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# Overexpression of a truncated human topoisomerase III partially corrects multiple aspects of the ataxia-telangiectasia phenotype

**(topoisomerase/DNA repair/genetic recombination)**

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**ABSTRACT** **Ataxia-telangiectasia (A-T) is a recessive human disease characterized by radiation sensitivity, genetic instability, immunodeficiency, and high cancer risk. We previously used expression cloning to identify CAT4.5, a human cDNA that partially suppresses multiple aspects of the A-T phenotype upon transfection into cultured cells. Sequencing CAT4.5 revealed a 1.1-kb intronic fragment followed by a related ORF of 2.5 kb that encodes the near full-length ORF for *hTOP3*, the first mammalian topoisomerase III to be identified. Endogenous expression of *hTOP3* was found in all human tissues tested. Both pCAT4.5 and an antisense *hTOP3* construct were able to inhibit spontaneous and radiation-induced apoptosis in A-T fibroblasts, whereas overexpression of a full-length *hTOP3* cDNA did not. We postulate that topoisomerase III may be deregulated in A-T cells and that CAT4.5 complements the A-T phenotype via a dominant-negative mechanism. Furthermore, functional correction of hyper-recombination in A-T cells by CAT4.5 supports the hypothesis that the hTOP3 topoisomerase is involved in the control of genomic stability, perhaps in concert with the Bloom or Werner syndrome DNA helicases.**

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by a pleiotropic phenotype that includes progressive cerebellar degeneration, immunodeficiency, premature aging, genetic instability, and a high incidence of cancer (1, 2). Heterozygote carriers also appear to be at increased risk for cancer, perhaps due to subtle defects in their responses to genetic damage (2). Cells from A-T homozygotes behave abnormally after exposure to ionizing radiation and radiomimetic drugs. The cells lack the normal radiation-induced $G_1$/S, S phase, and $G_2$/M cell cycle checkpoints and exhibit hypersensitivity to the clastogenic and cytotoxic effects of ionizing radiation, as manifested by increased chromosome aberrations, decreased colony survival, and excessive apoptosis (1). In normal cells, the p53 tumor suppressor protein mediates radiation-induced activation of both $G_1$/S cell cycle arrest and apoptosis (3). The finding that induction of p53 by ionizing radiation is impaired in A-T cells, together with evidence for multiple cell cycle checkpoint abnormalities and dysfunctional apoptosis in A-T, has led to proposals that the A-T protein might be a key component of a signal transduction network that mediates cellular responses to certain types of DNA damage (1, 4, 5).

These models are supported by sequence analysis of *ATM*, the gene responsible for A-T (6). The putative ATM protein shows similarities to the Tel1, Rad3, Mec1, and Mei41 eukaryotic cell cycle checkpoint proteins, all of which share a carboxyl-terminal kinase domain (7). It is not known at this time which proteins lie downstream or upstream of ATM in its signal transduction pathway. However, human cDNAs that complement the phenotypic defects of cultured A-T cells when constitutively overexpressed may encode new members of the ATM-dependent signal transduction network. Alternatively, complementing cDNAs may alter the production and/or repair of DNA lesions that trigger ATM-dependent responses. Identification of these genes and characterization of their potential roles in the ATM-dependent signal transduction pathway thus may yield insights as to the molecular pathology of the A-T defect and further our knowledge of how cells respond to DNA damage.

In an attempt to isolate both candidate A-T genes and secondary suppressors, we previously transfected A-T fibroblasts (AT5BIVA) with a human fibroblast cDNA library cloned into the episomal expression vector pRep5 (8). About 610,000 independent A-T cell clones stably transformed with members of the cDNA library were treated with the radiomimetic drug streptonigrin, and 9 unrelated cDNAs were recovered from 29 surviving streptonigrin-resistant clones. One of these cDNAs, CAT4.5, was recovered from two independent streptonigrin-resistant clones and found to complement several aspects of the A-T phenotype (8). For example, AT5BIVA fibroblasts transfected with pCAT4.5 demonstrate increased clonal survival after x-irradiation when compared with parental AT5BIVA cells (see Fig. 1*A*). pCAT4.5 can also suppress hyper-recombination in A-T cells, in that we found that AT5BIVA fibroblasts harboring the pCAT4.5 vector expressed a >20-fold reduction in their spontaneous rates of mitotic recombination (8). Finally, AT5BIVA fibroblasts transfected with pCAT4.5 lack the radioresistant DNA synthesis phenotype of their parent cell line (8).

