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Profiles of PGH-α, GTH I-β, and GTH II-β mRNA transcript levels at different ovarian stages in the wild female Japanese eel *Anguilla japonica*

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Abstract

The complete complementary DNA (cDNA) encoding pituitary gonadotropin II-β subunit (GTH II-β) of Japanese eel *Anguilla japonica* was cloned and sequenced, and the profiles of pituitary glycoprotein hormone α subunit (PGH-α), GTH I-β, and GTH II-β mRNA transcript levels at different stages of ovarian development before vitellogenesis in the wild females were investigated. The maturity of female eels was divided into four stages: juvenile, sub-adult, pre-silver, and silver stages based on ovarian development and skin color. The GTH II-β cDNA was cloned by reverse transcription and polymerase chain reaction (RT-PCR) amplification from total pituitary RNA. The full length GTH II-β cDNA was obtained using 5′- and 3′-rapid amplification of cDNA ends. The cloned eel GTH II-β cDNA consists of 646 bp nucleotides, including 53 bp nucleotides of 5′-untranslated region (UTR), 423 bp of open reading frame, and 170 bp nucleotides of 3′-UTR followed by a poly(A) tail. It encodes a 140-amino acid precursor molecule of GTH II-β subunit with a putative signal peptide of 24 amino acids and a mature peptide of 116 amino acids. RT-PCR analysis showed that the pituitary transcript levels of α subunit steadily increased during eel silvering. The expression of GTH I-β and II-β mRNA levels, however, varied in different ovarian developmental stages. The mRNA expression of both GTH I-β and GTH II-β were detectable in juvenile stage. The expression levels of GTH II-β mRNA, but not GTH I-β, were significantly increased in sub-adult stage. The transcript levels of GTH I-β and II-β subunits further increased in pre-silver and silver stages. We demonstrated for the first time that the differential transcription patterns of pituitary PGH-α, GTH I-β, and GTH II-β mRNAs occur during silvering of the wild female Japanese eels.

Keywords: Japanese eel (*Anguilla japonica*); Silvering; Ovarian development; GTH I-β subunit; GTH II-β subunit; α-Subunit; Gene expression

1. Introduction

Vertebrate pituitary synthesizes and secretes glycoprotein hormones (PGHs): gonadotropin (GTH) and thyrotropin (TSH). Two types of GTHs are existent in tetrapods: luteinizing hormone (LH) and follicle stimulating hormone (FSH). In teleost, the homologous counterparts of FSH and LH are GTH I and GTH II, respectively (Li and Ford, 1998). All PGHs are heterodimers consisting of α- and β-subunits. The α- and β-subunits are initially synthesized as separate glycoproteins from different genes, and are associated in cytoplasm by non-covalent bonding to form biologically active dimmer molecules (Gharid et al., 1990). α-Subunits are identical for GTHs and TSH within the same species; while the β-subunits are species and hormone specific. The duality of gonadotropins, GTH I and GTH II, was also identified in anguillid eels, the primitive

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teleosts (Yoshiura et al., 1999). However, the GTH II-β subunit peptide deduced from partial GTH II-β cDNA of the Japanese eel (Nagae et al., 1996b) showed a difference of two amino acid residues when compared with that deduced from the partial GTH II-β genomic DNA (GenBank Accession No. AF395603). Information concerning complete cDNA of eel GTH II-β subunit is not available yet. For investigating the existence of the diversity of GTH II-β subunit in Japanese eel, we therefore cloned and sequenced the complete GTH II-β subunit cDNA.

Anguillid eels are catadromous fishes with complex life cycle that includes both marine and freshwater habitats (Tesch, 1977). After living in rivers for years, eels undergo significant morphological metamorphosis and physiological changes from yellow (non-migratory) to silver (migratory) stages (also known as "silvering") (Egginton, 1986, 1987; Fontaine et al., 1995; Jessop, 1987; Lokman and Young, 1998a,b; Matsui, 1958; Matsui, 1972; Pankhurst, 1982; Pankhurst and Sorensen, 1984; Rohr et al., 2001; Sorensen and Pankhurst, 1988; Tesch, 1977; Yamada et al., 2001). They then migrate back to deep sea to spawn and die. Under conditions of cultivation, eels have immature gonads containing previtellogenic oocytes in the females or spermatogonia in the males due to a lack of GTH synthesis and release (Nagae et al., 1996b). However, the development of gonad of Japanese eels can be induced by exogenous endocrine factors; for example, multiple injections of salmon pituitary homogenates induce oogenesis (Yamamoto and Yamauchi, 1974) and a single injection of human chorionic gonadotropin induces spermatogenesis (Miura et al., 1991). More recently, the changes in expression of GTH II-β and GTH I-β mRNAs at various stages of ovarian development were investigated in Japanese eels following chronic induction with hormones (Nagae et al., 1996b; Suetake et al., 2002; Yoshiura et al., 1999). The pituitary mRNA levels for GTH I-β were high in immature yellow eels, but decreased in the hormone induced mature ones (spermiating males and ovulated females). On the contrast, the pituitary mRNA levels for GTH II-β were very low in the yellow eels, but increased markedly with the progression of ovarian development. Thus, the expression of GTH I-β and GTH II-β genes in the eels clearly differs from each other. The expression patterns of the GTH I-β and GTH II-β mRNAs during silvering in the wild female eels, however, are not clear. We therefore investigated the expression of the GTH I-β and GTH II-β mRNA levels at different stages of ovarian development from juvenile through silver stages of the wild female Japanese eels.

