The Topoisomerase-related Function Gene TRF4 Affects Cellular Sensitivity to the Antitumor Agent Camptothecin*

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Camptothecin is an antitumor agent that kills cells by converting DNA topoisomerase I into a DNA-damaging poison. Although camptothecin derivatives are now being used to treat tumors in a variety of clinical protocols, the cellular factors that influence sensitivity to the drug are only beginning to be understood. We report here that two genes required for sister chromatid cohesion, TRF4 and MCD1/SCC1, are also required to repair camptothecin-mediated damage to DNA. The hypersensitivity to camptothecin in the trf4 null mutant does not result from elevated expression of DNA topoisomerase I. We show that Trf4 is a nuclear protein whose expression is cell cycle-regulated at a post-transcriptional level. Suppression of camptothecin hypersensitivity in the trf4 mutant by gene overexpression resulted in the isolation of three genes: another member of the TRF4 gene family, TRF5, and two genes that may influence higher order chromosome structure, ZDS1 and ZDS2. We have isolated and sequenced two human TRF4 family members, hTRF4-1 and hTRF4-2. The hTRF4-1 gene maps to chromosome 5p15, a region of frequent copy number alteration in several tumor types. The evolutionary conservation of TRF4 suggests that it may also influence mammalian cell sensitivity to camptothecin.

Camptothecin is a plant alkaloid derived from the Chinese tree Camptotheca acuminata which was initially discovered to be active against murine leukemia (1). Interest in camptothecin was heightened with the discovery in 1985 that the drug caused DNA damage by specifically targeting DNA topoisomerase I (2). The drug was shown to stabilize a covalent reaction intermediate in which DNA topoisomerase I is linked via a tyrosine residue in the protein to the 3'-phosphoryl end of the broken DNA strand (3, 4). The reversible single strand nick that results is thought to lead to cytotoxicity by conversion into an irreversible lethal lesion, probably a double strand break, upon encounter of the damaged DNA with a DNA replication fork (5). If the resulting double strand break is not repaired, cell death results.

A rigorous demonstration that DNA topoisomerase I (TOP1) is the sole target of camptothecin came from studies of camptothecin action in yeast. Camptothecin was shown to kill the yeast Saccharomyces cerevisiae and to poison the action of yeast TOP1 as it does in mammalian cells. Significantly, yeast cells deleted for the TOP1 gene are completely resistant to killing by the drug (6, 7). Furthermore, expression of human topoisomerase I in the top1 deletion strain restores sensitivity to killing by camptothecin (6, 7). Thus, camptothecin kills both mammalian and yeast cells by turning TOP1 into a DNA-damaging agent.

Yeast has proven to be an extremely useful system in which to study the parameters affecting camptothecin-mediated cytotoxicity because of the availability of well characterized mutants in DNA repair pathways and facile genetic methods (7–9). One important insight was the observation that defects in double strand break repair cause hypersensitivity to killing by camptothecin (6, 7), supporting the notion that a double strand break generated during DNA replication is a major lethal lesion. Indeed, in yeast cells as in mammalian cells, agents that inhibit DNA synthesis also greatly reduce camptothecin-mediated killing (10). The RAD52 epistasis group is required for double strand break repair in S. cerevisiae, and mutations in RAD52 cause marked hypersensitivity to camptothecin. Defects in the DNA damage checkpoint pathways which cause hypersensitivity to camptothecin in mammalian cells also cause inviability in yeast cells expressing a top1 mutation that mimics the effects of camptothecin (11). In addition, sensitivity to camptothecin can be modulated through altered expression of multidrug resistance-related efflux pumps such as SNQ2 (12) or through dominant mutations in transcriptional regulators of these pumps (13). Studies in yeast are likely to lead to further insights into the mechanism of repair of this unusual type of DNA damage, provided the important pathways and genes are evolutionarily conserved.

