발생과 생식 제 4권 제 1호, 125~131 (2000)
Dev. Reprod. Vol. 4, No. 1, 125~131 (2000)

Increased mRNA Related Ovarian Maturation during Induction of Maturational Competence in Red Seabream, *Pagrus major*

Cheol-Young Choi[1†], Young-Jin Chang[2] and Fumio Takashima[1]

[1]*Department of Aquatic Biosciences, Tokyo University of Fisheries, Konan, Minato-ku, Tokyo 108-8477, Japan*

[2]*Department of Aquaculture, Pukyong National University, Nam-ku, Pusan 608-737, Korea*

참돔, *Pagrus major*의 성숙능력 유도시 증가된 난성숙 관련 mRNA

최철영[1†] · 장영진[2] · 隆島史夫[1]

[1]東京水產大學 資源育成學科 · [2]부경대학교 수산과학대학 양식학과

ABSTRACT: This study has used differential display-PCR, to amplify genes transcribed during the ovarian maturation induced by human chorionic gonadotropin (hCG). The cDNA expressed at the times of acquisition of oocyte maturational competence in red seabream (*Pagrus major*) following treatment with hCG was amplified and cloned. A full-length of cDNA for *P. major* was isolated using differential display-PCR and 5'RACE. This cDNA clone contained 2,662 nucleotides including the open reading frame that encoded 434 amino acids. Homology analyses, using the GenBank and EMBL general database searches, indicated that the nucleotides sequence of the cDNA does not have high homology with any other genes. This cDNA was judged to be a gene, which induction of maturational competence coincides with increase of mRNA related ovarian maturation. Consensus sequences which were consistent with protein kinase C phosphorylation sites and casein kinase II phosphorylation sites were identified. *In vitro*, the transcription level of mRNA related ovarian maturation increased between 9 hr and 24 hr following treatment of ovarian follicles with hCG. It was also increased after GtH-II (300 ng/ml) stimulation. Furthermore, *in vivo*, mRNA related ovarian maturation was rarely expressed prior to the acquisition of oocyte maturational competence, but was strongly expressed after the acquisition of oocyte maturational competence, suggesting that the hCG induction of maturational competence is brought about by the *de novo* synthesis of the mRNA related ovarian maturation in *P. major*.

Key words: Red seabream, *Pagrus major*, mRNA related ovarian maturation, Maturational competence.

요 약: Differential display-PCR 방법을 이용하여, hCG 처리에 의한 참돔, *Pagrus major*의 난성숙 능력의 획득 경과시간에 따라 새롭게 발현하는 cDNA를 해석하였다. Differential display-PCR과 5'RACE 방법을 이용하여, 2,662 염기와 434개의 아미노산을 코드하고 있는 cDNA의 전염기배열을 결정하였다. DNA의 데이터베이스인 GenBank 및 EMBL을 이용하여 상동성을 검색한 결과, 본 cDNA와 높은 상동성을 나타낸 유전자는 검색되지 않았다. 따라서 본 cDNA는 참돔의 난성숙 능력 유도와 함께 그 발현량이 증가하는 난성숙 관련 유전자로 판단되었다. 또한 본 cDNA에서는 protein kinase C 인산화 및 casein kinase II 인산화 consensus 배열의 존재가 확인되었다. 본 연구에서 cloning된 난성숙 관련 유전자는 난여포에 hCG 처리 9~24시간 후에 그 발현량이 증가하였으며, GtH-II (300 ng/ml)로 배양한 난여포에서 특이적으로 증가하였다. 또한 *in vivo* 실험에서 난성숙 관련 유전자는 난성숙 능력 획득 이전의 난소에서는 거의 발현하지 않았으나, 난성숙 능력을 획득한 난소에서 강하게 발현된 점으로 보아, hCG에 의한 난성숙 능력 유도에 성숙기간 중 새롭게 합성되는 난성숙 관련 유전자가 관여할 가능성이 높다.

INTRODUCTION

In teleost fishes, oocyte maturation is induced by maturation-inducing steroids (MIS), such as 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) (Nagahama, 1987) and 17α, 20β,21-trihydroxy-4-pregnen-3-one (20β-S) (Trant & Thomas, 1988). These steroids are produced by the follicular layer surrounding oocytes under stimulation of gonadotropic hormone (GtH)

†교신저자: Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 905-0227, Japan. (Tel)+81-980-47-6215 (Fax)+81-980-47-4919 e-mail: choi_cy@ hanmail.net