2. Materials and methods

2.1. Animals

Wild female Japanese eels were collected by eel traps in the lower reach of Kaoping River of southwest Taiwan (120°50′E and 22°40′N) as indicated time (Table 1). The captured eels were stunned by ice and immediately transported to the laboratory for examination. After measuring the total length (TL, ±0.1 cm) and body weight (BW, ±0.1 g), eels were decapitated and the gonad weight (GW, ±0.01 g) were measured and gonadosomatic index (I_G) was estimated according to the formula:

$$I_G = 100 \times [\text{GW (g)}/\text{BW (g)}].$$

Gonads were then fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin for histological examination. The mean oocyte diameters (OD, ±1 μm) were calculated from randomly selected 20 round oocytes with complete nucleus. Maturational stages of the oocytes were determined according to Yamamoto et al. (1974).

2.2. Classification of maturing status

In our previous investigation, the maturity of the wild female Japanese eel before and during silvering was divided into three stages (yellow, pre-silver, and silver) based on skin color and histological observations of ovarian development (Han et al., 2003). In the present

Table 1
Morphological and gonadal characteristics of the wild Japanese eels in different stages of ovarian development

	Yellow		Pre-silver	Silver	Tukey's HSD
	Juvenile	Sub-adult			
Sample size	11	17	9	9	
Time of collection	Dec. 2000	Aug., Oct. 2000	Oct. 2000	Oct., Dec. 2000	
	Feb. 2003	Feb. 2001	Jun. 2001	Feb. 2001	
TL (cm)	43.4 ± 1.0	53.7 ± 1.3	60.7 ± 1.6	65.1 ± 2.0	Ju < Sa < Ps = Sv
BW (g)	68.4 ± 3.0	197.2 ± 13.5	374.3 ± 40.0	474.0 ± 34.9	Ju < Sa < Ps = Sv
I_G (%)	0.15 ± 0.02	0.36 ± 0.02	0.59 ± 0.06	1.58 ± 0.18	Ju < Sa < Ps < Sv
OD (μm)	40.0 ± 0.95	73.8 ± 2.5	109.4 ± 3.4	181.1 ± 12.2	Ju < Sa < Ps < Sv

TL, total length; BW, body weight; I_G, gonadosomatic index; OD, oocyte diameter. Ju, juvenile; Sa, sub-adult; Ps, pre-silver; Sv, silver. $p < 0.001$ for all significance differences.

study, the yellow eels were further divided into juvenile and sub-adult stages based on I_G and OD for better comparison (Table 1). The I_G and OD of the female Japanese eel were significantly different among different ovarian developmental stages ($p < 0.001$, Table 1). Ovaries of juveniles contained mainly stage II (chromatin nucleolus stage) oocytes. The ovaries of sub-adults also contained stage II (chromatin nucleolus stage) oocytes predominantly, but with larger OD than those of juveniles (Table 1). In the pre-silver females, the oocytes grew rapidly and were mainly in stage III (perinucleolus stage). The initial oil drops became apparent at periphery of the oocytes. In the silver females, the oocytes continued to grow, and the oil drops accumulated and filled the whole cytoplasm. They were mainly in stage IV (oil-drop stage) (Han et al., 2003).

2.3. Designing of oligonucleotide primers

Oligonucleotides used as PCR primers for cloning the GTH II-β subunit cDNA of the Japanese eel are listed below and shown in Fig. 1. The sense primer (SP: 5′ → 3′) and antisense primer (ASP: 3′ → 5′) of GTH I-β and α subunits were designed from the conserved coding regions of the Japanese eel published (Nagae et al., 1996b; Yoshiura et al., 1999). The β-actin sequence of the Japanese eel was cloned by our laboratory.

