Biochemical and Biophysical Research Communications **287,** 282–287 (2001)
doi:10.1006/bbrc.2001.5580, available online at http://www.idealibrary.com on IDEAL®

M Phase-Specific Association of Human Topoisomerase IIIβ with Chromosomes

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Received August 13, 2001

Two isoforms, 1 and 2, of human DNA topoisomerase IIIβ were expressed in HeLa cells as a fusion protein to the C-terminus of green fluorescent protein (GFP). The fusion protein of the isoform 1 was found to be localized to the nucleus, and to be associated with chromosomes during metaphase and anaphase. As yeast *top3* mutants are known to exhibit phenotypes indicative of defective chromosome segregation, the result suggests that the isoform 1 of the human enzyme may also be involved in chromosome segregation. Two-hybrid screening for interaction partners of the isoform identified three candidate genes: *CENP-F*, a gene encoding a centromere protein and two genes of no known function, one of which was novel. The GFP fusion of the isoform 2 was found in the cytoplasm, indicating the nuclear localization signal sequence in the isoform 1 is in the C-terminal part that is different between the two isoforms.

Key Words: **DNA topoisomerase; green fluorescent protein; chromosome segregation; M phase; nuclear localization.**

Loss of topoisomerase III in eukaryotes results in defects in cellular processes. Delay in late S/G_2, increase in frequency of recombination between repeats, and failure in sporulation have been observed in topoisomerase III mutants of yeast *Saccharomyces cerevisiae*, in which the gene for the eukaryotic enzyme (*TOP3*) was discovered (1–3). In yeast *Schizosaccharomyces pombe*, *TOP3* has been shown to be essential for viability and the lethality of *top3* mutation is likely to result from defective chromosome segregation as manifested by 'torn' chromosomes in dividing cells (4, 5). Such phenotypic severity has also been observed for mammalian *TOP3* counterparts, *TOP3*α and *TOP3*β. Studies using knock-out mice have shown that *TOP3*α is essential for cell proliferation (6) and that *top3*β−/− mutants have a reduced life span (7).

Eukaryotic topoisomerases III have been shown to be a feeble supercoil relaxation activity (8–11). This indicates that the enzyme needs a single-stranded region for interaction with DNA, a common characteristic of type-IA topoisomerases (12). Genetic and biochemical evidence showing interaction between topoisomerase III and helicases of the RecQ type has suggested that topoisomerase III acts on DNA dupleces separated by a RecQ-like helicase(s) (2, 5, 13–15). However, the biochemistry of the suggested action by the two enzymes has not been fully characterized. Thus, it remains a conundrum precisely how a loss of topoisomerase III activity leads to the phenotypes indicative of defective chromosome segregation.

Of the two human topoisomerases III hitherto known, the more recently identified β gene (*hTOP3*β) was initially identified in the immunoglobulin λ gene locus by a genome sequencing project (16). Analyses of its mRNAs have shown that the primary transcript is alternatively spliced into three species (17). The predicted protein products (hTOP3β) of the three, isoforms 1–3, are 862, 730, and 707 amino acids in length, respectively, and they share the N-terminal catalytic domain that is conserved among the type-IA topoisomerases (9, 11, 12, 17). The tissue specificity of mRNA abundance was found to be different for the messages (17). Therefore, it is expected that the isoforms are expressed differently to interact with different proteins or with different DNA structures by the use of the alternative C-terminal domains (17).

We chose to collect some cytological data in order to gain insight into biochemical and phenotypic correlates of topoisomerase IIIβ and also to find differences between the isoforms 1 and 2 of hTOP3β. For this purpose, we expressed hTOP3β in HeLa cells as a green fluorescent protein (GFP) fusion and examined the intracellular location of the fusion protein. The results, together with those of a two-hybrid screening for interaction partners of hTOP3β, are presented below.

