Expression and Localization of Eel Testicular ZP-homologues in Female Japanese Eels (Anguilla japonica)

Authors: Kudo, Nozomi, Miura, Takeshi, Miura, Chiemi, and Yamauchi, Kohei

Source: Zoological Science, 17(9) : 1297-1302

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.1297

ZOOLOGICAL SCIENCE **17**: 1297–1302 (2000)

Expression and Localization of Eel Testicular ZP-homologues in Female Japanese Eels (*Anguilla japonica*)

Nozomi Kudo[1], Takeshi Miura[1,2]*, Chiemi Miura[1] and Kohei Yamauchi[1]

[1]*Division of Marine Biosciences, Graduate School of Fisheries Science, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan and*
[2]*"Unit Process and Combined Circuit" PRESTO Japan Science and Technology Corporation, Japan.*

ABSTRACT—In male Japanese eels, eel spermatogenesis-related substance (eSRS) 3 and 4, having high sequence-similarities to zona pellucida protein (ZP) 2 and ZP3, respectively, are down-regulated by gonadotropin stimulation, with their transcripts disappearing upon the initiation of spermatogenesis. Using Northern blot analysis, we investigated the expression of eSRS3 and 4 mRNA in the developing ovary and the liver of SPH (salmon pituitary homogenate)-injected female eels. Both transcripts were detected in the ovary, but not in the liver. When the eel ovary was subjected to *in situ* hybridization using eSRS3 and 4 cRNA probes, the cytoplasm of previtellogenic oocytes showed a strong signal in comparison with the weak signal in vitellogenic oocytes. Furthermore, stronger signals were observed in the chromatin-nucleolus and the perinucleolus stages than in the oil-droplet stage. Subsequently, we synthesized peptides that were deduced from eSRS3 and 4 cDNAs and generated specific antibodies against them. Staining of the cytoplasm of oocytes in the previtellogenic stage and of egg envelopes in the vitellogenic stage occurred when these antibodies were used in an immunohistochemical analysis. These expression patterns in the ovary suggest that eSRS3 and 4 are components of the eel egg envelope.

INTRODUCTION

The egg envelope in teleosts surrounds the oocyte and plays a major role in fertilization and embryogenesis. In particular, the inner layer of the envelope is well known to be highly homologous to the zona pellucida in mammalian eggs. In mammals, *Xenopus* and some teleosts, major glycoprotein components of the zona pellucida or egg envelope, are synthesized by the oocyte (Wassarman, 1988; Hedrick, 1996; Anderson, 1967; Begovac and Wallence, 1989; Chang *et al*., 1996, 1997), however, in several other teleost species these components have been found to be synthesized by the liver (Hamazaki *et al*., 1984; Yamagmi *et al*., 1994; Fujita *et al*., 1998; Shimizu *et al*., 1998). In the latter teleosts, the egg envelope components are generally observed in females. These substances have also been detected in the serum of male teleosts after estradiol-17β treatment and their transcripts have been observed in the livers of such males (Hamazaki *et al*., 1987; Murata *et al*., 1991; Hyller *et al*., 1991; Oppen-Berntsen *et al*., 1992a,b ; Larsson *et al*., 1994; Murata *et al*., 1995, 1997; Giacco *et al*., 1998a).

We have previously isolated two testicular cDNA clones, eel spermatogenesis-related substance (eSRS) 3 and 4, with sequence similarities to the ZP-domains of the mouse zona pellucida sperm binding protein (ZP) 2 and ZP3, respectively (Miura *et al*., 1998). Furthermore, we have found that eSRS3 and 4 mRNA are expressed in immature testes containing only premitotic spermatogonia (Miura *et al*., 1998).

Under culture conditions, Japanese eels do not initiate gonadal development. Gonadal maturation, however, can be induced by hormonal treatment such as that administered by injections of human chorionic gonadotropin (HCG) into males (Yamamoto *et al*., 1972; Miura *et al*., 1991) or of chum salmon pituitary homogenates (SPH) into females (Yamamoto and Yamauchi, 1974). In addition, eSRS3 and 4 transcripts have been detected in the immature testis and ovary (Miura *et al*., 1998). It is possible that eSRS3 and 4, which are ZP homologues and are expressed in the ovary, are components of the egg envelope. In this study, we investigated the expression and localization of eSRS3 and 4 in the ovary and liver of sexually immature and mature fish.