We now demonstrate that the CAT4.5 insert is a 1.1-kb intronic fragment fused to a nearly full-length cDNA of the human topoisomerase III (*hTOP3*) gene recently described by Hanai *et al.* (9). Northern blot analysis indicates that *hTOP3* is expressed in multiple somatic tissues. Transfection of either the pCAT4.5 vector or a *hTOP3* antisense construct inhibited spontaneous and radiation-induced apoptosis in A-T fibroblasts. In contrast, transfection of A-T fibroblasts with an expression vector containing the full-length *hTOP3* cDNA caused increased spontaneous apoptosis and failed to suppress radiation-induced apoptosis. Taken together, our results suggest that CAT4.5 may suppress the A-T phenotype by acting as a dominant-negative topoisomerase III mutant and provide the first direct evidence that *hTOP3*, like its yeast homologue, is involved in maintaining genetic stability.

## MATERIALS AND METHODS

**Cell Culture, Survival, and Apoptosis Assays.** AT5BIVA (GM5849) was established from a complementation group D

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Abbreviation: A-T, ataxia-telangiectasia.
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A-T patient, GM847 from a patient with Lesch–Nyhan syndrome, and GM639 from a normal individual. All three are simian virus 40 (SV40)-transformed fibroblast lines obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Routine tissue culture, transfections, and irradiation of cells were carried out as previously described (8). Survival of irradiated single cells was quantitated after 10–14 days incubation by counting surviving colonies of >50 cells. Apoptosis was measured 0–96 hr after irradiation. Cells collected at each time point were stained with a combination of 4′,6-diamidino-2-phenylindole, calcein acetoxymethyl ester, and ethidium homodimer (EthD-1); examined by fluorescent microscopy; and scored as living, apoptotic, or necrotic based on their characteristic morphologies (10). Results from four separate experiments were pooled for the $\chi^2$ analyses of spontaneous apoptosis. A total of 2,744, 2,987, and 3,889 cells were examined for the parental A-T line, the *hTOP3* antisense clones, and the *hTOP3* sense clones, respectively.

**Cloning of *hTOP3* Expression Constructs.** The cDNA plasmid pCAT4.5 was selected from a human cDNA library cloned into pRep5 (8). Cloning of full-length *hTOP3* cDNA was accomplished by reverse transcription–PCR amplification of its 5′ end with primers FOR51-*Xba*I (5′-TCATCTAGAGCTTCCCCAGGATGCGCCGGCAGC-3′) and REV9 (5′-CCACCCCGCTCTAGAATGCTC-3′) and cloning of the resulting 1.7-kb amplification product into the *Xba*I site in Bluescript, followed by insertion of a 0.9-kb *Eco*RI fragment into the bacterial expression vector RSET-B, which contained the 3′ end of the *hTOP3* cDNA from pCAT4.5. The resulting full-length *hTOP3* cDNA was inserted in sense and antisense orientation into the expression vector pRep5 after double digestion with *Xho*I/*Hin*dIII and *Kpn*I/*Hin*dIII, respectively.

**Northern Blot Analyses.** Northern blot analysis was carried out using a multiple-tissue Northern blot (CLONTECH) and following the manufacturer's recommended protocols. Briefly, the blot was prehybridized for 5 hr at 42°C in 5× standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/10× Denhardt's solution/100 μg/ml denatured herring sperm DNA/50% formamide/2% SDS. A 3-kb fragment covering the CAT4.5 ORF was labeled by the random-priming method (RediPrime, Amersham) and added to fresh hybridization solution at $1.5 \times 10^6$ cpm/ml. Hybridization proceeded for 22 hr. The blot was washed in 2× standard saline citrate (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.05% SDS at room temperature for 40 min, followed by a 5-min wash at 50°C in 0.1× SSC/0.1% SDS. The blot was exposed at −80°C on Hyperfilm (Amersham) with two intensifying screens for 5 days. Control hybridization was carried out using a labeled β-actin probe ($10^5$ cpm/ml) and a 5-day exposure.