Primer 1. SP for GTH II-β subunit: 5′-ATGTCAG TCTACCCAGAATGCA-3′.
Primer 2. ASP for GTH II-β subunit: 5′-AGACGT GTCCATGGTGCACAGGT-3′.
Primer 3. Gene specific primer (GSP) of GTH II-β subunit for 3′-RACE: 5′-TCCACGGTGTACCAGC GCGT-3′.
Primer 4. GSP of GTH II-β subunit for 5′-RACE: 5′-ACGCGCTGGTACACCGTGGACA-3′.
Primer 5. Adapter primer (AP) for 3′-RACE: 5′-GG CCACGCGTCGACTAGTACTTTTTTTTTTTTT TTT-3′.
Primer 6. Abridged universal amplification primer (AUAP) for 3′-RACE: 5′-GGCCACGCGTCGACT AGTAC-3′.
Primer 7. Abridged anchor primer (AAP) for 5′-RACE: 5′-GGCCACGCGTCGACTAGTACGGGI IGGGIIGGG IIG-3′, where I is the base inosine.
Primer 8. SP for GTH I-β subunit: 5′-ACAGCG CTGTGCTTGACATTG-3′.
Primer 9. ASP for GTH I-β subunit: 5′-GCAGCCAT TAACCATGCAAGACA-3′.
Primer 10. SP for GTH α subunit: 5′-ATGATGGTG TGTCCAGGAAAG-3′.
Primer 11. ASP for GTH α subunit: 5′-GCAGTGGC AGTCTGTGTGGTT-3′.
Primer 12. SP for β-actin subunit: 5′-GCTGTCCC TGTATGCCTCTGG-3′.
Primer 13. ASP for β-actin subunit: 5′-GTCAGGA TCTTCATGAGGTAGTC-3′.

2.4. Total RNA extraction and reverse transcription-polymerase chain reaction for GTH II-β cDNA sequence

Total RNA was extracted from the individual pituitary glands of each eels using the total RNA miniprep system kit (Viogene, Sunnyval, CA, USA). The concentration and quality of the extracted RNA were measured at $A_{260\ nm}/A_{280\ nm}$ (Kontron Spectrophotometer, UVIKON 810). Complementary DNA was synthesized from 1.0 μg total RNA with oligo$(dT)_{18}$ primer (100 ng) using the first-strand cDNA synthesis kit (Stratagene, CA, USA), according to the manufacturer's instructions. Reverse transcription was performed using moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Stratagene, CA, USA) for 35 min at 42 °C and later 70 °C for 10 min to heat-inactivate the MMLV-RT.

The PCR procedures were performed in 50 μl final volume containing 1 μl RT product, 5 μl of 10× reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, and 2.5 U Taq DNA polymerase (Gibco-BRL, MD, USA) using primers 1 and 2 (100 ng for each). After an initial 2 min denaturing step at 94 °C, 35 cycles of amplification were performed using a cycle profile of 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1 min. Elongation was extended to 10 min at 72 °C after the last cycle. The PCR products were sequenced by a Big Dye-Terminator Kit and analyzed on polyacrylamide gels with an ABI 377 automated sequencer (Perkin–Elmer Applied Biosystems).

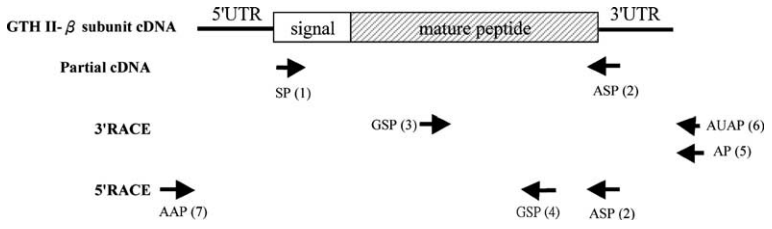

Fig. 1. Procedures of RT-PCR sequencing of GTH II-β subunit cDNAs from pituitary glands of the Japanese eel. Numbers in the parentheses denote the corresponding oligonucleotide primers listed in Materials and Methods; open box indicates the signal peptide; gray box indicates the mature peptide.

2.5. Rapid amplification of cDNA ends

The remaining 5′ and 3′-UTR sequences were obtained by Rapid Amplification of cDNA ends (RACE) using the RACE kit (Gibco-BRL, MD, USA). 3′-RACE was performed according to the manufacturer's instructions. Briefly, 1 μg pituitary total RNA was reverse-transcribed using AP (primer 5) by 200 U Superscript II reverse transcriptase, followed by PCR using primers 3 and 6. Similarly, for 5′-RACE, 1 μg pituitary total RNA was reverse-transcribed by 200 U Superscript II reverse transcriptase with primer 2. The acquired single-strand cDNA was column purified and then oligo(dC) tailed using terminal deoxynucleotidyl transferase. PCR was then performed using the AAP (primer 7) and the primer 4. The PCR condition and the products sequencing were described above.

