Mechanisms of DNA Topoisomerase I- Induced Cell Killing in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT: DNA topoisomerase I (Top1) catalyzes the relaxation of supercoiled DNA by a mechanism of transient DNA strand cleavage characterized by the formation of a phosphotyrosyl bond between the DNA end and active site tyrosine. Camptothecin reversibly stabilizes the covalent enzyme-DNA intermediate by inhibiting DNA religation. During S-phase, collisions with advancing replication forks convert these complexes into potentially lethal lesions. To define the DNA damage induced by alterations in Top1p catalysis and the cellular processes that mediate the repair of such lesions, the yeast *Saccharomyces cerevisiae* was used. Substitution of conserved residues N-terminal to the active site tyrosine (Tyr-727) produced alterations in the camptothecin sensitivity or catalytic cycle of DNA Top1. For example, substituting Ala for Thr-722 in Top1T722A increased the stability of the covalent enzyme DNA intermediate. As with camptothecin, Top1T722A-induced cytotoxicity was ascribed to a reduction in DNA religation. By contrast, enhanced covalent complex formation by Top1N726H resulted from a relative increase in the rate of DNA cleavage. Conditional yeast mutants were also selected that exhibit temperature-sensitive growth only in the presence of the self-poisoning Top1T722A enzyme. Subsequent analyses of these *tah* mutants identified 9 genes whose function suppresses the cytotoxic action of camptothecin and Top1T722A. These include genes encoding essential DNA replication proteins (*CDC45* and *DPB11*) and proteins involved in SUMO- or ubiquitination (*UBC9* and *DOA4*).

INTRODUCTION

Eukaryotic DNA topoisomerase I (Top1) catalyzes changes in DNA topology and plays an important role in cellular processes involving DNA, including replication, recombination, transcription, and chromosome condensation.[1–3] This monomeric enzyme is encoded by the *TOP1* gene and is highly conserved in terms of amino acid sequence, reaction mechanism, and sensitivity to anticancer agents, such as camptothecin (CPT).[1,2,4,5] Topoisomerase I binds duplex DNA and transiently cleaves a single DNA strand.[3,6,7] A phosphodiester bond in the DNA undergoes nucleophilic attack by the active tyrosine to generate a phosphotyrosyl linkage between the enzyme and the 3′ phosphate of the nicked DNA. The noncovalently bound end of

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DNA presumably rotates around the intact, nonscissile strand to affect changes in the linkage of the DNA strands. The formation of the covalent enzyme-DNA intermediate conserves the energy of the phosphodiester bond, so that religation of the nicked DNA via a second transesterification reaction does not require ATP.

Camptothecin interferes with the catalytic cycle of DNA Top1 by reversibly stabilizing the covalent enzyme-DNA intermediate.[1,4,5] However, the formation of this ternary drug–enzyme–DNA complex is insufficient to cause cell death. Rather, the collision of advancing replication forks with these drug-stabilized intermediates appears to produce the cytotoxic DNA lesions that signal cell cycle arrest and cause cell death. Such a model is further supported by numerous studies establishing the S-phase specificity of CPTs at pharmacologically relevant doses and the ability of replication inhibitors, such as aphidicolin, to suppress CPT cytotoxicity.[4,8–11]

In contrast to *Drosophila* and mouse, the *TOP1* gene in the budding yeast *Saccharomyces cerevisiae* is nonessential.[12–15] Genetic studies have established that other gene products, such as DNA topoisomerase II and Trf4p, can compensate for the loss of Top1p function. Nevertheless, DNA topoisomerase I is required for the cytotoxic action of CPT.[16–18] Yeast cells deleted for *TOP1* (*top1*Δ) are resistant to CPT, while expression of yeast or human *TOP1* sequences from plasmids is sufficient to restore drug sensitivity. These data establish DNA topoisomerase I as the cellular target of CPT and confirm that drug cytotoxicity results from the stabilization of the covalent complex, rather than the inhibition of a nonessential enzyme. Thus, camptothecins have been described as topoisomerase I poisons to contrast their action on enzyme function with drugs that inhibit catalytic activity. Although CPT inhibits the relaxation of supercoiled DNA catalyzed by human DNA topoisomerase I, the concentrations of drug needed exceeds those required to stabilize the covalent enzyme-DNA intermediate or induce cell death.[4,19] Moreover, CPT does not inhibit the catalytic activity of yeast Top1, yet expression of the yeast enzyme is sufficient to restore CPT sensitivity to *top1*Δ yeast cells and enhance CPT cytotoxicity in mammalian cells.[19, 20]