**Sequence Analysis and Comparison.** The complete insert of the plasmid pCAT4.5 (4.3 kb) was sequenced on one strand by primer walking using *Taq* FS DNA polymerase and fluorescent dideoxy terminators in a cycle sequencing method. The resulting DNA fragments were electrophoresed and analyzed using an automated Applied Biosystems 373A Stretch DNA sequencer. The first sequence was derived from a primer complementary to the pRep5 vector. Critical regions (e.g., at the start of the ORF) were confirmed by sequencing the opposite strand. Computer analysis was performed with the assistance of the Wisconsin Genetics Computer Group (GCG) programs; the ORF was determined by using MAP, and homology searches of DNA and derived amino acid sequences were done using FASTA.

**Genomic CAT4.5 Clones and Sequence.** Genomic clones for the CAT4.5 locus were obtained by screening a cosmid library constructed from flow-sorted chromosome 17 DNA (11) using a 3-kb probe covering the CAT4.5 ORF. Among six positive cosmids, c155D9 was found to contain the most 5′ part of the CAT4.5 ORF after hybridization to a 0.6-kb probe of the 5′ region. The reverse primer 5′-CAGGCTCGGTCAGGTTTTCAC-3′, located at nucleotide 1245 in the CAT4.5 ORF, was used for sequencing on the cosmid template in the 5′ direction.

## RESULTS

**pCAT4.5 Inhibits Radiation-Induced Apoptosis in A-T Fibroblasts.** We previously demonstrated that A-T cells transfected with pCAT4.5 were partially suppressed for radiosensitivity, as measured by colony survival (Fig. 1*A*). We recently found that the sensitivity of A-T fibroblasts to the cytotoxic effects of ionizing radiation is due to an unusually low threshold for late-onset apoptosis (10), suggesting that pCAT4.5 might block the cytotoxic effects of ionizing radiation on A-T cells by interfering with radiation-induced apoptosis. To test this hypothesis, fibroblast cultures were irradiated and assayed for morphologically apoptotic cells. Transfection of the A-T fibroblast line AT5BIVA with the pCAT4.5 vector was associated with a decrease in the proportion of cells undergoing apoptosis after exposure to 5 Gy when compared with either parental AT5BIVA cells (Fig. 1*B*) or pRep5 vector-transfected AT5BIVA controls (data not shown).

**Sequencing CAT4.5 Reveals an ORF of 2.5 kb Encoding *hTOP3*.** To identify the etiology of its complementing activity, we sequenced the cDNA insert of pCAT4.5. An ORF of 2.5 kb was found encoding a protein that is identical to amino acids 141–976 of the recently described hTOP3 protein (9), except for two conserved amino acid changes (Asp → Asn) at amino acid positions 434 and 717 of the full-length protein (Fig. 2). The CAT4.5 ORF thus resembles the near full-length *hTOP3* coding sequence, lacking only the 5′ portion that encodes the first 140 amino acids. Translation beginning at the first in-frame ATG of the CAT4.5 ORF (amino acid 153) would result in a protein of 694 amino acids. Based on sequence homology to other topoisomerases, amino acid 208 of the CAT4.5 ORF would be considered the active-site tyrosine (12). The size of the entire CAT4.5 ORF, as predicted by computer analysis, was confirmed by *in vitro* transcription and translation of a construct encoding amino acids 18–847 of the CAT4.5 ORF, which had been placed in-frame behind a vector-derived ATG start codon (data not shown). The CAT4.5/*hTOP3* sequence contains a 1.3-kb region with strong homology to the *Saccha-*

