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Research Article

A human homolog of the yeast gene encoding tRNA 2′-phosphotransferase: cloning, characterization and complementation analysis

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Abstract. The *Saccharomyces cerevisiae TPT1* gene plays a role in removing the 2′-phosphate from ligated tRNA during the maturation of pre-tRNA. Here we reported the cloning and characterization of the human *TRPT1* gene as a homolog of yeast *TPT1*. The *TRPT1* gene is located at human chromosome 11q13 and encodes a polypeptide of 253 amino acids. BLAST searches with its amino acid sequence revealed the ubiquitous occurrence of *TRPT1* homologs and their functional relationships with the presence of the DUF60/KptA domain. Northern analysis demonstrated that the gene is primarily expressed in heart and skeletal muscle, with lower or undetectable levels in other tissues studied. A plasmid-shuffling experiment showed that the human *TRPT1* gene could complement the *tpt1* mutation in *S. cerevisiae.*

Key words. Human *TRPT1*; *Saccharomyces cerevisiae TPT1*; tRNA splicing; DUF60 domain; Northern blot; functional complementation; plasmid shuffle.

tRNA splicing occurs ubiquitously in *Eubacteria*, Archaea and Eukarya as a required step in the maturation of intron-containing tRNA precursors [1–3]. In *Eubacteria*, all introns are self-splicing, involving only two RNA-catalyzed phosphotransfer reactions. By contrast, the splicing of tRNA introns in Eukarya and Archaea is enzyme mediated [4]. Details of the splicing reaction process are best understood in the yeast *Saccharomyces cerevisiae*. In yeast, an endonuclease is required at first to excise the pre-tRNA at its two specific sites to yield three fragments: the linear intron and two exons [5–7]. With the presence of GTP and ATP, the two exons are then ligated by tRNA ligase to form a mature sized tRNA with a 2′-phosphate group at the splicing junction [8–10]. During the next step, the splicing junction 2′-phosphate is removed by the tRNA 2′-phosphotransferase (Tpt1p) and transferred to NAD to produce ADP-ribose 1″, 2″-cyclic phosphate (Appr > p) and nicotinamide [11–13]. Finally, Appr > p is converted into ADP-ribose 1″-phosphate (Appr-1″ p) by a specific cyclic phosphodiesterase (CPDase) [14, 15].

The splicing pathway present in the yeast *S. cerevisiae* is also conserved in vertebrates [3, 16], although most of the tRNA splicing in these organisms appears to involve another RNA ligase that joins the two tRNA exons together directly without formation of the 2′-phosphate [17, 20]. The activity of endonuclease, tRNA ligase and tRNA 2′-phosphotransferase has been described in *Xenopus* and humans [3,16–19]. Thus the tRNA-splicing pathway of yeast could occur in *Xenopus* and humans.

The yeast *TPT1* gene encoding tRNA 2′-phosphotransferase is essential for vegetative growth in *S. cerevisiae* and is highly conserved [4, 13, 21, 22]. tRNA 2′-phosphotransferase activity in humans was first reported

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by Zillmann et al. [3, 16]: HeLa cell nuclear extracts were demonstrated to efficiently remove the 2′-phosphate created at the splicing junction to produce mature tRNA. In the present study, we report the cloning and characterization of the human tRNA 2′-phosphotransferase gene designated *TRPT1* according to the HUGO Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature).

Materials and methods

Yeast strains and media

The *S. cerevisiae* strain Fy1679-06c (*Matα ura3-52 leu2∅1 trp1∅63 his3∅200 GAL2*) was used in this study. Culture media for yeast growth were prepared as described in Burke et al. [23].

Plasmids

pHKB147, a cloning vector 5.8 kb in size, was constructed previously in this laboratory. It carried the yeast *URA3* gene and a centromere sequence of yeast chromosome II (*CEN II*) for manipulation in yeast, and the ampicillin resistance gene (Ap^r) for selection in *Escherichia coli*. The plasmid pDBLeu was from Invitrogen, containing a centromere fragment of yeast chromosome VI (*CEN VI*) for copy number maintenance and the *LEU2* marker gene for selection in yeast. Plasmid pFB2 was a derivative of pDBLeu whose GALDNA-binding domain was removed by religation after being cut with *Sal*I and *Hind*III. For the construction of the disruption cassette, plasmid pRS426 [24] was used.

