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Molecular Cloning, Identification and Characteristics of a Novel Isoform of Carbamyl Phosphate Synthetase I in Human Testis

Ran Huo, Hui Zhu, Li Lu, Lanlan Ying, Min Xu, Zhiyang Xu, Jianmin Li, Zuomin Zhou and Jiahao Sha*

Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, 210029, China

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A gene coding a novel isoform of carbamyl phosphate synthetase I (CPS1) was cloned from a human testicular library. As shown by cDNA microarray hybridization, this gene was expressed at a higher level in human adult testes than in fetal testes. The full length of its cDNA was 3831 bp, with a 3149 bp open reading frame, encoding a 1050-amino-acid protein. The cDNA sequence was deposited in the GenBank (AY317138). Sequence analysis showed that it was homologous to the human CPS1 gene. The putative protein contained functional domains composing the intact large subunit of carbamoyl phosphate synthetase, thus indicated it has the capability of arginine biosynthesis. A multiple tissue expression profile showed high expression of this gene in human testis, suggesting the novel alternative splicing form of CPS1 may be correlated with human spermatogenesis.

Keywords: A novel isoform of CPS1, Arginine biosynthesis, cDNA microarray, Human testicular library, Spermatogenesis

Introduction

Spermatogenesis is the main function of the testes. It is a well-characterized developmental process, from prospermatogonia to mature spermatozoon (Krawetz *et al*., 1999), regulated by programmed gene expression (Eddy, 1998). Studies on genes specifically expressed in the testes at different developmental stages may reveal new genes related to their function, especially spermatogenesis (McCarrey, 1998). In the seminiferous tubules of fetal testes there are only sertoli cells and undifferentiated spermatogonia cells. In adult testes spermatogenesis takes place in three major phases (i) proliferation and differentiation of spermatogonia, (ii) meiosis and (iii) spermiogenesis. The seminiferous tubules of adult human testes contain not only sertoli cells and spermatogenous cells, but also various spermatogenic cells. Due to these broad differences in histological structures, transcripts from fetal and adult testes can be used for comparison to identify the gene related to testis development and spermatogenesis.

Various approaches have been developed to obtain information on gene expression levels based on certain intensity measurements i.e., suppression subtractive hybridization (SSH) (Diatchenko *et al*., 1999), differential display technology (Liang and Pardee, 1992) and cDNA microarray (Diatchenko *et al*., 1999), etc. Of these, the cDNA microarray is a useful, high throughput method, which has been widely used in recent years. In our laboratory, using a human testis cDNA microarray, 386 unique genes differentially expressed between adult and embryo testes were identified. These genes may be related to testis development and spermatogenesis (detail information has been published in our laboratorys website). Herein, a gene coding a novel isoform of CPS1, which is more highly expressed in human adult than in embryo testes, is reported. Its characteristics and possible correlation with spermatogenesis are also discussed.

Materials and Methods

Samples In this study, testes from human adults (n = 2) and 6-month-old fetuses (n = 3) were collected from the deceased or naturally aborted. All the testis tissues were obtained under informed consent using forms approved by the Ethical Committee, Nanjing Medical University, P. R. China.

Preparation of human testis cDNA microarray 9,216 positive λ phage clones were randomly picked from the Human Testis Insert phage cDNA library (Hl5503U Clontech, Palo Alto, USA) and amplified by PCR. The PCR products were then spotted on a membrane to make a human testis cDNA microarray, as described in detail previously (Sha *et al*., 2002; Cheng *et al*., 2002).

*To whom correspondence should be addressed.
Tel: 86-25-86862908 Fax: 86-25-86862908
E-mail: shajh@njmu.edu.cn

Preparation of probe DNA using mRNA from human adult and embryo testes After homogenization of the testis tissues, the total mRNA was extracted using trizol RNA isolation protocol (Gibco BRL, Grand Island, USA), and quantified by UV spectrometry and electrophoresis. The PolyA+ mRNA was purified using an affinity column filled with poly (dT) resin (Qiagen, Hilden, Germany). The probes were prepared by incorporation of ^{33}P-labelled dATP in a reverse transcription reaction using 2 μl of the purified mRNA as the template, an oligo (dT) as the primer and M-MLV reverse transcriptase. Each labeling reaction was carried out with 200 μCi of α ^{33}P-dATP, according to the manufacturer's instruction (New Life Science, Boston, USA).