We have discovered that TOP1 functions, together with a novel gene called TRF4 (for Topoisomerase Related Function), in the process of mitotic chromosome condensation (14, 15). Furthermore, we have shown that the Trf4 protein is physically associated with Smc1p and Smc2p (14), proteins that bind directly to chromosomes and cause condensation (16). The Smc protein complex can alter DNA topology (17), which is likely to explain the functional redundancy with TOP1. Recent evidence from our laboratory shows that TRF4 is also required to maintain a different aspect of higher order chromosome structure, sister chromatid cohesion. Thus, two key aspects of higher order chromosome structure, sister cohesion and mitotic chromosome condensation, are mediated by Trf4p most likely through its association with Smc1p (14).

Here we report the surprising observation that the trf4 mutant is also profoundly hypersensitive to killing by camptothecin. We also show that mutation of another Smc1p-associated protein required for sister chromatid cohesion, Mcd1p/ScC1p, also results in dramatic camptothecin hypersensitivity. It is of great importance to understand the molecular mechanisms employed to repair DNA damage caused by camptothecin be-

1 I. B. Castaño and M. F. Christman, unpublished observation.
cause DNA repair deficiencies are likely to affect sensitivity of cancer cells to camptothecin (18). Specific tumors known to be defective in the relevant repair pathway might be significantly more responsive to camptothecin therapy (19). A detailed knowledge of the repair mechanisms used in tumors could lead to the identification of novel drug targets. Furthermore, because double-strand breaks are known to lead to genomic instability such as gene amplification (20), a thorough knowledge of the means by which these breaks are repaired is central to understanding tumorigenesis. Our data suggest that higher order chromosome structure may be a critical determinant of camptothecin toxicity.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Media**—Camptothecin was purchased from Sigma and dissolved in dimethyl sulfoxide at a stock concentration of 4 mg/ml. Methyl methanesulfonate (MMS),

hydroxyureas, and nocodazole were purchased from Sigma. α-Factor was purchased from Fluka. Yeast strains were routinely grown in YEP or synthetic complete (SC) medium lacking the appropriate supplements with a 2% final concentration of glucose (YPD) or galactose (YP-Gal).

**Plasmids and Strains**—The plasmids and yeast strains used in this study are listed in Tables I and II, respectively. All gel purifications were performed using Qiagen columns (Qiagen, Chatsworth, CA). All yeast transformations were performed using lithium acetate in the medium, synthetic complete medium; PCR, polymerase chain reaction; yeast transformations were performed using Qiagen columns (Qiagen, Chatsworth, CA).

**Drug Sensitivity Assays**—Equal numbers of cells of wild-type (CY184) and VG-906–1A, trf4::HIS3 (CY1000), and mec1-1 (CY1229) strains were serially diluted 10-fold, and 5-μl aliquots were spotted on YPD plates containing 10 μg/ml camptothecin and buffered with 25 mM HEPES, pH 7.2. Control plates contained final concentrations of 0.25% dimethyl sulfoxide and 25 mM HEPES, pH 7.2. Plates were incubated at 30 °C for 2 days. Sensitivity to MMS was assayed as described above for wild-type (CY184) and trf4::HIS3 (CY855) strains. Cells were spotted on YPD plates containing 0.025% MMS.

**UV Sensitivity Assay**—Equal numbers of cells of wild-type (CY184), trf4::HIS3 (CY1000), and rad3 (CY674) strains were plated on YPD plates and exposed to increasing doses of UV irradiation using a Stratagene UV Stratalinker. Plates were immediately wrapped in aluminum foil and incubated at 30 °C for 2 days. Colonies were counted, and percent viability was calculated based on the number of colonies arising without exposure to UV irradiation.