Cloning of the human *TRPT1* gene

The human *TRPT1* gene was cloned from the 18-week-old human fetal brain library constructed in a modified pBluescript II SK (+) vector during large-scale cDNA sequencing. The modified vector was constructed by introducing a DNA fragment containing *Sfi*IA (5′-GGCCATTATGGCC-3′) and *Sfi*IB (5′-GGCCGCCTCGGCC-3′) recognition sites between the *Eco*RI and *Not*I of pBluescript II SK (+) (Stratagene). Following the protocol of the SMART PCR cDNA library construction kit (Clontech), double-stranded cDNA from human fetal brain mRNA (Clontech) was cut and ligated into the *Sfi*I-digested modified vector. The cDNA sequencing and assembly were performed as described elsewhere [25].

Bioinformatics analysis

DNA sequence homology search and comparison were accomplished by the BLAST Sever at NCBI (http://www.ncbi.nlm.nih.gov/BLAST), and protein sequence alignment was performed using the online service Clustalw (http://www.expasy.ch) and GeneBee (http://www.genebee.msu.su).

Northern blot analysis of *TRPT1* expression

The human multiple tissue Northern blot membrane containing 1.0 μg poly(A)$^+$ RNA per lane was from Clontech. The encoding sequence of about 0.76 kb of full-length *TRPT1* cDNA was labeled with ^{32}P-dCTP by random-primer extension using the Rediprime TM II labeling system (Amersham Pharmacia Biotech). The blot was then hybridized with ^{32}P-labeled cDNA probe at 68 °C overnight, washed under stringent conditions and autoradiographed at −70 °C for 2 weeks.

Plasmid construction for gene disruption and functional complementation

In plasmid construction for *TPT1* gene expression, the open reading frame (ORF) sequence of *TPT1* was amplified with primers P_1F (5′-ACCCGGGATGCGCCAGGTACTACAA-3′) and P_1R (5′-AGCGGCCGCTTATATCTTTTCGAGCGG-3′) using total DNA of *S. cerevisiae* S288C as the template. The PCR product was digested with *Sma*I and *Not*I and followed by cloning into pFB2 (*CEN VI LEU2*) to generate plasmid pFB2-*TPT1* (*CEN VI LEU2* P_{ADH}-*TPT1*) in which the *TPT1* ORF was under the control of the yeast ADH promoter. After *Bam*HI digestion of pFB2-*TPT1*, the fragment containing the *TPT1* ORF was released and cloned into the *Bam*HI site of pHKB147 to generate the plasmid pHT8 (*CEN II URA3* P_{ADH}-*TPT1*).

To construct the plasmid for human *TRPT1* gene expression, the complete coding sequence of the gene was amplified by PCR using primers P_2F (5′-ACCCGGGATGAACTTCTCTGGAGGA-3′) and P_2R (5′-TGCGGCCGCTTATTGTTGGATCCTCCT-3′) and the product was ligated with the *Sma*I-*Not*I double-digested plasmid pFB2. The resulting plasmid was designated pFB2-*TRPT1* (*CEN VI LEU2* P_{ADH}- *TRPT1*).

For construction of the disruption cassette containing the *HIS3* gene to replace the yeast chromosomal *TPT1* gene, the yeast *HIS3* gene was amplified from *S. cerevisiae* S288C DNA with primers P_3F (5′-AGGATCCGCTGCACGGTCCTGTTC-3′) and P_3R (5′-TGAATTCG TTTCTTG GAGCTGGGAC-3′) and inserted into the plasmid pRS426 digested with *Bam*HI and *Eco*RI to produce plasmid pRS-*HIS3*. For occurrence of homologous recombination between disruption cassette and yeast chromosome, two other PCR fragments corresponding to the 5′- and 3′-flanking region of yeast *TPT1* gene were amplified with primers P_4F (5′-TGCGGCCGCACGAGTTGAATTAAGCT-3′), P_4R (5′-AGGATCCCAGTCATTCTGCTCAA-3′) and P_5F (5′-AGAATTCATCA AGCTCAACTTT TA-3′), P_5R (5′-AGTCGACAGTTAGACTGCT ACACCA-3′), respectively, and cloned at the 5′ and 3′ end of the *HIS3* gene in pRS-*HIS3*. The resultant

