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Tissue-specific subunit of the mouse cytosolic chaperonin-containing TCP-1

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Abstract We have cloned a novel *Tcp-1*-related mouse testis cDNA encoding a polypeptide of 531 amino acids which shares 81.2% identity with the ζ subunit of the mouse cytosolic chaperonin-containing TCP-1 (CCT). Immunoblot analysis of mouse testis CCT subunits separated by 2-dimensional gel electrophoresis indicates that this novel gene, *Cctz-2*, encodes a CCT subunit of M_r 57 000 and pI 7.1. *Cctz-2* mRNA is detected only in testis whereas the other *Cctz* gene, *Cctz-1*, is expressed in all tissues investigated. The CCTζ-2 subunit may have specific functions in the folding of testicular proteins and for interactions with testicular molecular chaperones.

Key words: Molecular chaperone; CCT; Mouse testis; Anti-peptide antibody; Tissue-specific subunit

1. Introduction

The chaperonins are a family of molecular chaperones involved in protein folding, assembly and transport. The chaperonin-containing TCP-1 (CCT), a recently discovered chaperonin [1], is abundant in eukaryotic cytosol [2], and averaged electron microscopic images of CCT show a double-torus-like structure with 8-fold rotational symmetry [3]. CCT facilitates the folding of actin, tubulin and firefly luciferase [4,5] concomitant with ATP hydrolysis in vitro, and CCT can be isolated bound to newly synthesized actin, tubulin and some other unidentified polypeptides in vivo [6]. In yeast, CCT is essential and mutations in individual CCT subunits affect assembly of tubulin and/or actin [1].

One of the characteristics of CCT which distinguishes it from other chaperonins is its hetero-oligomeric nature; in general, mammalian CCT is comprised of eight different polypeptide species [7,8] but in mouse testis CCT and bovine testis TRiC, nine or more subunit species are distinguishable by 2-D PAGE analysis [8,9]. We have already cloned the genes encoding the eight constitutively expressed CCT subunits; *Tcp-1* [10] (also called *Ccta*), and the seven *Tcp-1*-related genes *Cctb*, *Cctg*, *Cctd*, *Ccte*, *Cctz*, *Ccth* [8] and *Cctq* [11]. These genes encode the α, β, γ, δ, ε, ζ, η and θ subunits of CCT, respectively.

Here we report a novel gene encoding a ninth subunit of CCT. Analysis of the gene shows that it encodes a protein similar to CCTζ and we have named it *Cctz-2* encoding CCTζ-2. We renamed the previously cloned *Cctz* gene [8] as *Cctz-1* encoding CCTζ-1. The *Cctz-2* gene is expressed only in testis; we discuss the possible role of CCTζ-2 in protein folding in testicular cells.

2. Materials and methods

2.1. Solutions

SSPE, SSC, Denhardt's reagent, deionized formamide and salmon sperm DNA solution were prepared according to Sambrook et al. [12].

2.2. cDNA cloning and sequencing

A 1.0-kb partial *Tcp-1*-related cDNA (d005) cloned from a Swiss mouse testis cDNA library was previously reported with its 0.6-kb single-pass sequence [13]. A 129/Sv mouse testis cDNA library was screened using a ^{32}P-labelled d005 probe. Ten clones were purified and nucleotide sequence of the longest clone with 1.8-kb insert (pTζ2.2) was determined by the dideoxynucleotide chain termination method with fluorescently labelled primers using the PRISM kit and a 373A automated sequencer (ABI). Nucleotide and deduced amino-acid sequences were analyzed by UWGCG programs.

2.3. Purification and 2-D PAGE of CCT

Germ cells were isolated from the testes of MF1 mice and CCT was purified from the protein extract by sucrose gradient centrifugation followed by ATP-affinity column chromatography [7]. CCT subunits were resolved by isoelectric focusing followed by SDS-PAGE as described previously [8].

