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cpZPC, a newt ZPC molecule, localizes to the inner surface of the egg envelope

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ABSTRACT Zona pellucida-3 is an essential molecule for the binding of sperm to the egg envelope and for induction of the acrosome reaction in mice. Its homologous molecules, ZPCs, have been widely identified in the eggs of many vertebrates, except for urodeles. In this study, to investigate the participation of ZPC in newt fertilization, we cloned the cDNA of newt ZPC from the ovary of *Cynops pyrrhogaster* by reverse transcription polymerase chain reaction (RT-PCR). The cDNA was constructed from 1,397 nucleotides and included one open reading frame corresponding to a sequence of 439 amino acids. The deduced amino acid sequence had identities at 52, 47 and 45 % with *Xenopus* gp41, mouse ZP3 and medaka L-SF, respectively. It included four potential N-linked glycosylation sites and 12 highly conserved cysteine residues of mammalian ZP3/ZPC molecules. This result suggests that *Cynops* ZPC (cpZPC) has molecular features similar to those of mammalian ZP3/ZPCs. Messenger RNA for *cpZPC* was detected in the ovary and faintly in the testis. Two bands corresponding to 84 kDa and 70 kDa in the egg envelopes were detected by immunoblotting with an antiserum raised against a 9 aminoacid peptide in the C-terminus domain of cpZPC. The molecular size of 84 kDa fits with the size of a putative sperm-binding protein reported by Nakai *et al.* (1999), suggesting that cpZPC may contribute to sperm binding to the egg envelope in *C. pyrrhogaster*. The results of immunohistochemistry suggest that cpZPC was localized in the inner surface of the egg envelope. Similar localization is seen only in fish, suggesting that cpZPC is a unique molecule which may allow us to investigate the functional evolution of the egg envelope in vertebrates.

KEY WORDS: *newt, egg envelope, ZPC*

Introduction

Vitelline envelope is important for the sperm-egg interaction upon fertilization. It has been found to assure the species-specific binding of sperm in sea urchins (Aketa and Onitake, 1969; Aketa *et al.*, 1972; Schmell *et al.*, 1977; Vacquier and Moy, 1977; Glabe and Vaquier, 1977). After fertilization, the envelope is converted into a fertilization envelope by hardening and partial modification with the exudates from cortical granules and acts as the block to polyspermy (Vacquier *et al.*, 1973; Foerder and Shapiro, 1977; Glabe and Vacquier, 1978).

In mammals, the innermost egg envelope is called the zona pellucida, and it is composed of three components, ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980a; Shimizu *et al.*, 1983). In mouse, ZP3 is involved in the first binding of sperm to the envelope and the subsequent induction of the acrosome reaction (Bleil and Wassarman, 1980b; Florman *et al.*, 1984; Florman and Wassarman, 1985).

The homologous molecules of mouse ZP3, ZPCs, also have been identified in lower vertebrates such as *Xenopus laevis* gp43/41 (Kubo *et al.*, 1997; Yang and Hedrick, 1997) and medaka L-SF (Murata *et al.*, 1995). However, the function of these molecules is still under discussion.

In amphibians, the vitelline envelope is constructed in the ovary (Yamaguchi *et al.*, 1989). When the ovulated eggs of *Xenopus laevis* are passing through the oviduct, the egg envelope is partially digested with oviductin, the trypsin-like protease (Gerton and Hedrick, 1986; Hardy and Hedrick, 1992). Oviductin allows the eggs to become fertilizable. One of the vitelline envelope components, gp43, is digested and converted into a smaller form, gp41, in the oviduct (reviewed in Hedrick and Nishihara, 1991). Thus, this molecule is thought to be significant for fertilization in anurans (Katagiri *et al.*, 1999).

Newts, unlike anurans, undergo internal fertilization (Elinson, 1986; Wake and Dickie, 1998). Though their fertilization process is fundamentally similar to that of anurans, some regulating systems may be modified to fit the fertilization mode (Ukita *et al.*, 1999;

Abbreviations used in this paper: cpZPC, Cynops ZPC.

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Fig. 1. Nucleotide and deduced amino acid sequences of *Cynops ZPC* cDNA. *The cDNA is composed of 1397 bp and contains one open reading frame that encodes a polypeptide of 439 amino acids. The sequence indicated by a broken line indicates the poly(A)+ signal (AATAAA) overlapping the stop codon (asterisk). There are four potential N-linked glycosylation sites (Asn-X-Ser/Thr) (thick lines). Shaded boxes indicate the primer sites used for RT-PCR and 5'-RACE.*

Mizuno *et al*., 1999; Nakai *et al*., 1999; Onitake *et al*., 2000). Newt eggs are physiologically polyspermic. No cortical granule exists in the egg cortex, and the fertilization envelope is not formed during fertilization (Hope *et al*., 1963). In the newt, *Cynops pyrrhogaster*, the coelomic eggs can be fertilized when they are inseminated with sperm at high concentration (Matsuda and Onitake, 1984), suggesting that the newt egg envelope has a different function than that of anurans. However, little is known about the molecular features of egg envelopes in newts. In this study, we cloned the cDNA for ZPC of *C. pyrrhogaster* and examined the distribution of the ZPC homologous protein using the antiserum against a recombinant peptide. The results revealed a unique feature of the egg envelope of newts.

Results

Cloning of Cynops ZPC (cpZPC) *cDNA*

A cDNA fragment of 612 bp was obtained by RT-PCR with the degenerated primer based on the highly conserved region of *ZPCs*, in combination with oligo-(dT) primer. Base sequence of the fragment showed a high homology with *Xenopus gp41* and mammalian *ZP3/ZPC* sequences. To amplify the 5'-prime region of *cpZPC* cDNA, 5'-RACE was performed using the 5'-terminus 20 bp of the cDNA fragment as a gene-specific primer. This procedure gave a specifically amplified band of 917 bp, with the sequence overlapping 23 bp of the 5'-terminus region of the cDNA fragment of 612 bp.

