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Molecular Cloning of cDNA Encoding Two Subunits of Calcineurin from Scallop Testis: Demonstration of Stage-Specific Expression during Maturation of the Testis[1]

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Complementary DNAs encoding two subunits of scallop (*Patinopecten yessoensis*) testis calcineurin were cloned, and the nucleotide sequences of their coding regions were determined. The deduced amino acid sequences of the catalytic subunit, calcineurin A (486 amino acid residues, M_r 55,005.91), and the regulatory subunit, calcineurin B (170 residues, M_r 19,237.67), showed high similarity to those of mammalian calcineurins, especially to the brain-type ones rather than to the testis-specific isoforms. Northern blot analysis showed that only a single species for each subunit was expressed in testis and the expression of each subunit increased dramatically from January to March during the maturation stages of the one-year cycle. The period when the maximum amount of mRNAs for calcineurin was expressed corresponds to the one immediately after meiosis, that is, the maturation stage in which 20–80% of the average testis is occupied by spermatozoa. The result is consistent with the one as to the expression of the testis-specific isoform of calcineurin A in mouse, which occurs immediately after meiosis. This is the first report on the stage-specific expression of calcineurin in invertebrate testis and its sequence similarity to the mammalian brain-type isoforms may indicate that the mammalian testis-specific isoforms appeared in evolution after the divergence of mammals from the mollusks and then diverged rapidly for specific functions in testis.

Key words: calcineurin, calmodulin, cDNA cloning, phosphoprotein phosphatase 2B, testis-specific isoform of calcineurin.

The Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase, phosphoprotein phosphatase 2B, was first isolated from a mammalian brain, and was named calcineurin (*1–3*). Calcineurin functions as a heterodimer consisting of a catalytic subunit (calcineurin A, CNA) of 60 kDa and a regulatory subunit (calcineurin B, CNB) of 19 kDa (*4*). The primary structure of CNA comprises four functional domains, a phosphatase catalytic domain at its N-terminus, and the following three domains: a CNB binding domain, a calmodulin binding domain and an autoinhibitory domain near the C-terminus (*4, 5*). As an intrinsic subunit with "EF-hand" Ca^{2+} binding sites, CNB binds 4 mol of Ca^{2+} with high affinity, which results in low activation (*4*). Ca^{2+}-dependent binding of calmodulin to the calcineurin heterodimer is required for full activation. The details of the molecular structure of calcineurin in a region of the catalytic domain and the CNB binding domain with bound CNB were revealed by X-ray crystallography (*6–8*).

Mammalian CNA has been classified into three isoforms: the α-, β-, and γ-types. The α- and β-type isoforms have been isolated from human brain (*9, 10*) and rat brain (*11, 12*). In mouse, the α- and β-types have been isolated from brain (*13*) and thymus (*14*), respectively. Expression of the γ-type isoform is specific to testis, and it has been isolated from the tissues of man (*15*) and mouse (*16*). Two isoforms of CNB have been isolated from mammals: one associated with CNAα or CNAβ (a brain-type CNB) (*17*), and the other only expressed in testis (a testis-type CNB) (*18, 19*), whereas only one type has been reported in drosophila (*20*) and the budding yeast (*21*).

The physiological functions of calcineurin have been studied in tissues rich in this enzyme. In the brain, calcineurin is thought to be related to a synaptic mechanism like long-term potentiation and long-term depression (*22, 23*) in neuronal tissues (*24*). In the thymus, calcineurin has been shown to interact with a nuclear factor of activated T-cells (*25, 26*), which can activate transcription of the IL-2 (*27*) and IL-4 (*28*) genes during the immune response. In the testis, it has been reported that a large quantity of calcineurin is expressed immediately after meiosis (*16*), and it has only been located in the nuclei of round or elongated spermatids (*29*). Furthermore, calcineurin has been located in the postacrosomal region and the flagella of mammalian spermatozoa (*30*). Therefore, it has been thought that calcineurin in the testis is related to spermiogenesis and later

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB041523 for calcineurin A and AB041524 for calcineurin B

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Abbreviations BSA, fatty acid–free bovine serum albumin, CNA, calcineurin A; CNB, calcineurin B; MOPS, 3-morpholinopropanesulfonic acid; ORF, open reading frame, pfu, plaque-forming units; 1× SSC, 0.15 M NaCl, 0.015 M sodium citrate; UTR, untranslated region.

to the regulation of flagellar motility, the details of which have not been revealed yet.

We have been studying the role of calcineurin in the testis of scallop, a marine invertebrate, in which high amounts of calmodulin are expressed. The scallop, *Patinopecten yessoensis*, is cultured and easily available in Hokkaido, and has a large mature testis in the spring during the maturation stage of the one-year cycle (*31*). We have isolated cDNA clones encoding two subunits of scallop calcineurin, and found that calcineurin mRNAs are expressed maximally just before full maturation of the testis. We also found that the deduced amino acid sequences were more similar to those of the mammalian brain-type isoforms than those of the testis-specific isoforms. These results are consistent with the postulated roles of testis-specific calcineurin in mammals, and further indicate that the testis-specific isoforms of mammalian calcineurin might have appeared after divergence of scallop calcineurin from the line to mammalian brain-type calcineurin.