(Nagahama, 1987; 1990). It is known that priming with an injection of GtH influences the induction of oocyte maturation, *in vitro* (Patino & Thomas, 1990; Degani & Boker, 1992). However, this general concept does not always hold true as shown by studies of the oocyte maturational competence of daily spawners, such as kisu (*Sillago japonica*) (Kobayashi et al., 1988; Zhu et al., 1994), tobinumeri-dragonet (*Repomucenus beniteguri*) (Zhu et al., 1989; 1994), Atlantic croaker (*Micropogonias undulatus*) (Patino & Thomas, 1990), olive flounder (*Paralichthys olivaceus*) (Yoshikuni et al., 1992) and red seabream (*Pagrus major*) (Kagawa et al., 1994). The response of the oocytes to DHP could be induced *in vitro* by preincubation with human chorionic gonadotropin (hCG) (Patino & Thomas, 1990; Yoshikuni et al., 1992; Kagawa et al., 1994; Zhu et al., 1994), suggesting that DHP does not act as a mediator of hCG in the development of oocyte maturational competence. Moreover, in the oocytes of *M. undulatus* (Patino & Thomas, 1990) and *P. major* (Kagawa et al., 1994), actinomycin D has been found to inhibit GtH induced germinal vesicle breakdown (GVBD). These reports suggest that the production of new proteins through a mechanism of GtH-stimulated RNA synthesis is essential for the development of oocyte maturational competence (Patino & Thomas, 1990; Kagawa et al., 1994). Except for *M. undulatus* connexin 32.2 mRNA (Yoshizaki et al., 1994), however, the activities of genes related to oocyte differentiation or maturational competence has not been directly addressed in *P. major* or in any other vertebrate species.

The present study was designed to determine whether the change of mRNA levels in the ovarian follicles of *P. major* following treatment with hCG during the acquisition of oocyte maturational competence, using differential display-PCR. This method potentially amplifies genes transcribed during hCG-induced oocyte maturation.

MATERIALS AND METHODS

1. Fish and Tissue Collection

Mature *P. major* weighing 2.0~3.0 kg were purchased from fishermen in Gokasho Bay, Mie Prefecture, Japan and kept in a flow through outdoor tank (3 ton). The fish were anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO) and killed by decapitation at 08:00 hr (incompetent ovaries) and 16:00 hr (competent ovaries). Incompetent ovaries were removed and placed in ice-cold Ringer's solution (pH 7.4, supplemented with 10 g of NaCl, 0.22 g of KCl, 0.25 g of $CaCl_2$, 0.74 g of $MgSO_4 \cdot 7H_2O$, 1.19 g of HEPES, 0.9 g of glucose, 0.1 g of streptomycin, and 100,000 U of penicillin G calcium per liter). Ovaries were cut into small pieces in ice-cold Ringer's solution and the ovarian follicles were dispersed by pipetting the fragments.

2. Incubation Procedures

Clusters of ovarian follicles were removed by passage of the suspension through a stainless-steel wire mesh (pore diameter, 600 ㎛), and ovarian follicles at the tertiary yolk stage (about 500 ㎛ in diameter) (Matsuyama et al., 1988) were collected on a wire mesh (about 420 ㎛). Approximately 60 ovarian follicles were transferred to individual wells of 24-well culture plates, each of which contained 1 ml of Leibovitz's L-15 medium (Gibco-BRL, Gaithersburg, MD), pH 7.6, supplemented with 2.4 g of HEPES, 0.1 g of streptomycin, and 100,000 U of penicillin G calcium per liter. The oocytes were preincubated in 1 ml of Leibovitz's L-15 medium in the presence of human chorionic gonadotropin (hCG; Teikoku Zoki Ltd, Tokyo, Japan) at 10 IU/ml. At time 0 (untreated control), 3, 6, 9, 12 and 24 hr after hCG treatment, ovarian follicles were washed 3 times with fresh Leibovitz's L-15 medium, transferred to Leibovitz's L-15 medium containing 17α, 20β-dihydroxy-4-pregnen-3-one (DHP; 10 ng/ml; Sigma) and/or actinomycin D (1 ㎍/ml; Sigma) and incubated for 24 hr at 20℃. In addition, some ovarian follicles were preincubated with DHP (10 ng/ml), human insulin-like growth factor-I (IGF-I; 10 nM; human recombinant, Bachem Inc., Torrance, CA), *P. major* GtH-I (100 ng/ml, 300 ng/ml) (Tanaka et al., 1993), or *P. major* GtH-II (100 ng/ml, 300 ng/ml) (Tanaka et al., 1993) for 0 (untreated control), 3, 6, 9, 12 and 24 hr, transferred to medium containing DHP (10 ng/ml) and/or actinomycin D (1 ㎍/ml) and incubated for 24 hr at 20℃. After incubation, all the ovarian follicles were stored at −80℃ until used.