The combined cDNA was composed of 1397 nucleotides (Fig. 1); the putative ATG start codon existed at 25 nt, and there was a TAA stop codon at 1341nt, which encoded a protein of 439 amino acids. The deduced amino acid sequence comprised the ZP domain and four potential N-linked glycosylation sites (Asn-X-Ser) (Fig. 1). It included 13 cysteine residues, 12 of which were found to be conserved in the same positions as those in mammalian ZPCs.

In comparison with the ZPC proteins, the deduced amino acid sequence of cpZPC had 52, 47 and 45 % homology with *Xenopus* gp41 (Yang and Hedrick, 1997; Kubo *et al*., 1997), mouse ZP3 (Ringuette *et al*., 1988) and medaka L-SF (Murata *et al*., 1995), respectively (Fig. 2, Table 1). These results suggest that the cloned cDNA encodes the ZPC molecule in *Cynops pyrrhogaster*. The hydropathy indices of the cpZPC showed a similar pattern of hydrophobic or hydrophilic regions to those of mouse ZP3, particularly in the C-terminus domain (Fig. 3).

TABLE 1

AMINOACID IDENTITY OF cpZPC AGAINST THE ZPC HOMOLOGOUS MOLECULES IN VERTEBRATES

		Amino Acid Identity (%)						
		Cynops	*Xenopus*	Pig	Human	Mouse	Medaka	Carp
		whole molecule						
Cynops	ZP DOMAIN		46	40	38	38	32	30
Xenopus		58		37	39	35	29	33
Pig		52	50		72	64	32	28
Human		48	50	84		66	28	26
Mouse		48	46	72	72		27	25
Medaka		43	42	43	42	38		40
Carp		42	42	40	38	34	54	

note. Aminoacid sequences were refered to Kubo *et al.* (1996) for *Xenopus*, Yurewicz *et al.* (1993) for pig, Kipersztok *et al.* (1995) for human Ringuette *et al.* (1988) for mouse, Murata *et al.* (1995) lor medaka and Chang *et al.* (1996) for carp.

Expression of cpZPC transcripts

Northern blot analysis using total RNA from the ovary of *C. pyrrhogaster* revealed a 1.5-kbp transcript (Fig. 4A) that was consistent with the length of the *cpZPC* cDNA. No transcript was detected in the liver, spleen or heart.

By RT-PCR with the *cpZPC*-specific primers based on the cDNA sequence (Fig. 1), single bands were detected in total RNA from the ovary (Fig. 4B) and faintly in that from the testis. No transcript was detected in total RNA from the brain, heart, liver, kidney or muscle. A band was clearly detected in the total RNA from all tested organs by RT-PCR with the *ß-tubulin* primers used as a control.

Immunological Study

The antiserum against the c-terminus peptide of cpZPC was prepared, and immunoblotting was performed in the vitelline envelopes of coelomic and uterine eggs. In that of the coelomic eggs, bands of 94 kDa, 84 kDa and 70 kDa in molecular weight were detected (Fig. 5), whereas in that of the uterine eggs, the bands of 84 kDa and 70 kDa in molecular weight were detected. There are six major components in the egg envelope of *C. pyrrhogaster* (Nakai *et al.*, 1999). The 84 kDa band corresponded to the molecule previously described as a possible binding molecule in the egg envelope (Nakai *et al.*, 1999), though the molecular weight of the candidate differed slightly from that noted by Nakai *et al.* (1999).

The coelomic and uterine eggs were sectioned and immunostained with the antiserum. Strong staining was observed in the innermost area of the egg envelopes of both eggs (Fig. 6). No staining was observed in the control. These results indicate that cpZP3 is localized in the innermost area of the egg envelope in the coelomic eggs, and that the localization is unchanged by the egg passing through the oviduct.

