J. Microbiol. Biotechnol. (2003), **13**(5), 710–716

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation of cDNAs for Gonadotropin-II of Flounder (*Paralichthys olivaceus*) and Its Expressions in Adult Tissues

LEE, JAE HYUNG, SOO WAN NAM[1], AND YOUNG TAE KIM*

Department of Microbiology, Pukyong National University, Busan 608-737, Korea
[1]Department of Biotechnology and Bioengineering, Dong-Eui University, Busan 614-714, Korea

Received: February 14, 2003
Accepted: April 30, 2003

Abstract Gonadotropin (GTH) is a pituitary glycoprotein hormone that regulates gonadal development in vertebrates. In teleosts, two types of gonadotropins, GTH-I and GTH-II, are produced in the pituitary, and they comprised of common α and distinct β subunits. In the present study, the cDNAs encoding GTH α and GTH-IIβ subunits were cloned and sequenced from flounder (*Paralichthys olivaceus*) pituitary cDNA library. The nucleotide sequence of the α subunit was 619 bp long, encoding 124 amino acids, and that of the GTH-IIβ subunit was 538 bp long, encoding 145 amino acids. GTH subunits had well conserved cysteines, when aligned with other members of the glycoprotein family. The β subunit of gonadotropin II (GTH-IIβ) had a different N-linked glycosylation site. RT-PCR analysis showed an increase of GTH II mRNA levels in association with gonadal development, and also showed that the mRNA expression of the α subunit was detected only in tissues from pituitary glands.

Key words: Gonadotropin, pituitary glycoprotein, teleosts, cDNA library, flounder, expression, RT-PCR

Gonadotropins (GTH) are pituitary glycoproteins that play a central role in vertebrate reproduction. The vertebrate pituitary produces two forms of gonadotropins that are structurally related glycoproteins: the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) in tetrapods, and GTH-I and -II in fish [19]. All of these glycoproteins are non-covalently bound dimers of α and β subunits, as are other members of the vertebrate glycoprotein hormone families, including the thyroid-stimulating hormone (TSH) and inhibin. In teleosts GTH is required for the seasonal reproductive development of gonads [34]. GTH-I is expressed first in ontogeny, whereas GTH-II appears in the later stages of the reproductive cycle [33].

*Corresponding author
Phone: 82-51-620-6366; Fax: 82-51-611-6358;
E-mail: ytkim@pknu.ac.kr

Two forms of gonadotropins, GTH-I and GTH-II, have been isolated in a variety of species [12, 36]. In teleosts, GTH cDNAs have been cloned and sequenced from Chinook salmon *Oncorhynchus tschawytscha* [34], common carp *Cyprinus carpio* [1, 3], chum salmon *Oncorhynchus keta* [31], pike eel *Muraenesox cinereus* [24], silver carp *Hypophthalmichthys molitrix* [2], European eel *Anguilla anguilla* [30], African catfish *Clarias gariepinus* [20], killifish *Fundulus heteroclitus* [23], tuna *Thunnus obesus* [27], striped bass *Morone saxatilis* [8], Japanese eel *Anguilla japonica* [25], Pacific herring *Clupea pallasi* [29], and red seabream *Pagrus major* [7]. It appears that, at least in the salmonid species, gonadotropin I is associated with the early stages of spermatogenesis and vitellogenesis [35], while GTH-II is associated with oocyte maturation, ovulation and spermiation [26, 32]. However, knowledge of the molecular structure of GTH in the marine fishes is limited. Furthermore, the nature of the two GTHs in these fishes and their roles in the control of reproduction are still unclear.

The α subunit is common to GTH-I and -II, while the β-subunit is hormone specific [28]. The β-subunit is of considerable interest in research on the evolution of glycoprotein hormones because it is divergent during the evolution of vertebrate GTH [3]. Both the α- and β-subunits of GTH contain cross-linked disulfide bonds and are glycosylated in specific asparagine residues [8].

The flounder (*Paralichthys olivaceus*) is a commercially important marine aquaculture species in Korea, one of the most evolved teleosts, and has been the object of studies on various functional genes at the molecular level [4, 13, 22]. In addition, the flounders gonadal maturation is easily induced by long-term photoperiod and low-water-temperature treatment. Therefore, the flounder is a unique and crucial model for fish reproduction research. In the present study, we initially focused on the isolation of cDNAs encoding the α and β subunits of flounder GTH-II and characterized their expressions in adult tissues. Herein, we provide the

molecular characteristics and tissue expressions of our newly identified α subunit (GenBank accession number, AF268692) and GTH-IIβ subunit (GenBank accession number, AF268694) cDNAs from adult flounder. These data will provide a base of knowledge for the primary structure of GTH at the molecular level and the functional diversity of pituitary glycoprotein hormones.