**DNA Topoisomerase I Assay**—To prepare crude protein extracts, a single colony from wild-type (CY184), trf4::HIS3 (CY855), and top1–LEU2 (CY154) was resuspended in 30 μl of SED (1 mM sorbitol, 25 mM EDTA, 50 mM dithiothreitol), and spheroplasts were prepared by the addition of 4 μl of zymolase (5 mg/ml in 1 mM sorbitol). The spheroplasts were pelleted, lysed by the addition of 12 μl of yeast lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM KCl, 10% glycerol, 1 mM Na2EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and incubated for 30 min on ice. Cell debris was removed by centrifugation, and supernatants containing the crude protein extract was collected. The protein concentration was determined using a Bio-Rad assay. The extracts were diluted in buffer consisting of 100 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 5% glycerol to a final concentration of 5 mg/ml. DNA topoisomerase assays were performed using a standard relaxation assay (TopoGEN, Columbus, OH) using 1 μl of diluted crude protein extracts. Reactions were terminated at timed intervals by the addition of stop buffer (TopoGEN) and analyzed by electrophoresis on 0.8% agarose gels and ethidium bromide staining.

**Complementation of Cold Sensitivity**—To assess whether the TRF4-GFP fusion was functional, we tested complementation of the cold-sensitive phenotype of a trf4::HIS3 strain by integration of the pGAL-TRF4-GFP construct. Strains CY1112, CY1114, and control strains containing pRS305 integrated at the LEU2 locus were streaked on plates containing glucose and plates containing galactose. Duplicate plates were incubated at 17 °C for 6 days and 30 °C for 2 days. Functional pGAL-TRF4-GFP candidates were identified on the basis of galactose-dependent growth at 17 °C.

**Fluorescence Microscopy**—To prepare cells for fluorescence microscopy, cells were grown overnight in YPD or YP-Gal. Aliquots of cells were removed, treated with α-factor (25 μg/ml final concentration), hydroxyurea (100 mM final concentration), or nocodazole (15 μg/ml final concentration), diluted in buffer consisting of 100 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 5% glycerol to a final concentration of 5 mg/ml. DNA topoisomerase assays were performed using a standard relaxation assay (TopoGEN, Columbus, OH) using 1 μl of diluted crude protein extracts. Reactions were terminated at timed intervals by the addition of stop buffer (TopoGEN) and analyzed by electrophoresis on 0.8% agarose gels and ethidium bromide staining.

**TABLE I**

| Plasmids | Description | Source |
|----------|-------------|--------|
| pRS305   | LEU2 yeast integrating vector | Sikorski and Hieter |
| yGFP3    | GFP mutant 3 HindIII/PstI fragment in pUC19 | Dan Burke |
| CB636    | pGAL-TRF4 in YEp35 | Laboratory collection |
| CB1001   | GFP mutant HindIII/PstI fragment in pRS305 | This study |
| CB1007   | pGAL-TRF4-GFP in CB1001 | This study |
| CB3865   | URA3 2 μg yeast genomic library | David Pellman |

**TABLE II**

| Yeast strains | Description | Source |
|---------------|-------------|--------|
| CY143         | MATA ade2-1::ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rDNA::URA3 | Laboratory collection |
| CY154         | MATA top1-7::LEU2 in CY1413 | Laboratory collection |
| CY184         | MATA ade2-1::ura3-1 his3-11, 15 trp1-1 leu2-3, 112 can1-100 rDNA::ADE2 | (28) |
| CY180         | MATA rad3-2::his2 leu2 cys1 | (39) |
| CY155         | MATA trf4::HIS3 in CY184 | (28) |
| CY869         | MATA ade2-1::URA3-1 his3-11, 15 trp1-1 leu2-3, 112 can1-100 rDNA::ADE2 | (15) |
| CY1800        | MATA ade2-1::URA3-1 his3-11, 15 trp1-1 leu2-3, 112 | This study |
| CY1112        | MATA ade2-1::URA3-1 his3-11, 15 trp1-1 leu2-3, 112 | This study |
| CY1115        | MATA ade2-1::URA3-1 his3-11, 15 trp1-1 leu2-3, 112 | This study |
| VG906-1A      | MATA ade2-1::URA3-1 his3-11, 15 trp1-1 leu2-3, 112 | (26) |
| VG955-7D      | MATA ade2-1::URA3-1 his3-11, 15 trp1-1 leu2-3, 112 | (26) |
| CY1229        | MATA mcd1-1 bar1 trp1 leu2 gal1 | Cross of VG955-7D and CY869 |
TRF4 Affects Cellular Camptothecin Sensitivity