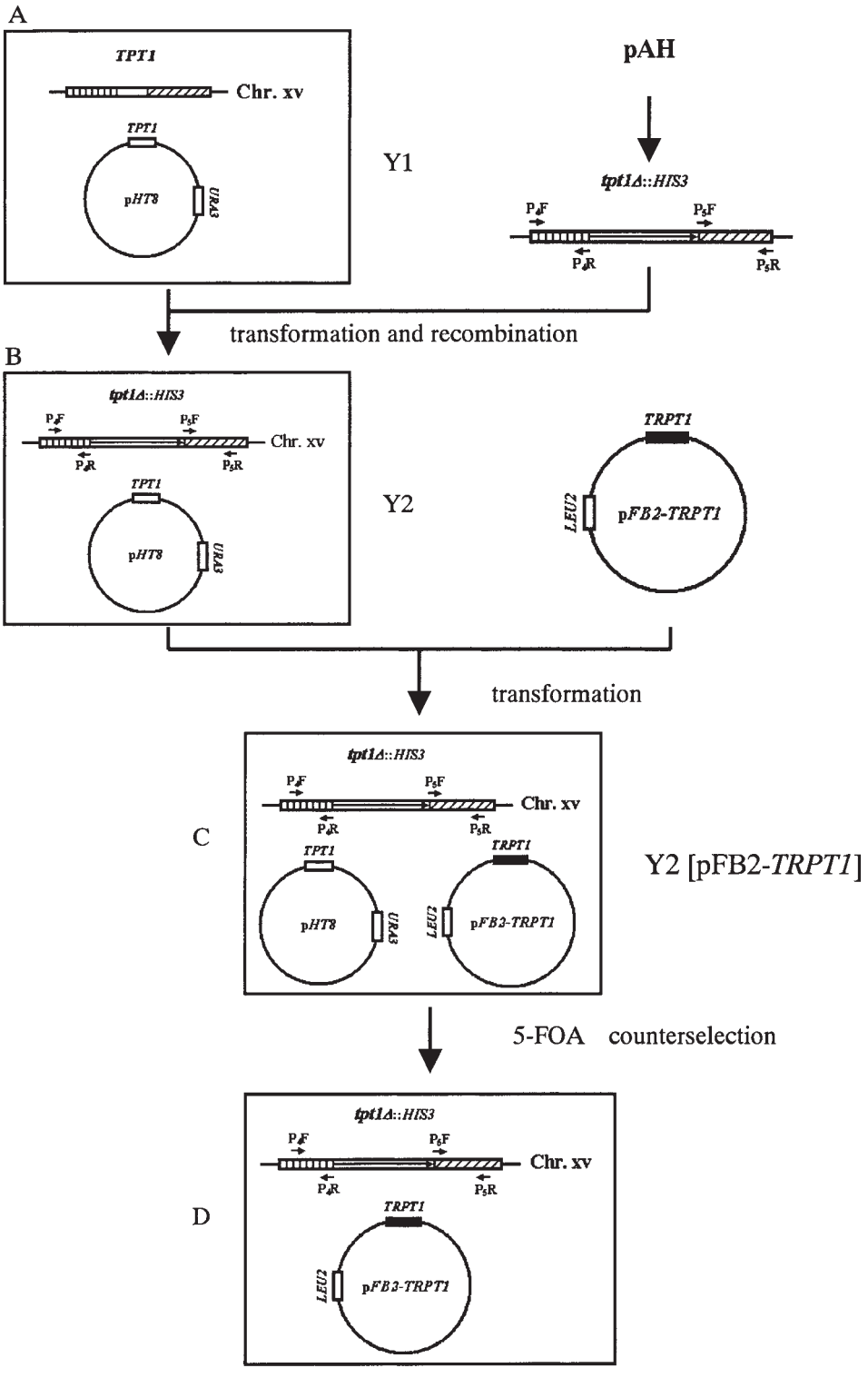

Figure 1. Gene disruption and plasmid shuffle for functional complementation. This diagram outlines the procedures for the *TRPT1* gene complementation experiment, leaving out the positive and negative controls. The panels are not drawn to scale. (*A*) Strain Y1. (*B*) Strain Y2 with the disrupted *tpt1* gene was generated by homologous recombination after transformation with disruption cassette. (*C*) *tpt1*-deleted strain Y2 transformed with the *TRPT1* gene for complementation. (*D*) Strain Y2 with the wild-type *TPT1* phenotype was rescued on a 5-fluoro-orotic acid (5-FOA) plate by the *TRPT1* gene borne on plasmid pFB-*TRPT1* after the loss of the *URA3*-containing plasmid pHT8.

plasmid containing the disruption cassette for recombination was designated pAH. Finally, the validity of all the above constructs was confirmed by sequencing and restriction site mapping.

Functional complementation of the yeast lethal *tpt1* mutant

A complementation experiment was carried out using the plasmid-shuffling technique [26]. Whose four steps are shown in figure 1. (A) *S. cerevisiae* strain Fy1679-06c was transformed with plasmid pHT8, plated onto SC-ura medium, and strain Y1 was obtained by selection of Ura^+ transformants; (B) Strain Y1 was then transformed by the disruption cassette prepared from plasmid pAH, and Ura^+ His^+ transformants were selected on an SC-his-ura plate. From these transformants, a yeast *TPT1*-defective mutant Y2 {*Matα ura3-52 leu2∅1 trp1∅63 his3∅200 GAL2 tpt1∅::HIS3* [pHT8 (*CEN II -URA3*)]} was selected and its validity checked by PCR. (C) The Y2 mutant was transformed with each of the plasmids pFB2-*TRPT1*, pFB2-*TPT1* and pFB2, and plated