MUTATIONS IN DNA TOPOISOMERASE I

The phenotypic consequences of CPT treatment are faithfully reiterated in yeast.[1,21,22] As in mammalian cells, drug treatment induces sister chromatid exchange and cell cycle arrest in G2. The enhanced CPT sensitivity of cells defective in double-strand break repair (due to deletion of the *RAD52* gene) argues for the involvement of DNA recombination in the repair of drug-induced DNA lesions.[16,17] The cytotoxic action of the drug is also highly S-phase dependent, as the DNA replication inhibitor, aphidicolin, abrogates CPT-induced cell killing.[10] However, in terms of investigating the cytotoxic mechanism of camptothecins, the genetically tractable yeast system offers the added advantage that the *TOP1* gene is dispensable for mitotic cell growth, yet essential for drug action.[13] Thus, the regulated expression of yeast or human *TOP1* from plasmid-borne sequences in *top1*Δ strains enables direct assessment of specific amino acid substitutions on DNA topoisomerase I function and CPT sensitivity in the absence of the endogenous enzyme.[1, 21]

The structures of an N-terminal deletion of human DNA topoisomerase I, in covalent and noncovalent complexes with DNA, were recently reported.[7,23,24] As with

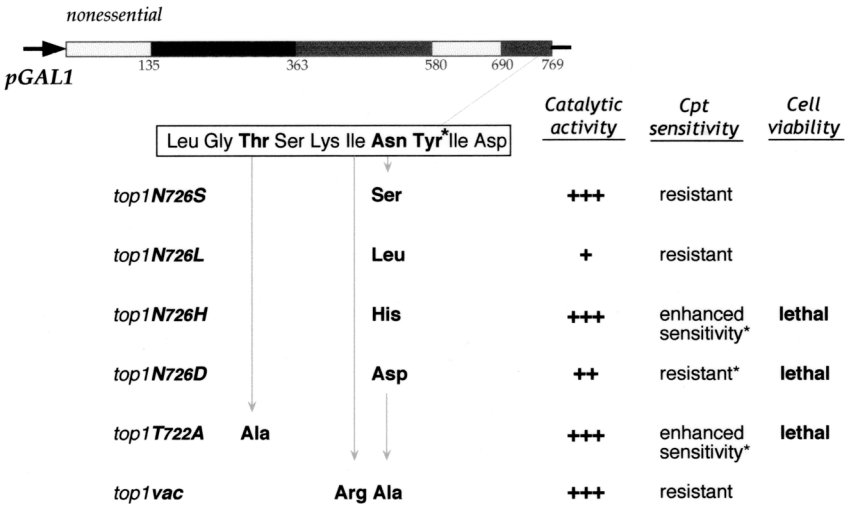

FIGURE 1. Substitutions of conserved residues around the active site tyrosine affect the catalytic activity and camptothecin sensitivity of DNA topoisomerase I. Conserved central and C-terminal domains of eukaryotic DNA topoisomerase I, essential for enzyme activity, are indicated by *gray shading*. The *boxed residues* around the active site tyrosine* (Tyr-727) in yeast Top1 are identical to those found at the corresponding position in the human enzyme, with the exception of isoleucine to leucine changes. The amino acid substitutions in each *top1* mutant are indicated with the allele designations on the left. For each *top1* mutant, any affects on cell viability or camptothecin sensitivity were assessed following galactose-induced expression of plasmid-borne sequences in *top1*△ cells, in the presence or absence of camptothecin. Following protein purification, the specific activity of equal concentrations of the mutant enzymes was determined in plasmid DNA relaxation assays. Each + indicates a 10-fold difference in activity relative to wild-type Top1 (+++). The ability of camptothecin to stabilize the covalent enzyme-DNA complex was also determined in DNA cleavage assays. In the case of the lethal *top1* mutants, *top1N726H*, *top1N726D*, and *top1T722A*, galactose induced a greater than 3-log drop in cell viability in the absence of camptothecin. Thus, relative enzyme sensitivity to camptothecin was solely defined *in vitro*.