MATERIALS AND METHODS

RNA Isolation and cDNA Library Construction

Mature flounders (*P. olivaceus*) were purchased from a nearby fish market, and thirty pituitary glands from both sexes were collected. Total RNA was isolated with a TRIzol reagent (Invitrogen, Carlsbad, U.S.A.). The RNA pellet was washed with 70% ethanol, dried, and dissolved in DEPC-treated water. Poly(A) RNA was isolated with a Micro-FastTrack™ 2.0 Kit (Invitrogen, Carlsbad, U.S.A.). The quantity of RNA was determined by measuring O.D. value at 260 nm. The construction of the pituitary cDNA library was performed using a ZAP-cDNA® Synthesis Kit (Stratagene, La Jolla, U.S.A.). The resulting library contained approximately 1×10^5 clones. The library was then amplified up to 3×10^9/ml.

Screening of GTH cDNA and DNA Sequencing

Conserved nucleotide sequences of GTH subunits among fish species were searched using the NCBI (National Center for Biotechnology Information) nucleotide and protein sequence database. Oligonucleotide degeneracy primers (Table 1) for screening gonadotropins were synthesized from GenoTech (Taejeon, Korea) and labeled with a DIG (oligoxigenin) oligonucleotide 3' end labeling kit (Roche, Mannheim, Germany). Approximately 1×10^5 plaques from the cDNA library were screened with the above probe. Positive plaques recovered from the first screening were further confirmed by the second screening [14, 15, 16, 17, 18]. Positive plaques were recovered from the second screening, and the phagemid containing the insert was excised according to the manufacturers instructions (Stratagene, La Jolla, U.S.A.). DNA sequencing of the excised phagemid was performed using the ABI PRISM™ DNA sequencing kit (Applied Biosystems, Foster, U.S.A.) and determined with ABI 377 Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems, Foster, U.S.A.).

Comparative Sequence Analysis of Fish GTH Subunits

To define the molecular evolution of GTH α subunit, several fish GTH α subunit sequences were imported from the SwissPort data bank/GenBank as follows: *Hippoglossus hippoglossus* (Atlantic halibut, CAD10503), *Pagrus major* (red sea bream, BAB18562), *Morone saxatilis* (striped bass, Q91119), *Acanthopagrus latus* (yellowfin seabream, P30970), *Cyprinus carpio* 1 (common carp 1, X56497), *Cyprinus carpio* 2 (common carp 2, M37380), *Katsuwonus pelamis* (bonito, AAB25414), *Anguilla anguilla* (European eel, A37198), *Muraenesox cinereus* (pike eel, S07091), *Oncorhynchus masou* 1 (cherry salmon 1, AAB30422), *Oncorhynchus masou* 2 (cherry salmon 2, AAB30422), *Clarias gariepinus* (catfish, AAB24036), and the *Thunnus obesus* (tuna, P37204) GTH α subunit sequence. Also, to define the molecular evolution of the GTH-IIβ subunit, the sequences of *Morone saxatilis* (striped bass, Q91121) and *Thunnus obesus* (tuna, P37206) GTH-IIβ subunit were imported from the SwissPort data bank/GenBank. Finally, all the DNA sequence data were analyzed, using Internet-based programs such as ClustalW, MAP, and FASTA through the ExPASy tools (Geneva, Switzerland).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In order to perform reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from pituitary, liver, kidney, muscle, spleen and testis from mature flounder (N=10; size: 45 cm ±10 cm, body weight: 900 g ±300 g; 3 years old) and from the head of immature flounder (N=5; size: 7 cm ±2 cm, body weight: 4 g ±1 g; 4 months old). Titan™ one tube RT-PCR system (Roche, Mannheim, Germany) was used. Master mix 1 contained 0.2 mM dNTPs, 5 mM dithiothreitol, 50 pmol upstream (GT-FS: Table 1) and downstream (GTH-S: Table 1) primers,

Table 1. Oligonucleotide primers used for this study.

Primer	Nucleotide sequence	Remark
GT-F	5'-G(C/T)TGCTTCTCC(A/C)(A/C/G)AGCGTACC-3'	Probe for GTH α-subunit, Forward
GT-R	5'-CA(A/C/G)TGGC(A/C/G)CT(A/C)CGTGTGGTTC-3'	Probe for GTH α-subunit, Reverse
GT-FS	5'-TTCTATCAACATGGTAACTGC-3'	GTH α-subunit RT-PCR, Forward
GTH-S	5'-TCATATCTTGTGATAATAGC-3'	GTH α-subunit RT-PCR, Reverse
GTH-B2F	5'-ACCAC(A/T/G/C)ATCTG(C/T)AGTGG(C/T)CACTG-3'	Probe for GTH-IIβ, Forward
GTH-B2R	5'-TGCAGGCTCTC(A/C/G)A(A/T)GGT(A/C/G)CAGTC-3'	Probe for GTH-IIβ, Reverse
LT-7	5'-TTGTAATACGACTCACTATAGGGC-3'	T7 modified primer
Actin-F	5-CACACCTTCTACAAGGAGCTG-3'	β-actin, Forward
Actin-R	5'-CGGTCAGGATCTTCATGAGG-3'	β-actin, Reverse