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0006-291X/01 $35.00

MATERIALS AND METHODS

Vector construction for expression of GFP-hTOP3β fusions in HeLa cells. A cDNA encoding the full-length isoform-1 hTOP3β was obtained as an expressed sequence tag clone (H30621). The nucleotide sequence of the entire cDNA was determined by the dideoxy termination method on an AlfExpress sequencer (Pharmacia) and deposited to the GenBank/EMBL/DDBJ database (AF017146). A nucleotide at the position 2447 was missing in the clone, compared with the genomic DNA sequence in the database (16). A region comprising the position was replaced with a DNA fragment PCR-amplified from a cDNA library and containing the missing nucleotide. The active-site tyrosine at the 336th amino-acid position (TAC) was mutated to phenylalanine (TTC) by site-directed mutagenesis using PCR. The mutated protein-coding region was placed in between the *Hin*dIII and the *Bam*HI sites of pEGFP-C3 (Clontech). The *Hin*dIII site, AAGCTT, was followed by GGCCAGATCCGTAAC and then by the hTOP3β coding region, thereby fusing the GFP protein and the isoform-1 protein with a 13 amino acid linker between them. The plasmid was designated pEGFP-C3-hTOP3β-1. A DNA segment containing the last exon of the isoform 2 of hTOP3β was PCR-amplified from genomic DNA. In the downstream PCR primer, a *Bam*HI site, GGATCC, was placed immediately after the TGA termination codon. The segment was used to replace a 3′ part of the isoform-1 cDNA and generate a clone encoding the isoform-2 protein. A plasmid to express a GFP fusion of the isoform-2, pEGFP-C3-hTOP3β-2, was constructed by replacing a 3′ part of the *hTOP3β* gene of pEGFP-C3-hTOP3β -1. In the above cloning steps, the nucleotide sequence was confirmed for every PCR segment.

Transfection of HeLa cells with plasmids expressing GFP-hTOP3β. A 200 μl suspension containing $0.9-4.0 \times 10^5$ trypsinized HeLa cells was diluted into 2 ml of serum medium in a chamber slide (Lab-Tek, NUNC) and cultured for 24 h at 37°C. Transfection of the cells with pEGFP-C3-hTOP3β-1 or pEGFP-C3-hTOP3β-2 was carried out by the use of the Effectene transfection kit (Qiagen) according to the manufacturer's instruction.

Fluorescence microscopy. Twenty-four hours after transfection, the cells were washed with PBS and fixed with 10% formaldehyde for 10 min. After a wash with PBS, the cells were strained with 0.2 μg/ml DAPI, re-washed with PBS, and observed under a fluorescence microscopy (BX80, Olympus) equipped with a cooled CCD image system (SenSys and IP-Lab, Roper Scientific).

Two-hybrid screening. Two-hybrid screening in *S. cerevisiae* was carried out according to Vidal (18). A "bait" plasmid expressing a fusion of the Gal4-DNA binding domain and the full-length isoform-1 hTOP3β was constructed as follows. A DNA segment comprising the hTOP3β gene was initially placed downstream of the *Nde*I site of pAS1-CYH2, also a two-hybrid "bait" vector (19). The mutant version of *hTOP3β*, changing the active site tyrosine to phenylalanine, was used. Following the *Nde*I site was a sequence, GCCAGATCCGTAACC, before the initiation codon of *hTOP3β*. A DNA fragment comprising a part of the gene for the Gal4-DNA binding domain and the *hTOP3β* gene was swapped into an equivalent part of pPC97. A *S. cerevisiae* strain MaV103 (18) was used for screening. Cells were transformed with the "bait" plasmid obtained as above and designated pPC97-hTOP3β-1, and then with a human T-cell cDNA library cloned in pPC86 kindly provided by M. Vidal. The cells were plated on SC-His-Trp-Leu plates containing 25 mM aminotriazole. After 3 days, the colonies were transferred to nitrocellulose membranes and assayed for β-galactosidase activity with X-gal. Library plasmids were recovered from the positives and re-tested for production of β-galactosidase in MaV103 transformed with pPC97-hTOP3β-1.

RESULTS AND DISCUSSION

Localization of hTOP3β Fused to GFP

hTOP3β genes for the isoforms 1 and 2 were fused downstream of the *GFP* gene in a mammalian expression vector pEGFP-C3 and expressed transiently in HeLa cells. To avoid possible complication due to DNA topoisomerase activity of the expressed gene products, the active-site tyrosine was mutated to phenylalanine. Cells transfected with the vector were observed under a fluorescence microscope to examine the location of the fusion protein. The results are shown in Fig. 1. In contrast to GFP expressed alone (panel B), GFP fused with the isoform 1 of hTOP3β was localized to the nucleus (panel E), clearly indicating that the protein is nuclear in accordance with its capacity of DNA transaction. Although some intranuclear structure was observable by phase-contrast microscopy (panel D), no further correlation of the fluorescence distribution to the structures or the distribution of DNA stained with DAPI (panels E and F) was seen. Although most of the observed cells were in the interphase, it was feasible to find cells in the M phase. In the prophase, with the breakdown of nuclear envelope (panel G), the fluorescence of the fusion protein spread through the cell (panel H). A this stage, the fluorescence was not associated with the condensed chromosomes but it was observed where there was not DNA (panel I). In the metaphase, the fluorescence of the fusion protein was observed in a restricted area in the cell (panels J and K) and more or less associated with DNA (panels K and L). This association was observed also in the anaphase (panels M-O). These results show that the isoform 1 of hTOP3β intimately associated with metaphase chromosomes.