other DNA topoisomerases, this eukaryotic type IB enzyme forms a protein clamp that circumscribes the DNA duplex. Several amino acid substitutions have been reported to affect the CPT sensitivity of yeast or human Top1.[1,4,7,25] Although widely scattered among conserved sequences, these residues cluster near the active site of the enzyme, along one face of the DNA in the crystal structures.

The conservation in enzyme mechanism and CPT sensitivity among cellular type IB enzymes is also reflected in amino acid sequence.[1,4,26] As shown in FIGURE 1, the residues immediately N-terminal to the active site tyrosine (Tyr727 in yeast and Tyr723 in human) are mostly identical. Indeed, substitution of these residues has profound effects on the catalytic activity and CPT sensitivity of DNA topoisomerase I. For instance, substituting Arg-Ala for the two residues preceding the active site tyrosine in yeast or human Top1 rendered the mutant enzymes resistant to CPT.[19,27] In these experiments, *top1*Δ strains were transformed with a single copy yeast vector expressing the indicated *top1* allele from the galactose-inducible *GAL1* promoter.

Cells expressing wild-type *TOP1* exhibit a greater than 3-log drop in viability when plated on selective media containing galactose and CPT. In contrast, cells expressing the yeast or human *top1vac* mutant were unaffected by the drug. Comparisons of wild-type and mutant proteins purified from these cells indicated that enzyme specific activities were indistinguishable in plasmid DNA relaxation assays. However, relative to wild-type Top1, the levels of CPT-stabilized, covalent enzyme-DNA complexes were dramatically diminished in DNA cleavage assays containing the mutant enzymes. Thus, the levels of drug-stabilized enzyme-DNA complexes *in vitro* corresponded with the cytotoxic activity of CPT in cells expressing the particular *top1* mutant.

Mutation of the conserved Thr-722 to Ala in yeast *top1T722A* had the surprising effect of mimicking the action of CPT, by increasing the stability of the covalent enzyme-DNA intermediate.[28,29] *GAL1*-promoted expression of *top1T722A* induced a rapid drop in cell viability and a terminal G2-arrested phenotype in the absence of CPT. Although the mutant enzyme was catalytically active, biochemical studies indicated a defect in DNA religation, resulting in higher concentrations of the covalent intermediate. This mirrors the cytotoxic mechanism ascribed to CPT.[30] The analogous substitution in human *top1T718A* produces similar alterations in enzyme function.[31]

To further investigate the contribution of active site residues to DNA topoisomerase I catalysis and drug sensitivity, we focused on several substitutions of Asn-726. In the active site of the human enzyme, this Asn is one of a few residues to interact with phosphate groups in the scissile DNA strand. In addition to studies of the *top1vac* and *top1N726L* mutants,[19,27] substitution of this residue with Ser in the human enzyme conferred CPT resistance with little affect on enzyme activity.[32] More recently, Asn-726 was mutated to His, Ser, or Asp in yeast Top1N726H, Top1N726S, and Top1N726D, respectively.[33] As described above for the *top1vac*, *top1N726L,* and *top1T722A* mutants, the consequences of these substitutions on cell viability, drug sensitivity, and enzyme function were assayed in *top1*Δ cells and in DNA relaxation and cleavage assays *in vitro*.