onto SC-his-ura-leu medium. Three kinds of transformants, Y2 [pFB2-*TRPT1*] (fig. 1C), Y2 [pFB2-*TPT1*], and Y2 [pFB2] were obtained. (D) For determination of *tpt1* mutant complementation, each of the transformants was grown separately in liquid SC-his-leu medium for 24 h. Cells were then harvested and plated on the same medium supplemented with 5-fluoro-orotic acid (5-FOA, 1 mg/ml) for Ura^- counterselection. After incubation for 3–5 days at 30 °C, colony formation was checked.

Results

Cloning and sequence analysis of *TRPT1*

We cloned a full-length cDNA of the human *TRPT1* gene from a human fetal brain cDNA library. Its nucleotide sequence and the predicted amino acids have been submitted to GenBank (accession number AY211494).

The entire *TRPT1* was 963 bp in length and contained an ORF from nt 171 to 932, encoding a predicted protein of 253 amino acids. An in-frame stop codon was found at the 5′ untranslated region (5′-UTR) and a consensus polyadenylation signal (aataaa) at the 3′-UTR. Thus we conclude that it is a genuine ORF in vivo. The theoretical molecular mass and isoelectric point (pI) of the deduced translation product were estimated as 27.8 kD and 10.13, respectively.

A BLAST search in the human genome database showed that the gene consisted of eight exons and seven introns and was mapped to contig NT37773.1, located at chromosome 11q13 spanning approximately 2.5 kb. The first exon contains the first 161-bp 5′-UTR of the cDNA, and the second exon contains 9 bp 5′-UTR and 75 bp of the coding region encoding 25 amino acids residues of TRPT1 protein. All exon-intron boundaries, with only the exception of the 5′-donor site of the first intron, were compatible with GT-AG rules [27] (table 1).