2.6. Sequence analysis of the GTH II-β subunit

The acquired GTH II-β cDNA (Accession No. AY082379) of the present study was deduced to the putative peptide sequences and aligned using Clustal W (v1.81) program with that of the *Anguilla japonica* (Nagae et al., 1996b) and those of the 8 anguillid eels deposited in the GenBank, which were cloned by J.P. Huang and two of the authors of the present study (Y.S. Han and W.N. Tzeng): *A. japonica* (AF395603), *Anguilla anguilla* (AF395598), *Anguilla rostrata* (AF395606), *Anguilla marmorata* (AF395604; AF395605), *Anguilla australis australis* (AF395601), *Anguilla reinhardti* (AF395600), *Anguilla celebesensis* (AF395602), and *Anguilla bicolor bicolor* (AF395599).

2.7. Transcript levels of PGH-α, GTH I-β, and GTH II-β mRNAs in different developmental stages

Since the mRNA levels of PGH-α, GTH I-β, and GTH II-β examined in the present study were too low to be detected by Northern blot analysis, a RT-PCR was thus used to examine their expression levels. One microgram of total RNAs from individual eel pituitaries of juvenile ($n = 11$), sub-adult ($n = 17$), pre-silver ($n = 9$), and silver ($n = 9$) stages was reverse-transcribed using oligod$(T)_{18}$ primer (100 ng) and MMLV-RT (Stratagene, CA, USA) following instructions recommended by the manufacturer. An optimal PCR amplification cycle (25 cycle) was chosen to observe the different cDNA levels based on parallelism of different PCR cycles (15, 20, 25, and 30 cycles). The primers 1 and 2 for GTH II-β subunit, 8 and 9 for GTH I-β subunit, and 10 and 11 for PGH-α subunit were used for PCRs. As an internal control in the RT-PCRs, β-actin was also amplified using the primers 12 and 13. PCR products were analyzed by 2.5% agarose gel electrophoresis. To validate the mRNA levels estimated by RT-PCR analysis, two pituitaries of eels selected from each ovarian stages, were analyzed by real-time quantitative PCR using the fluorescence dye SYBR Green 1 (Morrison et al., 1998).

2.8. Statistical analysis

Data were analyzed using the statistic software SPSS 10.0 (SPSS). Differences among morphometric characters or the transcript levels of PGH-α, GTH I-β, or GTH II-β at different stages of ovarian development were analyzed by Tukey's HSD multiple range test. Differences were considered significantly at $p < 0.05$.

3. Results

3.1. Sequencing analysis of the Japanese eel GTH II-β cDNA

The partial GTH II-β subunit cDNA was amplified from pituitary glands of Japanese eels by RT-PCR using the primers 1 and 2. This resulted in a 357-bp product, which was found to be a single band in 1.5% agarose gel and agreed with the amino acid sequences of GTH II-β subunits of other anguillids, as processed by BLAST program of the National Center for Biotechnology Information, USA. The complete cDNA sequences were determined using 3′- and 5′-RACE. The complete GTH II-β subunit cDNA of Japanese eel was 646 bp in total length, including 53 bp of the 5′-untranslated region (5′UTR), 423 bp of the coding region, and 170 bp of the 3′-untranslated region (3′UTR) followed by a 20-bp poly(A^+) tail (Fig. 2). The coding region encoded a peptide of 140 amino acids, which containing a putative signal peptide of 24 amino acids and a mature peptide of 116 amino acids (Fig. 2).

```
ATAAACCAAACTTTCCTGCGGCCAGCTGAGCCATTGGAATACATTCTCTAAGTATGTCAG   60
                                                  M  S          2
TCTACCCAGAATGCACCTGGCTCCTCTTTGTATGCTTGTGTCACCTCCTTGTTTCTGCTG  120
V  Y  P  E  C  T  W  L  L  F  V  C  L  C  H  L  L  V  S  A    22
GAGGCTCTTTACTGCTGCCTTGTGAGCCAATCAATGAAACTATTTCTGTGGAGAAAGATG  180
G  G  S  L  L  L  P  C  E  P  I  N  E  T  I  S  V  E  K  D    42
GATGTCCAAAATGTCTGGTGTTCCAAACATCCATCTGCAGTGGTCACTGCATCACCAAGG  240
G  C  P  K  C  L  V  F  Q  T  S  I  C  S  G  H  C  I  T  K    62
ACCCAAGCTACAAGAGCCCGCTGTCCACGGTGTACCAGCGCGTGTGCACGTACCGGGACG  300
D  P  S  Y  K  S  P  L  S  T  V  Y  Q  R  V  C  T  Y  R  D    82
TCCGCTACGAGACGGTGCGGCTGCCAGACTGCCGCCCCGGCGTGGATCCCCATGTGACTT  360
V  R  Y  E  T  V  R  L  P  D  C  R  P  G  V  D  P  H  V  T   102
TCCCCGTGGCTCTGAGCTGCGACTGTAACCTGTGCACCATGGACACGTCTGACTGCGCCA  420
F  P  V  A  L  S  C  D  C  N  L  C  T  M  D  T  S  D  C  A   122
TCCAGAGTCTGAGGCCCGACTTCTGCATGAGCCAGCGGGCCAGCCTCCCCGCGTAGGAGG  480
I  Q  S  L  R  P  D  F  C  M  S  Q  R  A  S  L  P  A  ★      140
AGCCCACGGACTGGTCCTCCCGCCCTCCACACCGCTCTTACCCAGCATGCATCTGCATTG  540
CCGAAGAGGGCCAACACATGCAGAGATGCTCCATGTGGAAGAAGCATAAAGAATAAGCAC  600
ATACTTTGGGCACATACTTCTGGGGGTTCCAGCAAGTGGTTTCAGCAAAAAAAAAAAAAA  660
AAAAA                                          (Ploy A)
```