Screening of genes differentially expressed in the embryo and adult human testis The adult testis cDNA microarray was hybridized with ^{33}P-labelled embryo testis and adult testis probes nylon membranes spotted with cDNA fragments were prehybridized with 20 ml prehybridization solution (6 × sodium chloride-sodium citrate buffer (SSC), 0.5% SDS, 5 × Denhardt compound and 100 μg/ml denatured salmon sperm DNA ml^{-1}) at 68ºC for 3 h. Overnight hybridization with the ^{33}P-labelled cDNA from the testis samples was carried out in 6 ml hybridization solution (6 × SSC, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA ml^{-1}), followed by stringent washing with 20 ml wash solution (10% SSC, 0.5% SDS) at 65ºC for 1 h. The membranes were exposed to phosphor screen overnight and scanned using a FLA-3000A fluorescent image analyser (Fuji Photo Film, Tokyo, Japan). The radioactive intensity of each spot was linearly scanned with a 65,536 gray-grade and a pixel size of 50 microns, and interpreted using the array gauge software (Fuji Photo Film, Tokyo, Japan). After subtraction of the background from an area where no PCR product was spotted, the clones with an intensity density > 10 were considered as positive signals. Hybridization data would be considered invalid if there was a difference of > 1.5-fold in the intensities of any of the 12 control spots for the same control cDNA between arrays. When the standard difference of the two dots for each DNA sample was < 0.3, the signals could be accepted by the software. The hybridization intensities of corresponding dots in adult and fetus were compared. If the difference in the spot intensity values in the adult and fetus was more than threefold higher or lower, the corresponding genes were considered as differentially expressed. Sequence identification and data analysis were then performed (Cheng *et al*., 2002).

Analysis of tCPS1 expression in different tissues After sequence identification and analysis, a novel isoform of the human carbamoyl-phosphate synthetase 1 (hCPS1) gene, named tCPS1, was found. To determine the tissue distribution of tCPS1, primers specific to the 5end of tCPS1, which overpasses an intron, were designed and amplified with cDNAs of sixteen tissues (testis, skeletal muscle, liver, pancreas, brain, lung, kidney, heart, placenta, spleen, thymus, prostate, ovary, small intestine, colon (mucosal lining) and leukocytes) (Human Multiple Tissue cDNA (MTC) Panel I and II kit, Cat#K1420-1 and K1421-1, Clontech). The forward and reverse primers were: 5' AAAGAACCACAAGGAA TG 3' (nt 91-nt 108) and 5' GACTGATGTAATGGTGGTAG 3' (nt 350-nt 369), respectively. The desired fragment was 279 bp. The plasmid containing the tCPS1 gene was used as a positive control for the PCR amplification. 20 μl PCR reaction mixture contained: 2 μl 10 × reaction buffer, 1.5 μl $MgCl_2$(25 mmol/L), 1.5 μl dNTP (2 mmol/L), 0.15 μl taq polymerase (5 U/ul), 2 μl cDNA template, 1 μl each primer 5 pmol/μl) and 11 μl water. The PCR conditions were as follows: denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 1 min. The first cycle had a denaturation period of 5 min. The last cycle had an extension period of 7 min. 35 cycles were performed. The PCR products were analyzed by electrophoresis.

RT-PCR was used to confirm the hybridization of the cDNA microarray. Human embryo (6 months) and adult testes cDNA that had been amplified in our laboratory were used as templates. The cDNA preparation was similar to that of the probe DNA described above, but using non-labeled dNTPs in the reverse transcription reaction. The primers and PCR procedures have been described above. β-actin mRNA was amplified as a control.

Results

Differential expression of tCPS1 in adult and fetal testes The results of the hybridization with fetal and adult testes probes indicated that in adult testes a clone, named tCPS1, was highly expressed. The hybridization intensities in the adult and fetal testis were 19.38 and 4.23, respectively (Fig. 1), with the expression level in adult testis being about 4.59 folds higher.

Features of cDNA and deduced protein The full cDNA length of tCPS1 was 3831 bp, with a 3149 bp open reading frame from nt 496-3642, which encodes a 1049 amino acids protein with a predicted molecular weight of 11.6 kDa and isoelectric point of 5.67 (Fig. 2). The methionine at nt 496-498 was almost certainly the site of initiation as there was an upstream stop code TAG at nt 304-306. The cDNA sequence of this clone was deposited in GenBank, with the accession number AY317138. A Blast search in the human genome database localized the tCPS1 gene to human chromosome 2. NT_005403.13/HS2_5560, a clone in chromosome 2 (length

