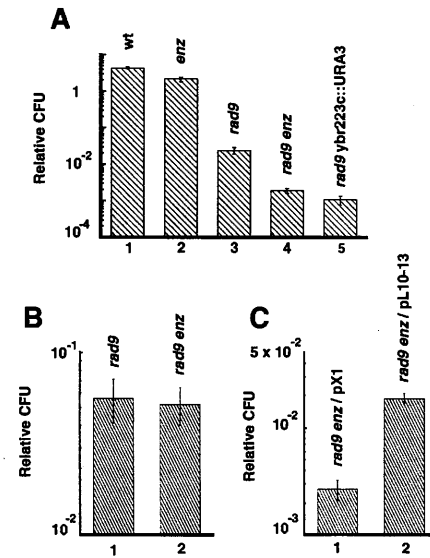


Fig. 2. Influence of TDP activity on cell survival after drug treatment. The indicated yeast strains were exposed to drug for 24 hours, diluted, and plated (9). Killing by the drug is calculated from the relative change in colony-forming units (CFUs), the number of colonies obtained from a portion of the culture after drug treatment divided by the number in a portion of the starting culture. (A) CPT was added at 100 μ g/ml to strains HNY102, E6, HNY243, HNY244, and HNY383 (bars 1 to 5). (B) MMS was added at 0.01% to strains HNY243 and HNY244 (bars 1 and 2). (C) CPT was added at 100 μ g/ml to strain HNY244 containing either a control plasmid, pX1, or plasmid pL10-13 (bars 1 and 2).

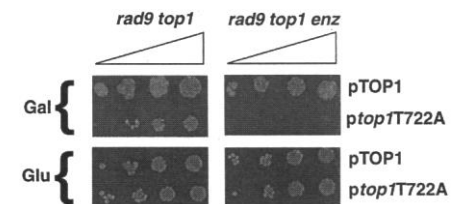


Fig. 3. Cell growth with a toxic topoisomerase. Strains HNY243 *top1* Δ and HNY244 *top1* Δ were transformed with derivatives of plasmid YCpGAL1 bearing either a wild-type *TOP1* gene or the Thr⁷²² \rightarrow Ala (T722A) mutant (17). These strains were serially diluted and spotted on uracil-deficient minimal plates containing either 2% glucose (Glu) or galactose (Gal) to repress or induce the plasmid-borne gene.

REPORTS

ruption of the *RAD9* gene, the CPT sensitivity of the low activity mutant (strain HNY244), was increased by a factor of 12 relative to the *rad9* derivative of the parental strain, HNY243 (Fig. 2A, bars 3 and 4); the same difference was seen after the mutant had undergone two additional rounds of back crossing (7).

The *RAD9* gene is needed both for the operation of DNA damage checkpoints and for expression of a set of DNA damage-inducible genes (10). The loss of these functions in a *rad9* mutant not only increases the sensitivity of the cell to killing by CPT, it apparently leaves TDP activity as a principal remaining source of repair of CPT-induced

damage. Under these circumstances, killing by CPT still reflects topoisomerase trapping; when the *TOP1* gene of HNY244 was disrupted, survival increased nearly 1000-fold (7). The mutant line was not sensitized to all sources of DNA damage; killing by methyl methane sulfonate (MMS), an alkylating agent, was indistinguishable in HNY243 and HNY244 (Fig. 2B).

Mutations in yeast topoisomerase I have been isolated that depress rejoining and thereby lead to accumulation of covalent complexes (11). We used these mutants for an independent test, one without recourse to drugs and the attendant questions concerning uptake, of the importance of TDP

activity for in vivo repair of topoisomerase-DNA adducts. Indeed, overexpression of a mutant (but not the wild-type) *TOP1* gene was more toxic to the strain with low TDP activity than to its control (Fig. 3). A second mutant, *top1R517G*, with a similar defect (11), was similarly hypertoxic in the strain with low TDP activity (7).

From a library of yeast genomic fragments screened (12) for the ability to improve the CPT resistance of HNY244 and restore its TDP activity, we obtained plasmid pL10-13 (Figs. 1B and 2C). Several subclones of the ~8-kb insert in this plasmid retained full activity (7). The smallest of these subclones contains a single open