3. Differential Display-PCR

Total RNA was extracted using a total RNA extraction kit (Pharmacia Biotech, Uppsala, Sweden) from each time of hCG-treated ovarian follicles. Differential display-PCR experiments were carried out using an RNA map™ mRNA differential

display system (GenHunter, Brookline, MA). Total RNA was reverse-transcribed with an anchor primer in the presence of Maloney murine leukemia virus (MMLV) reserve transcriptase (GenHunter) and then amplified with an 5' adaptor primer (5'-AAGCTTCGACGCT-3') and the anchor primer (5'-AAGCTTTTTTTTTTTA-3') in the presence of Ampli-*Taq* DNA polymerase (5 U/$\mu\ell$; Perkin-Elmer, Norwalk, CT). PCR was carried out for 40 cycles as follows; 94°C for 30 sec for denaturing; 40°C for 2 min for annealing; and 72°C for 30 sec for extension, followed by 1 cycle for 10 min at 70°C for extension. Three $\mu\ell$ loading dye was added to 5.25 $\mu\ell$ the amplification products, heated at 80°C for 2 min and then fractionated on a 6% DNA sequencing gel. The gel was run at 55 W constant powers for 3 hr, dried and then analyzed by staining of DNA silver staining kit (Pharmacia Biotech.). The PCR product which increased particularly during the oocyte maturation was excised from the gel, soaked in 100 $\mu\ell$ of H_2O for 10 min, boiled for 15 min and centrifuged at room temperature (20°C) for 2 min. DNA was precipitated from the supernatant by the addition of 10 $\mu\ell$ of 3 M sodium acetate, 5 $\mu\ell$ of glycogen (10 mg/ml; Sigma) and 350 $\mu\ell$ of 100% ethanol for 15 min at −80°C, and centrifugation for 10 min at 4°C. The pellet was then dissolved in 10 $\mu\ell$ of H_2O. For each band, 4 $\mu\ell$ of the extracted DNA was reamplified using the same primer set and PCR conditions used above. One fragment was isolated and ligated, cloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced.

4. Rapid Amplification of cDNA 5'ends (5'RACE)

The PCR production (179 bp) was particularly increased during the development of oocyte maturation. Ovarian follicles from each times of incubation with hCG (10 IU/ml) were found to have an adaptor primer (5'-AAGCTTCGACGCT-3') and the anchor primer (5'-AAGCTTTTTTTTTTTA-3') included with the RNA map™ mRNA differential display system. One clone isolated using differential display-PCR did not contain the complete 5'ends. Consequently, a fragment containing the 5'end of the transcript was amplified using a 5'RACE System for Rapid Amplification of cDNA Ends, Version 2.0 kit (Gibco-BRL). The total RNA (1 μg) was reverse-transcribed according to the kit protocol using a gene specific primer (GSP)1 (5'-ATGTGTGTTATGACAT-3') located within the coding sequence. Therefore, two GSPs were designed (GSP2; 5'-ACAGACTTTATATCTGAGGTACTACTGGACC-3' and GSP3; 5'-CACCAACATGGCCGCCAACTC-3'). The final PCR product was amplified and cloned and sequenced using the ALFexpress DNA sequencer (Pharmacia Biotech.), and the DNA sequence was analyzed using the GENETYX-WIN (Software Development Co., Japan) software package.

5. Northern Blot Analysis

To clarify whether isolated mRNA from ovarian follicles from each time of incubated with hCG (10 IU/ml) was differentially expressed specifically in the oocyte undergoing developmental maturation, Northern blot analysis was performed. The total RNA (30 μg) was electrophoresed according to the formaldehyde gel method (Sambrook et al., 1989) and transferred to nylon membranes (Hybond N^+) (Amersham, Buckinghamshire, U.K.) according to the manufacturer's instructions. The PCR product label used for the Oligolabelling kit (Amersham) with [α-^{32}P] dCTP. Post-hybridization washing was carried out in 2×SSC for 20 min at 65°C, in 1×SSC/0.5% SDS for 15 min at 65°C, and finally in 0.1×SSC/0.5% SDS for 15 min at 65°C. The hybridization signals were analyzed using FUJIX BAS 1000 Bio-Imaging Analyzer (Fuji Film, Japan).

6. Statistics

The data were expressed as mean±S.E.M. ANOVA followed by Duncan's multiple range tests was as applied for statistical analysis.