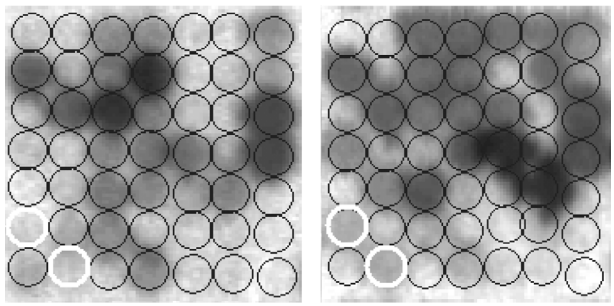

Fig. 1. Partial cDNA hybridization images showing the differential expression of tCPS1 in fetal and adult testes. Thick white rings indicate tCPS1 cDNA. The intensities in six-month fetal and adult testes were 4.23 and 19.38, respectively. The hybridization intensity was 4.59 folds higher in adult than fetal testes.

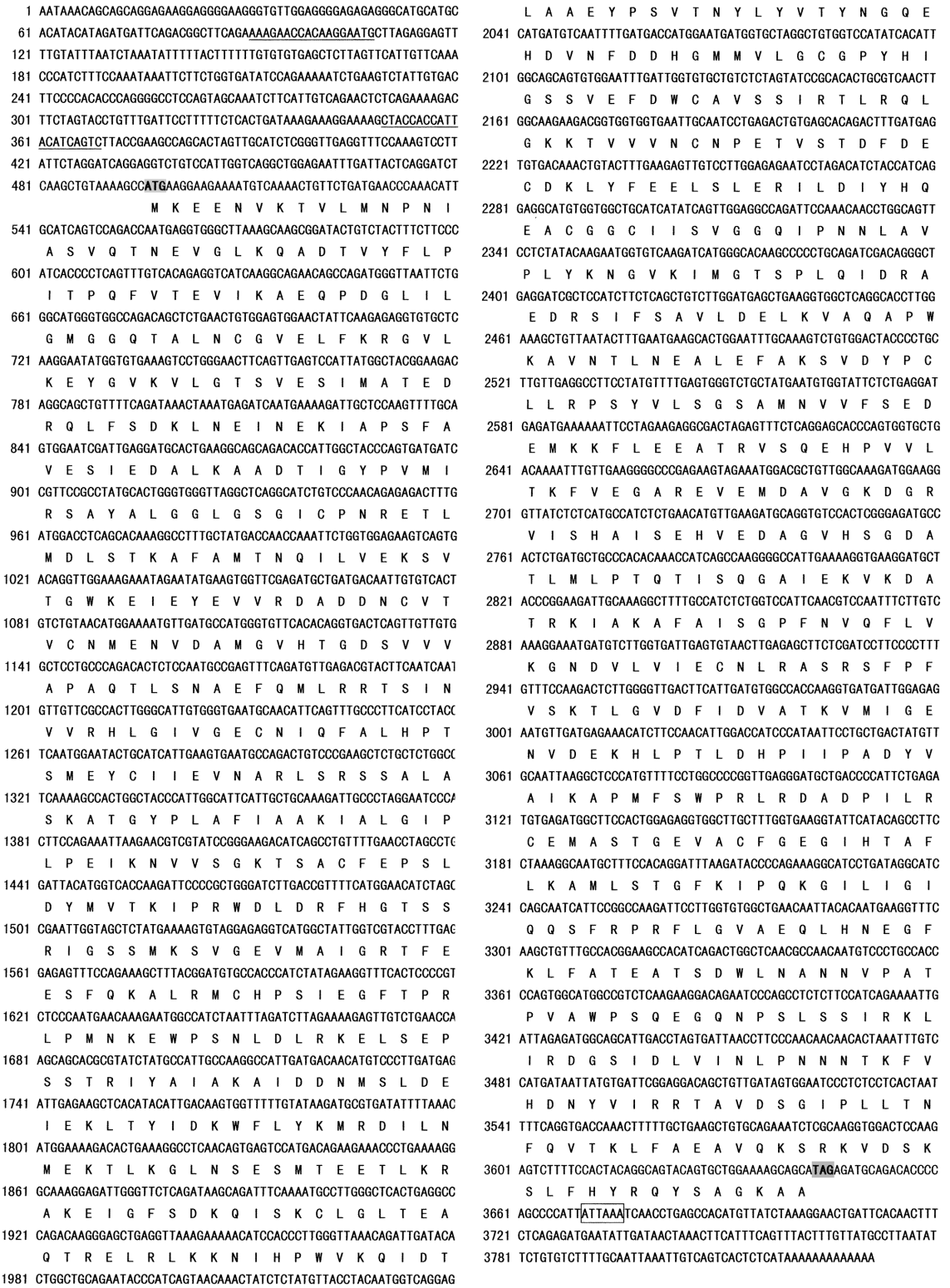

Fig. 2. Nucleotide and deduced amino acid sequences of tCPS1. The single-letter code translated amino acid sequence is indicated below the nucleotide sequence from positions 496 to 3645 (1049 amino acid residues). The initiation and termination codons are marked with shadows. The polyadenylation signal is boxed and the primers with which RT-PCR is performed are underlined. The upstream primer is located in the specific region of tCPS1. The downstream primer is homologous with hCPS1.

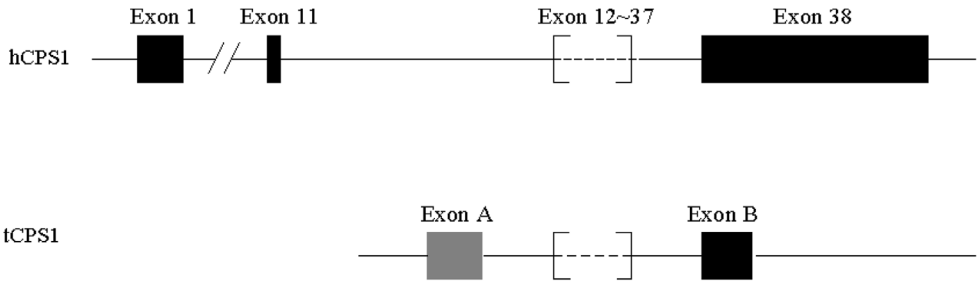

Fig. 3. Transcript and splicing comparison of hCPS1 and tCPS1. 26 identical exons in two sequences are bracketed and omitted. Exons 1-11 only existed in hCPS1. Exon A (303 bp) only existed in tCPS1 and exon B (274 bp) was short than exon 38 (1228 bp) of hCPS1.