Studies in yeasts have suggested that topoisomerase III is likely to resolve some structural problem of DNA incurred by RecQ-like helicase (2, 13). Failure to do so appears to be the cause of phenotypes exhibited by *top3* mutants especially in chromosome segregation (1–5). Thus, the present result seems to indicate that the isoform 1 of hTOP3β is also involved in resolution of structural problems at the time of chromosome segregation. We are aware that interpretation of the present data needs caveats. For instance, the level of expression of the GFP-hTOP3β fusion may have well exceeded the normal cellular level of hTOP3β protein, which has not been known. Fusing GFP to the N-terminus of hTOP3β may have hindered its proper interaction with other nuclear components Nevertheless, the present results at least demonstrate that there is some change in the chromosome structure that is detected by hTOP3β.

In contrast to the isoform 1, the isoform 2 was located to the cytoplasm (Fig. 1, panels P–R). In the isoform 2, the C-terminal 155 amino acids in the isoform 1 are replaced with a stretch of 23 amino acids

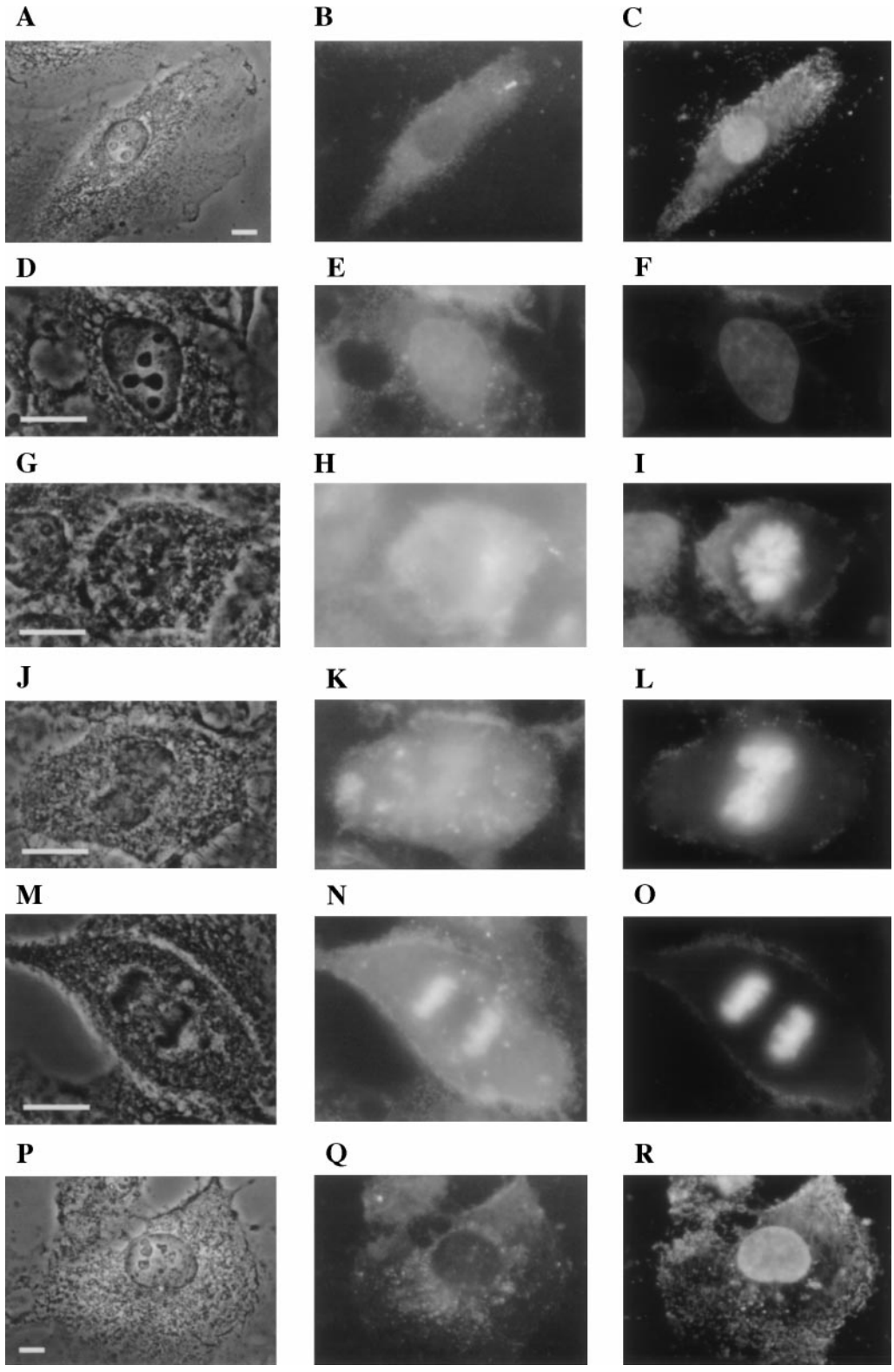

FIG. 1. HeLa cells producing fusion protein of hTOP3β and GFP. Twenty-four hours after transfection with a plasmid expressing a gene for the fusion protein, cells were fixed with formaldehyde, stained with DAPI, and observed under a fluorescence microscope. (A–C) Cells producing non-fusion GFP; (D–O) the isoform 1 of hTOP3β fused to the C-terminus of GFP; (P–R) the isoform 2 of hTOP3β fused to the C-terminus of GFP. Cells in A–F and P–R were in the interphase; G–I, in the prophase; J–L, in the metaphase; M–O, in the anaphase. A, D, G, J, M, and P are phase-contrast; B, E, H, K, N, and Q, GFP fluorescence; C, F, I, L, O, and R, DAPI fluorescence. The bars are 10 μm.

TABLE 1

Positives Obtained in a Two-Hybrid Screening for hTOP3β-Interacting Proteins