Fig. 2. Nucleotide and deduced amino acid sequences of the Japanese eel GTH II-β subunit. In right-hand column, upper numbers refer to the nucleotide sequence and lower numbers refer to the amino acid sequence. The start (ATG) and stop (TAG) codons are designated by boxes.

3.2. Variation of PGH-α, GTH I-β, and GTH II-β mRNA expressions at different ovarian stages of wild Japanese eel

The expression of PGH-α mRNA levels at different stages of ovarian development is shown in Fig. 3. The results were normalized with data from β-actin. PGH-α mRNA levels gradually increased with ovarian development, and the differences were significant ($p < 0.05$) between pre-silver and juvenile stages, and also between silver and juvenile/sub-adult stages. The expression of GTH I-β and II-β mRNA levels with ovarian development is shown in Fig. 4. For GTH I-β mRNA, the expression levels were higher in pre-silver and silver stages than in juvenile and sub-adult ones. For GTH II-β mRNA, however, the expression levels were highest in silver females, and lowest in juveniles (Fig. 4). In the silver females, the mRNA expression in GTH II-β, but not GTH I-β, was increased further. Representative real-time quantitative PCRs for PGH-α, GTH I-β and II-β mRNA expressions at different stages of ovarian development are shown in Fig. 5. The calculated mRNA levels of PGH-α, GTH I-β, and II-β mRNA expression at different ovarian stages are comparable to the corresponding mRNA levels estimated by the RT-PCR analysis.

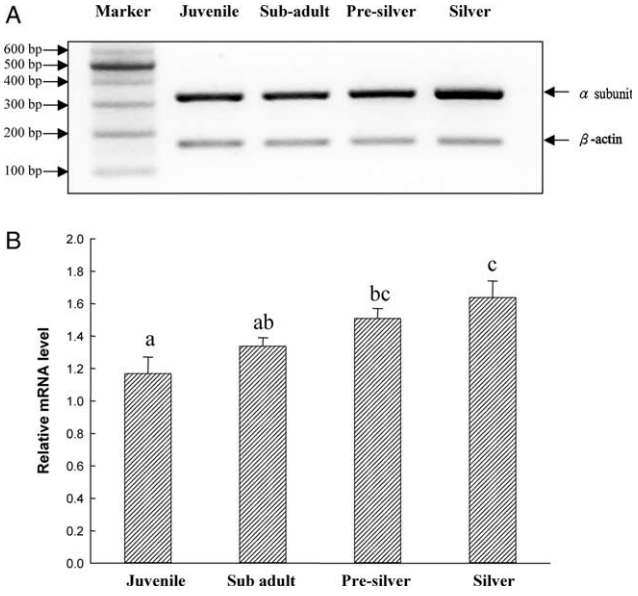

Fig. 3. Expressions of the pituitary PGH-α subunit mRNA in the wild Japanese eels at different stages of ovarian development. (A) Total RNA prepared from each stage was reverse-transcribed and subjected to PCR. Amplified products were analyzed on 2.5% agarose gel. β actin was used as control in each line. (B) The α subunit band intensities from different ovarian stages were analyzed by Kodak Digital Science ID image analysis software, Ver 3.0. Different letters above histograms indicate that the differences are statistically significant ($p < 0.05$).