MATERIALS AND METHODS

Materials—Live scallops, *Patinopecten yessoensis*, from the same batches, were cultured in Funka Bay and harvested every month for about 2 years (October 1997 to April 1998, and May 1998 to March 1999) at Yakumo. Testes were dissected from the live scallops, some of which were homogenized immediately to extract RNA, and the others were frozen quickly in liquid N_2 and stored at −80°C until use. Scallop testis calcineurin was prepared as described briefly by Moriya *et al* (*29*), and the details will be published elsewhere. The catalytic (CNA) and regulatory (CNB) subunits were isolated by gel-filtration in the presence of 8 M urea, and then subjected to protein sequencing after reduction/pyridylethylation. Total cellular RNA of scallop testis was prepared by the acid-guanidinium-phenol-chloroform procedure (*32*) using about 0.5 g of tissue. Polyadenylated RNA [poly(A)$^+$ RNA] was fractionated by chromatography on oligo(dT) cellulose (NEB). A random-primed cDNA library in the λgt10 vector (lambda gt10/*Eco*RI/CIAP-treated vector kit; Stratagene) was prepared from poly(A)$^+$ RNA using a TimeSaver cDNA synthesis kit with a Directional cloning toolbox (Amersham Pharmacia Biotech), and packaged using Gigapack II packaging extracts (Stratagene), according to the manufacturer's instructions.

Isolation of cDNA Clones for Calcineurin A—A scallop testis cDNA library (8×10^5 recombinant phage) was screened with a probe RBα corresponding to nucleotides 1048–1870 of rat brain calcineurin Aα (CNAα) (*11*) produced by the PCR method. Plaques from $2–3 \times 10^4$ plaque-forming units (pfu) in each NZY plate were transferred to a nylon membrane (Hybond-N$^+$; Amersham Pharmacia Biotech), and then probed with a ^{32}P-labeled RBα in hybridization buffer (30–50% formamide, 6× SSC, 40 μg/ml salmon sperm DNA, 0.5% SDS) after prehybridization in the same buffer containing 5× Denhardt's solution (1 mg/ml Ficoll, 1 mg/ml BSA, 1 mg/ml polyvinylpyrrolidone). After washing, the membrane was allowed to dry, and then exposed to an imaging plate (Type BAS-III; Fujix). Two positive clones out of 8×10^5 plaques were visualized with BAS-2000 (Fujix). Then ucleotide sequence of the cDNA insert in one of the positive clones, ST-1 (1,171 bp), was 71.5%-identical to a region of rat brain CNAα (*11*) exhibiting a deduced amino acid sequence identity of 82.8%. Therefore, the scallop testis cDNA library was screened with ST-1 as a probe.

Probes were labeled with ^{32}P using a random primer DNA labeling kit (Takara) in the presence of [α-^{32}P]dCTP with cDNA fragments of RBα or others as a template.

Phage DNA was purified by the liquid culture method (*33*). The inserted cDNA fragment was subcloned into pBluescript II SK+, and the resulting plasmid DNA was purified by the improved alkaline-SDS method (*33*). The nucleotide sequence of the subcloned cDNA was determined by the dideoxy chain termination method with an autosequencer (DNA sequencer SQ5500, Hitachi; LI-COR dNA sequencer model 4000L, Aloka).

Isolation of cDNA Clones for Calcineurin B—The oligo-(dT)-primed single-stranded cDNA, derived from scallop testis poly(A)$^+$ RNA with a cDNA cycle kit (Invitrogen), served as a template for PCR amplification. Two degenerated primers, 5′-TI(A/T)(G/C)IGTNGA(C/T)GA(A/G)TT(C/T)ATGAC-3′ (primer B-1s) and 5′-ACCAT(C/T)TT(C/T)TT-(A/G)TGNAC(A/G)TCCAT-3′ (primer B-3a), were prepared

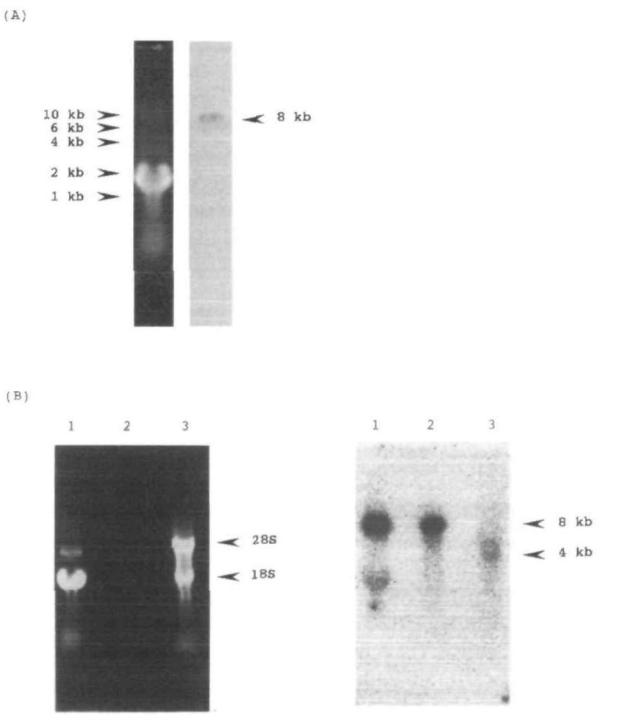
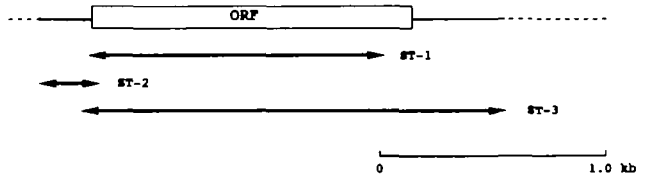