As summarized in FIGURE 1, each mutations had profound effects on various aspects on DNA topoisomerase I function.[33] In terms of DNA relaxation, substitution of Asn with an aliphatic residue (Leu) or an acidic residue (Asp) produced a 20–100-fold drop in specific activity. In contrast, a hydroxyl residue (Ser) or basic residue (His) had little effect on enzyme-catalyzed relaxation of supercoiled DNA. However, the requirements for CPT sensitivity were more stringent. Only enzymes containing the basic His (Top1N726H) or polar Asn (wild-type Top1) exhibited CPT-enhanced DNA cleavage *in vitro*. The presence of an acidic residue (Asp), aliphatic (Leu) or hydroxyl (Ser) residue immediately N-terminal to the active site tyrosine (Tyr-727) abrogated the ability of CPT to stabilize the covalent enzyme-DNA intermediate *in vitro*.

In *top1*Δ cells, galactose-induced expression of the CPT resistant Ser and Leu mutants also conferred a drug-resistant phenotype.[27,33] However, despite differences in specific enzyme activity and drug sensitivity *in vitro*, *GAL1*-promoted expression of the His and Asp mutants (*top1N726H* and *top1N726D*, respectively) induced cell death in the absence of CPT. Detailed biochemical studies of enzyme-DNA complexes in DNA cleavage assays, using full-length DNA substrates, suicide DNA

substrates, or nicked DNA molecules, indicated distinct mechanisms of enzyme-induced lethality. The His mutant (Top1N726H) exhibited increased covalent complex formation in the absence of CPT. However, in contrast to the decrease in DNA religation attributed to camptothecin or Top1T722A,[28,30] the His mutant exhibited increased rates of DNA scission.[33]

With Top1N726D, the presence of Asp at position 726 diminished enzyme binding of DNA.[33] Whereas this did not produce an increment in covalent complexes, the introduction of nicks in the nonscissile strand, downstream of the cleavage site, selectively destabilized the covalent enzyme-DNA intermediate. Wild-type and His mutant complexes were not affected. These data suggest that once the covalent linkage is formed between the Asp mutant enzyme and DNA, the deficit in DNA binding is localized to the noncovalently bound DNA, 3′ to the cleavage site. As a consequence, protein assemblies tracking along the DNA (such as replication forks) would have a greater probability of displacing the noncovalently bound DNA end before the phosphotyrosyl linkage is resolved.

Although additional studies are necessary to test various aspects of this model, the data clearly indicate novel mechanisms of DNA topoisomerase I-induced DNA damage result from single residue changes in the active site. Related questions concerning cellular responses to these lesions have also to addressed. Nevertheless, these findings suggest an opportunity to develop novel DNA topoisomerase I poisons that interfere with aspects of the catalytic cycle, distinct from that targeted by camptothecins.

CELLULAR FACTORS THAT MODULATE CAMPTOTHECIN SENSITIVITY

Yeast has also proven a valuable model to investigate cellular processes that function in modulating cellular responses to camptothecins.[1,10,21] Genetic and biochemical studies established DNA topoisomerase I as the cellular target of these drugs. Furthermore, in isogenic cell lines or yeast strains, the relative levels of DNA topoisomerase I correlate with the cytotoxic action of CPT.[20,22,27,34] However, in genetically diverse backgrounds, such as tumor cells or yeast strains defective in various aspects of DNA repair or DNA damage checkpoints, enzyme levels are not predictive of drug sensitivity.[11,16,28,35,36] For example, yeast cells defective in double-strand break repair (*rad52*Δ strains) or the DNA damage checkpoint (*rad9*Δ strains) are hypersensitive to CPT as well as the damage induced by the *top1T722A* mutant.[16–18,28] Furthermore, CPT-resistant mammalian cell lines have been isolated with no apparent alteration in DNA topoisomerase I activity.[4,11] Experiments in yeast and mammalian cells implicate recombinational repair in the resolution of CPT-induced DNA lesions during S-phase. Yet, the specific drug-induced DNA lesions and the cellular processes involved in the recognition and repair of such lesions remain poorly defined. These issues are of critical importance for the effective clinical application of camptothecins and in the development of more potent DNA topoisomerase I poisons.