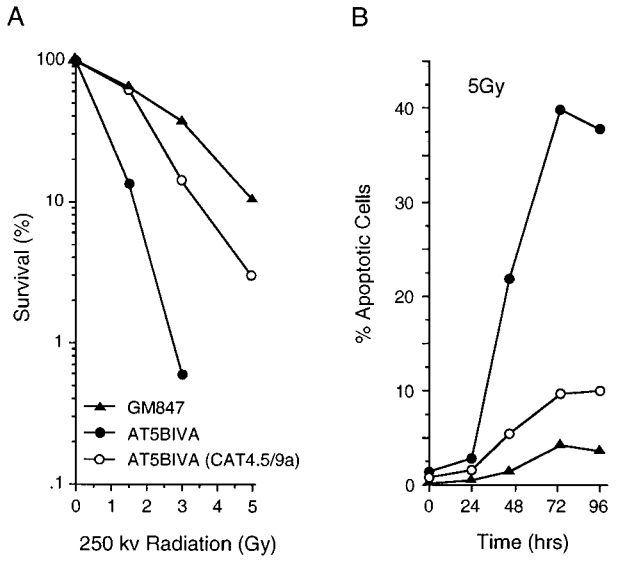


FIG. 1. Phenotypic complementation of A-T fibroblasts by pCAT4.5. (*A*) Colony survival of irradiated control cells (GM847), parental A-T cells (AT5BIVA), and A-T cells transfected with pCAT4.5 [AT5BIVA(CAT4.5/9a)]. (*B*) Induction of apoptosis by x-irradiation (5-Gy x-rays).

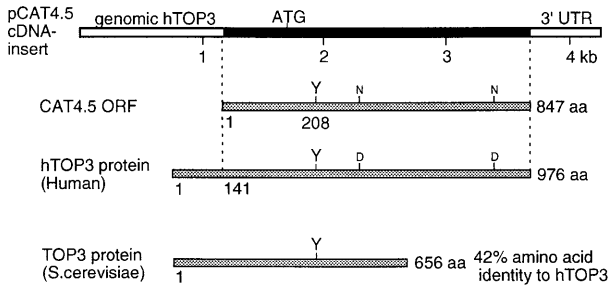


FIG. 2. Organization of pCAT4.5. The ORF starts at nucleotide 1,140, with the first in-frame ATG start codon at nucleotide 1,600. The derived amino acid sequence is identical to amino acids 141–976 of human TOP3 protein except for two conserved amino acid changes at positions 434 and 717 of hTOP3. The hTOP3 protein shares 42% amino acid identity with the yeast TOP3 protein. Nucleotides 1–1,139 upstream of the CAT4.5 ORF represent genomic sequence of the human *TOP3* gene. Y, active-site tyrosine; UTR, untranslated region.

*romyces cerevisiae* topoisomerase gene *TOP3* (13) at the nucleotide level (57% identity) and amino acid level (42% identity) (Fig. 2). *TOP3* encodes a prokaryotic-like type I DNA topoisomerase that is thought to be involved in maintaining genomic stability (14–16). The homology region between CAT4.5 and yeast *TOP3* shares common motifs with the *Escherichia coli* topoisomerases type I and III (both with 47% amino acid similarity), encoded by topoisomerase genes *topA* (17) and *topB* (18), respectively.

**CAT4.5 Contains Genomic *hTOP3* Sequence Upstream of the ORF.** CAT4.5 has previously been mapped to chromosome 17p11 by fluorescence *in situ* hybridization (8). Genomic clones containing the CAT4.5 ORF were obtained by screening a cosmid library constructed from flow-sorted chromosome 17 DNA (11) with a 3.0-kb probe spanning the whole ORF. The resulting positive cosmids mapped to the critical deletion interval for Smith–Magenis syndrome, a multiple-malformation syndrome associated with mental retardation and delayed growth (unpublished work). Among six positive cosmids, c155D9 was found to contain the most 5′ part of the CAT4.5 ORF after hybridization to a 0.6-kb probe of the 5′ region. To extend the ORF in the 5′ direction, the cosmid c155D9 was therefore used as a template for further sequence analysis. Sequencing from a reverse primer located at nucleotide 1,245 in the ORF and subsequent primer walking revealed that the derived cosmid sequence is identical to the first 1,140 bp of CAT4.5. CAT4.5 thus appears to be a partially spliced version of a *hTOP3* cDNA that contains intronic *hTOP3* sequence in front of the ORF (Fig. 2).