Fig. 1. **Northern blot analysis of mRNAs for the subunits of scallop testis calcineurin.** Twenty micrograms of total RNA from scallop testis or rat brain, or 2 μg of scallop testis poly(A)$^+$ RNA was electrophoresed in a 1%-agarose gel, and then probed with RBα for CNA or 1s-3a for CNB. Size markers are indicated by arrowheads. (A) Results for CNA. Left, ethidium bromide staining; right, autoradiogram. Total RNA from scallop testis was analyzed. (B) Results for CNB. Left, ethidium bromide staining; right, autoradiogram. Lane 1, scallop testis total RNA, lane 2, scallop testis poly(A)$^+$ RNA, lane 3, rat brain total RNA. On ethidium bromide staining, a typical band pattern of 28S/18S-ribosomal RNAs was not observed for the total RNA fraction from fresh scallops. This is a common result for marine mollusks such as scallop and mussel, and is thought to be due to some specific cellular components in these species and not a result of degradation of RNAs. An 8-kb band for CNB was also detected for the poly(A)$^+$ RNA fraction purified from the total RNA fraction.

based on the amino acid sequences of the scallop testis CNB, LSVDEFMT and MDVHKKMV, respectively, which were determined by peptide sequencing. A PCR product was purified by electrophoresis in a 6%-polyacrylamide gel and then subcloned into the pCRII vector using an Original TA cloning kit (Invitrogen) Nucleotide sequence analysis confirmed that the 343-bp PCR product encoded a peptide fragment of scallop CNB since the deduced amino acid sequence was identical to the one determined by peptide sequencing. The 343-bp cDNA fragment named 1s-3a was ^{32}P-labeled and used as a specific probe for CNB, with which the scallop testis cDNA library was screened.

Northern Blot Analysis—The formamide-denatured total cellular RNA (20 μg) was electrophoresed in a formaldehyde-1% agarose gel containing 1× MOPS (20 mM MOPS-NaOH, 5 mM CH_3COONa, pH 7.0) and 5.8% formaldehyde.

(A)



(B)