Comparisions of humanTRPT1 with its homologs from other organisms

A BLAST search with the protein sequence against the nonredundant (nr) database of GenBank revealed that a DUF60/KptA functional domain existed in the middle region from residue 25 to residue 203 of this protein. Homologs with different degrees of homology to TRPT1 were widely found in various organisms by the search. Comparing with TRPT1, there was 83, 40, 29, 29 and 28% identity to *Mus musculus* (NP_705825), *S. cerevisiae* (NP_014539), *E. coli* (P39380), *Fusobacteriam nucleatum* (NP_603999) and *Methanopyrus kanderi* AV19 (NP_614717) respectively, at the amino acid level. Functionally uncharacterized proteins with a DUF60 domain derived from *Rattus norvegicus* (XP_219539.1), *Schizosaccharomyces pombe* (NP_594515.1), *Arabidopsis thaliana* (NP_182058.1), *Streptomyces coelicolor* (NP_628137.1), *Clostridium perfringens* (BAB79850.1) and *Archaeoglobus fulgidus* (NP_070382.1) shared 79% identity (83% similarity), 42% identity (60% similarity), 36% identity (49% similarity), 35% identity (56% similarity), 32% identity (54% similarity) and 27% identity (43% similarity), respectively, with the TRPT1

Table 1. Exon-intron organization of the human *TRPT1* gene.

Exon	Size (bp)	5′-splice donor	3′-splice acceptor	Intron	Size (bp)
1	161	CCTTGGgcaagtctggatgct	tcactgccccccac**ag**GTCTTA	1	178
2	84	GAACAG**gt**gcgcgcctcctccc	cgctgggttctttc**ag**GACCGA	2	202
3	84	GAGCTG**gt**aagtaggggccttg	ccctgcctgcccac**ag**ATGGCT	3	528
4	172	CTGCAG**gt**cggggtgaggggac	ctacccgctcaacc**ag**GTACCT	4	79
5	178	TCAGTG**gt**cagtgccctccctt	ccagctgtctctgc**ag**GCATGC	5	200
6	57	TGGCAG**gt**gagtctggacaaag	ctggctctgtcagc**ag**ATGGAA	6	73
7	112	CTACCC**gt**gagaaccaccaccc	ctcttgtctttatc**ag**GAAAGC	7	130
8	121				

Exons are shown in uppercase, introns in lowercase. Donor and acceptor sequences are shown in bold.