RESULTS AND DISCUSSION

In this study, hCG (or GtH-II) induced oocyte maturational competence in the ovarian follicles of *P. major*. Kagawa et al. (1988) reported that GtH-II, but not GtH-I, is involved in the final maturation of *P. major* oocytes. Moreover, in the *M. undulatus* oocytes, actinomycin D blocked GtH-induction of oocyte maturational competence (Patino & Thomas, 1990). Kagawa et al. (1994) reported that hCG induced the oocyte maturational competence of *P. major* via synthesis of RNA and *de novo* protein synthesis. Therefore, newly-synthesized proteins are involved in hCG (or GtH-II)-induction of oocyte maturational competence. However, if hCG (or GtH-II) directly stimulates an

increase in oocyte maturational competence of ovarian follicles, this increase should not require de novo protein synthesis. In this study, cDNA expressed in ovarian follicles during the acquisition of oocyte maturational competence after treatment with hCG (or GtH-II) was cloned using differential display-PCR method. The differential display-PCR method was used to identify differences among subsets of mRNA samples, since it allows the amplification of genes the transcription of which is induced under unique conditions (Liang & Pardee, 1992; Liang et al., 1994; Zimermann & Schultz, 1994). Fig. 1 shows a base sequence of the cloned cDNA. It contained a 2,662 bp insert cDNA with an open reading frame of 1,302 bp that begins with the first ATG codon at position 3 bp and ends with a TGA stop codon at position 1,305 bp. A putative polyadenylation signal, AATAAA (Proudfoot & Brownlee, 1976) was recognized at position 2,634 bp. This open reading frame encodes a polypeptide containing 434 amino acids (Fig. 1). Homology analyses using the GenBank and the EMBL general database searches indicated that the nucleotide sequence of the cDNA did not have a high homology with any other genes. Therefore, this cDNA was judged to be a gene, which induction of maturational competence coincides with increase of mRNA related ovarian maturation. The physiological roles on production of the mRNA related ovarian maturation are not known.

In this study, Northern blot analyses revealed that the transcription level of mRNA related ovarian maturation increased with the time of incubation; the levels at 9 hr and 24 hr were significantly higher ($P<0.05$) than that at 0 hr (Fig. 2). Effects of hCG on *in vitro* induction of GVBD were examined using the ovarian follicles ($87.76\pm3.29\%$) of the oocytes had undergone GVBD at 12 hr (data not shown). Therefore, the processes of oocyte maturational competence and the induction of GVBD of the oocytes are well correlated. Furthermore, Northern blot analysis on transcription levels of mRNA related ovarian maturation, and on the β-actin probe for positive control, was carried out using 30 μg of total RNA extracted from ovarian follicles cultured for 12 hr in normal medium, DHP (10 ng/ml), human IGF-I (10 mM), *P. major* GtH-I (100 ng/ml, 300 ng/ml) or *P. major* GtH-II (100 ng/ml, 300 ng/ml) (Fig. 3). The mRNA related ovarian maturation was highly expressed in the presence of GtH-II, while DHP and IGF-I had no effect.

There is currently no information on the functional domain and consensus sequence of this gene. However, it is noteworthy that mRNA related ovarian maturation possesses the protein kinase C (PKC) phosphorylation site, (S,T)X(R,K) (Kishimoto et al., 1985; Woodget et al., 1986), and the casein protein kinase II phosphorylation site, $(S,T)X_2(D,E)$ (Pinna, 1990) (Fig. 1). These regulate phosphorylation of many systems (Hoh et al., 1991). Takeda et al. (1987) reported that rat connexin (Cx) 32 was phosphorylated by PKC *in vitro. M. undulatus* Cx32.2 also has PKC phosphorylation sites, and an increase in ovarian Cx32.2 mRNA production coincided with the induction of oocyte maturational competence (Yoshizaki et al., 1994). Moreover, phorbol 12-myristate 13-acetate (PMA), a PKC activator, inhibited GtH-II inducible oocyte maturational competence (Chang et al., 1999). Furthermore, Yoshizaki et al. (1994) reported that the transcription of Cx32.2 levels were negligible in incompetent ovarian tissue and increased substantially upon induction of competence by treatment with hCG. In the present study, in vivo Northern blot analysis was carried out using 30 μg of total RNA extracted from incompetent and competent ovaries (Fig. 4). The mRNA related ovarian maturation was highly expressed after, but not before, the acquisition of oocyte maturational competence. These results suggest that the gene activation of mRNA related ovarian maturation in *P. major* is related to the selective development of ovarian follicles to completion of oocyte maturational competence.

In summary, the present study found that the induction of oocyte maturational competence by GtH-II requires *de novo* synthesis of ovarian RNA and protein, and coincides with an increase in the production of a novel gene transcript, mRNA related ovarian maturation. On the other hand, the physiological roles of mRNA related ovarian maturation in the acquisition of oocyte maturational competence could not be clarified. Further research is needed to explain the role of steroids and secondmessenger systems in the regulation on the expression of mRNA related ovarian maturation.