2.4. In vitro transcription and translation

^{35}S-labelled proteins encoded by pTβ2, pTζ12 and pTζ2.2 (cDNA clones encoding CCTβ, CCTζ-1 and CCTζ-2, respectively, in pBluescript SK$^-$ vector [8]) were prepared in vitro using TNT coupled rabbit reticulocyte lysate system (Promega). Purified plasmid DNA of each clone (1 μg) was linearized by restriction enzyme digestion at the 3′ end of the insert cDNA. Transcription from the linearized DNA by T3 or T7 RNA polymerase followed by translation from the transcript was carried out in a total volume of 50 μl at 30°C for 1 h in the presence of 50% rabbit reticulocyte lysate, 1 μl RNA polymerase, 0.02 mM amino acid mixture minus methionine, 40 μCi [^{35}S]methionine (Amersham) and 40 units human placental RNAse inhibitor (Boehringer).

2.5. Immunoblot analysis

Rabbit polyclonal anti-peptide antibodies were produced and immunoblot analysis was performed as described previously [9].

2.6. Northern blot analysis

Total RNA was prepared from mouse tissues and cultured cells by guanidine isothiocyanate lysis followed by ultracentrifugation in CsCl solution [12]. The RNA samples (10 μg/lane) were electrophoresed on 2.2 M formaldehyde/1% agarose gels and transferred to GeneScreen

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Abbreviations: CCT, chaperonin containing TCP-1; *Ccta*, *Cctb*, *Cctg*, *Cctd*, *Ccte*, *Cctz-1*, *Cctz-2*, *Ccth* and *Cctq*, genes encoding α, β, γ, δ, ε, ζ-1, ζ-2, η and θ subunits of CCT, respectively; TCP-1, *t*-complex polypeptide 1; pI, isoelectric point; 2-D PAGE, 2-dimensional polyacrylamide gel electrophoresis; Hsp70, 70 kDa heat shock protein; Hsp60, 60 kDa heat shock protein

The sequence appearing in this paper is deposited under accession number Z50192 at the EMBL Databank.

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Plus. Membranes were hybridized independently with 1.5-kb ^{32}P-labelled PCR products of the mouse *Cctz-1* [8] and *Cctz-2* (pTζ2.2) cDNAs in Northern hybridization buffer (5×SSPE, 5×Denhardt's reagent, 1% SDS, 50% formamide) overnight at 42°C. Following hybridization, membranes were washed in 0.1×SSC/0.1% SDS at 68°C.

3. Results

3.1. Cloning and characterization of the mouse Cctz-2 gene

Previously during a mouse testis cDNA sequencing project, a 1.0-kb partial cDNA (clone d005) was recovered from a Swiss mouse testis cDNA library [13] and its 0.6-kb single pass sequence showed significant similarity to a partial human cDNA called HTR3 [14]. The partial amino acid sequence deduced from HTR3 shows 96% identity to the ζ subunit of the mouse CCT complex, deduced from the full-length 2.2-kb *Cctz* cDNA clone pTζ12 [8] and thus it is the human orthologue of CCTζ. Recently, the complete human cDNA for HTR3 has been cloned and renamed as TCP20 [15] and the corresponding yeast gene has been sequenced and named CCT6 [15,16].

Full-length cDNAs of clone d005 were recovered from a mouse 129/Sv testis cDNA library by hybridization screening. The nucleotide sequence of the longest clone, pTζ2.2 (1787-bp insert) was determined (EMBL accession number Z50192). The clone pTζ2.2 has an open reading frame encoding a polypeptide of 531 amino acids with a predicted molecular mass of 58 191 Da. The nucleotide sequence of the open reading frame shows 76.1% identity to that of mouse *Cctz*, clone pTζ12 [8] and the nucleotide substitutions between pTζ2.2 and pTζ12 are spread over the total length of the open reading frame. The deduced amino acid sequence of pTζ2.2 (Fig. 1) shows 81.2% identity to mouse CCTζ (531 amino acids) over its total length. The 5′- and 3′-noncoding regions show no significant similarity, and the 3′-noncoding region of *Cctz-2* (0.1 kb) is significantly shorter than that of *Cctz-1* (0.5 kb). Thus these two *Cctz* mRNA species may differ in their stability. These results indicate that pTζ2.2 and pTζ12 represent two different mouse genes encoding the ζ subunits of CCT. We name the novel gene *Cctz-2*, encoding CCTζ-2, and rename the original *Cctz* as *Cctz-1*, encoding CCTζ-1.