MATERIALS AND METHODS

Animals

Females of cultivated Japanese eels (*Anguilla japonica*) were purchased from a commercial eel supplier and kept in circulating seawater tanks with a capacity of 1000 liters at 20°C. Weekly injections of chum salmon pituitary homogenates (SPH) suspended in eel Ringer (NaCl, 3.0 mM; KCl, 3.0 mM; $MgCl_2$, 3.5 mM; $CaCl_2$, 5.0 mM; HEPES 10 mM, pH 7.4) were given intramuscularly to the female eels at a

* Corresponding author: Tel. +81-138-40-5546;
FAX. +81-138-40-5545.
E-mail: takeshi@pop.fish.hokudai.ac.jp

dose of 20 μg per gram body weight. Ovaries and livers were collected from immature and sexually mature eels.

Northern blot analysis

Total RNA was prepared from tissues using ISOGEN (Nippongene, Toyama, Japan), and poly $(A)^+$ RNA was purified with Oligo(dT)Latex beads (Oligotex-dT30; Takara, Ootu, Japan). Northern blot analysis was performed as described by Miura *et al.* (1998).

In situ hybridization

Eel ovaries from SPH-untreated fish were fixed in 4% parafolmaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2) at 4°C overnight, embedded in paraffin, and cut into 5 μm serial sections. Approximately 400 bps cDNA of eSRS3 and 4 were subcloned into pBluescriptsII KS-. Sense and anti-sense RNA probes were transcribed *in vitro* using digoxigenin-labeled UTP (Boehringer Mannheim, Germany) and T3 or T7 RNA polymerase (Gibco BRL, Gaithersburg, MD, USA). *In situ* hybridization was performed as described by Miura *et al.* (1999).

Peptide synthesis and immunization

The antigens were used the carboxyl-terminal peptide of eSRS3 (residues 360-380: CSSGKQRRSIAPVGKRSPEEK) and the amino-terminal peptide of eSRS4 (residues 79–94: SPVTPRPTFGRPGITQ). The sequence of the ZP domain is not included in these peptides. These amino acid sequences were characterized with regard to both secondary structure and hydrophilicity.

Synthesis was carried out with a solid-phase peptide synthesizer model 431A (Applied Biosystems, Forest City, CA, USA) using Fmoc chemistry. The synthesized peptides were cleaved from p-hydroxymethylphenoxymethyl polystyrene resin with 6% phenol, 2% 1,2-ethanedethiol and 4% thioanisole in trifluoroacetic acid. After lyophilization, the oligopeptides were coupled to bovine serum albumin (BSA) using N-(ε-maleimidocaproyloxy) succimide (Dojindo, Kumamoto, Japan). Male rabbits were immunized with 1 ml of PBS (pH 7.5) containing 1 mg of each peptide mixed with an equal volume of Freund's complete adjuvant. The rabbits received four immunizations at 7 day intervals. Sera were collected after the fourth injection. For affinity purification of antibodies, the peptides used for immunization were coupled to AminoLink affinity columns (Pharmacia) according to the manufacturer's instructions. The columns were washed with 1 M NaCl 10 mM PBS (pH 7.5), and the antibodies against each peptide were eluted by 0.1 M Glycine-HCl (pH 2.5). The eluted solution was neutralized with 1 M Tris. The purified antisera were used as the antipeptide antibodies. The specificity of the antibodies was tested by ELISA.

Western blot analysis

Frozen samples were homogenized with eel Ringer at 4°C. The homogenate was mixed with an equal volume of sample buffer (Tris-HCl (125 mmol/l), pH 6.8, 4%(W/V) SDS, 10%(V/V) 2-mercaptoethanol, 20%(V/V) glycerol, and 0.05%(W/V) bromophenol blue). The mixture was heated in a boiling water bath for 5 min, and centrifuged at 9,000 g for 5 min; the surpernatant was then collected as tissue extracts. Samples were separated using SDS polyacrylamide gel electrophoresis on 10% gels. The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blotting, the membranes were incubated and shaken for 30 min in 5% skim milk in 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl (TBS) to block non-specific binding sites. The blocked membranes were immersed overnight in 5% skim milk containing the primary antibodies diluted to 1:1000. After washing twice with TBS containing 0.025% Tween 20 (TTBS) and then with TBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Bio-Rad, South Richmond, CA, USA) diluted to 1:1000 in TBS for 2 hr. After washing, HRP activity was visualized using a freshly prepared solution of 0.06% 4-chloro-1-napthol in TBS containing 0.06% H_2O_2.