ACKNOWLEDGEMENTS

The authors would like to thank to Dr. John F. Leatherland, Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, for his valuable comments and critical readings of this manuscript. We also thank Dr. Akihiro

```
   1 GG
   3 ATGGCCCAGAACCAGGTGGTTCTGCAGTTCCGCTTTGCCACATTCGGGGACTCCATGCTGCAGAAGATGAACCTCCTACGTCACCAGAGA
     MetAlaGlnAsnGlnValValLeuGlnPheArgPheAlaThrPheGlyAspSerMetLeuGlnLysMetAsnLeuLeuArgHisGlnArg 30

  93 CGCTTCTGTGATGTCACCGTACGCATCAACCAGCTGGAGGTCCCCGGTCACAAGGTAGTGTTTGCCGCTGGCTCCTCGTTCCTGAGGGAC
     ArgPheCysAspValThrValArgIleAsnGlnLeuGluValProGlyHisLysValValPheAlaAlaGlySerSerPheLeuArgAsp 60

 183 CAGTTCATCCTGCAGCAGGATTCCAGGGCGGTTCAGATTTCCATGATCCAGGAGGCGGAGGTGGGCCGGCAGCTGCTGCTGTCCTGCTAC
     GlnPheIleLeuGlnGlnAspSerArgAlaValGlnIleSerMetIleGlnGluAlaGluValGlyArgGlnLeuLeuSerCysTyr  90

 273 ACAGGTCAGCTGGAGTTTCCCGAGCTGGAGCTGGTGCATTACCTGACAGTGGCCAGTTTCCTCCAAATGGGCCACATTGTGGAGCAATGC
     ThrGlyGlnLeuGluPheProGluLeuGluLeuValHisTyrLeuThrValAlaSerPheLeuGlnMetGlyHisIleValGluGlnCys 120

 363 ACTCAGGCACTCAGCAAGTTCATTAAACCCCAGGCTGCACGGCAGCTGGAGGTGGAGGTGGAGGTGGATATGAGGAGGGAGAAGAAGGAG
     ThrGlnAlaLeuSerLysPheIleLysProGlnAlaAlaArgGlnLeuGluValGluValGluValAspMetArgArgGluLysLysGlu 150

 453 GAGGGTTTATCCTCTCAGGCCCAGAGAGGGCAAGAGCACTCTCAGGTTCGGACTGTCCATGAGGAAGATGAGGACGTGGAGCATGTTGAG
     GluGlyLeuSerSerGlnAlaGlnArgGlyGlnGluHisSerGlnValArgThrValHisGluGluAspGluAspValGluHisValGlu 180
                                                  ● ● ● ●
 543 GACGACAACAATGGTGACGACGACGATGATGATGACGATGTGATCATACAGCCCAAGTCCCCTCTCCAGATCTTAGGCAGACGTCCAAGG
     AspAspAsnAsnGlyAspAspAspAspAspAspAspValIleIleGlnProLysSerProLeuGlnIleLeuGlyArgArgProArg 210

 633 CAGGGTATGGTGGAGAGTGACATCACTATAGTAAAAGTGGAGTCTGTGTCTGACGTAGCAGAAAACTCCATCACTGGTCATTTCCCCTCC
     GlnGlyMetValGluSerAspIleThrIleValLysValGluSerValSerAspValAlaGluAsnSerIleThrGlyHisPheProSer 240
                                                ● ● ● ●
 723 AGCCCACCTGCTGCCCTGCACTCTCCTGAGCCCCAGCACTCCCTCATCAACTCCACTGTTTACAGTCGTGGCAGTGAGATGGCGGTTCCA
     SerProProAlaAlaLeuHisSerProGluProGlnHisSerLeuIleAsnSerThrValTyrSerArgGlySerGluMetAlaValPro 270

 813 CCTGGCGTGGCCGGATACCCGCTCAGTCCGCCTCCTCCATCCCCTCCTGCGGAGAAACACAGCGGACACCAGAGGAACTACGACAAACCT
     ProGlyValAlaGlyTyrProLeuSerProProProProSerProProAlaGluLysHisSerGlyHisGlnArgAsnTyrAspLysPro 300

 903 CTACAATGGTACCACCAGTGCCCAAAATGTGCCCGGGTCTTCCGCCAGCTGGAGAACTACGCCAACCACCTCAAGATGCACAAGCTGTTC