Protein name	Database entry	Region of cDNA insert corresponding to the database entry	Number of identical clones	Color of X-gal product
CENP-F	NM_005196	5613rd–6407th	7	Dark
KIAA1564	AB046784	2857th–4396th	4	Dark
Novel	AB063180	1st–1758th	5	Dark
Epithelin	HSEPIT1	ca. 1450 bp from 728th	3	Medium
C1q	NM_001212	ca. 1100 bp from 91st	5	Medium
Fibulin	NM_006329	ca. 1350 bp from 769th	1	Pale
snRNP-B	HSSNRNPB	ca. 800 bp from 243rd	4	Pale

(17). Search for nuclear localization signal sequences by PSORT II (20) identified one at a stretch, RNPRRP-KDK, from the 845th to the 853th amino acid in the isoform 1. Since the stretch is lost in the isoform 2, the microscopy result indicates that the nuclear localization signal of the isoform 1 is likely to be the identified stretch. It has been shown that a 2.8-kb message encodes the isoform 1 and a 3.8-kb one encodes the isoform 2 (17). The shorter message was found to be more prominently expressed in testis, heart, and skeletal muscle, whereas the longer one in thymus, kidney, and pancreas (17). It is not known how the tissue-specificity is related to the difference in cellular localization between the two isoforms, as the function(s) of the isoform 2 in the cytoplasm is not known. Since the bacterial DNA topoisomerase III has been shown to be active in making a covalent complex with RNA (21), the possibility of the isoform 2 being an RNA topoisomerase can be entertained.

Screening for Putative Interaction Partner(s) of hTOP3β

To identify a factor(s) that associates the isoform-1 hTOP3β with chromosomes, two-hybrid screening in *S. cerevisiae* was carried out. The isoform 1 fused to the DNA-binding domain of Gal4 was used to screen 1×10^7 T-cell cDNA clones fused to the transcription activation domain of Gal4. 29 positives were obtained. Nucleotide sequencing identified seven cDNA species