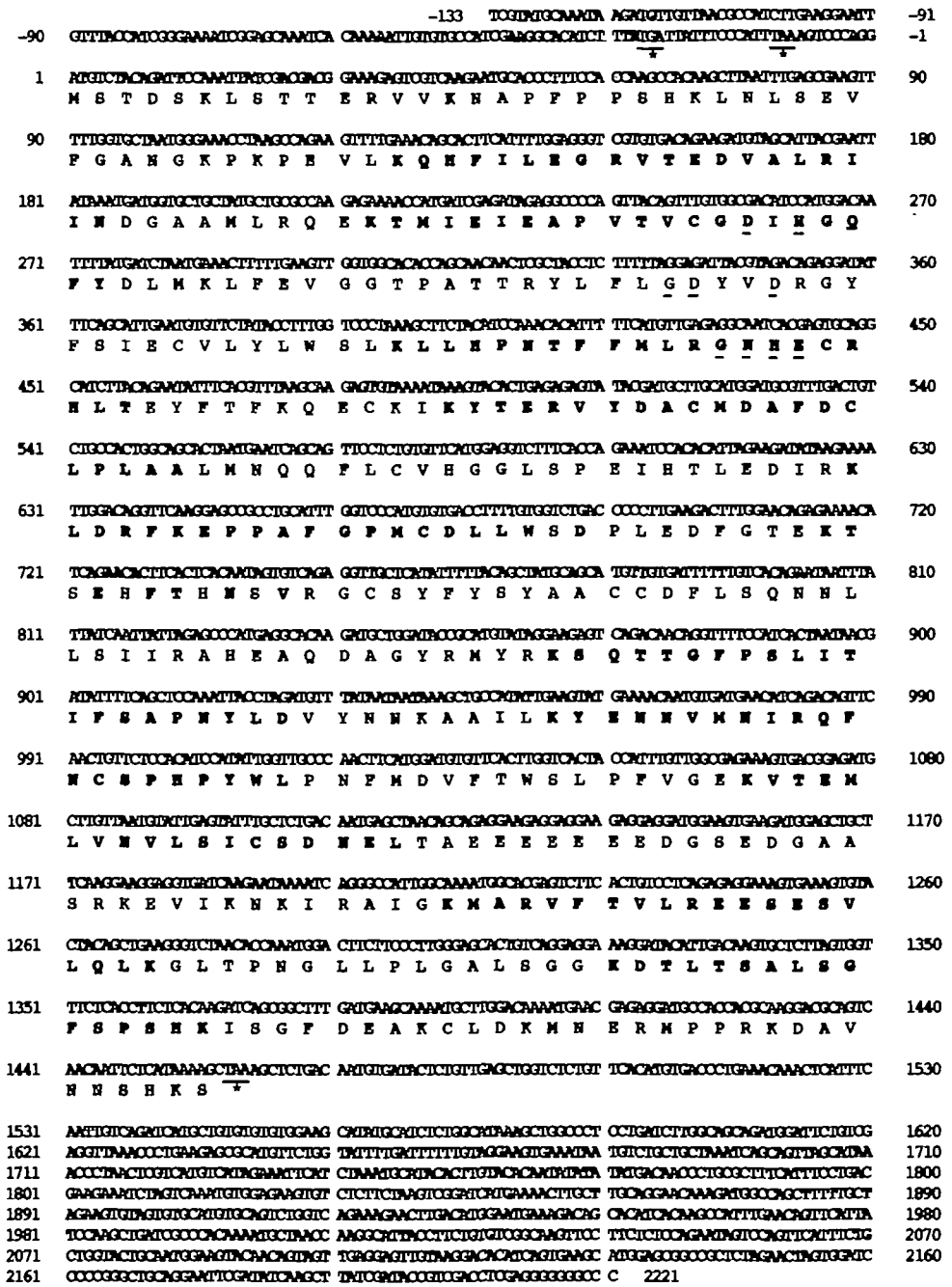


Fig. 2 **(A) Schematic representation of scallop testis CNA cDNA.** Three cDNA clones (ST-1, ST-2, and ST-3) are aligned to reconstruct the cDNA structure. **(B) Partial nucleotide sequence of the scallop testis CNA cDNA.** The whole sequence was constructed from the nucleotide sequences of the three isolated clones ST-1, nucleotides 268–1437, ST-2, nucleotides 133–318, and ST-3, nucleotides 186–2221 The termination codons are underlined with asterisks. The deduced amino acid sequence is shown under the nucleotide sequence, in which the residues determined by peptide sequencing are indicated by bold letters. Nine residues which can provide a scaffold for a dinuclear metal center in the active site are underlined

The RNAs in the gel were transferred to a nylon membrane (Hybond-N$^+$) in 20× SSC. The nylon membrane was prehybridized in hybridization buffer (37.5–50% formamide, 5× SSC, 0.05 M sodium phosphate (pH 6.8), 40 μg/ml salmon sperm DNA, 5× Denhardt's solution, 0.5% SDS) at 37 or 42°C for 2 h, and then hybridized at 37 or 42°C overnight with a ^{32}P-labeled probe, RBα, ST-1 or 1s-3a, in the same buffer. After washing 3 times at room temperature for 15-min each in 2× SSC containing 0.1% SDS, the blot was washed at 37 or 42°C overnight in 0.2× SSC containing 0.1% SDS, washed at room temperature for 10 min in the same solution, and then allowed to dry in air for 30 min. The blot was exposed to an imaging plate, and the hybridized band was detected with BAS-2000.

RESULTS

Isolation of cDNA Clones for Scallop Testis Calcineurin A—A total cellular RNA fraction of scallop testis was subjected to Northern blot analysis using a cDNA fragment of rat brain CNAα, RBα, as a probe, and expression of a sole 8-kb mRNA was confirmed (Fig. 1A). Then, a scallop testis