     LeuGlnTrpTyrHisGlnCysProLysCysAlaArgValPheArgGlnLeuGluAsnTyrAlaAsnHisLeuLysMetHisLysLeuPhe 330

 993 ATGTGCCTCCTGTGCGGCAAGACCTTCACGCAAGAGGGCAACCTGCACCGACACATGCGCGTGCACGCCGGCATCAAGCCCTTCCAGTGT
     MetCysLeuLeuCysGlyLysThrPheThrGlnGluGlyAsnLeuHisArgHisMetArgValHisAlaGlyIleLysProPheGlnCys 360

1083 AAAATCTGCGGCAAGACCTTCACCCAAAAGTGTTCTTTGCTGGATCACCTAAACCTGCACAGTGGGGACAAGCCACACCGCTGCAACTAC
     LysIleCysGlyLysThrPheThrGlnLysCysSerLeuLeuAspHisLeuAsnLeuHisSerGlyAspLysProHisArgCysAsnTyr 390
                                              ● ● ● ●
1173 TGTGACATGGTTTTCGCTCATAAGCCAGTTCTCCGCAAGCACCTCAAACAGATCCACGGCAAGAACAGCTTCGACAACGCAAACGAAGGC
     CysAspMetValPheAlaHisLysProValLeuArgLysHisLeuLysGlnIleHisGlyLysAsnSerPheAspAsnAlaAsnGluGly 420

1263 AGCCTGCACGAAGGGGGTCTTGACTTTGACTTTCGCCAAATATGAAATCATTGGACCCTTACGTTTTAGTAGCTTTGTTGGAAACCAGCC
     SerLeuHisGluGlyGlyLeuAspPheAspPheArgGlnIle *                                              434
      ● ● ● ●
1353 GTCATTTAAATGGAAATGGTGCGGGCTTTCTCTAAGATAGAAACTGAAGCTGATACAAAAATTTTCTTCGGTGAGAAGCCAGAAATGGCA
1443 CTATCAACGCTGGGCAGATGAGTGAACAGGACATGTCAGCCTCGTCCTGATGAGTTTAACTGGAGAGCATTAACGAGAAAGAGAAGTTTT
1533 CCTGGTCTAATGTCACTGTAGCCTCATTATCGTGGACTGTTTTTTATCGTTTTTTCTACAATACATTGTAGATGCCTTCATATCATACTT
1623 GAAAATTCAGCACTGAAACACAAATCGTAATGACTGTACATCATTAGTTTTATTAATGAATTAACATGTGTCTGCGTTGTTCAATGGAAA
1683 AAAACACAGAGAGCCGAAGTCACGCCCCTTCTGATTTTCCTCTGGGAAAAGAGAAAGACAGATAATTTTTTAATGCCATTTGAATTGTGT
1803 CATGAATCACACACATGATGTTTGTCGGTTTGGAGGATGGTTTTCCAAGTCAAGAAAGTCTTAATTTGTCATGAAATTAATGAAATGTGA
1863 GACTGAAAATACGGAATTACAAACACTATACAGCCAACAGTCTTGTTGAACCTTCACCAAAACCTGTAACTTAAACATTGTAGAGCTGCT
1983 CCTGTATATTAGCAGCTCACTGAACTGACCAGCTCTCCTTTCACATAGCTGCAGTTGTCAATATGACCCGATTTTTTGTGATTTTCATCT
2043 TTTTTAGTACCTTCTCTGTGCTGAGTTGGCGGCCATGTTGGTGTTTGTTGACGTGTGTTGAGCAAGATGATGTAGTTCAGTTTATGGCAA
2163 ACTGCAACTTTCTTGACTTGGAAAATTATCTTTTAAATTGACAAACATCACATATATCATCACATGTGTAAATTACAGCACAATTCAATT
2223 CAAATGTCTCTCCTTTGACTAAAATACCTAAAAGGTCCCATGGGGCAAACCGGAAGGAGCGGGACTTCGGCTCTCTATTGCAAAAAGGAT
2343 ATATATTCAAAATATTTATCATATTCATTTGTATATTGTGTATACAGTCTACTGTAGCCTTTCTTTCTTACCTGTGTGAATATGACAACA
2403 TCATAACTAAAAGAAACTTGAAAGAAGCTGGGTTGAAACATATTTTATTGTGGGAAAGCTACGCCGCTGATGGGGGATGCCAAAGTGTCT
2523 ATGTGAACTGAAATGGTTCTTTTATACAGACTTTATATCTGAGGTACTACTGGACCAAAGTGACATTGTACCTGTCCTGTGTTCATGCAA
2583 GTCGATGTGTGTTATGACATTAATAAACTCTTAATCAGTAAAAAAAAAAA
```