To define cellular factors that modulate cell sensitivity to CPT, we previously reported a yeast screen to identify mutations in genes other than *TOP1* that confer re-

sistance to CPT.[22] In these studies, wild-type *TOP1* was expressed from the *GAL1* promoter on a single copy vector, in *top1*Δ cells. Following mutagenesis and selection of drug-resistant colonies on galactose-containing media, the mutants were cured of the original vector and transformed with a plasmid that constitutively expressed *TOP1*. A secondary screen for CPT resistance eliminated *TOP1* mutants or mutations affecting efficient expression of the *GAL1* promoter. Each mutant strain contained a dominant mutation in the *PDR1* gene, resulting in a pleiotropic drug resistant phenotype.[35] *PDR1* encodes a transcription factor that regulates the expression of a network of yeast genes encoding ATP binding cassette (ABC) transport proteins.[37] These membrane-spanning proteins form membrane channels that transport a variety of molecules. Overexpression of ABC transporters has been linked to cytotoxic drug resistance, although CPT is not an effective substrate for p-glycoprotein or MRP. Only modest effects have been seen with topotecan and SN-38[5,21]; however, anion transporters have been implicated in the renal clearance of the carboxylate form of topotecan.[38] The ~1,000-fold CPT resistance of the yeast *PDR1* mutants required a specific transporter, Snq2.[35] Although ABC transporters exhibit some substrate specificity, 50-fold overexpression of a heterologous transporter also conferred CPT resistance. This raises the intriguing possibility that other, as yet unknown, transporters will be identified that modulate tumor cell sensitivity to various CPT analogues. Indeed, overexpression of the ABC transporter BCRP in ovarian cell lines selected for resistance to topotecan or mitoxantrone was recently shown to promote efficient efflux of topotecan.[39]

Although potential mechanisms of drug uptake/efflux are important considerations, this approach failed to illuminate events that occur downstream of the covalent complex. So to avoid the complications attendant with drug transport, we initiated a genetic screen for mutations that enhance cell sensitivity to the damage induced by DNA topoisomerase I mutant (*top1T722A*) that mimics the cytotoxic action of CPT.[36] The rationale for these studies was that low, constitutive levels of *top1T722A* expression, just like sublethal doses of CPT, still induce DNA damage. This is evidenced by increased rates of DNA recombination in repair-proficient strains and the inability of repair defective strains (i.e, *rad52*Δ cells) to tolerate even low levels of Top1T722A.[28,36] Thus, we reasoned that cellular processes that normally protect cells from DNA topoisomerase-I mediated DNA damage could be identified by isolating yeast mutants that exhibit temperature-dependent sensitivity to *top1T722A* (called *tah* mutants).

As depicted in FIGURE 2, *tah* mutants tolerate *top1T722A*-induced DNA damage at the permissive temperature (26°C).[36] However, at the high temperature (35°C), *tah* gene function is lost or diminished, and the cells are unable to survive the lesions induced by the Top1T722A mutant enzyme. To ensure that the *tah* temperature-sensitive (*tah*^{ts}) phenotype was plasmid linked, the cells were cured of the original *top1T722A* vector and rescreened for viability at 35°C. Only those cells exhibiting a *tah*^{ts} phenotype, whose viability at 35°C was unaffected by the expression of wild-type *TOP1* or the absence of DNA topoisomerase I function were selected. Twelve mutants were isolated exhibiting a stable *tah*^{ts} phenotype that segregated as a single, recessive gene mutation and enhanced sensitivity to CPT. This validated the use of *top1T722A* as a drug mimetic. Moreover, the large budded terminal phenotypes observed at 35°C were indicative of S-phase-induced lesions.