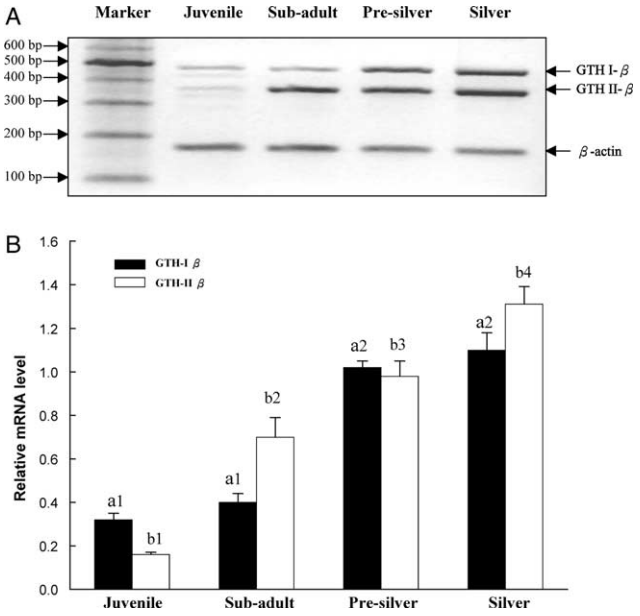

Fig. 4. Expressions of the GTH I-β and GTH II-β subunit mRNAs from pituitaries of the wild Japanese eel at different stages of ovarian development. (A) Total RNA preparation and agarose gel analysis were the same as Fig. 3. β actin was used as control in each line. (B) The GTH I-β and GTH II-β subunits band intensities in different developmental stages. Groups at the same alphabet letters with different numerals are statistically significant ($p < 0.05$).

4. Discussion

Alignment of the acquired GTH II-β subunit mature peptide in this study with those of other anguillid eels showed that the positions of all 12 cysteines and the putative single N-linked glycosylation site are conserved (Fig. 6). The deduced amino acid sequence of mature GTH II-β of the Japanese eel obtained in this study is identical to that of Japanese eel GTH II-β deposited in the GenBank (Accession No. AF395603), but exhibits a difference of two amino acids in comparison to that of the Japanese eel GTH II-β published by Nagae et al. (1996b) (Fig. 6). Amino acid sequence analysis revealed that all 8 eel species possess one common form of the GTH II-β peptide. However, *A. japonica* and *A. marmorata* have additional form which exhibiting a difference of two amino acid residues in comparison to the common form. According to the phylogenetic analysis constructed by the mitochondrial genes, it was inferred that the divergence time of anguillids was about 30–20 million years ago (Aoyama et al., 2001; Lin et al., 2001). The highly conserved GTH II-β subunit among anguillids implied the conserved evolution of the GTH II-β subunit.

As observed in the present study, the transcript levels of the PGH-α mRNA gradually increased during ovarian development of the Japanese eel (Fig. 3). Since the PGH-α subunit is expressed in both gonadotrops and thyrotrops of the pituitary gland, the measured PGH-α