Fig. 1. Base sequence of gene related ovarian maturation (GROM) in *P. major* and putative amino acid sequence. The nucleotide and amino acid residue number are shown on the left. The polyadenylation signal is boxed. Shaded amino acids show the predicted a casein protein kinase II phosphorylation sites. Underlines show predicted kinase C phosphorylation sites. This sequence data is available from DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB036921.

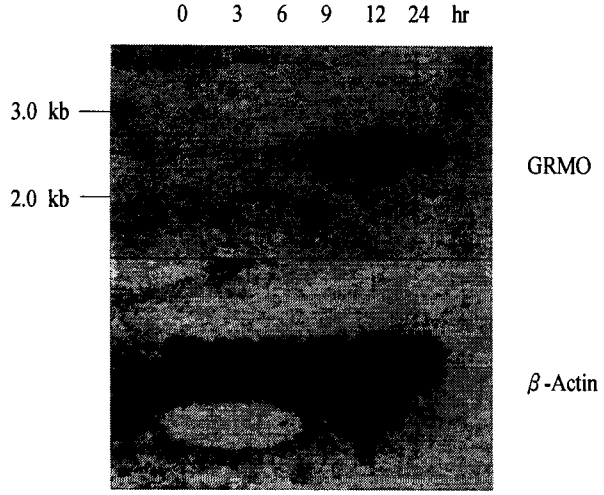

Fig. 2. Effects of hCG (10 IU/ml) on transcription levels of gene related ovarian maturation (GROM). Northern blot analysis of total RNA (30 μg) extracted from ovarian follicles incubated with hCG for 0, 3, 6, 9, 12 and 24 hr were hybridized with GROM and β-actin probes. The positions (kb) of RNA molecular weight markers are shown on left.

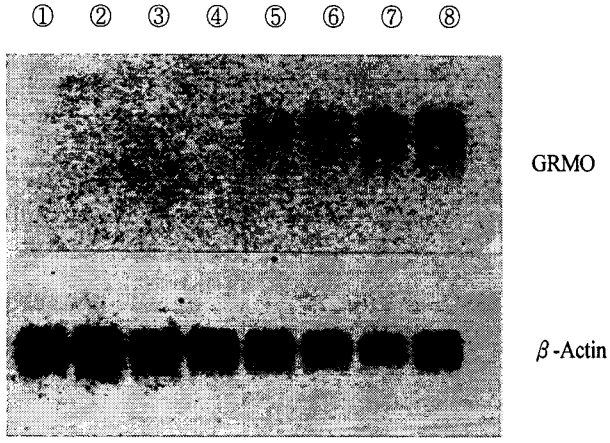

Fig. 3. Effects of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP, 10 ng/ml), human insulin-like growth factor-I (IGF-I, 10 nM), *P. major* GtH-I (100 ng/ml, 300 ng/ml) and *P. major* GtH-II (100 ng/ml, 300 ng/ml) on transcription levels of gene related ovarian maturation (GROM). Northern blot analysis of total RNA (30 μg) extracted from ovarian follicles was hybridized with P. major GROM and β-actin probes.

Takemura, Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus for his excellent suggestions and useful comments.

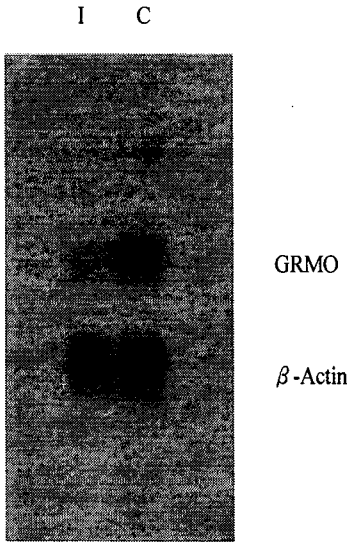

Fig. 4. *In vivo*, Northern blot analysis of total RNA extracted from incompetent (I) and competent ovaries (C). Incompetent and competent ovaries were collected at 08:00 hr and 16:00 hr, respectively. Total RNA (30 μg) was used for electrophoresis. After transfer to the membrane, the duplicated series of blots were separately used for probing with [α-^{32}P] dCTP-labelled gene related ovarian maturation (GROM) and β-actin probes.

REFERENCES

Chang X, Patino R, Thomas, P, Yoshizaki G (1999) Developmental and kinase-dependent regulation of ovarian connexin mRNA and oocyte maturational competence in Atlantic croaker. Gen Comp Endocrinol 114: 330-339.

Degani G, Boker R (1992) Sensitivity to maturation-inducing steroids and gonadotropin in the oocytes of blue gourami, *Trichogaster trichopterus* (Anabantidae, Pallas, 1770). Gen Comp Endocrinol 85: 430-439.

Hoh JH, John SA, Revel JP (1991) Molecular cloning and characterization of a new member of the gap junction gene family, connexin-31. J Biol Chem 266: 6524-6531.

Kagawa H, Tanaka H, Okuzawa K, Hirose K (1994) Development of maturational competence of oocytes of red seabream, *Pagrus major*, after human chorionic gonadotropin treatment *in vitro* requires RNA and protein synthesis. Gen Comp Endocrinol 94: 199-206.