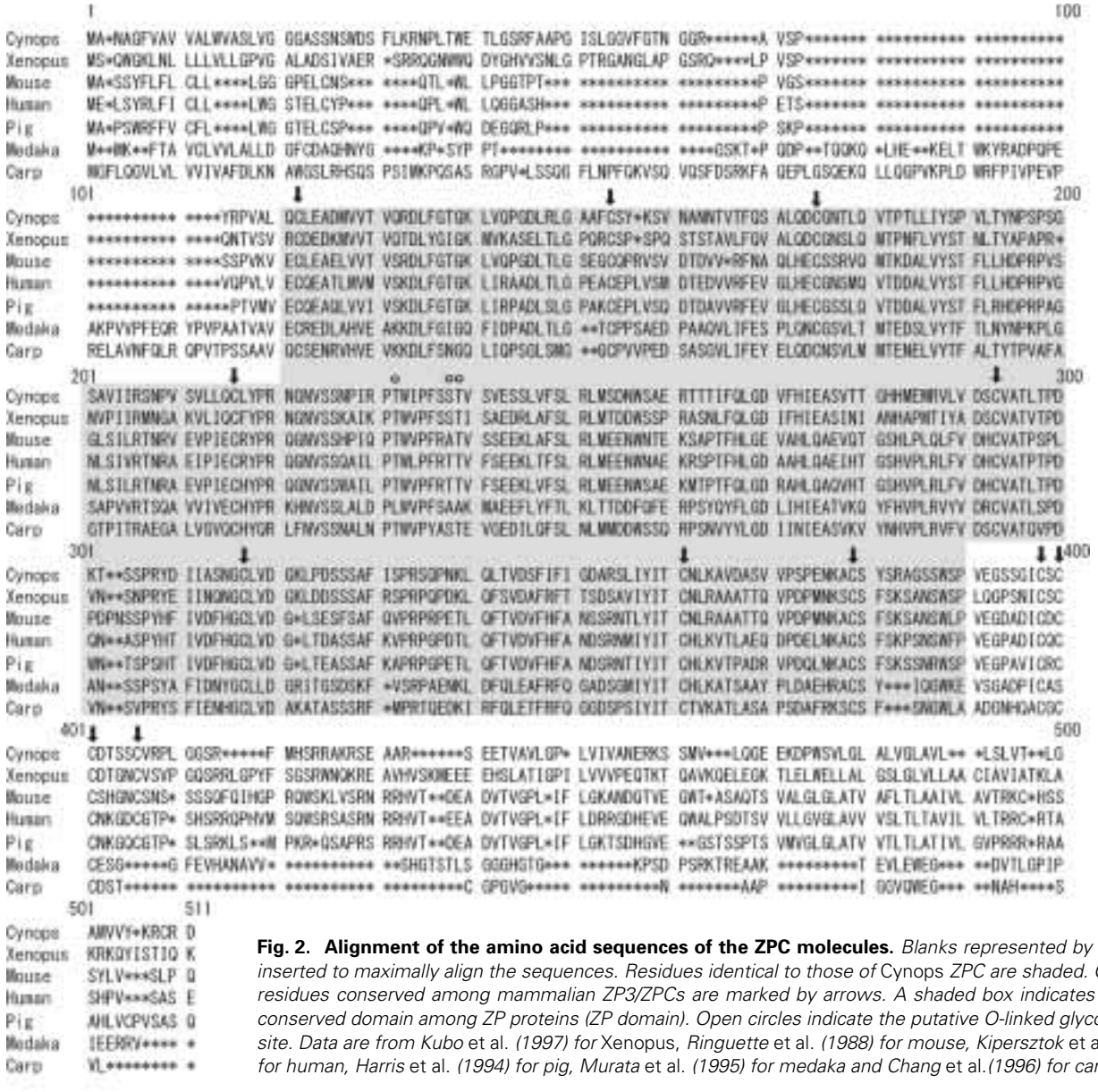

Fig. 2. Alignment of the amino acid sequences of the ZPC molecules. *Blanks represented by dots are inserted to maximally align the sequences. Residues identical to those of* Cynops *ZPC are shaded. Cysteine residues conserved among mammalian ZP3/ZPCs are marked by arrows. A shaded box indicates a highly conserved domain among ZP proteins (ZP domain). Open circles indicate the putative O-linked glycosylation site. Data are from Kubo* et al. *(1997) for* Xenopus, *Ringuette* et al. *(1988) for mouse, Kipersztok* et al. *(1995) for human, Harris* et al. *(1994) for pig, Murata* et al. *(1995) for medaka and Chang* et al.*(1996) for carp.*

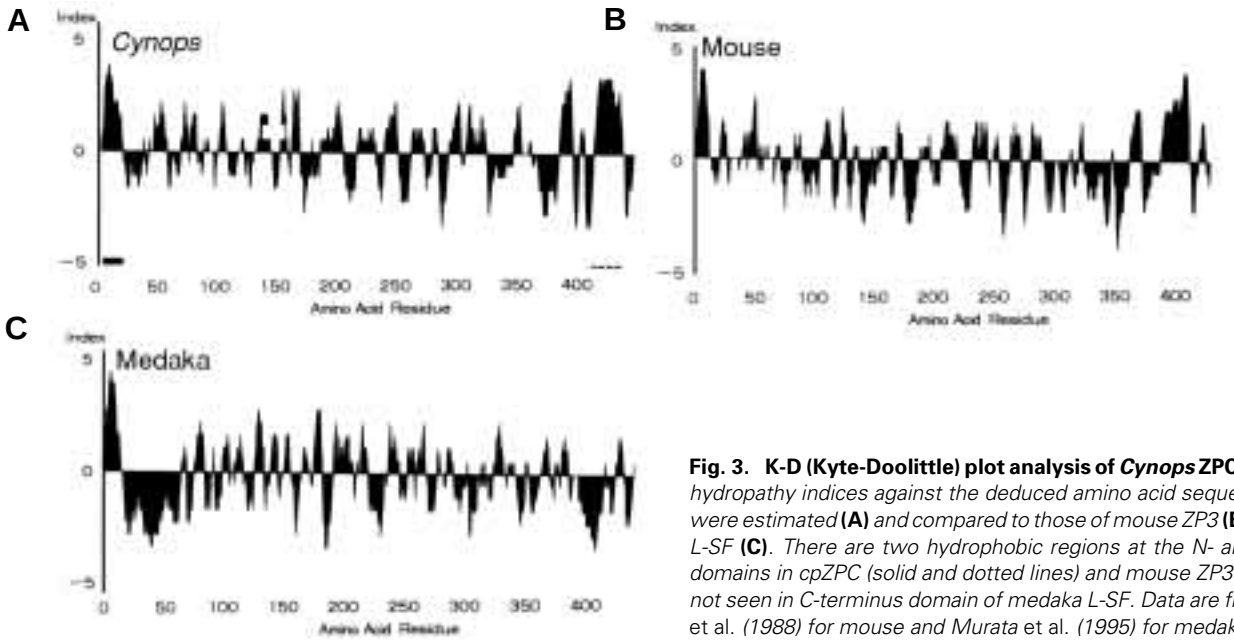

Fig. 3. K-D (Kyte-Doolittle) plot analysis of *Cynops* ZPC (cpZPC). *The hydropathy indices against the deduced amino acid sequence of cpZPC were estimated* **(A)** *and compared to those of mouse ZP3* **(B)** *and medaka L-SF* **(C)**. *There are two hydrophobic regions at the N- and C-terminus domains in cpZPC (solid and dotted lines) and mouse ZP3. The region is not seen in C-terminus domain of medaka L-SF. Data are from Ringuette* et al. *(1988) for mouse and Murata* et al. *(1995) for medaka.*

Discussion

Zona pellucida-C molecule exists widely in the egg envelopes of vertebrates from teleosts to mammals. However, its function is still under discussion, especially in lower vertebrates. In the present study, we identified the cDNA for ZPC of *Cynops pyrrhogaster*. This is the first report of *ZPC* expression in the newt gonad.

The cDNA of *cpZPC* is composed of 1397 bp nucleotides, which includes one open reading frame encoding 439 amino acids (Fig. 1). The deduced amino acid sequence of the cDNA was found to show 46% identity with *Xenopus gp41*, 38% with *mZP3* and 32% with medaka *L-SF* (Table 1). In the ZP-domain (Chamberlin and Dean 1990; Bork and Sander 1992), the sequence showed 58, 48 and 42 % homology with *gp41*, mouse *ZP3* and *L-SF*, respectively (Table 1). There are 12 cysteine residues conserved in mammalian ZPCs (Fig. 2).