template RNA, and 5U of Rnase inhibitor. Master mix 2 consisted of 5 x RT-PCR buffer and enzyme mix. Mix 1 and mix 2 were added to a 0.2-ml thin-walled PCR tube on ice. Then, the sample was placed in a thermocycler (Applied Biosystems, GeneAmp PCR system 2400) and incubated for 1 h at 50°C for reverse transcription followed by thermocycling. A temperature profile was on prereaction at 94°C for 5 min; 30 cycling reactions at 94°C for 30 sec., 52°C for 30 sec., and 72°C for 1 min; and finally for a 7 min extension at 72°C. To confirm that the absence of a GTH α subunit cDNA product was not due to any deficiency of the cDNA preparations, a β-actin gene which uses a widely expressed control gene was also amplified. This was done using the conditions described for GTH α (except for the use of an annealing temperature of 54°C) and the β-actin primers (Actin-F and Actin-R; Table 1).

RESULTS

Molecular Sequencing of the Pituitary Glycoprotein α Subunit

Conserved nucleotide sequences of GTH α subunit among fish species were determined using the NCBI nucleotide and protein sequence database, and the first round of PCR was carried out using a pair of the 'LT7' and 'GT-F' primers (Table 1). Consequently, the second round of amplification was performed with the GT-F and nested GT-R primers (Table 1) in order to prepare for the probe of screening α subunit, and this resulted in the 157 bp fragment whose identity was confirmed by nucleic acid sequencing (data not shown). The cDNA library was screened by colony hybridization with the above probe. The cDNA sequence of the flounder GTH α subunit gene was determined as shown in Fig. 1, and deposited at GenBank (AF268692). Also, the deduced amino acid sequence for the cDNA is shown in Fig. 1. The flounder GTH a subunit gene has 619 bp, including an open reading frame, 5'- and 3'-untranslated regions, encoding 124 amino acid residues. The cDNA consisted of 16 bp of 5'-untranslated region (UTR), 372 bp of coding region and 228 bp of 3'-UTR, followed by a poly(A) sequence. The 3' UTR contained polyadenylation signals (attaaa). There were two potential translation initiation sites (Fig. 1). The first ATG (CAAC**ATG**G; with an A in position-3 and a G in position 4) was the most likely initiator codon [21]. Also, the second ATG (CTCAA**TG**G) might be an initiator codon, however, the second ATG was not accepted as an initiator codon on the basis of the "first-AUG-rule" and a "purine" (most often A) in position-3. It gave a signal peptide of 30 amino acid residues, followed by a mature peptide of 94 amino acid residues.

Comparison of the flounder GTH α subunit gene with other species is shown in Fig. 2. For investigating the

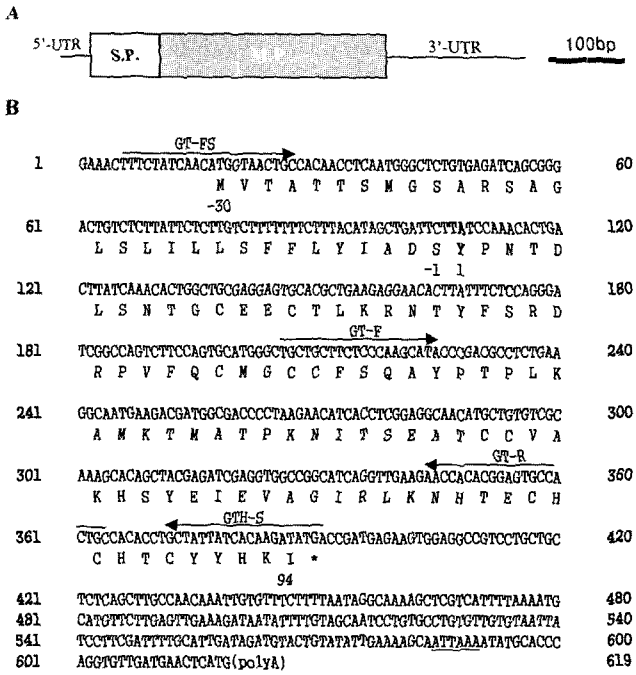

Fig. 1. (A) Structural location of flounder GTH a subunit cDNA.
5'UTR indicates 5'-untranslated region and 3'-UTR, 3'-untranslated region. S.P. indicates signal peptide and M.P., mature protein. (B) The full length of the nucleotide and deduced amino acid sequences of cDNA encoding flounder GTH α subunit. The nucleotide numbers are shown on both sides, and the amino acid residue numbers are shown under the amino acid sequence. The first amino acid of the putative mature GTH α (Y in bold type) is numbered as +1. Negative numbers are used to indicate the amino acids that comprise the signal peptide. An *asterisk* indicates the stop codon. The consensus polyadenylation signal is underlined.

similarity of flounder GTH α subunit gene with other species, the BLAST program was run. The flounder GTH a subunit gene has a high similarity in amino acid residues with other species, greater that 60% sequence identity. Also, putative N-linked glycosylation sites are located at positions 55 and 80 (Fig. 2).