Kagawa H, Tanaka H, Okuzawa H, Kobayashi M (1988) GTH-II but not GTH-I induces final maturation and development maturational competence of oocytes of red seabream *in*

vitro. Gen Comp Endocrinol 112: 80-88.

Kishimoto A, Nishiyama K, Nakanish H, Uratsuji Y, Nomura H, Takeyama Y, Nishizuka Y (1985) Studies on the phosphorylation of myelin basic protein-by-protein kinase C and adenosine 3':5'-monophosphate-dependent protein kinase. J Biol Chem 260: 12492-12499.

Kobayashi M, Aida K, Furukawa K, Law YK, Moriwaki T, Hanyu I (1988) Development of sensitivity to maturation-inducing steroids in the oocytes of the daily spawning teleost, the kisu, *Sillago japonica*. Gen Comp Endocrinol 72: 264-271.

Liang P, Pardee A (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967-971.

Liang P, Zhu W, Zhang X, Guo Z, O'Connell P (1994) Differential display using one-base anchored oligo-dT primers. Nucleic Acids Res 22: 5763-5764.

Matsuyama M, Adachi S, Nagahama Y, Matsuura S (1988) Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red seabream, Pagrus major during the spawning season. Aquaculture 73: 357-372.

Nagahama Y (1987) 17α, 20β-dihydroxy-4-pregnen-3-one: A teleosts maturation-inducing hormone. Develop Growth Differ 29: 1-12.

Nagahama Y (1990) Endocrine control of oocyte maturation in teleosts. In "Progress in Comparative Endocrinology" Eds by A Epple, CG Scanes, MH Stetson, Wiley-Liss, pp. 385-392.

Patino R, Thomas P (1990) Effects of gonadotropin on ovarian intrafollicular processes during the development of oocytes maturational competence in a teleost, the Atlantic croaker: Evidence for two distinct stages of gonadotropic control of final oocyte maturation. Biol Reprod 43: 818-827.

Pinna LA (1990) Casein kinase 2: An "eminence grise" in cellular regulation? Biochem Biophys Acta 1054: 267-284.

Proudfoot NJ, Brownlee GG (1976) 3'Non-coding region sequences in eukaryotic messenger RNA. Nature 263: 211-214.

Sambrook J, Fritsch EF, Maniatis T (1989) Electrophoresis of RNA through gels containing formaldehyde. In "Molecular Cloning: A Laboratory Manual Vol 1" Cold Spring Harbor Laboratory Press, New York, pp 743-745.

Tanaka H, Kagawa H, Okuzawa K, Hirose K (1993) Purification of gonadotropins (PmGTH-I and -II) from red seabream, Pagrus major and development of a homologous radioimmunoassy for PmGTH-II. Fish Physiol Biochem 10: 409-418.

Takeda A, Hashimoto E, Yamamura H, Shimazu T (1987) Phosphorylation of liver gap junction protein-by-protein kinase C. FEBS Lett 210: 169-172.

Trant JM, Thomas P (1988) Structure-activity relationships of steroids in inducing germinal vesicle breakdown of Atlantic croaker oocytes *in vitro*. Gen Comp Endocrinol 71: 307-317.

Woodget JR, Gould KL, Hunter T (1986) Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur J Biochem 161: 177-184.

Yoshikuni M, Nagahama Y, Adachi S, Ijiri S, Yamauchi K (1992) Gonadotropin induces an increase in 17α, 20β-DP levels and oocyte maturational competence in a teleost, the hirame, *Paralichthys olivaceus*. Zool Sci 9: 1162.

Yoshizaki G, Patino R, Thomas P (1994) Connexin messenger ribonucleic acids in the ovary of Atlantic croaker: Molecular cloning and characterization, hormonal control, and correlation with appearance of oocyte maturational competence. Biol Reprod 51: 493-503.

Zhu Y, Aida K, Furukawa K, Hanyu I (1989) Development of sensitivity to maturation-inducing steroids and gonadotropins in the oocytes of the tobinumeri-dragonet, *Repomucenus beniteguri*, Callionymidae (Teleostei). Gen Comp Endocrinol 76: 250-260.

Zhu Y, Kobayashi M, Furukawa K, Hanyu I (1994) Gonadotropin develops sensitivity to maturation-inducing steroid in the oocytes of daily spawning teleosts, tobinumeri-dragonet, *Repomucenus beniteguri* and kisu, *Sillago japonica*. Fish Sci 60: 541-545.

Zimmermann JW, Schultz RM (1994) Analysis of gene expression in the preimplantation mouse embryo: Use of mRNA differential display. Proc Natl Acad Sci USA 91: 5456-5460.