Southern blot analysis of mouse, rat, rabbit, bovine and human DNA suggests that both the *Cctz-1* and *Cctz-2* genes are present in these mammalian species (data not shown). Phylogenetic analysis based on the amino acid sequences of the CCT subunits supports this idea because the divergence time of the *Cctz-1* and *Cctz-2* genes is calculated to be more than 300 million years assuming that human and mouse diverged 80 million years ago (data not shown). These observations suggest that the *Cctz-2* gene function has been maintained in mammals and that CCTζ-2 protein may have an important conserved function in mammalian testis. Other vertebrates may also have both *Cctz-1* and *Cctz-2* since faint bands were observed by Southern blot analysis of DNA from other vertebrates (data not shown).

3.2. Analysis of the polypeptides encoded by the Cctz-1 and Cctz-2 genes

We have previously shown that mouse testis CCT contains nine subunit species (S1–S9) by 2-D PAGE analysis [8] and we have cloned mouse *Tcp-1* [10] (also called *Ccta*) and seven *Tcp-1*-related genes [8,11]. *Ccta*, *Cctb*, *Cctg*, *Cctd*, *Ccte*, *Cctz* (renamed as *Cctz-1*), *Ccth* and *Cctq* encode the α, β, γ, δ, ε, ζ (renamed as ζ-1), η and θ subunits of CCT, respectively. These gene products correspond, respectively, with the polypeptide spots S3, S4, S5, S9, S2, S6/S7, S8 and S1 of mouse testis CCT by 2-D PAGE analysis [8] and by immunoblot analysis using subunit-specific antibodies [9]. In order to determine which proteins are encoded by the two *Cctz* cDNAs, we have analyzed in vitro translation products of *Cctz-1* and *Cctz-2* by 2-D PAGE duplication (Fig. 2). This shows that *Cctz-1* and *Cctz-2* encode proteins of M_r 62 000, pI 6.90 and M_r 57 000, pI 7.1, respectively. The mobility of the CCTζ-1 in vitro translation product is consistent with it being S7; however, the CCTζ-2 polypeptide is significantly less acidic than both S6 and S7. An anti-peptide antibody (ZC-1) raised against the C-terminal 12 residues of CCTζ-1 (EIMRAGMSSLKG in single amino acid code) recognizes the

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CCTζ-2(MM) MAAIKIANPGAEVTRSQAALAVNICAARGLQDVLRPTLGPKGALKMLVSGAGDIKLTKDGNVLLHEMQIQHPTASIIAKVAAAQDHVTGDGTTSNVLIIG 100
CCTζ-1(MM) MAAVKTLNPKAEVARAQAALAVNISAARGLQDVLRTNLGPKGTMKMLVSGAGDIKLTKDGNVLLHEMQIQHPTASLIAKVATAQDDITGDGTTSNVLIIG
Tcp20(HS)  MAAVKTLNPKAEVARAQAALAVNISAARGLQDVLRTNLGPKGTMKMLVSGAGDIKLTKDGNVLLHEMQIQHPTASLIAKVATAQDDITGDGTTSNVLIIG
Cct6p(SC)  M-SLQLLNPKAESLRRDAALKVNVTSAEGLQSVLETNLGPKGTLKMLVDGAGNIKLTKDGKVLLTEMQIQSPTAVLIARAAAAQDEITGDGTTTVVCLVG

CCTζ-2(MM) ELLKQADLYISKGLHPRIITEGFDVAKTKALEVLDEIKVQK-EM--KREILLDVARTSLQTKVHAELADILTEAVVDSVLAI-RRPGVPIDLFMVEIVEM 200
CCTζ-1(MM) ELLKQADLYISEGLHPRIITEGFEAAKEKALQFLEQVKVSK-EM--DRETLIDVARTSLRTKVHAELADVLTEAVVDSILAI-RKKDEPIDLFMVEIMEM
Tcp20(HS)  ELLKQADLYISEGLHPRIITEGFEAAKEKALQFLEEVKVSR-EM--DRETLIDVARTSLRTKVHAELADVLTEAVVDSILAI-KKQDEPIDLFMIEIMEM
Cct6p(SC)  ELLRQARHFIQEGVHPRIITDGFEIARKESMKFLDEFKISKTNLSNDREFLLQVARSSLLTKVDADLTEVLTPIVTDAVLSVYDAQADNLDLHMVEIMQM