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GCGGGGTGGGACACCCCCATGCGGGATGAAGACTACGAGGGTGACATGGAGGAGGAGGTCGAGGAGGAAGAAGAGGGTGTGTTCTGGACCAGTGGCATGAGCAGGTCCAGCTGGGACAAC  120
 A  G  W  D  T  P  M  R  D  E  D  Y  E  G  D  M  E  E  E  V  E  E  E  E  E  G  V  F  W  T  S  G  M  S  R  S  S  W  D  N    40
ATGGACTATGTGTGGGAGGAGGAGGACGAGGAGGAAGACCTGGACTACTACTTGGGGGACATGGAGGAGGAGGACCTGAGGGGGGAGGATGAGGAGGACGAGGAGGAAGTGCTGGAGGAG  240
 M  D  Y  Y  V  W  E  E  E  D  E  E  E  D  L  D  Y  Y  L  G  D  M  E  E  E  D  L  R  G  E  D  E  E  D  E  E  E  V  L  E  E   80
GTTGAGGAAGAGGATCTAGACCCCGTCACCCCACTGCCCCCGCCTCCAGCCCCTCGGAGGTGCTTCACATGCCCTCAGTGCCGAAAGAGCTTTCCTCGGCGGAGCTTCCGCCCCAACCTG  360
 V  E  E  E  D  L  D  P  V  T  P  L  P  P  P  P  A  P  R  R  C  F  T  C  P  Q  C  R  K  S  F  P  R  R  S  F  R  P  N  L  120
CAGCTGGCCAATATGGTCCAGGTGATTCGGCAGATGCACCCAACCCCTGGTCGAGGGAGCCGCGTGACCGATCAGGGCATCTGTCCCAAACACCAAGAAGCCCTGAAGCTCTTCTGCGAG  480
 Q  L  A  N  M  V  Q  V  I  R  Q  M  H  P  T  P  G  R  G  S  R  V  T  D  Q  G  I  C  P  K  H  Q  E  A  L  K  L  F  C  E  160
GTAGACGAAGAGGCCATCTGTGTGGTGTGCCGAGAATCCAGGAGCCACAAACAGCACAGCGTGGTGCCATTGGAGGAGGTGGTGCAGGAGTACAAGGCCAAACTGCAGGAGCACGTGGAA  600
 V  D  E  E  A  I  C  V  V  C  R  E  S  R  S  H  K  Q  H  S  V  V  P  L  E  E  V  V  Q  E  Y  K  A  K  L  Q  E  H  V  E  200
CCACTGAGGAAGCACCTGGAGGCAGTGCAGAAGATGAAAGCCAAGGAGGAGAGGCGAGTGACAGAACTGAAGAGCCAGATGAAGTCAGAGCTGGCAGCGGTGGCCTCGGAGTTTGGGCGA  720
 P  L  R  K  H  L  E  A  V  Q  K  M  K  A  K  E  E  R  R  V  T  E  L  K  S  Q  M  K  S  E  L  A  A  V  A  S  E  F  G  R  240
CTGACACGGTTTCTGGCTGAAGAGCAGGCAGGGCTGGAACGGCGTCTCAGAGAGATGCATGAAGCCCAGCTGGGGCGTGCGGGAGCCGCGGCTAGTCGCCTTGCAGAACAGGCCGCCCAG  840
 L  T  R  F  L  A  E  E  Q  A  G  L  E  R  R  L  R  E  M  H  E  A  Q  L  G  R  A  G  A  A  A  S  R  L  A  E  Q  A  A  Q  280
CTCAGCCGCCTGCTGGCAGAGGCCCAGGAGCGGAGCCAGCAGGGGGGTCTCCGGCTGCTCCAGGACATCAAGGAGACTTTCAATAGGTGTGAAGAGGTACAGCTGCAGCCCCCAGAGGTC  960
 L  S  R  L  L  A  E  A  Q  E  R  S  Q  Q  G  G  L  R  L  L  Q  D  I  K  E  T  F  N  R  C  E  E  V  Q  L  Q  P  P  E  V  320
TGGTCCCCTGACCCGTGCCAACCCCATAGCCATGACTTCCTGACAGATGCCATCGTGAGGAAAATGAGCCGGATGTTCTGTCAGGCTGCGAGAGTGGACCTGACGCTGGACCCTGACACG 1080
 W  S  P  D  P  C  Q  P  H  S  H  D  F  L  T  D  A  I  V  R  K  M  S  R  M  F  C  Q  A  A  R  V  D  L  T  L  D  P  D  T  360
GCTCACCCGGCCCTGATGCTGTCCCCTGACCGCCGGGGGGTCCGCCTGGCAGAGCGGCGGCAGGAGGTTGCTGACCATCCCAAGCGCTTCTCGGCCGACTGCTGCGTACTGGGGGCCCAG 1200
 A  H  P  A  L  M  L  S  P  D  R  R  G  V  R  L  A  E  R  R  Q  E  V  A  D  H  P  K  R  F  S  A  D  C  C  V  L  G  A  Q  400
GGCTTCCGCTCCGGCCGGCACTACTGGGAGGAGCCTAAAGAACCCTCCTGGCCTCCAGCTCAGCCTTCTCTCACCTACTATGTCTGTCCAACAGACCGGCCAGAATTTAGCTTCACTTGA 1320
 G  F  R  S  G  R  H  Y  W  E  E  P  K  E  P  S  W  P  P  A  Q  P  S  L  T  Y  Y  V  C  P  T  D  R  P  E  F  S  F  T  *  440
GAGAGATCTGGAATGGTCGCCATGATTGAAACCACGCACCATTACATCATCATTACATTAATTACATCAACATAAATTATTTCTTCCCCCTTCCCTTTTCCAGCACTCAACCAAGGAGCA 1440
AAGCTCATCCCACCCCACACCCCTCCCAGGTCTGCTCACTGCCAGGCTCCTCTCCCCTTTGTTCAGTGGAGCTGGCTTTTCTCCCAGCCCCTTTCCATGCCTTTCACTCCATTTGGCAAG 1560
CTCTGAGGGGGAGCCTGGGGACGGGTTTGGGTCCCCAGGAGGAGAGCCTTGGGTATAATCTATTTTTCTAGGAGCCTCTTGCCTTGTCACTTGCAGCTTTCGCCCTCTGCTTTGATGGCT 1680
GAGGTGAACTCATGTTCTTTGGGAAAAGGGAAGGCGTGCTGTGGAAATAAAATGTTTATTTGCTTCTAAAAAAAAAAA                                          1758
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FIG. 2. The nucleotide and the predicted amino acid sequences of a novel gene identified in a two-hybrid screening for hTOP3β interaction partners. The 5′-end sequence including the initiation codon is likely to be missing. A B-box domain identified by PROSITE (143rd to 184th amino acid) is underlined. The nucleotide sequence has been deposited to GenBank/EMBL/DDBJ (AB063180).