**hTOP3 RNA Is Constitutively Expressed in Multiple Normal Tissues.** To examine *hTOP3* expression, a probe spanning the entire CAT4.5 ORF was hybridized to a Northern blot containing mRNA from different human tissues. Three distinct hTOP3 transcripts of about 7.2, 6, and 4 kb were detected at low abundance in all tissues (Fig. 3). Control hybridizations with an actin probe confirmed near equal mRNA amounts per lane and demonstrate that *hTOP3* is expressed to a similar low level in all tissues examined. [The 1.5-kb band in the skeletal muscle lane is known to be a false-positive as it has consistently appeared in multiple hybridizations using different probes (unpublished data)].

**Transfection of *hTOP3* Constructs Affects Apoptosis in A-T Cells.** As shown in Fig. 1*B*, expression of CAT4.5 in A-T cells is associated with inhibition of radiation-induced apoptosis. In addition, the proportion of spontaneous apoptotic cells is lower in cultures of CAT4.5-transfected A-T cells than in their corresponding parental controls (data not shown). To examine the mechanism of complementation by the truncated *hTOP3* cDNA encoded by CAT4.5, pRep5 derivatives containing a full-length *hTOP3* cDNA in both sense and antisense orientations were generated and stably transfected into AT5BIVA A-T fibroblasts. The proportion of spontaneously apoptotic cells was significantly higher in cultures of *hTOP3* sense-transfected A-T cells than in cultures of the parent A-T line (6.17% vs. 3.21%; $\chi^2 = 30.1$; $P < 0.0001$). At the same time, cultures of AT5BIVA fibroblasts transfected with the *hTOP3* antisense vector had significantly fewer spontaneously apoptotic cells than their parent line (1.91% vs. 3.21%; $\chi^2 = 9.8$; $P < 0.002$).

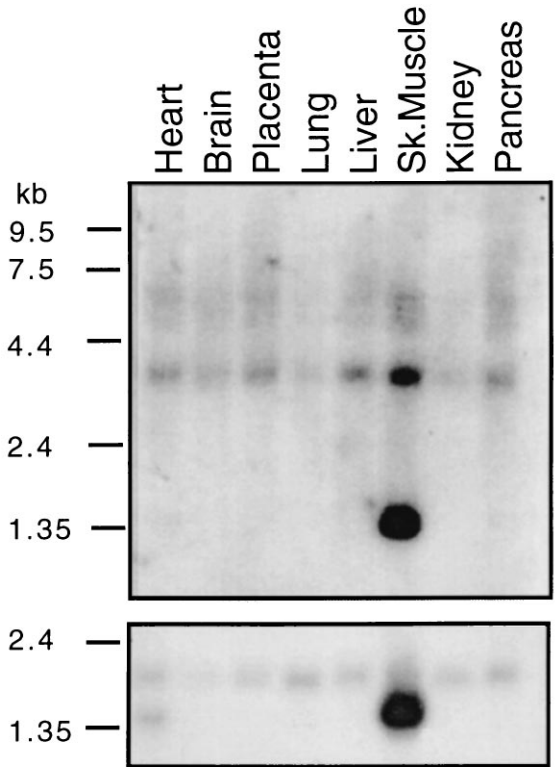


FIG. 3. Northern analysis of hTOP3 mRNA levels (*Upper*) and $\beta$-actin mRNA levels (*Lower*) in different human tissues.

Antisense *hTOP3*-transfected A-T clones exhibited less apoptosis than their parents after exposure to 3 Gy of $\gamma$-irradiation, whereas the hypersensitivity of A-T cells to radiation-induced apoptosis did not appear to be appreciably affected by transfection with the *hTOP3* sense construct (Fig. 4). Initial results suggest that resistance to radiation-induced apoptosis expressed by *hTOP3* antisense-transfected AT5BIVA cells is associated with an improvement in their clonal survival after irradiation, whereas neither the sense nor the antisense *hTOP3* constructs affect the clonal survival of the normal cell line GM639 (data not shown).