Molecular Sequencing of GTH-IIβ

Conserved nucleotide sequences of the GTH-IIβ subunit among the fish species were determined using the NCBI nucleotide and protein sequence database, and the first round of PCR was carried out using a pair of the 'LT7' and 'GTH-B2F' primers (Table 1). In order to prepare for the probe of screening GTH-IIβ subunit, the second round of amplification was consequently performed with the 'GTH-B2F' and nested 'GTH-B2R' (Table 1) and resulted in the 224 bp fragment. The resulting DNA fragment was sequenced and identified with a fragment homologous to the other GTH-IIβ subunits. Then, cDNA library was screened by colony hybridization with the above probe. The cDNA clones of the flounder GTH-IIβ subunit gene were isolated and sequenced. The sequences of the

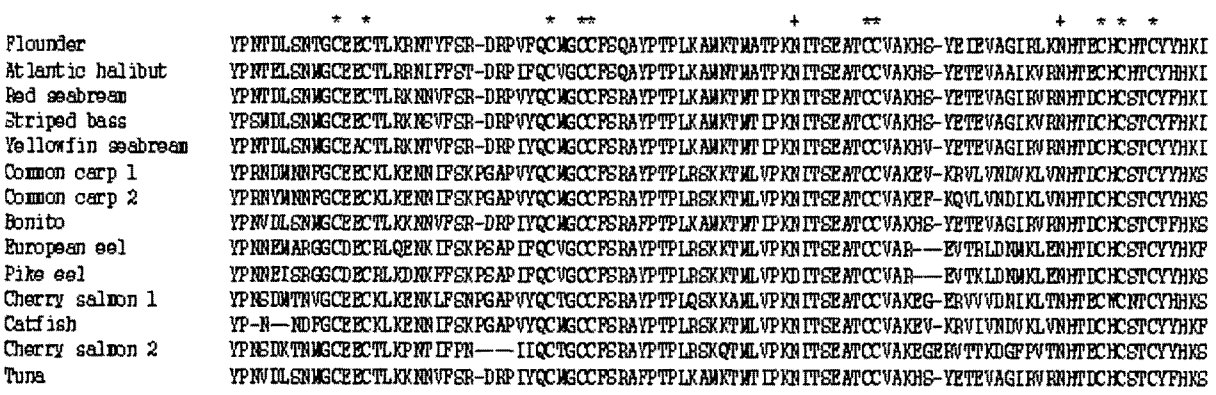

Fig. 2. Alignment of the deduced amino acid sequence of GTH α subunit from other fishes.
The fish GTH α subunit sequences were imported from the SwissPort data bank/GenBank as follows: *Hippoglossus hippoglossus* (Atlantic halibut, CAD10503), *Pagrus major* (red seabream, BAB18562), *Morone saxatilis* (striped bass, Q91119), *Acanthopagrus latus* (yellowfin seabream, P30970), *Cyprinus carpio* 1 (common carp 1, X56497), *Cyprinus carpio* 2 (common carp 2, M37380), *Katsuwonus pelamis* (bonito, AAB25414), *Anguilla anguilla* (European eel, A37198), *Muraenesox cinereus* (pike eel, S07091), *Oncorhynchus masou* 1 (cherry salmon 1, AAB30422), *Oncorhynchus masou* 2 (cherry salmon 2, AAB30422), *Clarias gariepinus* (catfish, AAB24036), and the *Thunnus obesus* (tuna, P37204) GTH α subunit sequence. The degree of shade shows the identities, which are identical to those of the flounder mature peptide sequence. *Asterisks* indicate cysteine residues. Putative N-linked glycosylation sites are indicated by plus (+) signs.

nucleotide and the deduced amino acids for the cDNA are shown in Fig. 3, and deposited at GenBank (AF268694).