RESULTS

The trf4 and mcd1-1 Mutants Are Hypersensitive to Killing by Camptothecin—An otherwise isogenic set of trf4::HIS3 and TRF4+ yeast strains was constructed by single step gene replacement (25) and then tested for sensitivity to the antitumor agent camptothecin. Killing by camptothecin was monitored on Petri plates with or without 10 μg/ml camptothecin incorporated into the agar (see "Experimental Procedures").

As shown in Fig. 1, the trf4::HIS3 mutant cells are roughly 3 orders of magnitude more sensitive to killing by camptothecin compared with the otherwise isogenic wild-type parent strain. In the absence of camptothecin the wild-type and trf4::HIS3 mutant cells grow equally well. A similar test was performed on congenic mcd1-1 and MCD1+ yeast strains. The MCD1/SCC1 gene product, like TRF4, is physically associated with SMC1 and MCD1 and required for sister chromatid cohesion (26, 27). Because MCD1/SCC1 is an essential gene, the test was performed at the semipermissive temperature of 30 °C. The mcd1-1 mutant was observed to be about 4 orders of magnitude more sensitive to camptothecin than the congenic parent strain (Fig. 1). The parent wild-type strains show slightly different camptothecin sensitivities because of their being derived from different strain backgrounds.

DNA Topoisomerase I Activity Is Not Elevated in the trf4 Mutant—It is known that overexpression of DNA topoisomerase I in yeast results in increased sensitivity to camptothecin-mediated killing because of the presence of higher concentrations of topoisomerase I enzyme (8, 9). Because TOP1 and TRF4 provide overlapping functions in yeast (14, 28), we reasoned that deletion of TRF4 might result in a compensatory elevation in TOP1 expression and thereby sensitize the trf4 mutant cells to camptothecin.

We tested this possibility directly by quantitatively measuring DNA topoisomerase I activity in crude extracts from isogenic trf4::HIS3 and TRF4+ cells. Topoisomerase I activity was measured by the ability to relax a negatively supercoiled plasmid DNA substrate in the absence of ATP as described under "Experimental Procedures." Under these conditions neither topoisomerase II nor topoisomerase III activity is detected.

Protein extracts were prepared from single yeast colonies. Approximately 5 μg of protein from each extract was added to a 20-μl reaction volume containing 1 μg of negatively supercoiled plasmid DNA. The negatively supercoiled substrate migrates faster than the relaxed reaction product on a 0.8% agarose gel. The reactions were incubated at 37 °C, and reaction progress was monitored by withdrawal of aliquots, addition of stop buffer containing EDTA, and electrophoresis on 0.8% agarose gels to separate negatively supercoiled substrate from the fully relaxed reaction product.

Fig. 2 shows that the relaxation reactions proceed with equivalent kinetics in the trf4::HIS3 and TRF4+ extracts over a 40-min period. Both reactions are nearly complete after 40 min, although some incompletely relaxed topoisomers can be observed in both extracts. Control reactions that were performed without added ATP or that contained a 5-fluoro-orotic acid reaction component revealed that camptothecin-resistant colonies were identified after 2 and 3 days of incubation at 30 °C. A 5-fluoro-orotic acid counterselection (23) was used to isolate rare segregants that had spontaneously lost the URA3-marked library plasmid, and the cells with no plasmid were then tested for camptothecin resistance. 133 of the 168 candidate suppressors were plasmid-dependent for the camptothecin-resistant phenotype. Library plasmids were rescued from yeast cells by "smash-n-grab" (24) and isolated from E. coli using Qiagen columns.