The hydropathy indices of cpZPC showed the corresponding signal sequence in the N-terminus domain (Fig. 3), suggesting that cpZPC is a secreted protein. A hydrophobic domain was also found to exist near the C-terminus region as in mammalian or anuran ZPCs. The hydrophobic domain is generally seen in membrane-spanning regions, though the mature ZPC protein is an extracellular molecule. This hydrophobic domain may play a role in interactions with other zona proteins or with the cytoplasmic membrane of oocytes (Ringuette *et al.*, 1988). It has not been observed in the fish ZPC molecules (Murata *et al.*, 1995). These results suggest that cpZPC is close to the mammalian ZPCs in conformation.

The mRNA for *cpZPC* was found to be expressed in the ovary and faintly in the testis (Fig. 4B). It was not detected in the other tested organs, suggesting that the cpZPC gene is specifically expressed in the gonads, especially in the ovary, of *C. pyrrhogaster*. It was reported that a gene homologous to ZPC expressed in fish testis (Miura *et al.*, 1998). On the other hand, expressions of female-specific gene may be induced by some environmental condition. Further study is needed to understand the *cpZPC* expression in the testis of *C. pyrrhogaster*.

The mRNA for mammalian ZPC is expressed in the ovary (Philpott *et al.*, 1987), while mRNA for *L-SF*, a *ZPC* gene in medaka fish, is expressed in the liver (Murata *et al.*, 1995). In medaka fish, the components of chorion, the egg envelope in fish, are produced in the liver and carried through blood flow to the ovary (Murata *et al.*, 1994). In *Xenopus*, the mRNA for *ZPC* is expressed in the ovary (Kubo *et al.*, 1997), suggesting that the egg envelope is formed in a different manner between the fish and anurans. In the present study, Northern blot analysis indicated that the mRNA for *cpZPC* was expressed not in the liver but in the ovary (Fig. 4), suggesting that egg-envelope formation occurs in the same manner in *C. pyrrhogaster* as in higher vertebrates.

All the ZPC homologous molecules reported are glycoproteins (Bleil and Wassarman, 1980b; Chamberlin and Dean, 1990; Yurewicz *et al.*, 1992; Murata *et al.*, 1995; Chang *et al.*, 1996; Kubo *et al.*, 1997). In the deduced amino acid sequence of cpZPC, four potential N-linked glycosylation sites (Asn-X-Ser) were also found (Fig. 1). One of the sites (aligned residues Asn223, Val224, Ser225) is conserved in mouse, human, pig, *Xenopus*, medaka and carp (Ringuette *et al.*, 1988; Harris *et al.*, 1994; Kipersztok *et al.*, 1995; Murata *et al.*, 1995; Chang *et al.*, 1996; Yang and Hedrick, 1997; Kubo *et al.*, 1997; Fig. 2) and is actually glycosylated in pig.

In pig, O-linked carbohydrates are also present at aligned positions Thr233, 239 and 240 (Yurewicz *et al.*, 1992), and these clustered O-glycosylation sites are conserved in other ZPCs, except for those of mouse and medaka fish (Yang and Hedrick, 1997; Fig. 2). In mammals, O-linked carbohydrates in ZP3 are involved in the sperm binding to the egg envelope and induction of the acrosome reaction (Bleil and Wassarman, 1980b; Florman and Wassarman, 1985). The clustered glycosylation sites may act as sperm-binding sites or stabilize the molecular conformation (Yurewicz *et al.*, 1991; Yang and Hedrick, 1997). In cpZPC, the potential O-linked glycosylation sites were found to be conserved in the same position (Fig. 2), suggesting that cpZPC can act as a sperm-binding molecule.

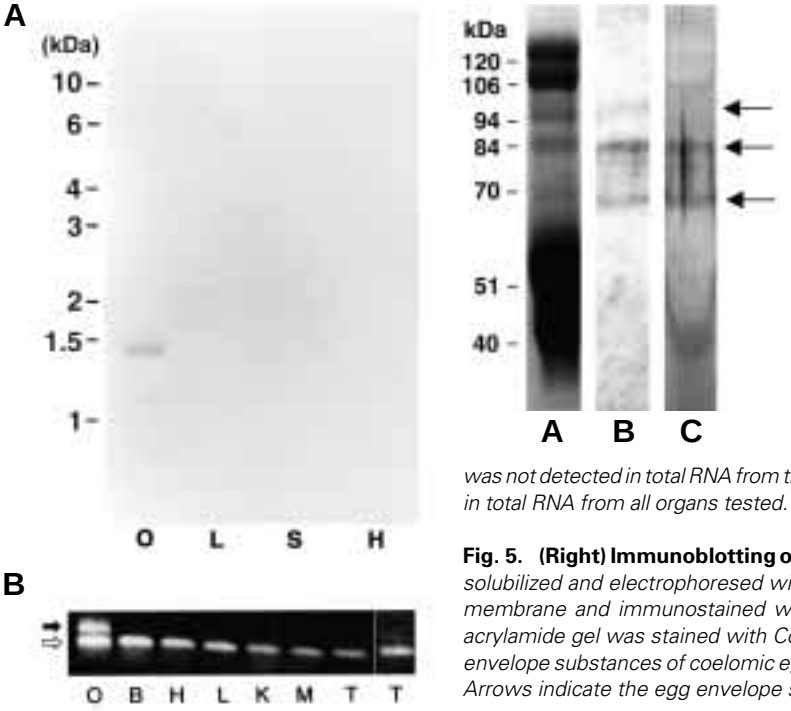

Fig. 4. (Left) Expression of *cpZPC* mRNA in *Cynops pyrrhogaster*. (A): *Ten μg of total RNA was prepared from the ovary, liver, spleen and heart from females of* Cynops pyrrhogaster. *The RNA was electrophoresed with a formaldehyde agarose gel and blotted on a nylon membrane. The samples were hybridized with a DIG-labeled* cpZPC *cDNA fragment (816-1,397 nt). The transcript of 1.5 kbp was detected only in the ovary. O, Ovary; L, Liver; S, Spleen; H, Heart.* **(B)** *One μg of total RNA was prepared from the ovary, brain, heart, liver, kidney, muscle and testis. This was reverse-transcribed with oligo-(dT) primer and amplified with* cpZPC*-specific primers or* β-tubulin*-specific primers as the control. PCR products were electrophoresed with 2% agarose gel and appeared in 1% ethidium bromide. A* cpZPC*-specific band (solid arrow) was detected in total RNA from ovary and faintly detected in that from testis (right column with under exposing). The band was not detected in total RNA from the other organs. A* β-tubulin*-specific band (open arrow) was detected in total RNA from all organs tested. O, ovary; B, brain; H, heart; L, liver; K, kidney; M, muscle; T, testis.*