Immunohistochemistry

Sections were deparaffinized in xylene, hydrated in a graded ethanol series, and washed in TBS. To block endogenous peroxidase activity, they were incubated for 30 min in methanol containing 1% H_2O_2 and then washed in TBS. To reduce non-specific binding, the sections were treated with 5% skim milk in TBS for 30 min at room temperature. Primary antiserum at a 1:1000 dilution was applied to sections overnight at 4°C. After washing with TBS, tissues were incubated with a solution of biotinylated goat anti-rabbit IgG at a 1:400 dilution (Dako, Glostrup, Denmark) for 2 hr at room temperature. After washing with TBS, the streptavidin and biotinylated HRP complex (Dako) was applied for 30 min, and the sections were then washed with TBS. The final reaction product was visualized using 3, 3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.6) containing 0.03% H_2O_2.

RESULTS

Expression and localization of eSRS3 and 4 mRNA in females

The expression of eSRS3 and 4 mRNA in various tissues from female eels was analyzed by Northern blot analysis. Intense signals were detected only in the ovary (Fig. 1). Both eSRS3 and 4 transcripts were detected in the ovary throughout the SPH-injection experimental period. The tran-

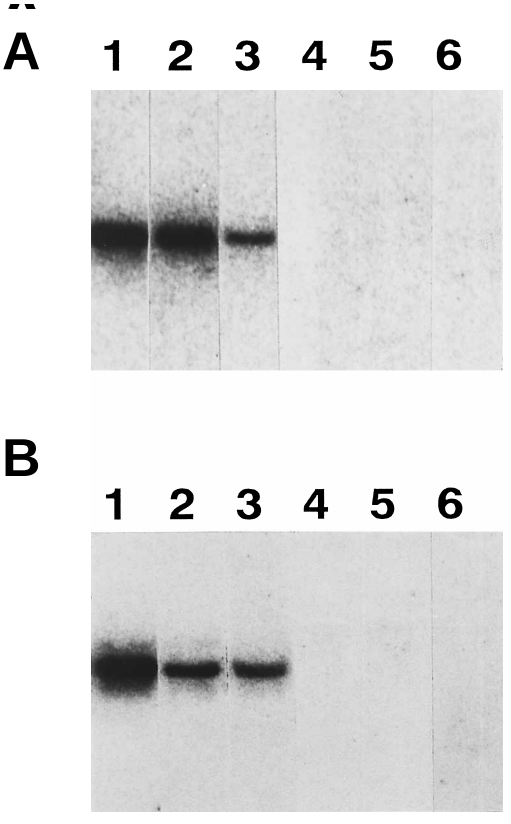

Fig. 1. eSRS3 (A) and eSRS4 (B) mRNA expression in the developing ovary and liver after SPH-injection of cultivated Japanese eel. One microgram of poly $(A)^+$ RNA was applied to each lane. Lane 1, immature ovary; lane 2, vitellogenic stage of ovary; lane 3, mature ovary; lane 4, liver of immature stage; lane 5, liver of vitellogenic stage; lane 6, liver of mature stage.