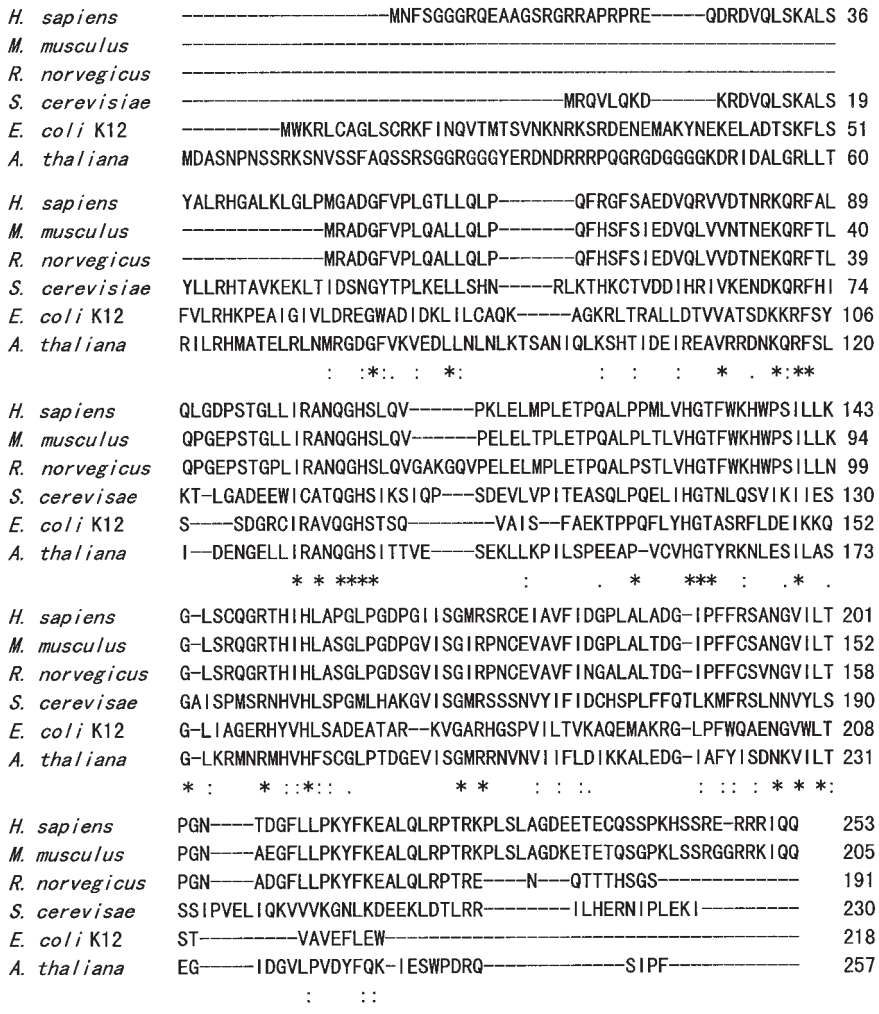

Figure 2. Alignment of the human *TRPT1* gene product and some of its homologs. Numbers on the right refer to the residue number of homologs. Gaps are introduced to maximize alignment. *, : and . indicate identical, strongly and weakly similar residues, respectively. Proteins aligned are: *H. sapiens*; *M. musculus*, NP_705825; *R. norvegicus*, Loc293704; *S. cerevisiae,* NP_014539; *E. coli* K12, NP_418751; *A. thaliana*, NP_182058.

protein sequence. Some with lower homology to TRPT1 were also observed in the Database. Figure 2 shows the alignments of the human TRPT1 protein and some sequences of its homologs. From the alignment, a large number of amino acid residues are identical among these proteins.

Human *TRPT1* gene expression pattern

The expression pattern of the human *TRPT1* gene examined by Northern blot analysis is shown in figure 3. Of the 12 human tissues investigated, one band of about 1.0 kb was detected in most of the tissues except spleen, small intestine, lung and peripheral blood leukocytes. The highest level of *TRPT1* transcript was found in skeletal muscle, and the expression level was also high in heart. Intermediate levels of the transcript were observed in kidney and liver, with low levels in thymus and placenta. However, in brain and colon, almost nothing was detectable.

Complementation analysis

The result of BLASTP searches revealed that the human TRPT1 protein shares 40% identity and 55% similarity with yeast Tpt1p in their entire amino acid sequences. This led us to ask whether the human *TRPT1* gene can functionally complement the yeast *tpt1* mutation. To address this question, a complementation experiment was conducted using plasmid shuffling, since the yeast *tpt1* mutant is lethal. As described in Materials and methods, we successfully constructed a *tpt1* mutant strain Y2 with homologous recombination between the disruption cassette and chromosome XV, in which the chromosomal *TPT1* ORF was replaced by the *HIS3* gene. Then we used this mutant strain Y2 to investigate the complementation of yeast *tpt1* by human *TRPT1* gene. As shown in figure 4, the yeast mutant strain Y2 transformed with either yeast *TPT1* or human *TRPT1* could grow on the 5-FOA plate, while the transformants of plasmid pFB2 were un-