CCTζ-2(MM) RHKSETDTQLIRGLVLDHGARHPRMRKQVRDAYILTCNVSLEYEKTEVSSGFFYKTVEEKEKLVKAERKFIEDRVQKIIDLKQKVCA-ESNKGFVVINQK 300
CCTζ-1(MM) KHKSETDTSLIRGLVLDHGARHPDMKKRVENAYILTCNVSLEYEKTEVNSGFFYKSAEEREKLVKAERKFIEDRVKKIIELKKKVCG-DSDKGFVVINQK
Tcp20(HS)  KHKSETDTSLIRGLVLDHGARHPDMKKRVEDAYILTCNVSLEYEKTEVNSGFFYKSAEEREKLVKAERKFIEDRVKKIIELKRKVCG-DSDKGFVVINQK
Cct6p(SC)  QHLSPKDTTFIKGLVLDHGGRHPDMPTRVKNAYVLILNVSLEYEKTEVNSGFFYSSADQRDKLAASERKFVDAKLKKIIDLKNEVCGMDPDKGFVIINQK

CCTζ-2(MM) GIDPVSLEMLAKHNIVALRRAKRRNLERLTLACGGLAVNSFEGLSEECLGHAGLVFEYALGEEKFTFIEDCVNPLSVTLLVKGPNKHTLIQIKDALRDGL 400
CCTζ-1(MM) GIDPFSLDALAKEGIVALRRAKRRNMERLTLACGGIALNSFDDLNPDCLGHAGLVYEYTLGEEKFTFIEKCNNPRSVTLLVKGPNKHTLTQIKDAIRDGL
Tcp20(HS)  GIDPFSLDALSKEGIVALRRAKRRNMERLTLACGGVALNSFDDLSPDCLGHAGLVYEYTLGEEKFTFIEKCNNPRSVTLLIKGPNKHTLTQIKDAVRDGL
Cct6p(SC)  GIDPMSLDVFAKHNILALRRAKRRNMERLQLVTGGEAQNSVEDLSPQILGFSGLVYQETIGEEKFTYVTENTDPKSCTILIKGSTHYALAQTKDAVRDGL

CCTζ-2(MM) RAVKNAIEDGCVVPGAGAVEVAIAEALVN---YKHRVQGRVRLGIQAFADALLIIPKVLAQNSGYDLQETLIKIQTKHAESKE-----LLGIDLNTGEPM 500
CCTζ-1(MM) RAVKNAIDDGCVVPGAGAVEVALAEALIK---YKPSVKGRAQLGVQAFADALLIIPKVLAQNSGFDLQETLVKVQAEHSESGQ-----LVGVDLSTGEPM
Tcp20(HS)  RAVKNAIDDGCVVPGAGAVEVAMAEALIK---HKPSVKGRAQLGVQAFADALLIIPKVLAQNSGFDLQETLVKIQAEHSESGQ-----LVGVDLNTGEPM
Cct6p(SC)  RAVANVLKDKNIIPGAGAFYIALSRYLRSANMNKLGAKGKTKTGIEAFAEALLVIPKTLVKNSGFDPLDVLAMVEDELDDAQDSDETRYVGVDLNIGDSC

CCTζ-2(MM) AAAEAGIWDNYCVKKHLLHSCTVIATNILLVDEIMRAGMSSLRD--- 547
CCTζ-1(MM) VAAEMGVWDNYCVKKQLLHSCTVIATNILLVDEIMRAGMSSLKG---
Tcp20(HS)  VAAEVGVWDNYCVKKQLLHSCTVIATNILLVDEIMRAGMSSLKG---
Cct6p(SC)  DPTIEGIWDSYRVLRNAITGATGIASNLLLCDELLRAGRSTLKETPQ
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Fig. 1. The amino acid sequence of CCTζ-2 deduced from the nucleotide sequence of pTζ2.2 (EMBL accession number Z50192) is aligned with those of mouse CCTζ-1 [8], human Tcp20 and budding yeast Cct6p [15,16]. A dash indicates an amino acid gap, and amino acids conserved in all these sequences are indicated by bold characters. MM, mouse; HS, human; SC, budding yeast.