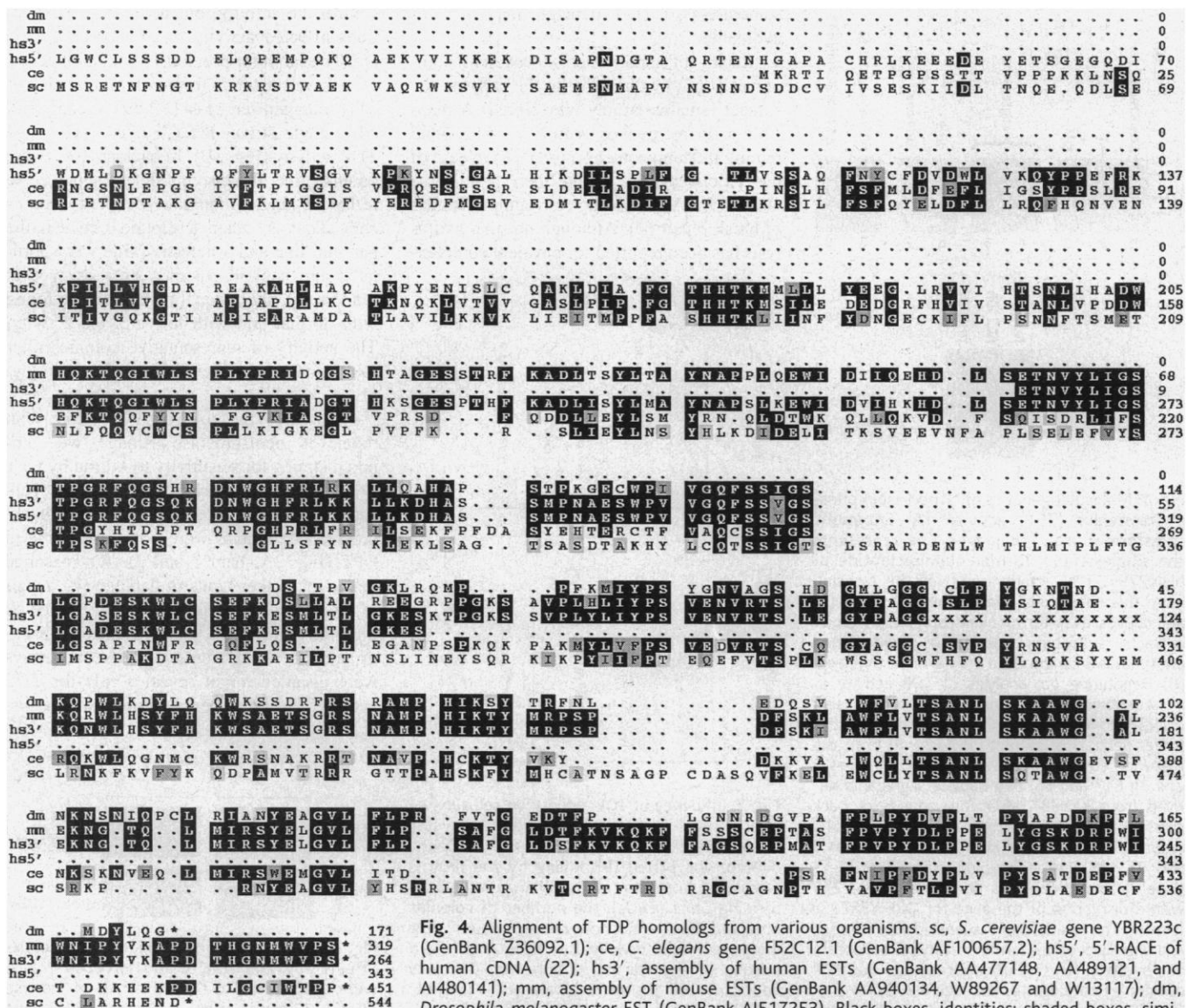


Fig. 4. Alignment of TDP homologs from various organisms. sc, *S. cerevisiae* gene YBR223c (GenBank Z36092.1); ce, *C. elegans* gene F52C12.1 (GenBank AF100657.2); hs5', 5'-RACE of human cDNA (22); hs3', assembly of human ESTs (GenBank AA477148, AA489121, and A1480141); mm, assembly of mouse ESTs (GenBank AA940134, W89267 and W13117); dm, *Drosophila melanogaster* EST (GenBank A1517253). Black boxes, identities; shaded boxes, similarities. A region of uncertain sequence in GenBank entry AA489121 is marked by x residues.

Together with the 5'-RACE, the product of a 3'-RACE (22) confirms the sequence of the human ESTs and shows that the sequence in the region of ambiguity is identical to that shown for the mouse protein. The product of the 5'-RACE extends for 79 amino acids upstream of the sequence shown but still may not include the start of the full-length human protein. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

reading frame (ORF), YBR223c, a protein of 544 amino acids and relative molecular mass ~ 62,000. Into strain HNY243, we generated by polymerase chain reaction (PCR) (13) a disruption that removed all but the first 32 amino acids of the ORF. The resulting strain had an enzymatic defect and CPT sensitivity very similar to that of HNY244 (Figs. 1B and 2A), indicating that YBR223c is involved in TDP activity. To distinguish whether YBR223c encodes or controls TDP activity, we introduced a histidine-tagged version (14) into *Escherichia coli*, which by itself has no detectable TDP activity. Induction of bacteria bearing this construct (but not a control construct) apparently resulted in massive overproduction of TDP because crude extracts of such cells had a specific activity >10,000 times as high as that of extracts from a standard yeast strain (Fig. 1, B and C). Moreover, most of the induced activity could be bound to a tag-specific column; specific elution released >75% of the bound activity, resulting in a fraction with a single Coomassie-stainable band of the expected molecular size (7). We conclude that YBR223c encodes the enzyme and have accordingly renamed its gene *TDP1*.