Fig. 5. (Right) Immunoblotting of the egg envelope of *Cynops pyrrhogaster*. *Egg envelopes were solubilized and electrophoresed with 10% acrylamide gel. They were blotted onto the nitrocellulose membrane and immunostained with antiserum against the c-terminus domain of cpZPC.* **(A)** *The acrylamide gel was stained with Coomassie brilliant blue. Six major bands were detected.* **(B,C)** *Egg envelope substances of coelomic egg (B) and uterine egg (C) were immunoblotted with the antiserum. Arrows indicate the egg envelope substances of 94, 84 and 70 kDa in molecular weight.*

In *C. pyrrhogaster*, sperm can bind to the egg envelope that passes through the oviduct (Nakai *et al.*, 1999). The binding is mediated by heparin/heparan sulfate of the binding molecule, and a molecule in the egg envelope is detected by heparin-affinity chromatography. The egg envelope of *C. pyrrhogaster* is composed of six major molecules. In the present study, the antiserum against the c-terminus domain of cpZPC specifically bound to the 94 kDa, 84 kDa and 70 kDa molecules in the coelomic eggs and 84 kDa and 70 kDa molecules in the uterine eggs (Fig. 5). It is unknown why the 94 kDa molecule was lost in the uterine eggs. It is probable that the precursor molecule was detected in the coelomic eggs or that the 94 kDa molecule in coelomic eggs was digested in the oviduct. In *Xenopus laevis*, the ZPC molecule, gp43 was digested to gp41 in the oviduct (reviewed in Hedrick and Nishihara, 1991). The 84 kDa band corresponded to the molecule with heparin affinity; suggesting the probability that cpZPC is involved in sperm binding to the egg envelope. The 70 kDa band is a minor component that is faintly detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5A). In *Xenopus*, the ZPA molecule has been identified as a sperm-binding molecule (Tian *et al.*, 1997a,b; 1999) that has two isoforms, gp69 and gp64, with different glycosylation sites and amino acid sequences (Tian *et al.*, 1999). It is probable that two isoforms also exist in cpZPC molecules though the 70 kDa molecule does not show heparin-binding activity (Nakai *et al.*, 1999). To examine this hypothesis, we investigated the amino acid sequences of the two kinds of molecules. However, we could not confirm the presence of the two isoforms because the other molecule of similar molecular weight existed in the egg-envelope. Further study is needed to characterize the 70 kDa molecule.

The vitelline envelope of *C. pyrrhogaster* is composed of three sublayers, which is detected immunologically (Watanabe and Onitake, 2002). The cpZPC molecules were localized in the inner surface of the egg envelope (Fig. 6), suggesting that cpZP3 cannot be involved in the first binding of sperm to the egg-envelope. It is known that in mouse, a two-step mechanism of

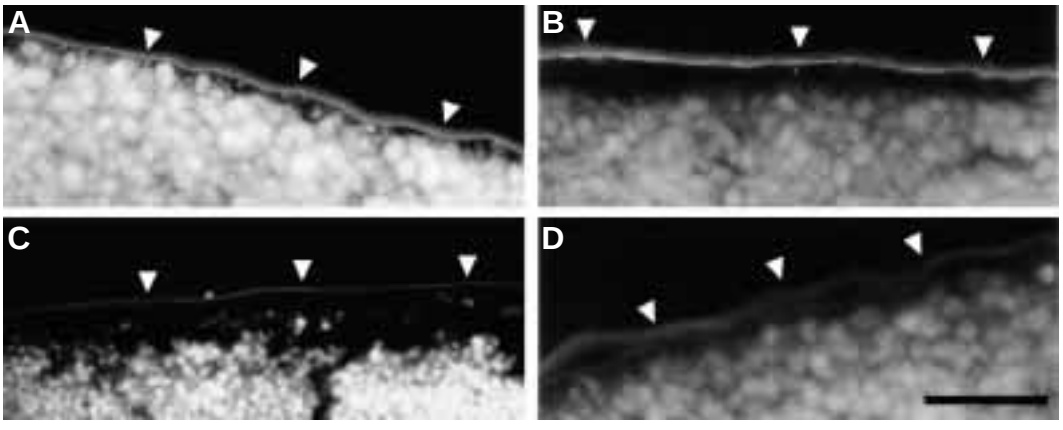

Fig. 6. Immunostaining of the egg envelope in *Cynops pyrrhogaster*. *Dejellied eggs were fixed in methanol fixative and 6 μm thick sections were prepared. They were immunostained with the antiserum against the c-terminus domain of cpZPC. The vitelline envelopes of coelomic eggs* **(A,C)** *or uterine eggs* **(B,D)** *were stained with antiserum against the c-terminus domain of cpZPC (A,B) and the pre-immunized serum as the control (C,D). Arrowheads indicate vitelline envelopes. Strong autofluorescence was observed in yolk granules. Bar, 50 μm.*

sperm binding to the egg envelope acts with ZP2 and ZP3, which correspond ZPA and ZPC, (Bleil and Wassarman, 1980b; Bleil *et al.*, 1988) and that these homologous molecules also demonstrate the sperm binding activity in *Xenopus* (Vo and Hedrick, 2000). To understand the mechanism by which sperm binds to the egg envelope of *C. pyrrhogaster*, it is necessary to identify the ZPA molecule and elucidate the sperm-binding activity in both molecules.

The results of the present study suggest that cpZPC has molecular features similar to those of mammalian ZPCs, although it may localize in the inner surface of the egg envelope as in some fishes. Mammalian ZPCs act as sperm-binding molecules, while sperm binding to the egg envelope does not occur in fishes. Because the newt egg envelope has sperm-binding activity, cpZP3 may be a good tool for elucidating the functional evolution of the egg envelope in vertebrates.