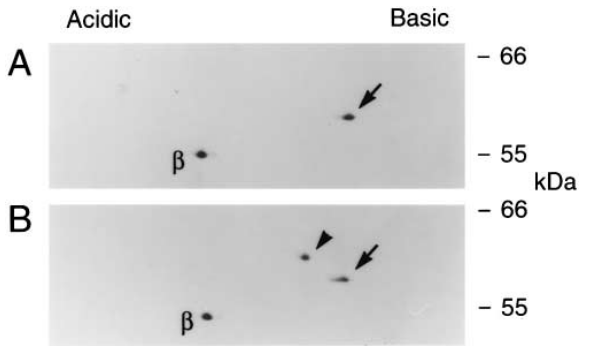

Fig. 2. 2-D PAGE analysis of the proteins encoded by mouse *Cctz-1* and *Cctz-2* cDNAs. In vitro translation from transcripts of *Cctz-1*, *Cctz-2* or *Cctb* cDNA were carried out in rabbit reticulocyte lysate in the presence of [^{35}S]methionine; their product proteins, CCTζ-1, CCTζ-2 and CCTβ subunits, respectively, were analysed by isoelectric focusing followed by SDS-PAGE. A: Mixture of CCTβ and CCTζ-2. B: Mixture of CCTβ, CCTζ-1 and CCTζ-2. The acidic side of pH gradient in the isoelectric focusing dimension is to the left. The apparent mobility of CCTζ-1 is the same as S7 (pI 6.90, M_r 62 000) and the mobility of CCTζ-2 is pI 7.1 and M_r 57 000. The Greek letter β indicates CCTβ. CCTζ-1 and CCTζ-2 are shown by arrowhead and arrow, respectively. The mobility of molecular mass standards (kDa) is indicated on the right.

spots S6 and S7 [9] but not the novel polypeptide, CCTζ-2. In order to distinguish the various polypeptides encoded by *Cctz-1* and *Cctz-2*, rabbit polyclonal antibodies were raised against the C-terminal four residues (Fig. 1) of CCTζ-1 (SLKG) and CCTζ-2 (SLRD). By immunoblotting, the antibody against SLKG recognizes both the S6 and S7 spots (Fig. 3B), indicating that these proteins are the *Cctz-1* gene products probably produced from one to the other by modification, and this idea is supported by peptide mass fingerprinting data which shows that S6 and S7 are closely related [17]. On the other hand, the antibody against SLRD recognizes a polypeptide of M_r 57 000 and pI 7.1 (Fig. 3C), which is the tenth CCT subunit polypeptide species separated by 2-D PAGE in mouse testis. We have assigned S10 to CCTζ-2, since S1–S5, S8 and S9 have already been characterized and CCTζ-2 is different from S6, S7 and a co-purifying polypeptide, p63 (M_r 63 000, pI 6.93) [9]. The mobility of CCTζ-2 by 2-D PAGE is very similar to that of CCTδ and CCTη (S8 and S9, M_r 56 000–57 000, pI 7.1–7.2) and thus CCTζ-2 cannot be clearly resolved away from these basic subunit species.