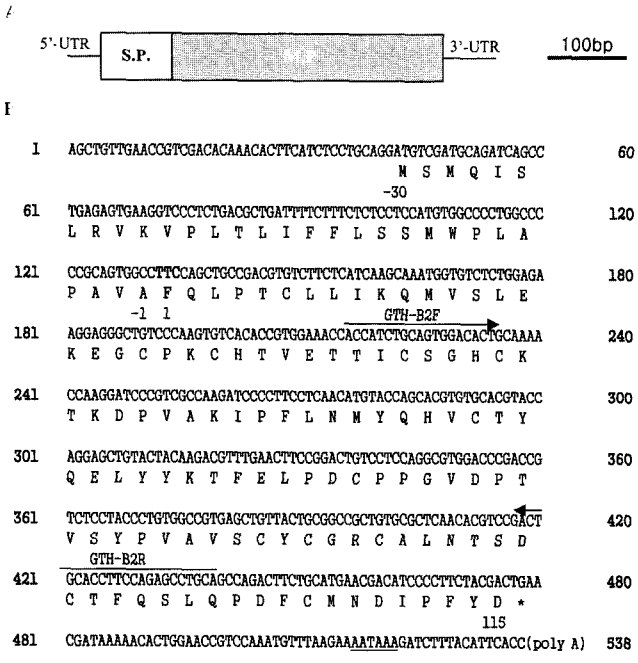

Fig. 3. (A) Structural location of flounder GTH-IIβ subunit gene. 5'UTR indicates 5'-untranslated region and 3'-UTR, 3'-untranslated region. S.P. indicates signal peptide and M.P., mature protein. (B) The full length of the nucleotide and deduced amino acid sequences of cDNA encoding flounder GTH-IIβ subunit. The nucleotide numbers are shown on both sides, and the amino acid residue numbers are shown under the amino acid sequence The first amino acid of the putative mature GTH-IIβ (F in bold type) is numbered as +1. Negative numbers are used to indicate the amino acids that comprise the signal peptide. An *asterisk* indicates the stop codon. The consensus polyadenylation signal is underlined.

The flounder GTH-IIβ subunit gene consists of 41 bp of 5'-UTR, 435 bp of coding, and 59 bp of 3' UTR, followed by a poly(A) sequence. The coding region encodes a 30 amino acid signal peptide and a 115 amino acid mature beta-subunit. The 3' UTR contained polyadenylation signals (aataaa).

Comparison of the flounder GTH-IIβ subunit gene with striped bass (*Morone saxatilis*) and tuna (*Thunnus obesus*) shows them to be aligned (Fig. 4). From the comparison data, it is noteworthy that the putative site of N-linked glycosylation is located at position 93.

Tissue Expressions for GTH

The gonadotropin requires both the α- and the β-subunits heterodimeric subunits for its regulatory function. The GTH α subunit is common to two forms of gonadotropins, GTH-I and -II. Therefore, understanding of the nature of

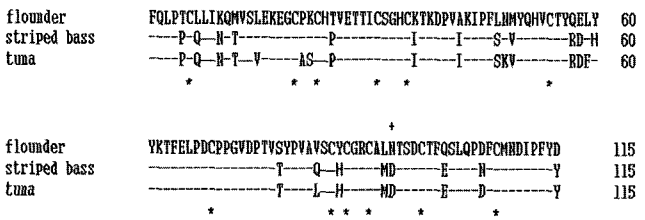

Fig. 4. Alignment of the deduced amino acid sequence of GTH-IIβ subunit from other fishes.
The sequences of *Morone saxatilis* (striped bass, Q91121) and *Thunnus obesus* (tuna, P37206) GTH-IIβ subunit were imported from the SwissPort data bank/GenBank. Dashes indicate amino acid residues, which are identical to those of the flounder mature peptide sequence. Dashes indicate amino acid residues, which are identical to those of the flounder mature peptide sequence. *Asterisks* indicate cysteine residues. Putative N-linked glycosylation sites are indicated by plus (+) signs.

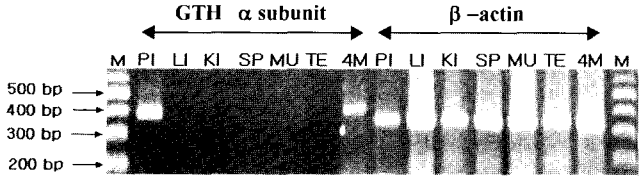

Fig. 5. Patterns of the expression of GTH α subunit and β-actin gene detected by RT-PCR.
Lane "M" indicates a molecular marker. "PI" indicates pituitary; "LI" (liver); "KI" (kidney); "SP" (spleen); "MU" (muscle); "TE" (testis) and "4M" (4 months old flounder).

gonadotropins and their roles in the control of reproduction in teleosts is important. The GTH α subunit gene will be used as a critical probe for the evaluation of the tissue expressions of GTH. To characterize the tissue expressions of gonadotropins, the reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers specific for the GTH α subunit gene using total RNAs isolated from flounder tissues as a template. As shown in Fig. 5, the patterns of DNA fragments amplified from RT-PCR provided evidence for the expressions of the GTH genes. The expression of the GTH α subunit gene was detected only from the pituitary tissues isolated from both adult and four-month-old immature flounder, but not in the liver, kidney, muscle, spleen and testis among the tissues investigated. Also, we used β-actin gene as a control gene to confirming that the absence of the GTH α subunit cDNA product was not due to any deficiency in the cDNA preparations. The message of both the α- and the β-subunits flounder β-actin gene was ubiquitously expressed in the tissues investigated as shown in Fig. 5. Thus, flounder gonadotropin gene is likely to be expressed only in pituitary glands in the developmental stages.