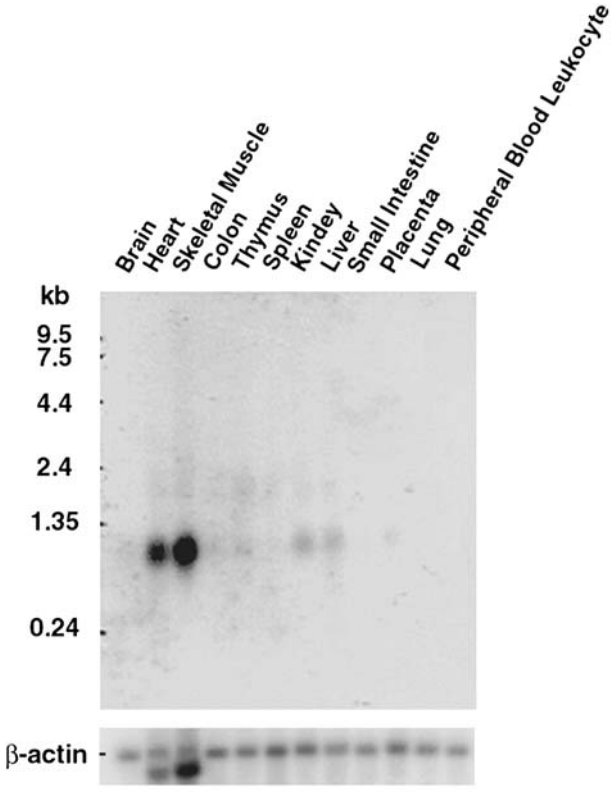

Figure 3. Expression profile of *TRPT1* mRNA in various human adult tissues. Molecular size markers are indicated on the left. *β-actin* is shown as a control.

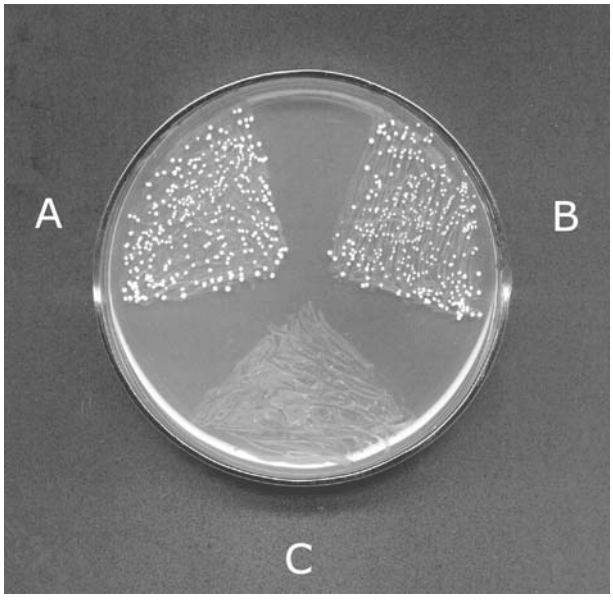

Figure 4. Functional complementation of the yeast *tpt1* mutant phenotype by *TRPT1*. Mutant strain Y2 was transformed with plasmid pFB2-*TPT1* containing wild -type yeast *TPT1* (*A*), plasmid pFB2-*TRPT1* containing human *TRPT1* (*B*) and plasmid pFB2 (*C*), as described in Materials and methods.

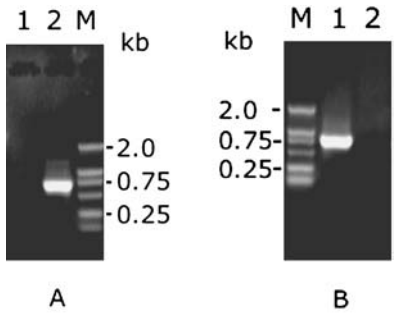

Figure 5. Agarose gel electrophoresis of PCR products for confirmation of *TRPT1* complementation. The primers used for amplification were P_2F-P_2R (lane 1) and P_1F-P_1R (lane 2) and the plasmids as template were isolated from colonies in figure 4A (*A*) and figure 4B (*B*), respectively.