Restriction digests of the remaining plasmids revealed the presence of Sau3A inserts in vector pSEY18 (URA3.5μ) was used to isolate suppressors. Library DNA was isolated from E. coli by Wizard Midi columns (Promega) and used to transform trf4::HIS3 (CY1000). Transformants were selected on SC-ura. After 3 days of incubation at 30 °C, approximately 270,000 total transformants were pooled, concentrated in 15% glycerol, and stored at −70 °C. Aliquots of transformants were periodically thawed, and the cells were diluted and plated on camptothecin plates. To prepare camptothecin plates, 375 μl of a camptothecin stock solution at 4 mg/ml in dimethyl sulfoxide was spread on YPD plates buffered with 25 mM dimethyl sulfoxide and 90% glycerol and placed on glass slides. Cells were examined using a Nikon Eclipse 800 microscope with differential interference contrast optics and DAPI and fluorescein isothiocyanate filters. Images were captured digitally using a Princeton Instruments CCD camera with IP Lab spectrum software and colorized using Adobe Photoshop. Relative fluorescence intensities were determined using a Chroma GFP filter and IP Lab spectrum software.

Fig. 1. trf4 and mcd1-1 mutants are hypersensitive to killing by camptothecin. Equal numbers of cells from trf4 (CY1000), wild-type (CY184 and VCG656-1A), and mcd1-1 (CY1229) strains were serially diluted 10-fold and then spotted on plates containing camptothecin (CPT) and control plates containing no drug and incubated at 30 °C for 2 days. The wild-type strains for trf4 and mcd1-1 are from different genetic backgrounds and show slightly different camptothecin sensitivities.

DNA Topoisomerase I activity is not elevated in trf4 relative to wild-type strains. DNA topoisomerase I activity was examined in trf4::HIS3 (CY855, TRF4−) and wild-type (CY184, TRF4+) crude protein extracts using a standard relaxation assay (TopoGEN). Reactions were terminated at timed intervals and analyzed by gel electrophoresis. Control lanes include the same substrate with top1–7::LEU2 (CY184) extract and no extract.

Protein extracts were pooled, concentrated in 15% glycerol, and stored at −70 °C. Aliquots of transformants were periodically thawed, and the cells were diluted and plated on camptothecin plates. To prepare camptothecin plates, 375 μl of a camptothecin stock solution at 4 mg/ml in dimethyl sulfoxide was spread on YPD plates buffered with 25 mM dimethyl sulfoxide and 90% glycerol and placed on glass slides. Cells were examined using a Nikon Eclipse 800 microscope with differential interference contrast optics and DAPI and fluorescein isothiocyanate filters. Images were captured digitally using a Princeton Instruments CCD camera with IP Lab spectrum software and colorized using Adobe Photoshop. Relative fluorescence intensities were determined using a Chroma GFP filter and IP Lab spectrum software.
percent viability was calculated. More than 200,000 transformants from the library were screened for growth on the camptothecin plates during several rounds of screening. Trf4 can suppress the camptothecin sensitivity of rad3, the trf4::HIS3 mutant host strain (CY1000), initially selecting only for the DNA library marker gene URA3. 2-μm plasmids are maintained at approximately 20–50 copies/cell and consequently overexpress most of the genes present on the insert (31). More than 200,000 transformants from the library were pooled, diluted, and then replated on plates containing camptothecin. At a low concentration of camptothecin wild-type cells are able to form healthy single colonies, whereas trf4::HIS3 mutant cells are killed (Fig. 5A). We examined 460,000 trf4::HIS3 mutant cells containing library plasmids for growth on the camptothecin plates during several rounds of screening. Plasmid DNA was recovered from 133 plasmid-dependent suppressors and transformed into E. coli.

