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Cloning and characterization of vitellogenin cDNA from the common Japanese conger (*Conger myriaster*) and vitellogenin gene expression during ovarian development

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Abstract

The major yolk protein precursor, vitellogenin (VTG) was detected in plasma from vitellogenic females and estradiol-17β (E_2)-treated immature females, but not in males and immature females by Western blotting in common Japanese conger *Conger myriaster*. Its molecular mass was approximately 180 kDa under denaturing and reducing conditions. The common Japanese conger VTG cDNA was cloned from the liver of vitellogenic female. It contains 5110 nucleotides including an open reading frame that encodes 1663 amino acids. The deduced amino acid sequence of the common Japanese conger VTG shares 80% identity with that of eel *Anguilla japonica* VTG-1, and 45–55%, 32–34% and 27–29% identity with the deduced amino acid sequences of other fish, amphibian and avian VTG with polyserin domain, respectively. In female common Japanese conger, VTG gene was highly expressed in the liver of this species similar with other oviparous vertebrates. The expression levels of VTG gene in the liver increased from the oil droplet stage to the tertiary yolk globule stage and were maintained until the migratory nucleus stage.

Keywords: Anguilliformes; Common Japanese conger; *Conger myriaster*; Liver; Oocyte development; RACE, real-time PCR; Vitellogenesis; Vitellogenin

1. Introduction

The common Japanese conger (*Conger myriaster*) of the order Anguilliformes is widely distributed along the coasts of Japan and Korea and the East China Sea. This species is an important fisheries resource, but fish catches have decreased year by year. The many aspects of the life history of this species are unclear and information on the reproductive biology and physiology is considerably restricted, because spawning areas have not been identified yet and mature females have not been caught in nature. Therefore, some studies on the elucidation of reproduction in this species were carried out using field research, as well as experiments on rearing conditions and hormone administration. The knowledge gained from those studies is as follows: (1) maturation of the female fish begins from autumn to winter on the coast of the Atsumi peninsula in Aichi Prefecture in Japan and then progresses offshore (Okamura et al., 2000); (2) the process of oogenesis is almost identical to that of other anguillid fish (Utoh et al., 2003); (3) this species ovulates pelagic eggs (Horie et al., 2001); (4) the external shape of hatched larvae is similar to that of the Japanese eel (*Anguilla japonica*) (Horie et al., 2002); (5) oocytes developed to the tertiary yolk globule stage under rearing conditions without hormone administration, unlike *Anguilla* species (Utoh et al., 2005). However, fertilized eggs have not been obtained without hormone administration until now. Furthermore, it is difficult to obtain fertilized eggs and hatched larvae even with hormone administration.

Researches on the seed production of this species have just begun in the latter half of the 1990s. Compared with Japanese

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eel, the history of those researches is very short. Improvements are still left particularly in the method of rearing and hormone administration. To obtain good-quality fertilized eggs and hatched larvae at high frequency, we need to understand further the mechanisms of oocyte development.

Vitellogenin (VTG) is the precursor of major yolk protein detected in oviparous vertebrates and invertebrates. It is a large-molecular-weight glycolipophosphoprotein synthesized under the control of estrogen, mainly estradiol-17β (E_2), in vertebrate females during vitellogenesis (Wallace, 1985; Byrne et al., 1989). E_2 is produced by ovarian follicles in response to gonadotropin release into the bloodstream. The complex of estrogen and its receptor recognizes estrogen-responsive elements (EREs) located upstream of the *VTG* gene and regulates transcriptional activity (Wahli, 1988). VTG, once secreted into the bloodstream, is transported to the ovary and is incorporated into growing oocytes by receptor-mediated endocytosis (Schneider, 1996). Subsequently, it is enzymatically cleaved into smaller proteins, lipovitellin and phosvitin in amphibians and avians (Wallace, 1985; Byrne et al., 1989), and lipovitellin, phosvitin and β′-component in fish (Matsubara and Sawano, 1995; Hiramatsu and Hara, 1996), and is used as an important source of nutrition during early embryonic development. In general, plasma VTG levels tend to increase during vitellogenesis in female fish (Van Bohemen et al., 1981; Pacoli et al., 1990; Mañanós et al., 1994; Bon et al., 1997; Kokokiris et al., 2001). Therefore, the plasma VTG level is used as an indicator in the process of oocyte development. In the present study, we describe the cloning and characterization of VTG cDNA and the pattern of *VTG* gene expression in the liver during oocyte development under rearing conditions and upon hormone administration, as the initial step in elucidating the female reproductive physiology of this species.