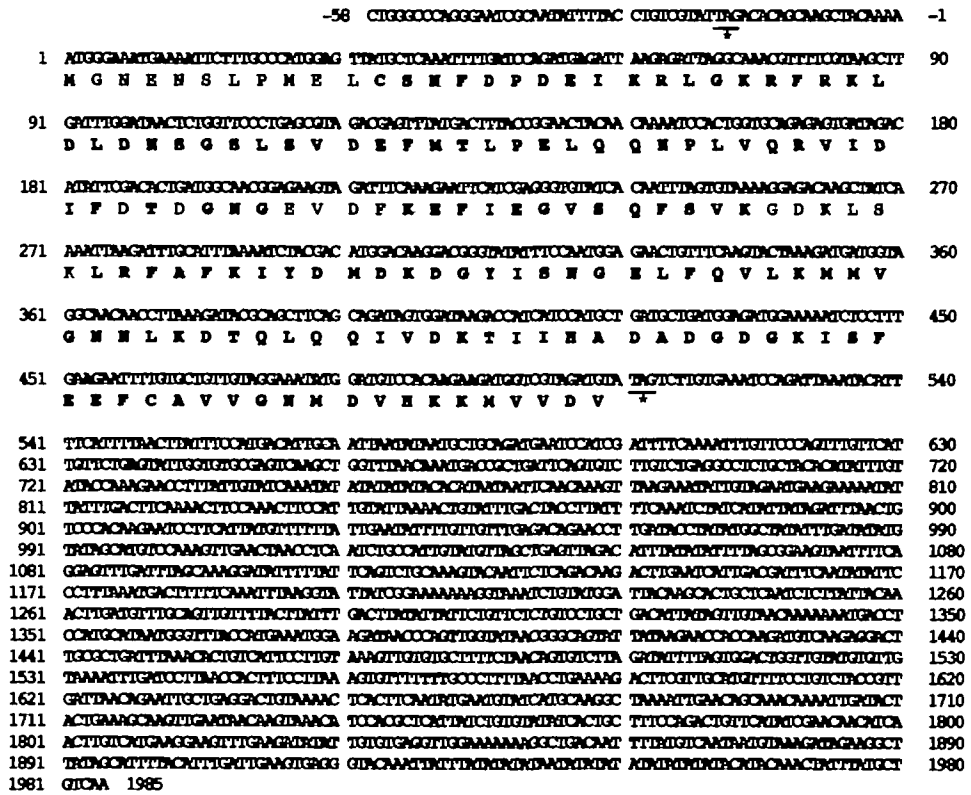


Fig. 3. **Partial nucleotide sequence of a cDNA for scallop testis CNB.** The termination codons are underlined with asterisks The deduced amino acid sequence is shown under the nucleotide sequence, in which the sequences determined by peptide sequencing are indicated by bold letters. Primer B-1s hybridizes to nucleotides 113–134 of the coding strand and primer B-3a hybridizes to nucleotides 478–500 of the complementary strand.

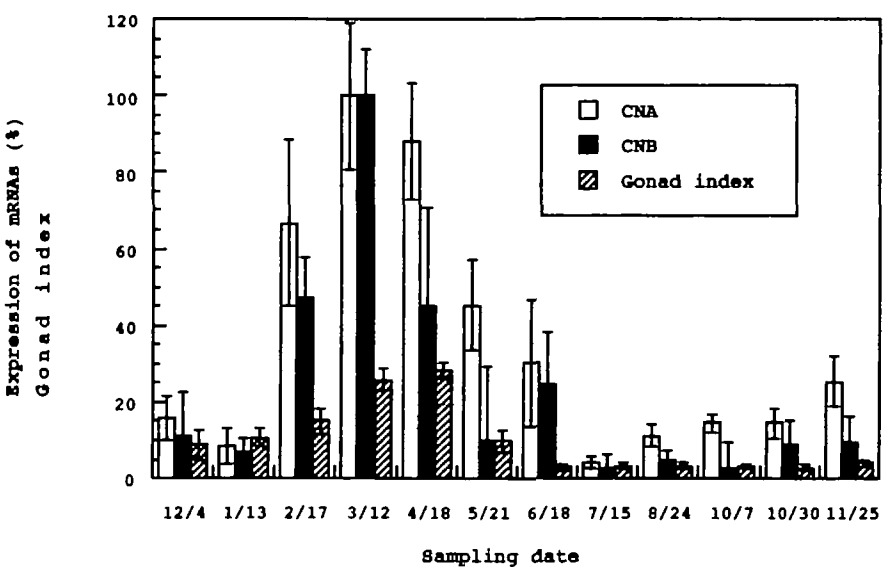


Fig. 4. **Expression of calcineurin mRNAs during the maturation cycle of scallop testis.** Scallops from the same batch were harvested every month, and the expression of mRNAs for CNA (open bars) and CNB (solid bars) in their testes was quantified by Northern blot analysis using 20 μg of total RNA and ^{32}P-labeled specific probes. The expression levels normalized as to the maximum expression value were compared with the normalized testis weight (hatched bars): gonad index = (weight of testis/body weight without shells) × 100 The results for five to ten independent scallops were averaged each month, and the standard deviations are shown by bars.

cDNA library was screened with RBα, 2 positive clones out of 8×10^5 plaques being yielded. A cDNA insert from one of the positive clones, ST-1, was subcloned into a *Not*I site of pBluescript II SK+ for sequencing. A 1,171-bp insert in ST-1 showed sequence similarities of 71.5 and 82.8% at the nucleotide and deduced amino acid levels, respectively, to rat brain CNAα, and the scallop testis cDNA library was screened with ST-1 as a scallop CNA-specific probe. Three positive clones were isolated, one of which, clone ST-2, was confirmed to encode a 5′-upstream region of ST-1. A nucleotide fragment containing a 3′-terminal region of clone ST-1 was isolated by *Ava*II/*Eco*RI (plasmid-origin) digestion of the ST-1 subclone in pBluescript II SK+. The cDNA library was screened with this fragment as a probe, and two more positive clones were isolated, of which clone ST-3 had the longest insert (Fig. 2A). DNA inserts in the positive clones were isolated for sequencing and an overall sequence of 2,354 bp was determined (Fig. 2B). The ATG codon at nucleotide 134 was assigned to an initiator for the following reasons: (i) the 5′-upstream region of the ATG codon contained two in-frame stop codons, and (ii) the nucleotide sequence surrounding this ATG codon agreed with the consensus sequence for initiation of translation often found in eukaryotes (*34*). A TAA codon at nucleotide 1592 was assigned to the termination codon. As a result this sequence was revealed to consist of an open reading frame (ORF) of 1,458 bp, a 5′-untranslated region (UTR) of 113 bp, and a 3′-UTR of 677 bp in which a polyadenylation signal has not been found yet. An amino acid sequence of 486 residues was deduced from the nucleotide sequence of ORF (Fig. 2B). The molecular mass of scallop CNA determined from the deduced sequence is 55,006 Da, which agreed with the value of 55,000 Da estimated on SDS-PAGE of the purified scallop testis calcineurin.