Database searches failed to reveal homology between *TDP1* and any genes of known function. Even individualized comparisons to motifs identified in various phosphodiesterases and phosphatases were, at best, marginal. On this basis, we conclude that *TDP1* encodes a previously unknown enzyme. However, eukaryotic (but not prokaryotic) databases contain several unannotated sequences that match *TDP1*, a finding consistent with the distribution of enzyme activity (5). The complete genome sequence of the nematode *Caenorhabditis elegans* contains a single ORF with substantial similarity to *TDP1*. Probing expressed sequence tag (EST) databases with the yeast and nematode proteins revealed many unambiguous matches (Fig. 4). In mouse and man, there are several EST entries that can be aligned to make up a single ORF with substantial similarity to the carboxyl-terminal half of *TDP1*. To see if the homology extends further, we carried out a PCR on a collection of human cDNAs (Marathon-Ready; Clontech Laboratories, Palo Alto, CA) with a primer complementary to the human EST sequence and a primer complementary to the tag affixed to the 5' end of the cDNAs. We cloned the resulting 5'-RACE (rapid amplification of cDNA ends) products; the sequence of one of the longest clones (Fig. 4) aligns well to most of the 5' half of the yeast and nematode ORFs. We conclude that the *TDP1* gene is highly conserved in eukaryotes.

Isolation of the *TDP1* gene will allow

studies of the enzymology and cell biology of a kind of DNA repair that has previously been hard to analyze. The gene also provides a potential tool to improve chemotherapy with camptothecins and other topoisomerase I poisons. Although these are promising anticancer drugs, their value is often limited by resistance of tumor cells or sensitivity of nontumor cells (or both). Repair of the topoisomerase lesion is likely to be one of the factors that determine the level of cellular sensitivity to topoisomerase poisons (15). With the *TDP1* gene in hand, one can readily assess the expression of this enzyme in individual patients and possibly predict the likelihood of therapeutic success. Moreover, if genetic or biochemical techniques can be used to alter enzyme activity, the efficacy and safety of the drugs may be improved.

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8. A *MATa leu2 ura3 ise1* strain, designated here as HNY102, was obtained from J. Nitiss. The *ise1* mutation inactivates the *ERG6* gene and thereby renders the strain permeable to CPT (17). HNY102 was treated with ethylmethane sulfonate, and colonies were chosen that showed poor growth when replicated to YPD plates (containing yeast extract, peptone, and dextrose) doped with CPT (3 µg/ml; Sigma). Midlogarithmic cultures of ~60 candidates were extracted and assayed for removal of the tyrosine moiety from oligonucleotide oHN279Y (5). A single colony was found that reproducibly yielded very low TDP activity. The mutant line, KYY337, was back crossed to HNY115, a *MATa* derivative of HNY102, made by transient introduction of a GAL-HO plasmid (18). Haploid segregants from the resulting diploid were picked at random and assayed as above; one low-activity segregant was used for a subsequent back cross. After four such rounds, hypersensitivity to growth on CPT-containing plates was lost, as was the hypersensitivity to cycloheximide and slow growth on YPD that also characterized KYY337. These phenotypes presumably reflected adventitious mutations that were unlinked to the one, denoted *enz*, causing low TDP activity. The *enz* mutation does not affect phosphatase activity. This is evidenced by similar amounts of hydrolysis of terminal processing when extracts of HNY102, KYY337, and the back-crossed derivatives are presented with a substrate oligonucleotide synthesized to have a 3'-phosphate (7). Standard protocols were used for yeast growth, mutagenesis, mating, and sporulation (19).
9. Because preliminary experiments showed that mitochondrial status influences killing by CPT (7), cells were grown to near saturation in medium

with glycerol in place of dextrose (YPG) to ensure a starting population with few or no petite derivatives (19). These cells were resuspended at optical density at 650 nm (OD_{650}) = 0.4 in YPD, grown for 2 hours, and diluted again in YPD to OD_{650} = 0.4. Drug was then added, and samples were withdrawn immediately and after 24 hours at 30°C. After dilution and plating on YPD, surviving colonies were counted after 3 to 4 days of growth. When plasmid-containing strains were assessed for CPT sensitivity, YPD was replaced throughout by uracil-deficient minimal medium (19) to ensure plasmid retention.

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22. The 5'- and 3'-RACE products of the human *TDP1* homolog have been deposited as GenBank AF182002 and AF182003, respectively.
23. We thank M. Lichten for advice about yeast manipulations, S.-w. Yang for early TDP assays, A. Hinnebusch for the yeast genomic library, and L. Rasmussen and R. Sugarek for DNA sequencing. K.C.Y. was supported by the Research Scholars Program of the Howard Hughes Medical Institute.

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