3.3. Similarities between mouse CCTζ-1, CCTζ-2, and other CCTζ proteins

Li et al. [15] reported the human and yeast homologues of CCTζ, named Tcp20 and CCT6, respectively [16]. The yeast CCT6 gene is essential for growth. Mouse CCTζ-1 and CCTζ-2, respectively, show 96.8 and 81.2% amino acid identity to human TCP20, which indicates that human Tcp20 and mouse CCTζ-1 are orthologues. Thus *Cctz-2* is a novel mammalian gene encoding a CCTζ-like subunit. Mouse CCTζ-1, CCTζ-2, and human TCP20, respectively, show 57.9, 53.2 and 57.9% identity to yeast Cct6p. The similarities of CCTζ subunits between mammals and yeasts are comparable to those of other CCT subunits [1,8,15], and the conserved amino acids in CCTζ subunits (Fig. 1) distribute not only in the putative ATPase domains but also in the putative polypeptide binding domains [1]. These observations indicate that CCTζ-1 and CCTζ-2 of mouse and the CCTζ subunits of human and yeast probably have overlapping functions for polypeptide binding.

3.4. Expression of Cctz-1 and Cctz-2 genes

Northern blot analysis of *Cctz-1* and *Cctz-2* mRNA shows that *Cctz-1* is expressed in a variety of mouse tissues and that *Cctz-2* is expressed only in testis (Fig. 4). No *Cctz-2* mRNA expression in tissues other than testis was detected by overexposure of the blot (data not shown). It is known that *Tcp-1/Ccta* is expressed in almost all cell types and that the expression pattern of *Cctz-1* is similar to that of *Tcp-1/Ccta* [1]. Both genes are expressed highly in testis and F9 cells and lower in brain, heart, spleen. Thus *Cctz-1* is likely to be a CCT subunit gene constitutively expressed in most cell types. On the other hand, *Cctz-2* mRNA was detectable only in testis. These observations are consistent with the CCT subunit expression patterns in other mammals. Testis is the only tissue known to have nine or more subunit species; other tissues have eight subunit species although not necessarily in equimolar ratio [1].

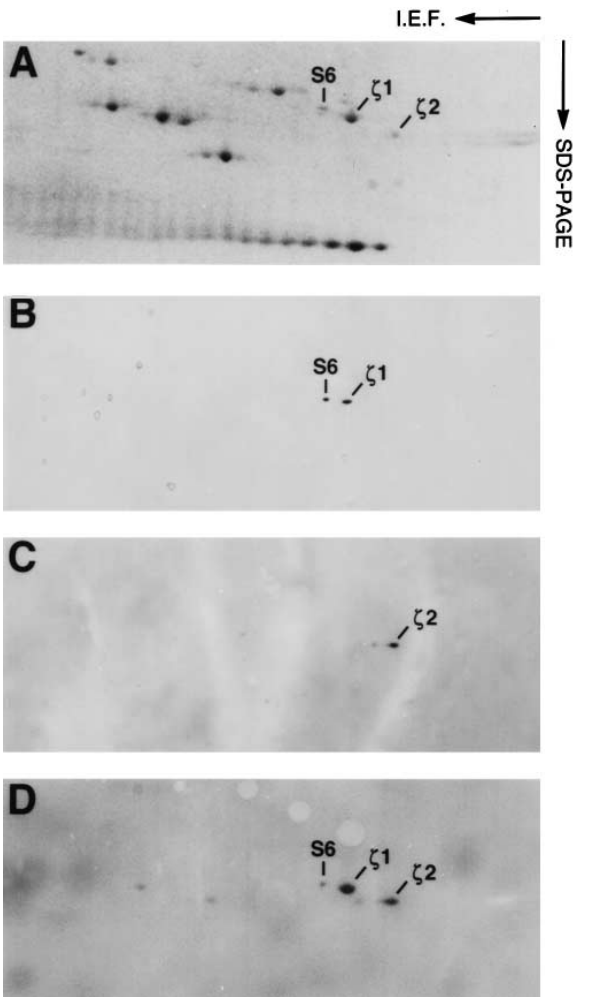

Fig. 3. Immunoblot analysis of CCTζ-1 and CCTζ-2. The subunits of mouse testis CCT were separated by 2-D PAGE and proteins were visualised by silver staining (A). CCT subunits were immunoblotted with rabbit polyclonal antibodies against the C-terminal four residues of CCTζ-1 (B), CCTζ-2 (C) and a mixture of these two antibodies (D). The train of spots in (A) is a carbamylated pI marker protein (creatine phosphokinase). S6, ζ1 and ζ2 indicate the S6, CCTζ-1 and CCTζ-2 subunits, respectively, of mouse testis CCT complex.