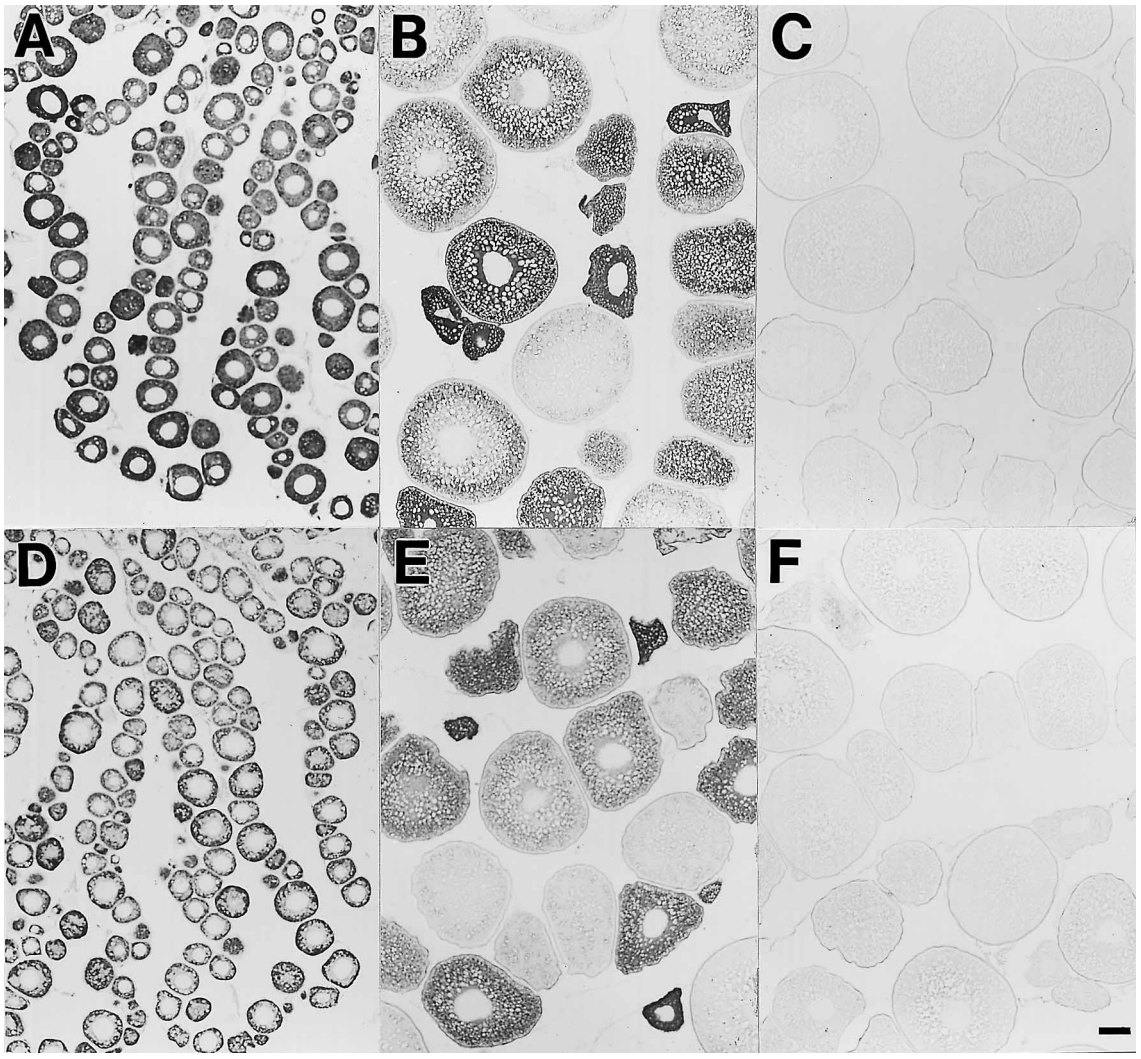

Fig. 2. *In situ* hybridization of eel ovarian tissue using eSRS3 (A, B, C) and eSRS4 (D, E, F) probes. (A, D) pre-vitellogenic ovary, and (B, E) vitellogenic ovary hybridized with anti-sense probes. (C, F) sections hybridized with sense probes as negative control. Bar, 100 μm.

scripts of eSRS3 and 4 in the ovary decreased simultaneously with oogenesis. No signals were detected in the liver throughout the SPH-injection experimental period.

To investigate the localization of eSRS3 and 4 mRNA, ovaries were subjected to *in situ* hybridization using the eSRS3 and 4 ribonucleotide probes. The signal from the cytoplasm of previtellogenic oocytes was strongly positive, whereas that of vitellogenic oocytes was weak (Fig. 2). Among the previtellogenic oocytes, those of the chromatin-nucleolus stage and the perinucleolus stage displayed stronger signals than those of the oil-droplet stage. However, no signal was detected in any other ovarian tissue. On the other hand, sense probes used as negative controls did not hybridize with any of the samples.