2. Materials and methods

2.1. Fish and sampling procedure

Common Japanese congers with of about 20 cm total length and 20 g body weight were caught in Mikawa Bay, central Japan, in September 1999. The fish were reared in outdoor tanks supplied with flowing seawater (temperature: 10–20 °C, salinity: 30–31‰) and fed with frozen Japanese sand lance *Ammodytes personatus*. Sampling was carried out from June 2000 to June 2001. The total length and body weight of sampled fish were ranged from 48.6 to 80.8 cm and from 235 to 922 g, respectively. The fish were anesthetized with 0.04% 2-phenoxyethanol and dissected. The gonad and liver were removed and weighed for calculation of the gonadosomatic index (GSI: the gonad weight/body weight × 100) and the hepatosomatic index (HSI: the liver weight/body weight × 100). The developmental stages of the oocytes were assigned according to Utoh et al. (2003). The livers were rapidly placed in guanidinium thiocyanate homogenization buffer (4 M guanidinium thiocyanate, 0.75 M sodium citrate pH 7.0, 0.5% *N*-lauroylsarcosine, 0.1 M 2-mercaptoethanol) for extraction of total RNA.

Two Japanese eels were caught in Mikawa Bay in September and November 2001. The fish were anesthetized with 0.08% 2-phenoxyethanol and dissected. The gonad was removed and weighed for calculation of the GSI. The total length and body mass of sampled fish were 71.8 and 83.0 cm, and 578 and 815 g, respectively.

The blood samples from both species were collected from the bulbus arteriosus using heparinized syringe and centrifuged at 1630 ×*g* for 10 min at 4 °C. The plasma was frozen at −40 °C until use.

2.2. Hormone treatment

To induce ovarian development, fish that reached the primary or secondary yolk globule stage were injected with 100 IU/kg human chorionic gonadotropin (hCG) (Sankyo, Tokyo, Japan) at the dorsal muscle every two weeks (Horie et al., 2001). Fish were kept in circulating seawater tanks at 10 or 12 °C and were not fed throughout the experimental period. After five or six injections, the liver was removed and immediately homogenized in guanidinium thiocyanate homogenization buffer to obtain total RNA.

2.3. Vitellogenin induction

Immature female common Japanese congers were injected with estradiol-17β dissolved in isopropyl alcohol (10 mg/kg body mass) twice a week. After 4 times injection, blood samples were collected from the bulbus arteriosus using heparinized syringe and centrifuged at 1630 ×*g* for 10 min at 4 °C. The plasma was frozen at −40 °C.

2.4. Gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out according to the method by Laemmli (1970) with 4.75% stacking and a 10% or 15% separating polyacrylamide gel in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 0.05% SDS) at 20 mA constant. Plasma samples were diluted 2-fold (only plasma from E_2-treated fish was diluted 6-fold) with TBS (20 mM Tris, 137 mM sodium chloride; pH 7.6). Samples were mixed 1 : 1 (v/v) with sample buffer (containing 4% SDS and 5% 2-mercaptoethanol) and heated for 5 min at 95 °C, after which 2 μL was loaded into each well. After electrophoresis, the protein bands were stained with CBB-R250.

2.5. Western blotting

Western blotting was carried out using ECL Western blotting reagent packs (Amersham Pharmacia Biotech, Little Chalfont, England). The gel was electroblotted onto a PVDF membrane (Amersham Pharmacia Biotech) for 1.5 h at 100 V. The membrane was washed briefly in TBS (20 mM Tris, 137 mM NaCl; pH 7.6), and then non-specific binding was blocked with an over night incubation at 4 °C in a solution containing 5% membrane blocking agent in TBST (TBS with 0.1% (v/v) Tween-20). The membrane was incubated with an antiserum for Japanese eel (*Anguilla japonica*) VTG diluted in TBST (1 : 10,000) for 1 h at