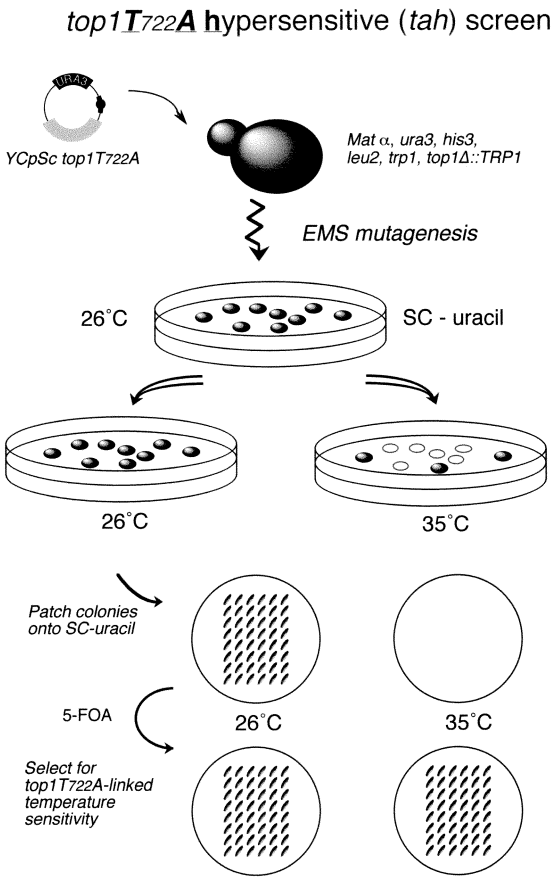

FIGURE 2. Isolation of *top1T722A* hypersensitive (*tah*) mutants.

The *tah* mutants exhibited a pleiotropic pattern of hypersensitivity to other forms of DNA damage at the nonpermissive temperature, in the absence of *top1T722A*. Most showed enhanced sensitivity to the inhibition of DNA replication induced by hydroxyurea (HU), consistent with the S-phase toxicity of CPT. Some were also hypersensitive to the alkylating agent MMS and UV irradiation. However, the spectrum of alterations in drug sensitivity was confined to DNA damaging agents. Furthermore, the temperature-sensitive phenotypes could not be attributed to alterations in DNA topoisomerase I function, as drug sensitivity was assessed in the absence of *TOP1*.

Wild-type *TAH* alleles were cloned by complementation. A yeast genomic DNA library in a single copy vector was transformed into individual mutants and plasmids capable of suppressing the *tah*^{ts}, or HU hypersensitive phenotypes were isolated. Subsequent subcloning identified individual *TAH* genes. These studies were facilitated by annotated databases containing the sequence of the yeast genome (*Saccha-*