Immunodetection of eSRS3 and 4 proteins

The specificity and titer of antibodies was checked by ELISA. The antisera for eSRS3 and 4 specifically recognized respective immunogens and did not cross-react with each other. Further, they reacted to recombinant eSRS3 and 4 produced in *Escherichia coli* (data not shown). Analysis of eSRS3 and 4 proteins in female eels was carried out using these antibodies.

The translations of eSRS3 and 4 were analyzed by Western blotting. Intense signals for both eSRS3 and 4 translations were only detected in the ovary throughout the SPH-injection experimental period (Fig. 3). A major 49 kDa or 54 kDa band was detected throughout oogenesis. The molecular masses, 49 kDa and 54 kDa, were highly similar to those predicted from eSRS3 and 4 cDNA, respectively. In addition, other weak bands were observed. No signals were detected in the liver. In contrast, the preimmune serum used as a negative control did not react to any of the samples.

To investigate the localization of the eSRS3 and 4 proteins, ovaries were subjected to immunohistochemistry by using eSRS3 and 4 antibodies. The antibodies stained the cytoplasm of previtellogenic oocytes and the egg envelope in oocytes at the oil-droplet stage (Fig. 4). However, immuno-

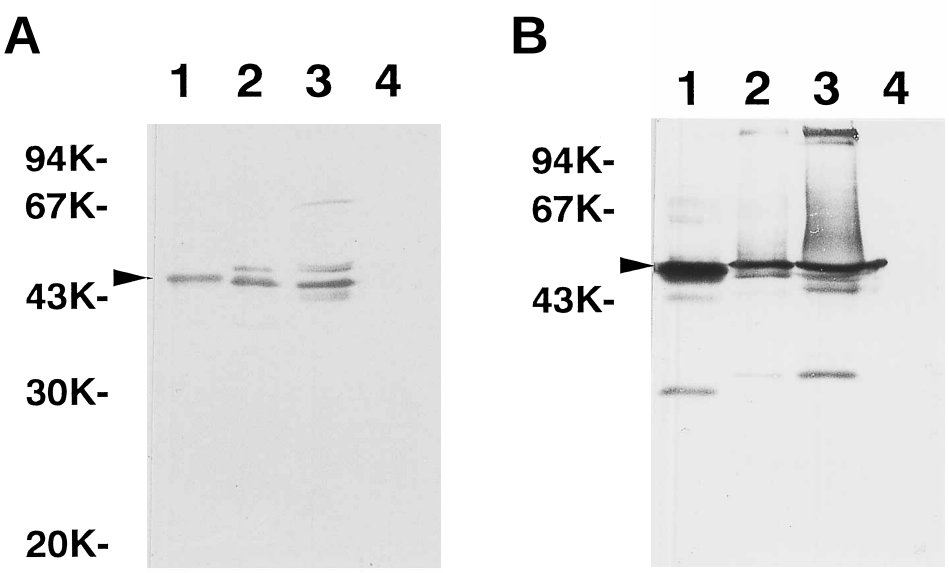

Fig. 3. Western blot analysis of the developing ovary and liver of cultivated Japanese eel after SPH-injection using anti-eSRS3 (A) and anti-eSRS4 (B). Fifty micrograms of protein was applied to each lane. Lane 1, immature ovary; lane 2, vitellogenic stage of ovary; lane 3, mature ovary; lane 4, liver. Positions of molecular weight markers are indicated on the left side of the figure. Arrowheads refer to the position of each main band.