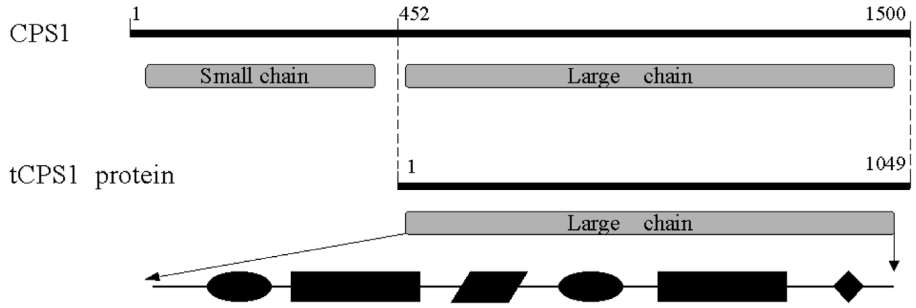

Fig. 4. Protein domains comparison of tCPS1 and CPS1. Compared to CPS1, the putative protein of tCPS1 loses 451 amino acids at its N terminus, which compose the small chain of CPS1. The same region between the tCPS1 and CPS1 proteins contains all the conserved motifs which form the large chain of CPS1. (● indicates CPS_L_chain domain, ■ indicates CPS_L_D2 domain, ▰ indicates CPSase_L_D3 domain and ◆ indicates MGS like domain).

= 84131122 bp), contains the genomic sequence of tCPS1. The tCPS1 gene is spliced to 28 exons and 27 introns, encompassing an 84,793 bp genomic DNA (from 61,617,429 bp to 61,702,222 bp) in NT_005403.13. Blast-nr showed it was highly homologous with the hCPS1 gene. A splicing comparison between tCPS1 and hCPS1 showed the two sequences to have 27 identical exons in the middle of their cDNAs, with eleven only existing in the 5' of hCPS1 cDNA, one merely in the 5' of tCPS1 cDNA and the last exon (274 bp) was shorter than that of hCPS1 (1229 bp), as showed in Fig. 3. Therefore, the difference between tCPS1 and hCPS1 may be caused by alternative splicing.