in the positives, as summarized in Table 1. Of the proteins encoded by the cDNAs, epithelin, C1q, and fibulin are not nuclear, and therefore their interaction with hTOP3β is fortuitous. Although snRNP-B is a nuclear protein, its involvement in splicing disqualifies it as a factor linking hTOP3β to chromosomes. Compared with these four, the remaining three, CENP-F, KIAA1546, and a hitherto uncharacterized gene, showed darker blue of the X-gal hydrolysis product (Table 1). When checked further for interaction specificity by the yeast two-hybrid system, the three genes did not activate the reporter β-galactosidase gene in conjunction with the control genes tried, *Rb* (22) and *dDP* (23). CENP-F is a protein associated with kinetochores during mitosis. It is accumulated in G2 and M phase and quickly degraded upon completion of mitosis. The protein is 3210 amino acids in length and is predicated to be a coiled-coil protein interspersed with non-coil domains (24). The region corresponding to the present cDNA fragment (Table 1) fell into one of the non-coil domains. KIAA1546 is a gene identified in a sequencing project for large mRNAs. No results of functional analysis of the gene or a homologue have been reported. Nucleotide sequencing of the new cDNA revealed that it encoded a new member of the B-box family of proteins (Fig. 2; 25, 26). Since many B-box containing proteins are involved in transcription control and since a closely related protein, RET finger protein, has been shown to be present in the nuclear matrix (25), the gene identified here is also likely to be associated with chromosomes. A Blast search of the current data-base indicated that the present cDNA clone lacked the very 5′ end segment and also that there were alternatively spliced species of the transcript.

It has been shown that eukaryotic topoisomerases III interact with RecQ-like helicases (2, 13–15). Five *recQ*-like genes have been identified in the human: *RECQ1* (27, 28), *BLM* (29), *WRN* (30), *RECQ4* (31), and *RECQ5* (15). Interaction between BLM and hTOP3α has been reported (14). Besides, RecQ5 has been reported to interact both with hTOP3α and hTOP3β (15). The present results of a two-hybrid screening suggest that interaction partners of human topoisomerases III may not be limited to helicases of the RecQ type. Interestingly, one of the candidates is a kinetochore-associated protein and another is likely a protein associated with chromosome. Further analysis of the interactions, and of the structure of the newly discovered gene, is in progress.

ACKNOWLEDGMENTS

We thank Dr. Marc Vidal for providing with a two-hybrid screening system. We thank Dr. Akira Shimamoto for providing with DNA materials and Dr. Masao Ono for instructions on cell culture methods. We are grateful to Dr. James C. Wang for collaboration in characterization of the hTOP3β gene and for communicating results prior to publication. We also thank Mayumi Tanaka for assistance in DNA sequencing. This work was supported by a grant from the Japanese MECSST (13680751).

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