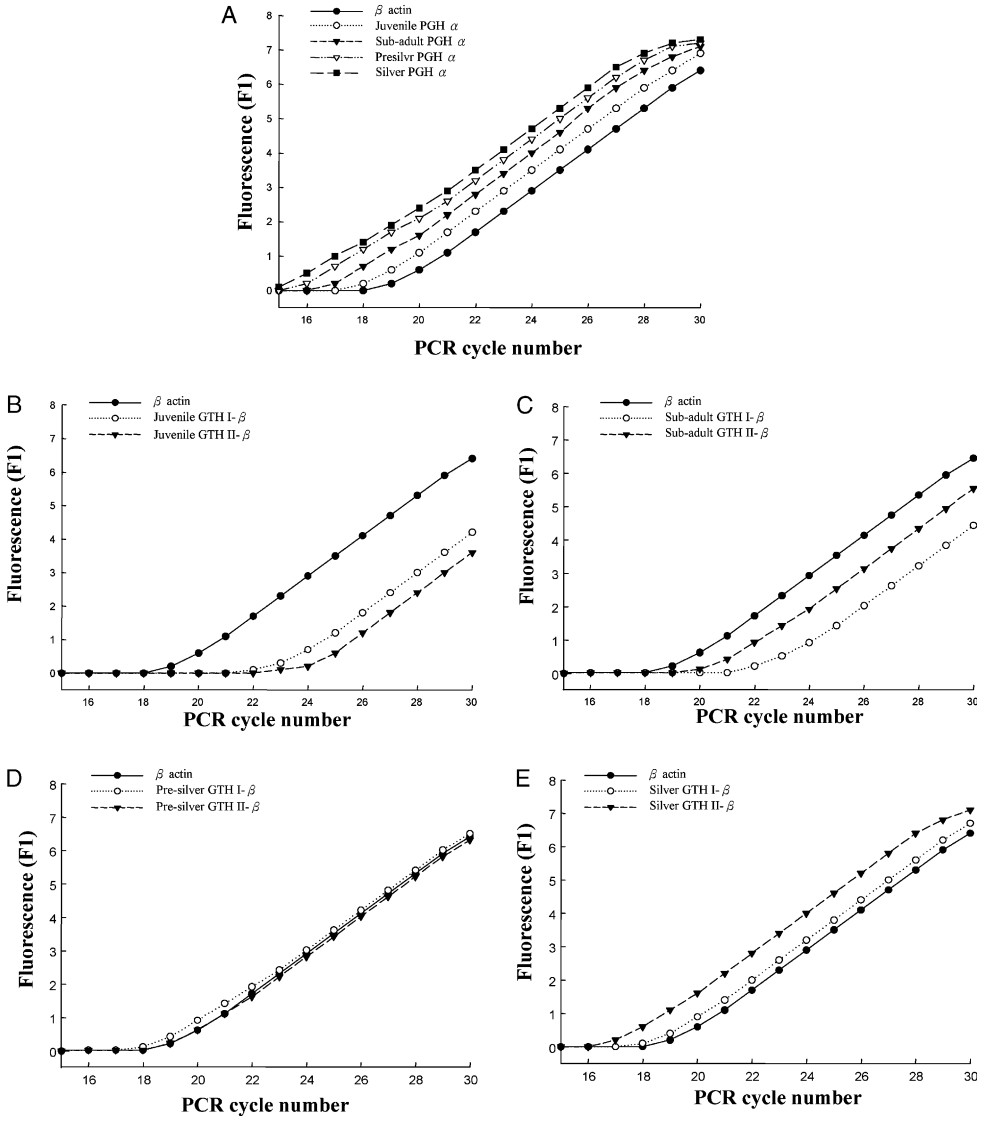

Fig. 5. Representative real-time PCRs of the PGH-α, GTH I-β, and II-β mRNAs from pituitaries of the wild female Japanese eels at different stages of ovarian development. (A) PGH-α of different stages. (B) GTH I-β and GTH II-β of juvenile stage. (C) GTH I-β and GTH II-β of sub-adult stage. (D) GTH I-β and GTH II-β of pre-silver stage. (E) GTH I-β and GTH II-β of silver stage.

mRNA levels thus would represent the expression of PGH-α subunits from both cells. However, histological evidences indicated that the pituitary thyrotropic cells of the Japanese eel are constant from pre-vitellogenic to mid-vitellogenic stages (Nagae et al., 1996b). The increased PGH-α mRNA expression at sub-adult, pre-silver, and silver stages of the eels observed in the present study thus would reflect the increasing activity from gonadotrops rather than from thyrotrops.

The regulation of PGH-α subunit expression is complex. Various factors have been shown to have effect on its expression in teleosts. The stimulatory factors include gonadotropin-releasing hormone, thyrotropin-releasing hormone, testosterone, estradiol-17β, neuropeptide Y, activin, and others (Breton et al., 1998; Counis et al., 1987; Dickey and Swanson, 1998; Kobayashi et al., 2000; Quérat et al., 1991; Yaron et al., 2001; Yu et al., 2002). Nagae et al. (1996b) reported that PGH-α mRNA of the Japanese eels increases almost linearly during ovarian development induced by injection of chum salmon pituitary homogenates, and suggested that the increase in PGH-α mRNA is probably due to the positive feedback of testosterone and estradiol-17β produced by ovarian follicles in response to the GTHs contained in salmon pituitary homogenates.

Previous studies by the method of Northern blot analysis showed that GTH I-β gene was only expressed in immature Japanese eel (Yoshiura et al., 1999), while GTH II-β gene was expressed in the mature eel of late-vitellogenic and migratory nucleus stages (Nagae et al., 1996b). These findings suggest that different regulation of the two GTHs exists in Japanese eels. In the present

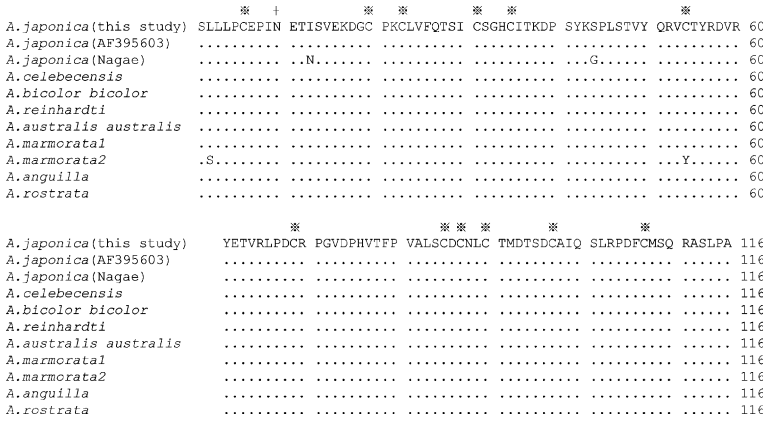

Fig. 6. Alignment of the putative mature peptide sequences of the GTH II-β subunit from eight eel species and subspecies. The top sequence (*A. japonica*) is from this study, and those of other two *A. japonica* sequences are from Nagae et al. (1996b) and the GenBank (AF395603), respectively. Dots indicate the consensus residues. Cysteine residues are indicated by asterisks (※). The putative N-linked glycosylation site is indicated by the plus signal (+).