DISCUSSION

In the present study, the cDNA encoding flounder pituitary glycoprotein gonadotropin α subunit was cloned and its nucleotide sequence was determined. We also isolated the GTH-IIβ subunit gene, because the sequence of the GTH-IIβ subunit gene has unique characteristics in its nucleotide sequences. Therefore, we report here the complete forms of the gonadotropins, since GTHs require the α subunit as a common subunit for their function. The sequence of the flounder GTH α subunit gene is reported for the first time in this paper. Comparison of the deduced amino acid sequence of the α-subunit of flounder with those of other teleost species shows greater than 60% sequence identities. The ten half-cystines and the two potential N-linked glycosylation sites are completely conserved. The presence of two types of α-subunit in several teleost species was reported [6, 10]. However, we were unable to find different types of cDNA encoding α subunit, suggesting that flounder possesses only a single type of alpha form subunit.

Furthermore, the GTH-IIβ subunit isolated in this study had nucleotide sequences and N-linked glycosylation sites (N-X-S/T) different from those reported previously [11]. N-linked glycosylation is important for it's function: A primary function of N-linked glycosylation is to ensure proper folding of glycoprotein [5] and also important for stability of the glycoprotein [9]. As shown in Fig. 6, the flounder GTH-IIβ had a N-linked glycosylation site (93 position) different from the site (10 position) determined by Kajimura *et al.* [11]. These differences are also shown in recently isolated Atlantic halibut (*Hippoglossus hippoglossus*) at a GenBank (accession number; AJ417769). The N-linked glycosylation site of GTH-IIβ in *Hippoglossus hippoglossus* was identical to the GTH-IIβ in this study. *Hippoglossus hippoglossus* and *Paralichthys olivaceus* are identical with suborder "*Pleuronectoidei*". We attribute these differences to the fact that the flounder may differ in character from the salmonid and mammalian species. In addition, the flounder GTH-IIβ had well conserved cysteines, when aligned with other members of the glycoprotein families (Fig. 4). Other conserved features, that are thought to be important in binding to the α subunit and shared with the GTH-IIβ subunits in other fishes included a teleost-specific Cys-Ser-Gly-His (CSGH) amino acid sequence (positions: 31-34) between the fourth and fifth cysteines and a Pro-Val-Ala (PVA) sequence before cysteine 8 [29].

In order to determine the expression of gonadotropins in flounder tissues, RT-PCR was used, and the expression

```
                                                    * *
Flounder          -MSMQISLRVKVPLTLIFFLSSMWPLAPAVAFQLPTCLLIKQMVSLEKEGCPKCHTVETTICSGHCKTKDPVAKI
Japanese flounder -MSMQISLRVKVPLTLIFFLSSMWPLAPAVAFQLPTCLLINQTVSLEKEGCPKCHTVETTICSGHCKTKDPVAKI
Atlantic halibut  METEQISVRVKLPLTLIFFLSSMWPLAPAVAFQLPKCQLIKQMVSLEKEGCPKCHTVETTICSGHCNTKDPVIKI
                                                  1       10                                  44
                                                                    +
Flounder          PFLNMYQHVCTYQELYYKTFELPDCPPGVDPTVSYPVAVSCYCGRCALNTSDCTFQSLQPDFCMNDIPFYD
Japanese flounder PFLNMYQHVCTYQELYYKTFELPDCPPGVDPTVSYPVAVSCYCGRCALNTSDCTFQSLQPDFCMNDIPFYD
Atlantic halibut  PFLNVYQHVCTYQELYYKTFELPDCPPGVDPTVSYPVAVSCYCGRCALNTSDCTFESLQPDFCMNDIPFYD
                  45                                                  93
```

Fig. 6. Alignment of deduced amino acid sequence of the flounder GTHIIβ subunit.
An asterisk indicates the distinct amino acids. Putative N-linked glycosylation sites are indicated by plus (+) signs.

was detected only in the pituitary glands, but not in the liver, kidney, muscle, spleen and testis among the tissues investigated. In the case of flounder, it usually takes adult species 3 years to spawn. We also investigated whether the GTH hormone, expressed using 4 months old immature fish, and the result of RT-PCR shows that flounder at least 4 months old expressed GTH hormones. A comparison of adult to immature flounder showed that the expression of GTH hormones increased as the gonadal developmental stages progressed.