Cloning and Sequencing of cDNA for Scallop Testis CNB—Recombinant phages of 8×10^6 pfu were screened with 1s-3a as a scallop testis CNB-specific probe. Eight out of 15 positive clones were subcloned for sequence analysis (Fig. 3). Considering the overlapping of the determined sequences, a 2,043-bp sequence of cDNA encoding scallop testis CNB was determined, which consisted of an ORF of 510 bp, a 5′-UTR of 58 bp, and a 3′-UTR of 1,472 bp Since

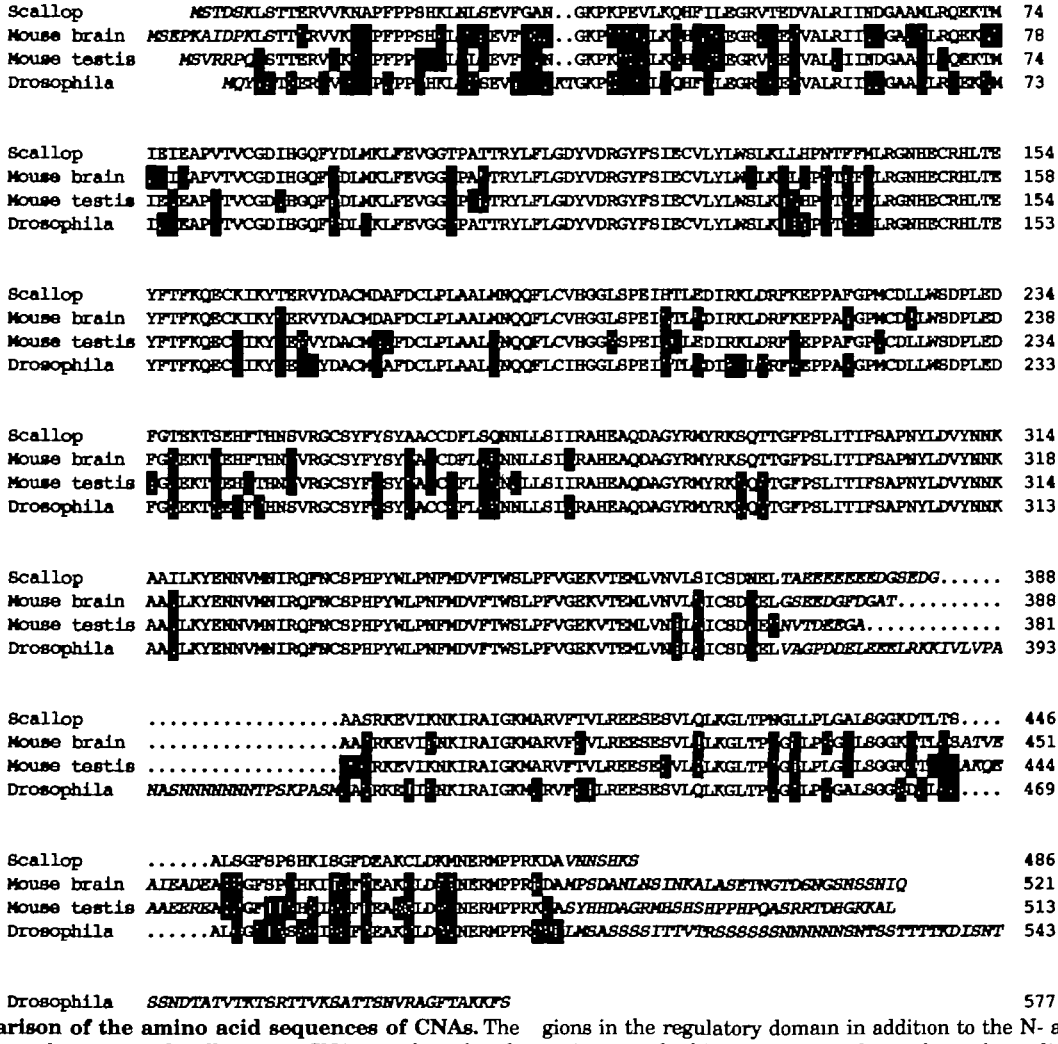


Fig 5 **Comparison of the amino acid sequences of CNAs.** The deduced amino acid sequence of scallop testis CNA was aligned with those of mouse brain-type (*13*), mouse testis-specific type (*16*), and drosophila 21EF (*20*) CNA. Gaps are indicated by periods. Two regions in the regulatory domain in addition to the N- and C-terminal regions are highly divergent and are shown by italics. Substituted residues are shown as white letters on a black background.