Diagnostic PCRs were used to eliminate those clones that contained the TRF4 gene itself. One such clone, 16-2, is shown in Fig. 5B. Limited restriction mapping placed the remaining library clones into three classes, and a member of each class was sequenced. One class of suppressor carried inserts containing the TRF4-related gene, TRF5 (15). This demonstrates that at least two genes from the evolutionarily conserved TRF4 gene family affect cellular sensitivity to camptothecin. The two other suppressor classes represented separate regions of chromosome XIII which encoded related genes called ZDS1 (clone 23-2
in Fig. 5B) and ZDS2 (clone 26-2 in Fig. 5B). Suppression of the camptothecin hypersensitivity in the trf4 mutant was observed to be considerably stronger for ZDS2 than for ZDS1. Both ZDS genes have been identified previously in other genetic screens (variously called NRC1, ZDS1, and OSS1), many of which were designed to identify products important for chromosome structure or cell cycle progression (32–34), although no molecular mechanism has yet been identified for their action.

**Isolation of hTRF4-1 and hTRF4-2, Human Homologs of TRF4/5—**

With the knowledge that two yeast TRF4 family members can profoundly affect cellular sensitivity to camptothecin and the fact that TRF4 is the canonical member of an evolutionarily conserved gene family, we sought to identify human genes related to TRF4. The presence or absence of human TRF4 expression in tumor cells may likewise affect tumor cell sensitivity to camptothecin. Searches of the existing data bases with yeast TRF4 revealed the presence of two human expressed sequence tags (h90950 and h85548) with high homology to TRF4 over short regions. However, the data base contained only 396 base pairs of sequence for h90950 and 382 base pairs from h85548. We obtained the clones from which the limited DNA sequences were derived from the ATCC archives and sequenced each clone in its entirety on both strands. The h85548 clone contained an insert of 3842 base pairs with a potential TRF4-related open reading frame of 517 amino acids (nucleotides 2–1152), containing a potential initiator methionine at position 41, which includes all of the regions of high evolutionary conservation in the TRF4 gene family. Thus, h85548 is likely to encode a full-length human TRF4 homolog. We have designated this full-length human clone hTRF4-1 (GenBank accession number AF089896). S. cerevisiae TRF4 and hTRF4-1 are 39% identical and 51% similar over 310 amino acids in the central region of both proteins, demonstrating a very high degree of evolutionary and likely functional conservation (Fig. 6). For comparison, the human and yeast DNA topoisomerase I gene products are 44% identical and 54% similar, indicating that the TRF4 gene family has been conserved to a similar degree. The hTRF4-1 gene is also 49% similar and 35% identical to the S. cerevisiae TRF5 gene over evolutionarily conserved gene family, we sought to identify human genes related to TRF4. The presence or absence of human TRF4 expression in tumor cells may likewise affect tumor cell sensitivity to camptothecin. Searches of the existing data bases with yeast TRF4 revealed the presence of two human expressed sequence tags (h90950 and h85548) with high homology to TRF4 over short regions. However, the data base contained only 396 base pairs of sequence for h90950 and 382 base pairs from h85548. We obtained the clones from which the limited DNA sequences were derived from the ATCC archives and sequenced each clone in its entirety on both strands.

**FIG. 4. Trf4-GFP protein is nuclear and cell cycle-regulated.** Cells containing TRF4-GFP under control of the native TRF4 gene promoter (CY1115) were arrested in G1, S, and G2/M phases of the cell cycle and examined for GFP signal by fluorescence microscopy. Images captured with the differential interference contrast filter show cell morphology, the DAPI filter shows DNA, and the fluorescein isothiocyanate (FITC) filter was used to visualize the GFP signal. The efficacy of cell cycle arrest was determined by examining cell morphology for ≥ 50 cells at each arrest point.