study, however, the expression of GTH II-β mRNA appeared in the immature juvenile stage. Such inconsistent findings are likely due to that the quantitative RT-PCR analysis, used in our study, is more sensitive than Northern blot analysis employed by previous investigators (Nagae et al., 1996b; Yoshiura et al., 1999), especially when the transcript level of the target gene is low. Recently, Suetake et al. (2002) using real-time quantitative PCR for measurement of the mRNA expression of GTH β subunits during ovarian maturation of Japanese eels, induced by repeated injection of salmon GTH, had identified the expression of GTH II-β in yellow eels, although its transcription level was significantly lower than that of GTH I-β. The more sensitive for RT-PCR analysis than Northern blot analysis is also true that the β-actin, used as an internal control, was expressed constantly by the RT-PCR analysis employed in the present study but virtually undetectable by Northern blot analysis (Nagae et al., 1996b; Yoshiura et al., 1999). In fish, GTH I is responsible for the survival and proliferation of oogonia; whereas GTH II is mainly responsible for final maturation and ovulation of oocytes (Mather et al., 1990; Tyler et al., 1991). In the present study, the juvenile females were in the stage of oogonia proliferation; therefore, the expression of GTH I-β mRNA should be more dominant than that of GTH II-β mRNA.

The early occurrence of GTH II-β mRNA in yellow stages examined in the present study is rather different from the findings of previous studies of Japanese eel (Nagae et al., 1996a,b); where they concluded that the occurrence of GTH II-β mRNA appeared only after the mid-vitellogenic stage. In teleosts, GTH II is more potent than GTH I in stimulation of gonadal production of sex steroids, which support the gematogenesis and also the second sexual characters (Goos and Schulz, 1997). In anguillids, the process of silvering is a necessary step before starting their seagoing spawning migration, and the high levels of blood androgens (mainly testosterone and 11-ketotestosterone) are proposed to be related to the silvering in both sexes (Lokman and Young, 1998a,b; Rohr et al., 2001). The pituitary GTH II-β positive cells were found in pre-vitellogenic stage with relatively low numbers, but their numbers were comparable to those of the GTH I-β positive cells in early vitellogenic Japanese eels (Ikeuchi et al., 1999). Thus, we suppose that the early occurrence of the GTH II-β mRNA before vitellogenesis of females may promote the androgen production for preparing the process of silvering.

The mRNA expressions of both GTH I-β and GTH II-β were markedly increased in the pre-silver stage in comparison to juvenile/sub-adult stages. In salmonids, both GTHs can stimulate the production of 17β-estradiol and maturation-inducing steroid (Suzuki et al., 1988; Swanson et al., 1989), suggesting their potential complementary roles to each other. The increases of sex steroids during eel silvering were found in the New Zealand freshwater eels (Lokman and Young, 1998b). Therefore, the relatively higher transcript levels of both GTH I-β and II-β subunits in pre-silver stage observed in the present study may contribute to the production of sex steroids for triggering eel metamorphosis and gametagenesis.

In the present study, the transcript levels of GTH II-β, but not GTH I-β, further increased from pre-silver to silver stages (Fig. 4). In induced sexually mature Japanese eel, the mRNA expression of PGH-α and GTH II-β increased gradually, while that of GTH I-β almost disappeared (Nagae et al., 1996a,b; Suetake et al., 2002; Yoshiura et al., 1999). Meanwhile, the blood sex steroids gradually increased during vitellogenesis and reached plateau around ovulation (Ijiri et al., 1995). Similar increase of blood sex steroid levels during induced

maturation was also found in the New Zealand longfinned eel (Lokman et al., 2001). In vitro studies of the European eel also indicated that sex steroids could upregulate GTH II-β mRNA expression (Huang et al., 1997). All these findings suggest that the expression profiles of GTH I-β and GTH II-β during eel vitellogenesis are likely opposite; with increasing expression of GTH II-β mRNA and with decreasing expression of GTH I-β mRNA.

In conclusion, we have cloned and sequenced the complete cDNA of the GTH II-β subunit of the Japanese eel, and analyzed the variation of the mature peptides of GTH II-β subunit among anguillids. This is the first report of the pituitary transcript profiles of PGH-α, GTH I-β, and GTH II-β subunits from immature juvenile stage to the maturing silver stage of wild female Japanese eels.

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