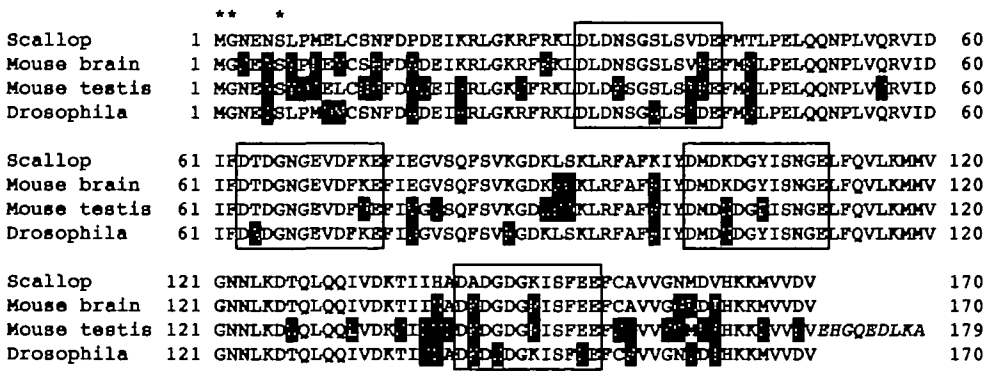


Fig. 6. **Comparison of the amino acid sequences of CNBs.** The deduced amino acid sequence of scallop testis CNB was aligned with those of mouse brain-type (*19*), testis-specific type (*19*), and drosophila (*20*) CNB Asterisks indicate the consensus sequence for myristoylation. The Ca^{2+} binding loops of the EF-hand motif are boxed Substituted residues are shown as white letters on a black background

TABLE I **Comparison of calcineurins from various sources.** The deduced amino acid sequences of calcineurins were aligned with the one of scallop testis calcineurin, and identical residues were counted, except for residues in the characteristic divergent regions indicated by italics in Figs 5 and 6 The sequences of human brain (*9*), human testis (*15*), and xenopus (*43*) CNA were aligned for analysis in a similar manner to as in Fig. 5 The sequences of human brain (*44*), rat brain (*35*) and rat testis (*18*) CNB were aligned for analysis in a similar manner to as in Fig 6.

Source	Calcineurin A (458 positions)					Calcineurin B (170 positions)				
	Identity (%)	Substituted positions				Identity (%)	Substituted positions			
		Total	Common	Brain	Testis		Total	Common	Brain	Testis
Human brain	83.4	76	59	17		89 4	18			
Human testis	80 1	91	59		32					
Mouse brain	83.6	75	52	23		88 8	19	15	4	
Mouse testis	80.1	91	52		39	76 5	40	15		25
Rat brain						89 4	18	16	2	
Rat testis						75 9	41	16		25
Xenopus	80 8	88								
Drosophila	78 2	100				87 7	21			

a polyadenylation signal has not been detected yet, the 3′-UTR of cDNA for scallop testis CNB could be much longer. An amino acid sequence of 170 residues with a calculated molecular mass of 19,237 Da was deduced from the nucleotide sequence of ORF. The estimated molecular mass of 16,200 Da on SDS-PAGE was smaller than the calculated mass, as observed for CNB from other species. The deduced amino acid sequence was identical to partial sequences of 149 residues determined by peptide sequence analysis (Fig. 3).

Northern Blot Analysis of CNB—The expression of mRNA for CNB in scallop testis and rat brain was examined by Northern blot analysis using 1s-3a as a CNB-specific probe. In total RNA from rat brain a major RNA species of 4 kb was hybridized consistent with the previous report (*35*), while an mRNA species with an approximate size of 8 kb was detected in total RNA or poly(A)$^+$ RNA from scallop testis (Fig. 1B). Thus, the mRNAs for scallop testis CNB and CNA are similar in size.

Expression of Calcineurin mRNAs during the Maturation Cycle of Scallop Testis—In the summer through the end of autumn, the scallop testis is small and thin, and looks translucent. From the beginning of winter through spring, it grows, becoming larger and thicker with a rounded shape. With an increase in the temperature of the seawater, the scallop usually begins to spawn at the beginning of May. During the process of maturation of the scallop testis, germ cells in the testis change dynamically for proliferation, meiosis, and spermiogenesis. As one of many Ca^{2+}/calmodulin-dependent enzymes, calcineurin is expected to function during the maturation process, and the expression levels of mRNAs for CNA and CNB were quantified by Northern blot analysis with samples harvested every month over the maturation cycle of one year. Twenty micrograms of the total RNA fraction of each testis was analyzed using cDNA clones for each subunit as probes. Only an 8-kb signal was detected for each subunit over the period and the expression levels are shown in Fig. 4. The expression of each 8-kb mRNA increased dramatically from January to March, and then decreased sharply. Since maturation of the scallop testis peaked in April, expression of the two subunits peaked about 1-month earlier than the maturation of the testis (Fig. 4).