In conclusion, these comparative data of the amino acid sequences of vertebrate GTHs indicate that the α subunit gene is highly conserved, similar to the GTH α subunit genes of other species, while the β subunit is diversified during the molecular evolution of vertebrate GTH. This result suggests that gonadotropin protein may function similarly to the way it functions in other species in vertebrate reproduction. Therefore, the present study conducted on fish contributes to deeper understanding of the mechanisms involved in gonadotropins regulated reproduction.

Acknowledgment

This work was supported by Korea Research Foundation Grant (KRF-2001-002-H00021).

References

1. Chang, Y. S., C. J. Huang, F. L. Huang, and T. B. Lo. 1988. Primary structures of carp gonadotropin subunits deduced from cDNA nucleotide sequences. *Int. J. Pept. Protein. Res.* **32:** 556-564.
2. Chang, Y. S., F. L. Huang, C. S. Liu, and T. B. Lo. 1990. Purification, characterization and molecular cloning of gonadotropin subunits of silver carp *Hypophthalmichthys molitrix*. *Gen. Comp. Endocrinol.* **78:** 23-33.
3. Chang, Y. S., F. L. Huang, and T. B. Lo. 1992. Isolation and sequence analysis of carp gonadotropin β-subunit gene. *Mol. Mar. Biol. Biochem.* **1:** 97-105.
4. Cho, J. J., B. K. Sung, J. H. Lee, J.-K. Chung, T.-J. Choi, and Y. T. Kim. 2001. cDNA for an immune response gene encoding low molecular weight polypeptide from flounder, *Paralichthys olivaceus*. *Mole. Cells* **11:** 226-230.
5. Ellgaard, L., M. Molinari, and A. Helenius. 1999. Setting the standards: Quality control in the secretory pathway. *Science* **286:** 1882-1888.
6. Gen, K., O. Maruyama, T. Kato, K. Tomizawa, K. Wakabayashi, and Y. Kato. 1993. Molecular cloning of cDNAs encoding two types of gonadotropin alpha subunit from the masu salmon, *Oncorhynchus masou*: Construction of specific oligonucleotides for the alpha 1 and alpha 2 subunits. *J. Mol. Endocrinol.* **11:** 265-273.
7. Gen, K., K. Okuzawa, B. Senthilkumaran, H. Tanaka, S. Moriyama, and H. Kagawa. 2000. Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. *Bio. Reprod.* **63:** 308-319.
8. Hassin, S., A. Elizur, and Y. Zohar. 1995. Molecular cloning and sequence analysis of striped bass (*Morone saxatilis*) gonadotropin-I and -II subunits. *J. Mol. Endocrinol.* **15:** 23-35.
9. Imperiali, B. and S. E. O'Connor. 1999. Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr. Opin. Chem. Biol.* **3:** 643-649.
10. Itoh, H., K. Suzuki, and H. Kawauchi. 1990. The complete amino acid sequences of alpha subunits of chum salmon gonadotropins. *Gen. Comp. Endocrinol.* **78:** 56-65.
11. Kajimura, S., Y. Yoshiura, M. Suzuki, and K. Aida. 2001. cDNA cloning of two gonadotropin β subunits (GTH-Iβ and -IIβ) and their expression profiles during gametogenesis in the Japanes flounder (*Paralichthys olivaceus*). *Gen. Comp. Endocrinol.* **122:** 117-129.
12. Kawauchi, H., K. Suzuki, H. Itoh, P. Swanson, N. Naito, Y. Nagahama, M. Nozaki, Y. Nakai, and S. Itoh. 1989. The duality of teleost gonadotropin. *Fish Physiol. Biochem.* **7:** 29-38.
13. Kim, D. S. and Y. T. Kim. 1999. Identification of an embryonic growth factor IGF-II from the central nervous system of the teleost, flounder, and its expressions in adult tissues. *J. Microbiol. Biotech.* **9:** 113-118.
14. Kim, H.-S. and T. J. Crow. 2002. Identification and phylogeny of the human endogenous retrovirus HERV-W LTR family in human brain cDNA library and Xq21.3 region. *J. Microbiol. Biotechnol.* **12:** 508-513.
15. Kim, Y.-J., N.-R. Lee, S.-Y. Choi, and K.-H. Min. 2002. Molecular cloning of the *nahC* gene endcoding 1,2-dihydroxynaphthalene dioxygenase from *Pseudomonas fluorescens*. *J. Microbiol. Biotechnol.* **12:** 172-175.
16. Kim, Y. T. and C. C. Richardson. 1993. Bacteriophage T7 gene 2.5 protein: An essential protein for DNA replication. *Proc. Natl. Acad. Sci. USA* **90:** 10173-10177.
17. Kim, Y. T. and C. C. Richardson. 1994. Acidic carboxyl-terminal domain of gene 2.5 protein of bacteriophage T7 is essential for protein-protein interactions. *J. Biol. Chem.* **269:** 5270-5278.
18. Kim, Y. T., Y. H. Song, and J. E. Churchich. 1997. Recombinant brain 4-aminobutyrate aminotransferases: Overexpression, purification, and identification of Lys-330 at the active site. *Biochim. Biophys. Acta.* **1337:** 248-256.
19. Koide, Y., H. Itoh, and H. Kawauchi. 1993. Isolation and characterization of two distinct gonadotropins, GTHI and GTHII, from bonito (*Katsuwonus plelamis*) pituitary glands. *Int. J. Pept. Protein. Res.* **41:** 52-65.
20. Koide, Y., T. Noso, G. Schouten, J. Peute, M. A. Zandbergen, J. Bogerd, R. W. Schulz, H. Kawauchi, and H. J. Goos. 1992. Maturational gonadotropin from the African catfish, *Clarias gariepinus*: Purification, characterization, localization, and biological acitivity. *Gen. Comp. Endocrinol.* **87:** 327-341.