**TABLE III**

Quantitation of TRF4-GFP signal under galactose induction

Cells containing pGAL-TRF4-GFP integrated at the LEU2 locus (CY1112) were grown overnight in YP-galactose, arrested in G1, S or G2/M phase of the cell cycle, and examined for GFP signal by fluorescence microscopy. Relative GFP intensity was quantitated using IP laboratory spectrum software. The fluorescence intensity of equal areas of both nucleus and cytoplasmic background was measured, and the relative GFP intensity was determined by subtracting the cytoplasmic background from the nuclear signal for each cell examined. The values have been normalized to the S phase intensity, which was arbitrarily set at 100.

| Relative GFP intensity | No. of Cells examined |
|-----------------------|-----------------------|
| G1                    | 13                    |
| S                     | 100                   |
| G2/M                  | 56                    |

The h85548 clone contained an insert of 3842 base pairs with a potential TRF4-related open reading frame of 517 amino acids (nucleotides 2–1152), containing a potential initiator methionine at position 41, which includes all of the regions of high evolutionary conservation in the TRF4 gene family. Thus, h85548 is likely to encode a full-length human TRF4 homolog. We have designated this full-length human clone hTRF4-1 (GenBank accession number AF089896). S. cerevisiae TRF4 and hTRF4-1 are 39% identical and 51% similar over 310 amino acids in the central region of both proteins, demonstrating a very high degree of evolutionary and likely functional conservation (Fig. 6). For comparison, the human and yeast DNA topoisomerase I gene products are 44% identical and 54% similar, indicating that the TRF4 gene family has been conserved to a similar degree. The hTRF4-1 gene is also 49% similar and 35% identical to the S. cerevisiae TRF5 gene over evolutionarily conserved gene family, we sought to identify human genes related to TRF4. The presence or absence of human TRF4 expression in tumor cells may likewise affect tumor cell sensitivity to camptothecin. Searches of the existing data bases with yeast TRF4 revealed the presence of two human expressed sequence tags (h90950 and h85548) with high homology to TRF4 over short regions. However, the data base contained only 396 base pairs of sequence for h90950 and 382 base pairs from h85548. We obtained the clones from which the limited DNA sequences were derived from the ATCC archives and sequenced each clone in its entirety on both strands.

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|-----------------------|-----------------------|
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| S                     | 100                   |
| G2/M                  | 56                    |
the same conserved region of 310 amino acids.

The h90950 clone contained an insert of 1479 base pairs with a potential TRF4-related open reading frame of 381 amino acids (nucleotides 3–1145). Therefore, it is likely to represent only a partial sequence. We have designated this human gene hTRF4-2 (GenBank accession number AF089897). The hTRF4-1 and hTRF4-2 gene products are 65% identical and 72% similar over the entire length of hTRF4-2 (381 amino acids). S. cerevisiae TRF4 and hTRF4-2 are 37% identical and 48% similar over 222 amino acids. The S. cerevisiae TRF5 gene is 48% similar and 34% identical to hTRF4-2 over a 221-amino acid region. An alignment of TRF4 family members from S. cerevisiae, human, Schizosaccharomyces pombe, and Caenorhabditis elegans reveals a high degree of conservation across many species (Fig. 6). Homology to the TRF4 family is also evident in a number of additional expressed sequence tags derived from mouse, rat, pufferfish, and Arabidopsis.

hTRF4-1 Maps to a Region of Frequent Copy Number Alteration in Several Tumor Types—A search of the GenBank STS data base with the hTRF4-1 nucleotide sequence revealed a 100% nucleotide sequence match over 200 base pairs with the Whitehead Genome Project physical marker STS WI-6634. This STS maps to chromosome 5 between 16 and 18 centimorgans from the end of the short (or “p”) arm, placing hTRF4-1 at cytogenetic location 5p15. We have shown previously that this region of 5p is among the most common regions amplified in small cell lung tumor cell lines (35) and in primary small cell tumors (36). In addition, amplifications in this region are frequently found in high grade ovarian tumors (37). The amplifications identified to date are large and contain many genes. Our data will provide a first step toward determining whether

FIG. 5. ZDS1 and ZDS2 overexpression can suppress the camptothecin hypersensitivity of a trf4 mutant. A 2-μm-based yeast genomic library was introduced into a trf4::HIS3 strain (CY1000), and rare camptothecin-resistant transformants were isolated. The suppressing library plasmids were isolated and analyzed by PCR, restriction digest, and sequencing. The genes identified include ZDS1 and ZDS2. Panel A, hypersensitivity of the trf4::HIS3 mutant to camptothecin under the conditions of the screen. Panel B, suppression of camptothecin hypersensitivity in trf4::HIS3 by overexpression of TRF4 (16-2), ZDS1 (23-2), and ZDS2 (26-2).