Compared to hCPS1, the putative protein of tCPS1 loses 451 amino acids at its N terminus, with the residual peptide sequence being the same as the sequence from the 452nd amino acid to the end of hCPS1, having an identity rate of 100%. SMART software (http://www.smart.embl-heidelberg.de/) showed that the lost 451 amino acids of the tCPS1 protein when compared to the hCPS1 contained a CPSase_sm_chain (Carbamoyl-phosphate synthase small chain, CPSase domain) domain and a GATase (Glutamine amidotransferase) domain. Furthermore, the SMART software identified two CPSase_L_chain (Carbamoyl-phosphate synthase L chain, N-terminal domain) domains, two CPSase_L_D2 (Carbamoyl-phosphate synthase L chain, ATP binding domain) domains, one CPSase_L_D3 (Carbamoyl-phosphate synthetase large chain, oligomerisation domain) domain and one MGS (methylglyoxal synthetase) like domain in the same region between the tCPS1 and hCPS1, as shown in Fig. 4.

mRNA expression of tCPS1 The expression profile of tCPS1 in various human tissues was studied using multi-tissue PCRs. Of the sixteen tissues examined, the tCPS1 was highly expressed in the testis, lung, liver and pancreas, moderately in the small intestine and colon and weakly in the ovary and leukocyte, as shown in Fig. 5.

The RT-PCR results also indicated that the tCPS1 was differentially expressed between human fetal and adult testes, which confirmed the hybridization result of the cDNA microarray, with a stronger signal in adult testes than in the embryos (Fig. 6).

Discussion

The ATP-dependent synthesis of carbamoyl phosphate from glutamine, or ammonia and bicarbonate, is catalyzed by carbamyl phosphate synthetase. In *Escherichia coli*, carbamyl phosphate synthetase is a heterodimer (Trotta *et al*., 1971). The small chain is required for the hydrolysis of glutamine and the delivery of the ammonium intermediate to the large subunit (Rubino *et al*., 1986). The large chain can catalyze the formation of carbamoyl-P in the absence of the small subunit, but only when ammonia is used as the nitrogen source (Trotta

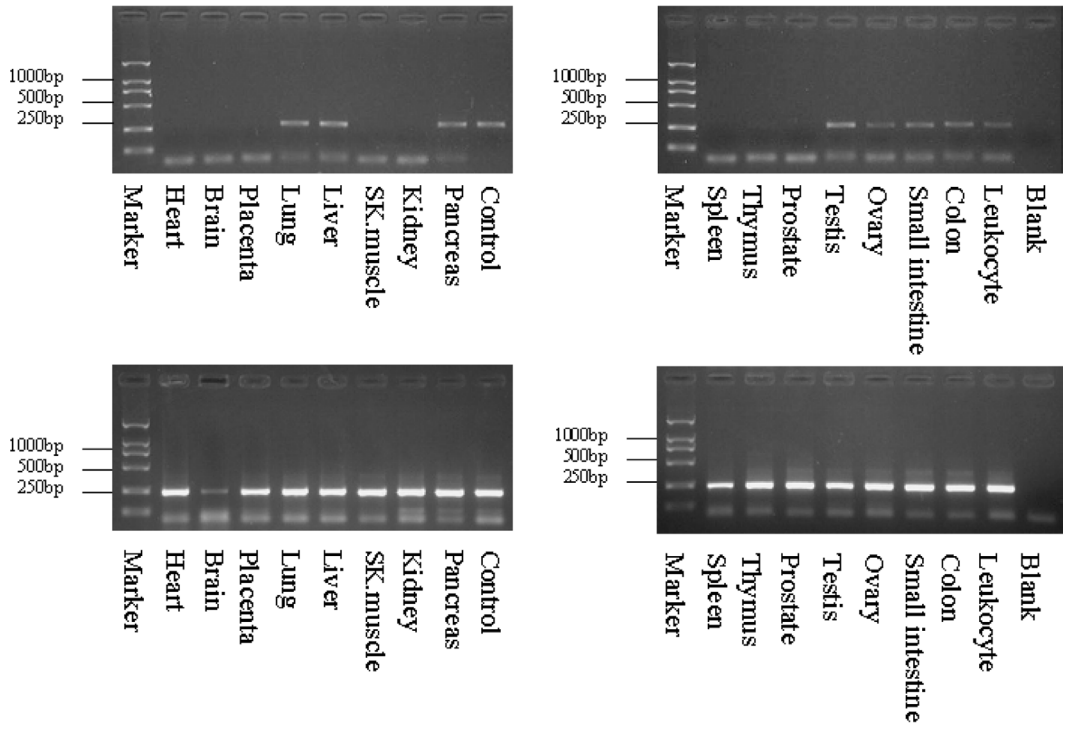

Fig. 5. The tissue distribution of tCPS1 mRNA. Electrophoresis showing expression profiles: Top panel: amplification of tCPS1. tCPS1 sequence-specific primers (F: 5' AAAGAACCACAAGGA ATG 3' and R: 5' GACTGATGTAATGGTGGTAG 3') were amplified with cDNAs of sixteen different tissues. The tCPS1 plasmid was used as a positive control of the PCR amplification; purified water was used as the negative control. The PCR product was 279 bp. tCPS1 was highly expressed in the testis, lung, liver and pancreas, moderately in the small intestine and colon and weakly in the ovary and leukocyte. Bottom panel: amplification of β-actin as a control. All organs expressed β-actin (247 bp).