## DISCUSSION

We conclude that CAT4.5 contains genomic and coding sequence for a human topoisomerase III gene, based on strong sequence homologies to the *S. cerevisiae TOP3* gene, the *E. coli topB* gene, and the recently described *hTOP3* gene. Furthermore, overexpression of a truncated hTOP3 protein partially corrects multiple aspects of the A-T phenotype, including spontaneous and radiation-induced apoptosis as well as hyperrecombination, a defect which also is seen in yeast *top3* and bacterial *topB* mutants (19).

Northern blot analysis indicates that *hTOP3* is constitutively expressed in multiple somatic tissues. The ability of CAT4.5 to suppress the radiation sensitivity of A-T cells suggests that the ubiquitously expressed *hTOP3* gene affects the cell's response to DNA damage and provides new support for the proposal

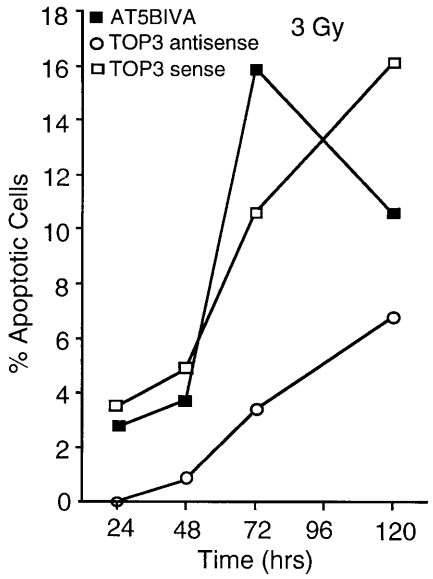


FIG. 4. Induction of apoptosis by 3-Gy γ-irradiation in parental A-T cells (AT5BIVA) and A-T cells transfected with antisense *hTOP3* vector or sense *hTOP3* vector. The numbers of apoptotic cells after irradiation were calculated after subtraction of the corresponding nonirradiated control of each time point and represent the mean values of four independent experiments.

that topoisomerases are involved in the repair and processing of DNA damage (20–22). There are several possible explanations for the suppression of radiosensitivity by pCAT4.5. The underlying defect in A-T now is thought to be an inability to trigger cell cycle checkpoints and other cellular functions in response to certain types of DNA damage (1, 4, 5, 10, 23). If *hTOP3* lay downstream of *ATM* in this signal transduction network, *hTOP3* regulation might be defective in A-T cells. Experimental modulation of topoisomerase III activity resulting from pCAT4.5 transfection might then allow irradiated A-T cells to activate damage responses despite a defective *ATM* gene, thus preventing the triggering of p53-mediated apoptosis, the principal cause of radiosensitivity in A-T cells (10).

Arguing in favor of this possibility, we previously demonstrated a >2-fold enhancement in the amount of the CAT4.5 mRNA expressed in A-T vs. normal fibroblasts (8). Taken together with our identification of CAT4.5 as *hTOP3*, these results suggest that basal levels of topoisomerase III activity are elevated in A-T cells. In this context, the ability of pCAT4.5 to suppress the radiosensitivity of A-T cells without affecting the radiosensitivity of normal cells may be the result of pCAT4.5 directing expression of a truncated hTOP3 protein that acts in a dominant-negative manner so as to reduce the overall level of topoisomerase III activity in A-T cells toward normal. Alternatively, even normal levels of hTOP3 protein activity may be detrimental to $ATM^{-/-}$ cells, thereby contributing to the A-T phenotype. In this case, pCAT4.5 transfection could partially suppress the A-T phenotype by depressing topoisomerase III activity to subnormal levels.

Such dominant-negative mechanisms of complementation are supported by our finding that overexpression of an antisense-*hTOP3* construct was able to block radiation-induced apoptosis in A-T cells. In addition, a full-length *hTOP3* construct failed to protect A-T cells from radiation-induced apoptosis, arguing against the possibility of complementation by a truncated but fully active version of *hTOP3* encoded by pCAT4.5. Further evidence for a dominant-negative mechanism of complementation is provided by the observation that both pCAT4.5 and the *hTOP3* antisense vector suppressed spontaneous apoptosis in A-T cells, whereas the *hTOP3* sense vector enhanced spontaneous apoptosis.