FIG. 6. Alignment of the TRF4 gene family. An alignment among six members of the TRF4 gene family was made using the Wisconsin sequence analysis package program “Pileup,” and the alignment was formatted using the program “Alscript.” The order of alignment from top to bottom is hTRF4-2, hTRF4-1, S. cerevisiae TRF4, S. cerevisiae TRF5, S. pombe TRF4, and C. elegans TRF4. The amino acid number corresponding to the S. cerevisiae TRF4 sequence is shown at the right of each row.

3 Eric Lander and Thomas James Hudson, unpublished data.
hTRF4-1 contributes to tumorigenesis or affects camptothecin sensitivity in tumors with 5p15 amplifications.

**DISCUSSION**

We have shown that *S. cerevisiae* cells deficient in either TRF4 or MCD1/SCC1, genes required for sister chromatid cohesion and mitotic chromosome condensation, are also hypersensitive to DNA damage caused by the antitumor agent camptothecin and MMS. The hypersensitivity to camptothecin killing in the trf4 mutant does not result from elevated expression of DNA topoisomerase I. The abundance of TRF4 is greatest during S phase, the cell cycle phase at which cells are most readily killed by camptothecin and the likely point at which sister chromatid cohesion is established. Suppression of camptothecin hypersensitivity in the trf4 mutant by gene overexpression resulted in the isolation of an additional *S. cerevisiae* member of the TRF4 gene family, TRF5, which is 55% identical and 72% similar to TRF4. Two genes that may influence higher order chromosome structure, ZDS1 and ZDS2, were also isolated. To begin to determine whether TRF4 function in tumor cells also influences camptothecin sensitivity we have isolated and sequenced two human TRF4 family members, hTRF4-1 and hTRF4-2. The hTRF4-1 gene maps to 5p15, a region that is frequently amplified in a variety of tumor types. The high degree of evolutionary conservation of the TRF4 family suggests that these proteins are likely to function similarly in all eukaryotes, and therefore amplifications of the hTRF4-1 gene in tumor cells may alter sensitivity to camptothecin in these tumors.

The primary lethal lesion generated by camptothecin is likely to be a replication-coupled double strand break (8). Two known repair pathways are important in the repair of DNA double strand breaks: homologous recombination and direct joining of nonhomologous ends. Mutations in the homologous recombination pathway cause dramatic hypersensitivity to MMS, a phenotype also observed in the *trf4* mutant. Thus, TRF4 might be required for recombinational repair of camptothecin damage. Alternatively, TRF4 and MCD1/SCC1 may function in DNA repair but not as part of an Smc protein complex.

The proteins encoded by the trf4 suppressors ZDS1 and ZDS2 are 38% identical over their nearly 1,000 amino acid lengths but do not otherwise contain informative primary sequence motifs. Screens in which these genes were identified previously include those designed to enhance replication origin function (34), to suppress defects resulting from histone mutations (33), and to suppress mutations in *CDC20*, a gene required for chromosome segregation. It has been suggested that these genes may have been found in different screens because of their ability to alter chromosome structure globally (34). This is consistent with the notion that higher order structure may be an important and largely unrecognized determinant of camptothecin toxicity.

Yeast has proven to be a powerful system to study the targets and repair of DNA damage caused by camptothecin and other antitumor agents (7–9). The fact that evolutionarily conserved cohesion proteins are required to repair DNA damage caused by camptothecin in yeast suggests that this will also be true in mammalian cells. The advent of clinical protocols that include camptothecin heightens the importance of understanding those cellular functions that affect cytotoxicity. Future studies on the functions of the human TRF4 genes hTRF4-1 and hTRF4-2, whose isolation is reported here, will address this directly.

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