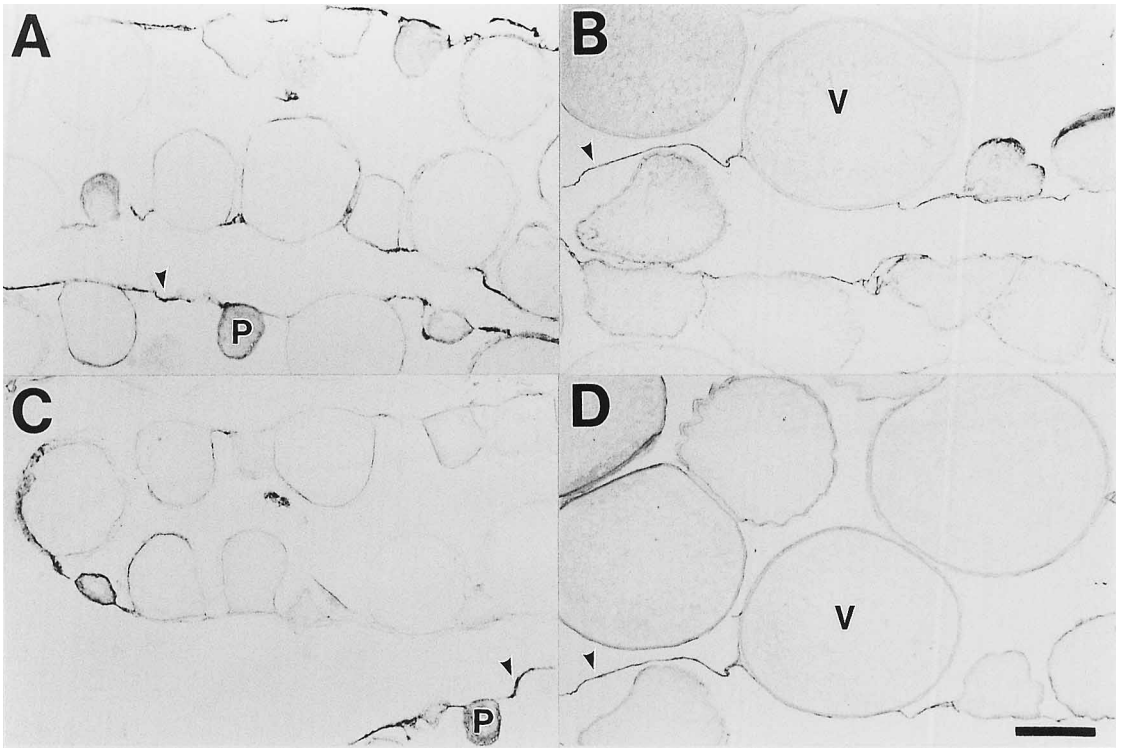

Fig. 4. Immunohistochemistry of eel ovarian tissue using anti-eSRS3 (A, B) and anti-eSRS4 (C, D). (A, C) immature ovary, (B, D) vitellogenic ovary. P; previtellogenic oocytes, V; vitellogenic oocytes. Arrowheads indicate the immunostained connective tissue. Bar, 100 μm.

staining by both antibodies was observed in the connective tissues of ovaries at all developmental stages.

DISCUSSION

The substances eSRS3 and 4 detected in the testis of the Japanese eel display high sequence similarities to ZP2, ZP3, and egg envelope components of other teleosts (Miura *et al.*, 1998). ZP have been reported to play an important role in fertilization as sperm receptors in mammals (Wassarman, 1988). In mammals, ZP2 and ZP3 are synthesized by the oocyte (Bleil and Wassarman, 1980; Shimizu *et al.*, 1983). In

teleosts, however, the homologues are synthesized either by the ovary or by the liver (Anderson; 1967; Hamazaki *et al*., 1984; Begovac and Wallence, 1989; Yamagami *et al*., 1994; Chang *et al*., 1996, 1997; Fujita *et al*., 1998; Shimizu *et al*., 1998). In this study, the expression and localization of eSRS3 and 4 were investigated during ovarian development and in the liver of female eels using antibodies against synthetic peptides corresponding to the amino acid sequence deduced from eSRS3 and 4 cDNA.

eSRS3 and 4 mRNA were expressed only in the ovary throughout oogenesis. These transcripts decreased during the progress of oogenesis as evidenced by their expression in previtellogenic but not in vitellogenic oocytes. Western blot analysis showed that both antibodies recognized main bands corresponding to the predicted molecular weight of eSRS3 and 4 cDNA, but that they also recognized several other bands throughout oogenesis. Immunohistochemical analysis showed that the antibodies specifically recognized the cytoplasm of oocytes in both the chromatin-nucleolus and perinucleolus stage and in the egg envelope of oocytes in the oil-droplet stage. Even though the antibodies immunoreacted to the connective tissue, eSRS3 and 4 mRNA were not detected in these tissues. Therefore, it is thought that this is a non-specific reaction or that these antibodies immunoreacted to some extracelluar matrix similar to eSRS3 and 4. These results indicate that eSRS3 and 4 are synthesized in the ovary, specially in immature oocytes before vitellogenesis, and become components of the egg envelope during the progression of oogenesis.