able to grow on the same plate. This result demonstrated that the human *TRPT1* gene could functionally complement the yeast *tpt1* mutant phenotype. The inviability of the strain transformed with pFB2 truly showed the lethality of the *tpt1* mutation for the vegetative growth of *S. cerevisiae*. To further confirm our study, plasmids were isolated from the colonies on the 5-FOA plate and amplified by PCR with primers specific to *TRPT1* (P_2F-P_2R) and *TPT1* (P_1F-P_1R). Analysis by agarose gel electrophoresis of PCR products showed that the DNA band specific to *TPT1* (ca. 0.70 kb in length) had appeared only in the PCR product which was amplified by using plasmid DNA isolated from colonies shown in figure 4A as template (fig. 5A, lane 2). In contrast, the DNA band specific to *TRPT1* (ca. 0.76 kb in length) appeared only in the PCR product which was amplified by using plasmid DNA isolated from colonies shown in figure 4B as template (fig. 5B, lane 1). The 0.70-kb and 0.76-kb fragments were also verified by sequencing and were in agreement with *TPT1* and *TRPT1*, respectively. Comparing the results in figure 5A and B, we believe that the plasmid pHT8 with the *TPT1* gene was cured from the strain Y2 [pFB2-*TRPT1*] (fig. 1C) after 5-FOA counterselection and the correction of the lethal phenotype of the *tpt1* mutation in figure 4B was really due to the existence of the *TRPT1* gene carried on plasmid pFB2-*TRPT1*.

Discussion

In yeast and other eukaryotes, splicing of tRNA precursors via a 3′,5′-phosphodiester, 2′-phosphomonesterforming pathway generates tRNA intermediates bearing a 2′-phosphate at the splicing junction. The 2′-phosphate is then transferred to NAD to form Appr>p by a specific 2′-phosphotransferase encoded by the *TPT1* gene [11, 13, 14]. The *TPT1* gene was first identified and cloned in yeast by Culver et al. [13] and McCraith and Phizicky [22]. In humans, the 2′-phosphotransferase activity capa-

ble of removing the 2′-phosphate formed at the splicing junction was first reported and characterized by Zillmann et al. [3, 16]. However, the gene that encodes the tRNA 2′-phosphotransferase remains unknown. During large scale sequencing of a human fetal brain library, we have, for the first time, cloned a human homolog of the yeast *TPT1* gene and designated it as *TRPT1*. Its 253-amino acid coding sequence is close to that of yeast *TPT1* (230 amino acids). Bioinformatic analysis of *TRPT1* and *TPT1* showed high homology for their amino acid sequences and the presence of a conserved DUF60/KptA domain in the middle section of TRPT1 and Tpt1p, indicating a possible functional closeness of the two genes. Using the plasmid-shuffling technique, the *TRPT1* gene was demonstrated to fully complement the yeast *tpt1* mutant phenotype. Therefore, human *TRPT1* is indeed a genuine homolog of the yeast *TPT1* gene, which codes for tRNA 2′-phosphotransferase.

BLAST analysis indicated that different proteins with high similarity to TRPT1 are widely distributed in a number of organisms from Archaea to Eukarya. To our surprise, all of them have the same pattern of DUF60/KptA in their protein sequences and many conserved amino acids are found in this domain (fig. 2), which strongly suggesting a functional relationships. The DUF60 domain is a distinguishing feature of this protein family and must have functional significance. Our study suggested that the DUF60 domain or DUF60-containing proteins are functionally, at least, related to the maturation of precursor tRNA. In addition, the ubiquitous occurrence of TRPT1 homologs in a wide range of organisms showed that *TRPT1* is evolutionarily conserved.

Northern blot analysis showed that the expression of the *TRPT1* gene differed among adult tissues. The detected band of about 1.0 kb in the blot was almost the same size as *TRPT1* cDNA, suggesting that our *TRPT1* gene indeed represents the full-length transcribed message in vivo. The differences in *TRPT1* mRNA expression raise the question whether the two splicing pathways of tRNA precursor exhibit tissue specificity. In other words, the splicing pathway of pre-tRNA with 2′-phosphate at the junction site might occur in some human tissues, while the regular 3′,5′-phosphodiester-forming splicing pathway may only occur in other tissues. More experiments are needed to evaluate this possibility.

In summary, we have cloned and characterized the human *TRPT1* as a homolog of the yeast *TPT1* gene. The *TRPT1* gene can functionally complement the yeast *tpt1* mutation and plays a role in transfering the 2′-phosphate during the maturation of human tRNA precursors. Northern blot showed the distribution of the *TRPT1* transcript in various tissues. To our knowledge, this is the first identification of the human tRNA 2′-phosphotransferase gene implicated in the splicing of pre-tRNA.

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