Materials and Methods

Preparation of Ovarian cDNA

Sexually mature Japanese newts, *C. pyrrhogaster*, were collected in Yamagata prefecture, Japan. Ovulation was induced in a female by twice injecting it with gonadotropin (Teikoku Zoki Inc., Tokyo, Japan) at a dose of 100 IU every 24 h. Ovaries were dissected out and immediately frozen in liquid nitrogen. Poly(A)+ RNA was prepared from the ovaries, and cDNA was synthesized with oligo-(dT) primer.

Cloning of Cynops ZPC (cpZPC) *cDNA*

To amplify the cpZPC fragment, we designed a degenerate primer using a highly conserved domain of ZPC molecules (mouse: Ringuette *et al.*, 1988; human: Chamberlin and Dean, 1990; marmosets: Thillai-Koothan *et al.*, 1993; pig: Harris *et al.*, 1994; carp: Chang *et al.*, 1996; *Xenopus*: Yang and Hedrick, 1997; Kubo *et al.*, 1997).

The first polymerase chain reaction (PCR) examination was executed with 0.5 μM ZPC-specific primer (0.5 μM degenerate primer (5'-GGWTGYYTKGTRGATGSSAA-3') and 0.5 μM oligo-(dT) adapter primer (5'-GGCCACGCGCGTCGACTAGTAGCTTTTTTTTTTT-3') in 50 μl of solution containing 1.25 U Taq DNA polymerase (Borhringer Mannheim, Germany) and 0.36 μg cDNA using a GeneAmp PCR System 2400 (PerkinElmer, USA). The apparatus was programmed to deliver 30 amplification cycles involving thermal denaturation at 95°C for 1 min, primer annealing at 40°C for 2 min and primer extension at 72°C for 2 min in one cycle, followed by completion of the PCR reaction at 72°C for 10 min. This PCR gave a specifically amplified DNA fragment of approximately 600 bp. For DNA sequencing, the amplification products were subcloned into the pCRTMII plasmid vector (Invitrogen Co., USA). The fragment was sequenced by the dideoxy chain-termination method with T7 primer or SP6 primer using a DNA sequencer (SQ-5500: Hitachi Co., Japan).

We performed 5'-rapid amplification of cDNA ends (5' RACE) using a 5'-RACE system version 2.0 (Life Tech. Inc., USA). One microgram of total RNA from the ovary was used as a template. The first strand cDNA was amplified by a gene-specific primer (5'-GGCATCGCCAATGAAGAT-3'). The amplification of the 5' end region of the cDNA was performed using a nested gene-specific primer (5'-GGGTTGGGACCGTGGCGAGATGAA-3') and an abridged anchor primer (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') (Life, Tech, Inc.). Amplification products were sub-cloned and sequenced.

Northern Blotting

The ovary, liver, spleen and heart were dissected out from females, and total RNA was prepared from each organ. Ten micrograms of the total RNA was electrophoresed on 1% agarose/formaldehyde gels, followed by blotting on nylon membrane (Borhringer Mannheim, Germany). The DNA probe was prepared from a cDNA fragment of cpZPC (816 to 1397 nt) with digoxigenin (DIG)-labeled deoxynucleotide triphosphates. An aliquot of the DIG-labeled probe was hybridized with RNA on the nylon membrane at 43°C overnight. The membrane was washed twice with 0.2 x SSC/0.1% SDS at 60°C for 30 min, three times with sol. 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 15 min and once with 0.5% skim milk in sol. 1 for 30 min. It was reacted with anti-DIG-AP Fab fragments (Boehringer Mannheim, Germany; 2000-fold dilution in sol. 1) for 1 h and then washed three times with sol. 1 for 15 min. The band emerged in 330 μg/ml NBT, 165 μg/ml BCIP, 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$ (pH 9.5).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from the ovary, brain, heart, liver, kidney, muscle and testis lysed by guanidinium thiocyanate. For RT-PCR analysis, 1 μg of the RNA was used for the cDNA synthesis using oligo-(dT) primer. For amplification of a cDNA corresponding to *cpZPC* mRNA, primers (F, 5'-GGGTGTCTGGTAGATGGGAA-3', and R, TCTCGACATCTCTTGTACAC-3'), were prepared and PCR was performed for 30 cycles involving thermal denaturation at 95°C for 1 min, primer annealing at 49°C for 2 min and primer extension at 72°C for 2 min. For amplification of a cDNA to *ß-tubulin* mRNA, the primers described in Yazawa *et al.* (2000) were prepared and PCR was performed under the same conditions as those for cpZPC. The PCR products were separated on 2% agarose gel, and the bands appeared in 1% ethidium bromide.

Antiserum

A hydrophilic region was selected in the C-terminus domain of cpZPC cDNA using the hydropathy indices of the Kyte-Doolittle (K-D) plot. A nine-amino acid peptide in the region was synthesized and conjugated with keyhole limpet hemocyanin by a glutaraldehyde method (Borel *et al.*, 1984). A synthetic peptide of 500 μg was emulsified with 0.25 ml Freund's complete adjuvant and injected into thighs of rats. The rats were boosted three weeks after the first injection and every two weeks after the second injection. Serum was collected one week after the final boost.

Immunoblotting

Egg envelopes were mechanically removed from coelomic eggs or uterine eggs and collected by centrifugation. Twenty to fifty egg envelopes were solubilized in 2% SDS, 0.0625 M Tris-HCl (pH 6.8) at 100°C for 10 min and electrophoresed with 10% polyacrylamide gel as described by Laemmli (1970). The envelopes were transferred onto a nitrocellulose membrane, washed three times with 0.5% skim milk-PBS and incubated with the antiserum against the cpZPC peptide (1:200 dilution with 0.5% skim milk-PBS) at room temperature for 2 h. The membrane was washed three times and incubated with biotin-conjugated antibodies against rat IgG (Bio Source Res., Inc., USA). After the membrane was washed with 0.5% skim milk-PBS, it was incubated with horseradish peroxidase (HRP)-conjugated avidin. The band emerged by the treatment of the membrane with 0.02% diaminobenzidine , 0.03% H_2O_2 and 2.5 mM Tris-HCl (pH 7.4).