Rather than playing a downstream role in an ATM-dependent signal transduction network, it is possible that hTOP3 may function independently of ATM in the processing and repair of DNA damage. In this model, changes in DNA topology mediated by altering endogenous topoisomerase III activity would prevent the triggering of apoptosis in A-T fibroblasts by DNA damage, perhaps by facilitating access to spontaneous and induced DNA lesions by repair complexes or otherwise promoting the correct repair of damaged DNA by ATM-independent means. Alternatively, CAT4.5 overexpression may confer radioresistance by preventing misrepair and/or the processing of DNA lesions into forms that might activate the cell death program in A-T cells.

Our identification of CAT4.5 as a human homologue of the *S. cerevisiae TOP3* topoisomerase lends new support to the concept that diverse species, from *E. coli* to *Homo sapiens*, use topoisomerases to help maintain genome stability. Eukaryotic topoisomerase III first was identified in *S. cerevisiae* by its ability to suppress mitotic recombination between repetitive sequences. The phenotype of *top3* mutant yeast includes increased intrachromosomal recombination, a high frequency of aneuploidy, slow growth, and telomere instability (13, 14). Sequence analysis of yeast *TOP3* revealed considerable structural homology to *E. coli* topoisomerases I and III, suggesting that TOP3 is a type I topoisomerase. This has been confirmed by biochemical studies using the purified TOP3 enzyme that demonstrated type I DNA relaxation activity *in vitro* (24). Based on the hyper-recombination phenotypes of yeast *top3* mutants and bacterial *topB* mutants that lack a functional topoisomerase III, it has been proposed that a primary function of topoisomerase III is to suppress genetic recombination (25, 26).

Human topoisomerase III may function as an anti-recombination protein in a manner similar to TOP3. In yeast, the TOP3 protein interacts with the putative DNA helicase SGS1 *in vivo*, and *sgs1* mutants can suppress the hyper-recombination phenotype of *top3* mutants (15). At the same time, *sgs1* mutants also exhibit hyper-recombination (15). Taken together, these observations suggest that the TOP3 and SGS1 proteins act in concert to maintain genetic stability, and that imbalances in the relative proportion of TOP3 topoisomerase and SGS1 helicase activities can result in elevated rates of spontaneous mitotic recombination.

Overexpression of the CAT4.5 cDNA suppresses the high rates of intrachromosomal mitotic recombination seen in A-T fibroblasts (8, 27). In our dominant-negative model of CAT4.5 complementation, endogenous topoisomerase III activity is recombinogenic in $ATM^{-/-}$ cells, and pCAT4.5 transfection results in reduced hTOP3 activity, thus suppressing somatic hyper-recombination.

*SGS1* shares extensive sequence homology with *BLM*, the Bloom syndrome gene (28) and *WRN*, the Werner syndrome gene (29). Like A-T, these human genetic diseases are associated with hyper-recombination (28–31). The known physical interactions between the TOP3 and SGS1 proteins suggest that the human topoisomerase III may interact directly with the BLM and/or WRN DNA helicases, perhaps forming a complex with reverse gyrase activity. We propose that the hTOP3 topoisomerase, acting in concert with BLM and WRN helicases, helps maintain genetic stability in human cells. The mechanism by which these proteins work together to suppress recombination may be similar to that proposed for the TOP3 and SGS1 proteins (15); either they facilitate separation of the intertwined strands of recombination intermediates before they can proceed to completion and/or their actions bias the resolution of Holliday structures toward parental duplexes. The hTOP3 protein also may reduce recombination by pre-

venting the formation of Z-DNA and other recombinogenic structures.

Further studies to characterize the biochemical properties of human topoisomerase III, its possible interactions with the BLM and WRN helicases, as well as its role in correcting the A-T phenotype are needed. At the same time, characterizing this novel human topoisomerase may aid the development of new therapeutic drugs for the treatment of cancer.

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