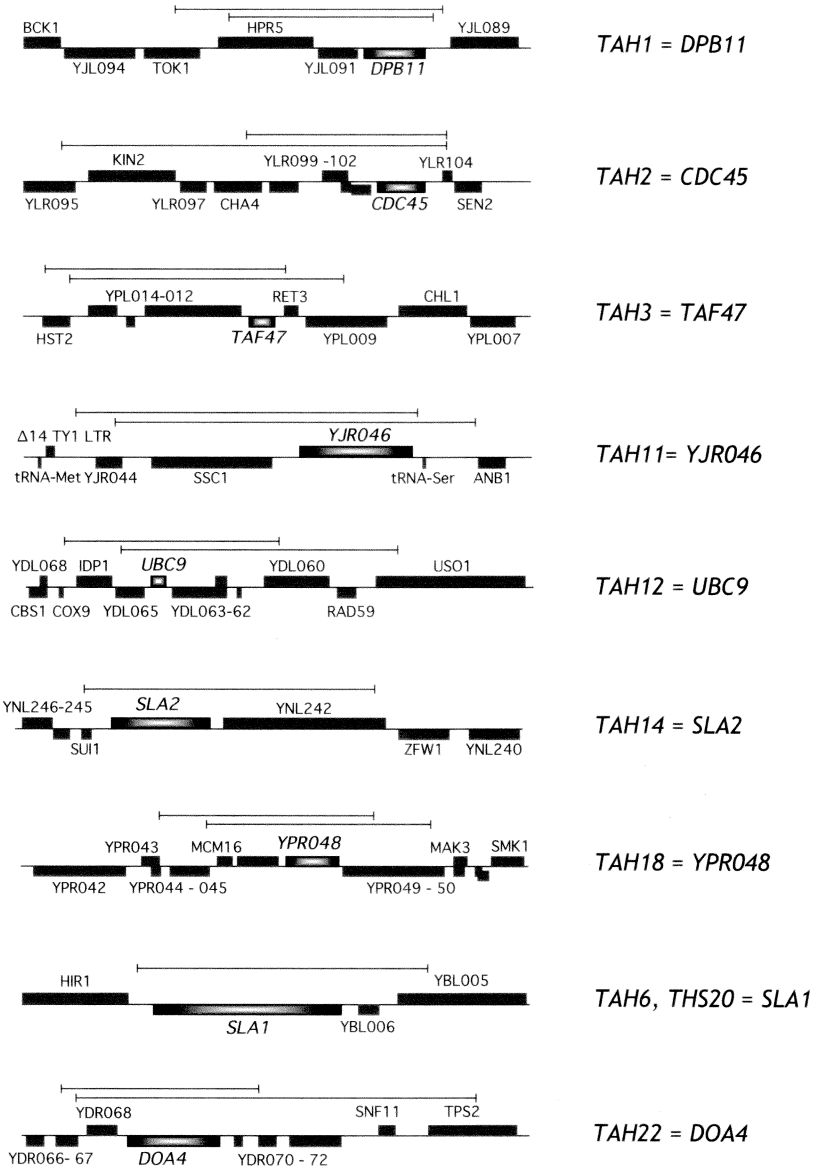

FIGURE 3. Wild-type *TAH* alleles cloned by complementation. Inserts of plasmids containing yeast genomic DNA capable of restoring tah mutant cell growth at the nonpermissive temperature in the presence of either *top1T722A* or HU are shown relative to their location on the indicated yeast chromosome. Subcloning and subsequent genetic studies confirmed the identity of the wild-type *TAH* gene within each plasmid.

romyces Genome Database [http://genome-www.stanford.edu/Saccharomyces/]. As depicted in FIGURE 3, the *TAH* genes encode proteins involved in DNA replication (*CDC45*–initiation of DNA replication,[40,41] *DPB11*–DNA replication, and the S-phase checkpoint[43]), SUMO- or ubiquitination (*UBC9*, *DOA4*) and transcription (*TAF47*), as well as cortical actin components (*SLA1*, *SLA2*) and two unknowns (*TAH11* and *TAH18*).

Experiments are currently underway to define the specific role that each gene product plays in modulating cell sensitivity to CPT. However, in the case of the *cdc45-10* and *dpb11-10* mutants, the construction of strains bearing both mutations (*cdc45-10, dpb11-10* cells) revealed a synthetic lethal interaction.[36] At 35°C, in the absence of *top1T722A* or any other DNA damaging agent, the double mutants were inviable. In synchronized cultures at the nonpermissive temperature, each of the single mutants (*cdc45-10* or *dpb11-10*) exhibited a delay in early S-phase transit. This coincided with a transient accumulation of Okazaki-sized DNA fragments in asynchronous cultures. Yet, the S-phase checkpoint was functional in each mutant. In contrast, the double *cdc45-10, dpd11-10* mutant exhibited a persistent accumulation of Okazaki-sized DNA fragments and a drop in viability as the cells transited S-phase. Taken together, these data suggest that *CDC45* and *DPB11* are required for processive DNA replication, possibly in mediating the switch from priming to processive DNA polymerases. In terms of CPT sensitivity, the accumulation of Okazaki fragments behind the advancing replication fork would preclude recombinational repair of the lesions resulting from collision of the fork with the drug-stabilized enzyme-DNA complexes.

Further genetic analyses will decipher the function of other *TAH* genes in modulating cell sensitivity to CPT-induced DNA damage. Despite the apparent complexity of gene functions uncovered in this screen, similarities in terminal phenotype suggest common action during S-phase, consistent with the cytotoxic action of CPT. However, preliminary studies also suggest that the DNA damage induced by the different self-poisoning forms of DNA topoisomerase I described above[33] may involve distinct signaling or repair processes. Thus, despite a unique cellular target (DNA topoisomerase I), various CPT analogues or structurally unrelated Top1 poisons may elicit DNA lesions with distinct cellular consequences, which may be exploited in the development of new antitumor agents.

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