Immunohistochemistry

Uterine eggs were mechanically dejellied with fine forceps. The uterine eggs and coelomic eggs were fixed in methanol fixative. The eggs were then embedded in paraffin, and sections of 6 μm were prepared. The sections were washed three times with 0.5% skim milk-PBS and incubated with the antiserum against the cpZPC peptide (1:200 dilution with 0.5% skim milk-PBS) at room temperature for 2 h. They were then washed three times and incubated with biotin-conjugated antibodies against rat IgG (Bio Source Res., Inc., USA). After the sections were washed with 0.5% skim milk-PBS, they were incubated with horseradish peroxidase (HRP)-conjugated avidin. The staining emerged by the treatment with 0.02% diaminobenzidine, 0.03% H_2O_2 and 2.5 mM Tris-HCl (pH 7.4).

References

AKETA, K. and ONITAKE, K. (1969). Effect on fertilization of antiserum against sperm-binding protein from homo- and heterologous sea urchin egg surface. *Exp. Cell Res.* 56: 84-86.

AKETA, K., ONITAKE, K. and TUZUKI, H. (1972). Tryptic disruption of sperm-binding site of sea urchin egg surface. *Exp. Cell Res.* 71: 27-32.

BLEIL, J.D. and WASSARMAN, P.M. (1980a). Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* 76: 185-202.

BLEIL, J.D. and WASSARMAN, P.M. (1980b). Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* 20: 873-882.

BLEIL, J.D., GREVE, J.M. and WASSARMAN, P.M. (1988). Identification of a secondary sperm receptor in the mouse egg zona pellucida: Role in maintenance of binding of acrosome-reacted sperm to eggs. *Dev. Biol.* 28: 376-385.

BOREL, H., SASAKI, T., STOLLAR, D.B. and BOREL, Y. (1984). Conjugation of DNA fragments to protein carriers by glutaraldehyde: immunogenicity of oligonucleotide-hemocyanin conjugates. *J. Immunol. Methods* 67: 289-302.

BORK, P. and SANDER, C. (1992). A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. *FEBS Lett.* 300: 237-240.

CHAMBERLIN, M.E. and DEAN, J. (1990). Human homologue of the mouse sperm receptor. *Proc. Natl. Acad. Sci. USA.* 87: 6014-6018.

CHANG, Y.S., WANG, S.C., TSAO, C.C. and HUANG, F.L. (1996). Molecular cloning, structural analysis, and expression of carp ZP3 gene. *Mol. Reprod. Dev.* 44: 295-304.

ELINSON, R.P. (1986). Fertilization in amphibians: The ancestry of the block to polyspermy. *Int. Rev. Cytol.* 101: 59-97.

FLORMAN H.M., BECHTOL, K.B. and WASSARMAN, P.M. (1984). Enzymatic dissection of the functions of the mouse egg's receptor for sperm. *Dev. Biol.* 106: 243-255.

FLORMAN, H.M. and WASSARMAN, P.M. (1985). O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* 41: 313-324.

FOERDER, C.A. and SHAPIRO, B.M. (1977). Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine crosslinks. *Proc. Natl. Acad. Sci. USA.* 74: 4214-4218.

GERTON, G.A. and HEDRICK, J.L. (1986). The coelomic envelope to vitelline envelope conversion in eggs of *Xenopus laevis*. *J. Cell Biochem.* 30: 341-350.

GLABE, C.G. and VACQUIER, V.D. (1977). Species specific agglutination of eggs by bindin isolated from sea urchin sperm *Nature* 267: 836-837.

GLABE, C.G. and VACQUIER, V.D. (1978). Egg surface glycoprotein receptor for sea urchin sperm binding. *Proc. Natl. Acad. Sci. USA* 75: 881-885.

HARDY, D.M. and HEDRICK, J.L. (1992). Oviductin. Purification and properties of the oviductal protease that processes the molecular weight 43,000 glycoprotein of the *Xenopus laevis* egg envelope. *Biochemistry* 31: 4466-4472.

HARRIS, J.D., HIBLER, D.W., FONTENOT, G.K., HSU, K.T., YUREWICZ, E.C. and SACCO, A.G. (1994). Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. *DNA Seq.* 4: 361-393

HEDRICK, J.L. and NISHIHARA, T. (1991). Structure and function of the extracellular matrix of anuran eggs. *J. Electron Microsc. Tech.* 17: 319-335.

HOPE, J., HUMPHRIES, A.A. Jr. and BOURNE, G.H. (1963). Ultrastructural Studies on developing oocyte of the Salamander *Trituus viridescens*: 1. The relationship between follicle cells and developing oocytes. *J. Ultrastruct. Res.* 9: 302-324.

KATAGIRI, CH., YOSHIZAKI, N., KOTANI, M. and KUBO, H. (1999). Analyses of oviductal pars recta-induced fertilizability of coelomic eggs in *Xenopus laevis*. *Dev. Biol.* 210: 269-276.

KIPERSZTOK, S., OSAWA, G.A., LIANG, L.F., MODI, W.S. and DEAN, J. (1995). POM-ZP3, a bipartite transcript derived from human ZP3 and a POM121 homologue. *Genomics* 25: 354-359.

KUBO, H., KAWANO, T., TSUBUKI, S., KAWASHIMA, S., KATAGIRI, Ch. and SUZUKI, A. (1997). A major glycoprotein of *Xenopus* egg vitelline envelope, gp41, is a frog homologue of mammalian ZP3. *Develop. Growth Differ.* 39: 405-17.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

MATSUDA, M. and ONITAKE, K. (1984). Fertilization of newt coelomic eggs in the absence of jelly envelope material *Roux's Arch. Dev. Biol.* 193: 64-70.