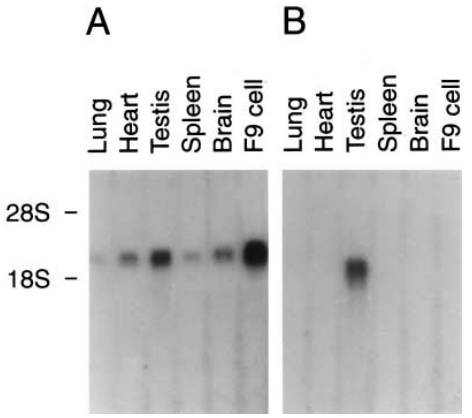

Fig. 4. Northern blot analysis of *Cctz-1* and *Cctz-2* mRNA. Duplicates of total RNA samples (10 μg/lane) were electrophoresed and blotted onto a membrane. The membrane was cut into two pieces and one piece was hybridized with the 1.5-kb ^{32}P-labelled PCR products of *Cctz-1* (A) and the other with *Cctz-2* (B) overnight. The hybridized membranes were washed in 0.1×SSC/0.1% SDS at 68°C. The mobility of 28S and 18S rRNA is indicated on the left.

In rat and guinea pig it is known that testis CCT has one more subunit species when compared with brain CCT [18]. Bovine testis CCT (TRiC) seems to possess nine subunit species [8] whereas rabbit reticulocyte CCT (cytosolic chaperonin) has eight subunit species including only one CCTζ species (polypeptide no. 1, [7]). This subunit is probably the rabbit orthologue of CCTζ-1 since the amino acid sequence analysis shows an identical amino acid sequence to mouse CCTζ-1 but not to CCTζ-2 (a substitution of F to Y at residue 465 in Fig. 1); the same residue position in human TCP20 (CCTζ-1) is F also. It is notable that human [14,15] and mouse [8] *Cctz-1* cDNAs were cloned from somatic cells and that the mouse *Cctz-2* cDNA fragment was cloned from testis by differential hybridization (positive in testis and negative in brain, [13]). Thus, we conclude that CCTζ-2 is testis-specific and that CCTζ-1 is common to testis and other tissues.

4. Discussion

We have shown that there are two CCTζ subunit species, CCTζ-1 and CCTζ-2, in the CCT molecular chaperone of testis, which are encoded by independent genes. These are the first subunit species identified to be closely related (81% identity) in amino acid sequence. There are seven other mouse CCT subunit species and their amino acid sequences have been determined [8,11] but no members of them are closely related. The different CCT subunits, CCTα, β, γ, δ, ε, ζ-1, η and θ, show approximately 30% identity with each other. The identical amino acids are mostly located in the putative ATP-binding domains while these subunits are highly diverged in the putative polypeptide binding domains [1]. Each CCT subunit gene is as old as the origin of eukaryotes and highly conserved from mammals to yeasts, suggesting an independent role for each subunit species in chaperonin function [8]. Since the amino acid sequences of CCTζ-1 and CCTζ-2 are closely related, this suggests that they have similar functions; however, their distinctive expression patterns suggest that they may each possess slightly different functions in testis. Since CCTζ-1 is common to all tissues investigated, it probably plays a fundamental role in CCT function, but since CCTζ-2 is only expressed in testis, it may have a specific function assisting in the biosynthesis of particular testicular proteins.

One possible candidate for such a protein substrate may be tubulin; α-tubulin has constitutively expressed subtypes and testis-specific subtypes [19,20], and we know tubulin is folded by CCT [6,21]. Another possibility may be the contribution to the interaction between CCT and other chaperones or co-factors. The 70 kDa heat shock protein (Hsp70) is known to have testis-specific subtypes [22,23] and it has been suggested that CCT interacts with Hsp70 [2,4,8]. We propose that in testis, the CCTζ-1 and CCTζ-2 subunits can interchange in the CCT complex. This substitution of CCT subunits is reminiscent of a model for the 20S proteasome which is another multi-toroidal, multi-subunit complex.

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