Regarding the origin of the egg envelope, Giacco *et al*. (1998 a, b) have hypothesized of the existence of a common ancestor in which the oocyte was the only site of synthesis for egg envelope components. In carp, goldfish, and zebrafish, egg envelope components have been reported to be synthesized by the ovary (Chang *et al*., 1996, 1997; Giacco *et al*., 1998 a). According to Nelson (1994), the Japanese eel belongs to the Elopomorpha subdivision, while carp, goldfish, and zebrafish belong to the Ostariophysi superorder and the Euteleostei subdivision. In species belonging to Elopomorpha and Ostariophysi, egg envelope components have not been detected in the serum after estradiol-17β (Larsson *et al*., 1994). In addition, in carp, ZP3 transcripts have not been detected in the liver after estrogen treatment (Chang *et al*., 1996). In contrast, in species not belonging to the previous two groups, egg membrane components have been detected in both the liver and serum after estradiol-17β treatment (Hamazaki *et al*., 1987; Murata *et al*., 1991; Hyller *et al*., 1991; Oppen-Berntsen *et al*., 1992a, b; Larsson *et al*., 1994; Fujita *et al*., 1998; Shimizu *et al*., 1998). Futhermore, it has recently been reported of medaka that ZP2 and ZP3 homologues are expressed in the ovary at 5 days after hatching but cannot be detected in the liver of maturing female fish (Kanamori, 2000). These results suggest that there may be two kinds of ZP homologues in one species, one originating from the ovary and another from the liver, where the egg membrane components originating from the ovary are expressed earlier than those from the liver. As previously mentioned, eSRS3 and 4 are synthesized only in the ovary and not in the liver at any developmental stage. It is possible, therefore, that egg envelope components other than eSRS3 and 4 are produced by the liver.

The results of the present study suggest that eSRS3 and 4 are egg envelope components due to their primary amino acid structure and their expression patterns in the ovary. eSRS3 and 4 may also play other roles independent of the construction of eel-egg envelopes, since the expression of both substances in the ovary has been observed earlier than the vitellogenic stage, when vigorous egg envelope formation occurs. Furthermore, their transcripts are expressed in immature testes containing only premitotic spermatogonia. It is possible, therefore, that eSRS3 and 4 have the same function in both males and females during the early developmental stages of the gonads. Further research on the function of eSRS3 and 4 is therefore required for us to develop a more complete understanding of early gametogenesis in the Japanese eel.

ACKNOWLEDGMENTS

We would like to thank Dr. M. R. Nader for reading the manuscript. This reseach was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists and the Ministry of Agriculture, Forestry and Fisheries of Japan (BDP 99-IV-2-6).

REFERENCES

Anderson E (1967) The formation of the primary envelope during oocyte differentiation in teleosts. J Cell Biol 35: 193–212

Begovac PC, Wallace RA (1989) Major vitelline envelope proteins in pipefish oocytes originate within the follicle and are associated with the Z3 layer. J Exp Zool 25: 56–73

Bleil JD, Wassarman PM (1980) 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

Chang YS, Wang SC, Tsao CC, Huang FL (1996) Molecular cloning, structural analysis, and expression of carp ZP3 Gene. Mol Reprod Dev 44: 295–304

Chang YS, Hsu CC, Wang SC, Tsao CC, Huang FL (1997) Molecular cloning, structural analysis, and expression of carp ZP2 Gene. Mol Reprod Dev 46: 258–267

Fujita T, Takemura A, Takano K (1998) Immunochemical detection of precursor proteins of yolk and vitelline envelope, and their annual changes in the blood of *Diodon holocanthus*. J Fish Biol 52: 1229–1240

Giacco LD, Vanoni C, Bonsignorio D, Duga S, Mosconi G, Santucci A Cotelli F. (1998a) Identification and spatial distribution of the mRNA encoding the gp49 component of the gilthead sea bream, *Sparus aurata*, egg envelope. Mol Reprod Dev 49: 58–69