DISCUSSION

An 8-kb mRNA for CNA and another 8-kb mRNA for CNB were detected in the scallop testis (Fig. 1). Throughout the cycle of 1 year only an 8-kb signal could be detected for each subunit. The expression of this 8-kb signal peaked in March, a little earlier than the maximal maturation of the testis, which peaks in April (Fig. 4). At this stage of maturation, spermatid cells produced immediately after meiosis change in shape to form spermatozoa, which account for 20 to 80% of the germ cells in the central parts of the testicular tubules (*31*). Therefore, expression of the mRNAs for scallop testis CNA and CNB peaks immediately after meiosis, which is consistent with the results as to the expression of the testis-specific isoforms of mouse CNA (*16*) and CNB (*19*), as well as the results as to immuno-histochemical localization of calcineurin in the mouse testis (*29*). Considering the results as to immuno-histochemical localization of

the mammalian calcineurin in the postacrosomal region of the head and in the flagellum of the tail of a spermatozoon (*30*), the scallop testis calcineurin encoded by the cDNAs cloned in this work is most probably related to spermiogenesis and flagellar motility, as proposed for the mammalian testis-specific calcineurins.

The large sizes of the mRNAs for scallop testis CNA and CNB (Fig. 1), 8 kb each, are interesting, since the reported sizes of mRNAs for CNA are within the range of 2.4 to 4.4 kb (*11*, *12*, *16*, *36*), and those for CNB between 4.2 kb (*35*) and 3.6 kb (*37*). They are extremely large compared with those for proteins expressed in scallop muscle, such as those for myosin light chains, 0.5–0.7 kb (*38*); troponin C, 2.0 kb (*39*); troponin T, 1.5 kb (*40*); and myosin heavy chain with M_r of 200,000, 6.8 kb (*41*). So far sequences of 677 bp for 3′-UTR with a 113-bp 5′-UTR and 1,458-bp ORF for CNA, and 1,472 bp for 3′-UTR with a 58-bp 5′-UTR and 518-bp ORF for CNB have been determined, in which the polyadenylation signals have not been confirmed yet (Figs. 2 and 3) The messenger RNAs for the two subunits of scallop testis calcineurin may have extremely long 3′-UTR, and the long 3′-UTR may be involved in the translational regulation of calcineurin in germ cells (*42*). Experiments to confirm such a regulatory role of the 3′-UTR are in progress, with use of specific antibodies against scallop testis calcineurin.

The deduced amino acid sequences for scallop testis CNA and CNB were aligned and compared with those for mammalian testis, mammalian brain and other sources (Figs. 5 and 6). On alignment of CNA, putative functional domains of scallop testis CNA could be assigned as follows (*4*): a catalytic domain, Ala66–Val324; a CNB binding domain, Asp344–Val364; a calmodulin binding domain, Ser391–Arg414; and an autoinhibitory domain, Ile457–Ala479. In the catalytic domain, residues providing a scaffold for an active site dinuclear metal center are completely conserved in scallop CNA (Fig. 2), and they can serve as ligands for the formation of the Fe^{3+}/Zn^{2+}-dinuclear center of the catalytic site (*6*, *7*). In addition to these functional domains, some stretches of sequences in the flanking regions are also conserved. As found previously in CNA sequences, the insertion of two divergent sequences is also observed in scallop CNA as well as the divergent sequences in the N-terminal and C-terminal regions. Except for these divergent regions, shown by italics in Fig. 5, the CNA sequences are highly similar, and the results of quantitative analysis of their similarity are shown in Table I. As shown in Table I, sequence similarity was also indicated by comparing the numbers of positions occupied by non-identical residues. Compared with the sequence of human testis CNA, scallop testis CNA showed 80.1%-identity, which is lower than the value, 83.4%-identity, in the case of human brain CNA. Similar results were obtained on comparison with mouse CNAs. The results indicate that scallop testis CNA, which exhibits the expected functions of mammalian testis-specific isoforms, is more similar to the mammalian brain-type isoforms. These results can be more clearly shown by comparing the numbers of non-identical residues between them. Compared with the sequence of human testis CNA, scallop testis CNA has 91 different residues ("Total" in Table I), and 59 of the substitutions are at identical positions to where substitutions were observed on comparison with human brain CNA ("Common" in Table I). Therefore, 32 positions with testis-specific substitutions ("Testis" in Table I) are about twice the 17 positions for brain-specific substitutions ("Brain" in Table I). A similar relation was observed on comparison with the two isoforms of mouse (Table I). Scallop testis CNA is more closely related to those of mammalian brain-type CNA in spite of the similarity in possible physiological function to mammalian testis-specific isoforms.

The results of quantitative analysis of the sequence similarity of CNBs are also shown in Table I. Compared with the results for CNA, the deduced amino acid sequence of scallop testis CNB showed higher similarity to mammalian brain-type CNBs, whereas it showed lower similarity to mammalian testis-specific isoforms (Fig. 6 and Table I). Comparison of the substituted positions indicated that the number of testis-specific positions with nonidentical residues is more than six times higher than that of brain-type-specific positions (Table I).

The present results indicate that scallop testis calcineurin could share physiological functions with the mammalian testis-specific isoforms of calcineurin, and that its amino acid sequence is more similar to those of brain-type isoforms. Since the results of Northern blot analysis indicated a single type of mRNA for the two subunits, the present results also suggest that the mammalian testis-specific isoforms appeared after the divergence of invertebrate mollusks in the evolutionary process and then diverged rapidly for specific functions in mammalian testis.

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