MIURA, T., KUDO, N., MIURA, C., YAMAUCHI, K. and NAGAHAMA, Y. (1998). Two testicular cDNA clones suppressed by gonadotropin stimulation exhibit ZP2- and ZP3-like structures in Japanese eel. *Mol. Reprod. Dev.* 51: 235-242

MIZUNO, J., WATANABE, A. and ONITAKE, K. (1999). Initiation of sperm motility of the newt, *Cynops pyrrhogaster*, is induced by the heat-stable component of egg-jelly. *Zygote* 7: 329-334.

MURATA, K., IUCHI, I. and YAMAGAMI, K. (1994). Synchronous production of the low- and high-molecular-weight precursors of the egg envelope subunits, in response to estrogen administration in the teleost fish *Oryzias latipes*. *Gen. Comp. Endocrinol.* 95: 232-239.

MURATA, K., SASAKI, T., YASUMASU, S., IUCHI, I., ENAMI, J., YASUMASU, I. and YAMAGAMI, K. (1995). Cloning of cDNAs for the precursor protein of a low-molecular-weight subunit of the inner layer of the egg envelope (chorion) of the fish *Oryzias latipes*. *Dev. Biol.* 167: 9-17.

NAKAI, S., WATANABE, A. and ONITAKE, K. (1999). Sperm surface heparin/heparan sulfate is responsible for sperm binding to the uterine envelope in the newt, *Cynops pyrrhogaster*. *Develop. Growth Differ.* 41: 101-107.

ONITAKE, K., TAKAI, H., UKITA, M., MIZUNO, J., SASAKI, T. and WATANABE, A. (2000). Significance of egg-jelly substances in the internal fertilization of the newt, *Cynops pyrrhogaster*. *Comp. Biochem. Physiol. part B* 126: 121-128.

PHILPOTT, C.C., RINGUETTE, M.J. and DEAN, J. (1987). Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. *Dev. Biol.* 121: 568-575.

RINGUETTE, M.J., CHAMBERLIN, M.E., BAUR, A.W., SOBIESKI, D.A. and DEAN, J. (1988). Molecular analysis of cDNA coding for ZP3, a sperm binding protein of the mouse zona pellucida. *Dev. Biol.* 127: 287-95.

SCHMELL, E., EARLES, B.J., BREAUX, C. and LENNARZ, W.J. (1977). Identification of sperm receptor on the surface of the eggs of the sea urchin, *Arbacia punctulata*. *J. Cell Biol.* 72: 35-46.

SHIMIZU, S., TSUJI, M. and DWAN, J. (1983). *In vitro* biosynthesis of three sulfated glycoprotein of murine zonae pellucidae by oocytes grown in follicle culture. *J. Biol. Chem.* 258: 5853-5863.

THILLAI-KOOTHAN, P., VAN DUIN, M. and AITKEN, R.J. (1993). Cloning, sequencing and oocyte-specific expression of the marmoset sperm receptor protein, ZP3. *Zygote* 1: 93-101.

TIAN, J., GONG, H., THOMSEN, G.H. and LENNARZ, W.J. (1997a). *Xenopus laevis* sperm-egg adhesion is regulated by modifications in the sperm receptor and the egg vitelline envelope. *Dev. Biol.* 187: 143-153.

TIAN, J., GONG, H., THOMSEN, G.H. and LENNARZ, W.J. (1997b). Gamete interaction in *Xenopus laevis*. Identification of sperm binding glycoproteins in the egg vitelline envelope. *J. Cell Biol.* 136: 1099-1108.

TIAN, J., GONG, H. and LENNARZ, W.J. (1999). *Xenopus laevis* sperm receptor, gp69/64 glycoprotein is a homologue of the mammalian sperm receptor ZP2. *Proc. Natl. Acad. Sci. USA.* 96: 829-834.

UKITA, M., ITOH, T., WATANABE, T., WATANABE, A. and ONITAKE, K. (1999). Substances for the initiation of sperm motility in egg-jelly of he Japanese newt, *Cynops pyrrhogaster*. *Zool. Sci.* 16: 793-802.

VACQUIER, V.D., TEGNER, M.J. and EPEL, D. (1973). Protease release from sea urchin eggs at fertilization alters the vitelline layer and aids in preventing polyspermy. *Exp. Cell Res.* 80: 111-119.

VACQUIER, V.D. and MOY, G.W. (1977). Isolation of bindin: the protein responsible for adhesion of sperm to sea urchin eggs. *Proc. Natl. Acad. Sci. USA* 74: 2456-2460.

VO, L.H. and HEDRICK, J.L. (2000). Independent and hetero-oligomeric-dependent sperm binding to egg envelope glycoprotein ZPC in *Xenopus laevis*. *Biol. Reprod.* 62: 766-774.

WAKE, M.H. and DICKIE, R. (1998). Oviductal structure and function and reproductive modes in amphibians. *J. Exp. Zool.* 282: 477-506.

WATANABE, A. and ONITAKE, K. (2002). The urodele egg-coat as the apparatus adapted for the internal fertilization. *Zool. Sci.* 19: 1341-1347.

YAMAGUCHI, S., HEDRICK, J.L. and KATAGIRI, C. (1989). The synthesis and localization of envelope glycoprotein in oocyte of *Xenopus laevis* using immunocytochemical methods. *Develop. Growth Differ.* 31: 85-94.

YANG, J.C. and HEDRICK, J.L. (1997). cDNA cloning and sequence analysis of the *Xenopus laevis* egg envelope glycoprotein gp43. *Develop. Growth Differ.* 39: 457-567.

YAZAWA, T., YAMAMOTO, T., NAKAYAMA, Y., HAMADA, S. and ABE, S. (2000). Conversion from mitosis to meiosis: Morphology and expression of proliferating cell nuclear antigen (PCNA) and Dmc1 during newt spermatogenesis. *Develop. Growth Differ.* 42: 603-611.

YUREWICZ, E.C., PACK, B.A. and SACCO, A.G. (1991). Isolation, composition, and biological activity of sugar chains of porcine oocyte zona pellucida 55k Glycoproteins. *Mol. Reprod. Dev.* 30: 126-134.

YUREWICZ, E.C., PACK, B.A. and SACCO, A.G. (1992). Porcine oocyte zona pellucida Mr 55,000 glycoproteins: Identification of o-glycosilated domains. *Mol. Reprod. Dev.* 33: 182-188.

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