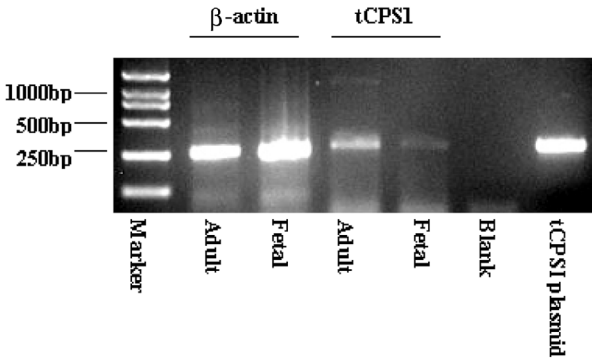

Fig. 6. RT-PCR analysis of the tCPS1 mRNA expression in human embryo and adult testes. Human embryo (6 months) and adult testes cDNA amplified in our laboratory was used as a template. Primer sequences are given in the legend of Fig. 5. A stronger signal is detectable in adult testes. β-Actin was also amplified as a control.

et al., 1971). In humans, there is a special type of carbamyl phosphate synthetases-mitochondrial CPS1. Unlike all known carbamyl phosphate synthetases, CPS1 uses ammonia as the nitrogen donor (Metzenberg, 1957; Marshall *et al*., 1961). The small subunit of CPS1 loses the critical cysteine residue-Cys269, which is essential for amido transferase activity, so this subunit is no longer active (Thoden *et al*., 1998). CPS1 is an important enzyme that initiates the biosynthesis of arginine (Simmer *et al*., 1990), which is primarily expressed in the liver and small intestine. To our knowledge, there have been no reports of studies investigating the relationship between CPS1 and testis function.

In the present study, a cDNA microarray was used to identify the genes related to the development of human testis and spermatogenesis; a novel splice variant of the CPS1 gene, with a higher expression in adult testes than in the embryo, was identified. As it was cloned from the human testis cDNA library; it was named testicular CPS1 (tCPS1). Consistent with its potential role in testes, tCPS1 was highly expressed in human testis. Sequence analysis of the tCPS1 protein showed it was shorter than CPS1 at its amino terminus. The truncated segment contained a CPSase_sm_chain domain and a GATase domain, which formed the small chain of carbamyl phosphate synthetase. Furthermore, the peptide sequence of the tCPS1 protein contained all the conserved domains of the large chain of CPSase. The inactivity of the small chain of CPS1 allows us to suggest that the tCPS1 protein can also carry out the function of arginine biosynthesis in the same way as CPS1.

Arginine takes part in sperm formation, and has been found to be a basic component of the nucleoprotein of spermatozoa of various species (Adnan, 1970). The later part of sperm

development, termed spermiogenesis, is characterized by striking morphological and molecular transformations (Meistrich, 1993). In elongating and condensing spermatids, a major restructuring of the somatic chromatin takes place (Lee *et al*., 2003), where the lysine-rich histones are first replaced by a group of transition proteins, which in turn are replaced by protamines (Meistrich, 1989). The protamines, which are small basic proteins containing much arginine, bind more tightly to the minor groove of DNA than histones, resulting in compaction of the chromatin in the sperm nucleus. As the biosynthetic rate of protamine production is very high during the spermiogenic process (Kaneko *et al*., 2002), large amounts of arginine are required for these cells. Furthermore, it has been demonstrated that chromatin condensation is disturbed when lysine-rich somatic histones are not sufficiently substituted by arginine-rich protamines during spermatogenesis (Meistrich *et al*., 1978).

In conclusion, the presence of tCPS1 mRNA in the human testis and its development-dependent expression profile, which accompanies its deduced function of arginine production, revealed that it was correlated to, and might play an important role in, spermiogenesis. Further work is needed to provide evidence for the exact role of tCPS1 in spermatogenesis.

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