Giacco LD, Binsignorio D, Cotelli F (1998b) ZPC homologues in teleost fish egg envelope. Zugote 6: 90

Hamazaki T, Iuchi I, Yamagami K (1984) Chorion glycoprotein-like immunoreactivity in some tissues of adult female medaka. Zool Sci 1:148–150

Hamazaki TS, Iuchi I, Yamagami K (1987) Production of a "Spawning Female-Specific Substance" in hepatic cells and its accumulation in the ascites of the estrogen-treated adult fish, *Oryzias latipes*. J Exp Zool 242: 325–332

Hedrick JL (1996) Comparative structural and antigenic properties of

zona pellucida glycoproteins. J Reprod Fertil Suppl 50: 9–17
Hyllner SJ, Oppen-Bertsen DO, Helvik JV, Walther BT, Haux C (1991) Oestradiol-17β induces the major vitellin envelope proteins in both sexes in teleosts. J Endocrinol 131: 229–236
Kanamori A (2000) Systematic identification of genes expressed during early oogenesis in medaka. Mol Reprod Dev 55: 31–36
Larsson DGJ, Hyllner SJ, Haux C (1994) Induction of vitelline envelope proteins by estradiol-17β in 10 teleost species. Gen Comp Endocrinol 96: 445–450
Miura C, Miura T, Kudo N, Yamashita M Yamauchi K (1999) cDNA cloning of a stage-specific gene expressed during HCG-induced spermatogenesis in the Japanese eel. Develop Growth Differ 41: 463–471
Miura T, Yamauchi K, Nagahama Y, Takahashi H (1991) Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool Sci 8: 63–73
Miura T, Kudo N, Miura C, Yamauchi K, 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
Murata K, Hamazaki TS, Iuchi I, Yamagami K (1991) Spawning fermale-specific egg envelope glycoprotein-like substances in *Oryzias latipes*. Develop Growth Differ 33: 553–562
Murata K, Sasaki T, Yasumasu S, Iuchi I, Enami J, Yasumasu I, 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
Murata K, Sugiyama H, Yasumasu S, Iuchi I, Yasumasu I, Yamagami K (1997) Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion). Proc Natl Acad Sci USA 94: 2050–2055
Nelson JS (1994) Fishes of the world, 3rd Ed. John Wiley & Sons, New York
Oppen-Berntsen DO, Gram-Jensen E, Walther BT (1992a) Zona radiata-proteins are synthesized by rainbow trout (*Oncorhyncus mykiss*) hepatocytes in response to oestradiol-17β. J Endocrinol 135: 293–302
Oppen-Berntsen DO, Hyllner SJ, Haux C, Helvik JV, Walther BT (1992b) Eggshell zona radiata-proteins from cod (*Gadus morhua*): Extra-ovarian origin and induction by estradiol-17β. Int J Dev Biol 36: 247–254
Shimizu S, Tsuji M, Dean J (1983) In vitro biosynthesis of three sulfated glycoproteins of murine zonae pellucidae by oocytes grown in follicle culture. J Biol Chem 258: 5858–5863
Shimizu M, Fujita T, Hiramatsu N, Hara A (1998) Immunochemical detection, estrogen induction and occurrence in serum of vitelline envelope-related proteins of Sakhalin taimen *Hucho perryi*. Fisheries Sci 64: 600–605
Wassarman PM (1988) Zona pellucida glycoproteins. Annu Rev Biochem 57: 415–442
Yamagami K, Murata K, Hamazaki TS, Iuchi I (1994) Intrahepatic formation of SF substances, the precursors of egg envelope proteins, in response to estrogen administration in fish. In "Perspectives in Comparative Endocrinology" Ed by KG Davey, RE Peter, SS Tobe, Nat Res Council Canada, Ottawa, pp 316–324
Yamamoto K, Hiroi O, Hirano T, Morioka T (1972) Artificial maturation of cultivated male Japanese eels by Synahorin injection. Bull Jpn Soc Sci Fish 38: 1083–1090
Yamamoto K, Yamauchi K (1974) Sexual maturation of Japanese eel and production of eel larvae in the aquarium. Nature 251: 220–222

(Received April 17, 2000 / Accepted June 17, 2000)