21. Kozak, M. 1984. Compilation and analysis of sequences upstream from the translational site in eukaryotic mRNAs. *Nucleic Acids Res.* **12:** 857-872.
22. Lee, E.-Y., H.-H. Park, Y. T. Kim, and T.-J. Choi. 2001. Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralithys olivaceus*). *Gene* **274:** 237-243.
23. Lin, Y. W. P., D. A. Rupnow, R. M. Greenberg, and R. A. Wallace. 1992. Fundulus heteroclitus gonadotrophin. 3. Cloning and sequencing of gonadotropic hormone (GTH) I and II β-subunits using the polymerase chain reaction. *Mol. Cell. Endocrinol.* **85:** 127-139.
24. Liu, C. S., F. L. Huang, Y. S. Chang, and T. B. Lo. 1989. Pike eel (*Muraenesox cinereus*) gonadotropin. Amino acid sequences of both alpha and beta subunits. *Eur. J. Biochem.* **186:** 105-114.
25. Nagae, M., T. Todo, K. Gen, Y. Kato, G. Young, S. Adachi, and K. Yamauchi. 1996. Molecular cloning of the cDNAs encoding pituitary glycoprotein hormone α- and gonadotrophin II β-subunits of the Japanese eel, *Anguilla japonica*, and increase in their mRNAs during ovarian development induced by injection of chum salmon pituitary homogenate. *J. Mol. Endocrinol.* **16:** 171-181.
26. Nozaki, M., N. Naito, P. Sqanson, W. W. Dickhoff, Y. Nakai, K. Suzuki, and H. Kawauchi. 1990. Salmonid pituitary gonadotropins. II. Ontogeny of GtH-I and GtH-II cells in the rainbow trout (*Salmon gairdneri irideus*). *Gen. Comp. Endocrinol.* **77:** 358-367.
27. Okada, T., I. Kawazoe, S. Kimura, Y. Sasamoto, K. Aida, and H. Kawauchi. 1994. Purification and characterization of gonadotropin I and II from pituitrary glands of tuna (*Thunnus obesus*). *Int. J. Pept Protein Res.* **43:** 512-515.
28. Pierce, J. G. and T. F. Parsons. 1981. Glycoprotein hormones: Structure and function. *Annu. Rev. Biochem.* **50:** 465-495.
29. Power, M. E., J. Carolsfeld, G. P. Wallis, and N. M. Sherwood. 1997. Isolation and characterization of a cDNA for gonadotropin II-beta of Pacific herring, an ancient teleost. *J. Fish. Biol.* **50:** 315-323.
30. Quérat, B., M. Jutisz, Y. A. Fontaine, and R. Counis. 1990. Cloning and sequence analysis of the cDNA for the pituitary glycoprotein hormone α-subunit of the European eel. *Mol. Cell. Endocrinol.* **71:** 253-259.
31. Sekine, S., A. Saito, H. Itoh, H. Kawauchi, and S. Itoh. 1989. Molecular cloning and sequence analysis of chum salmon gonadotrophin complementary DNAs. *Proc. Natl. Acad. Sci. USA* **86:** 8645-8649.
32. Suzuki, K., A. Kanamori, Y. Nagahama, and H. Kawauchi, 1988. Development of salmon GtH-I and GtH-II radioimmunoassays. *Gen. Comp. Endocrinol.* **71:** 459-467.
33. Swanson, P., K. Suzuki, H. Kawauchi, and W. W. Dickhoff. 1991. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol. Reprod.* **44:** 29-38.
34. Trinh, K.-Y., N. C. Wang, C. L. Hew, and L. W. Crim. 1986. Molecular cloning and sequencing of salmon gonadotropin β subunit. *Eur. J. Biochem.* **159:** 619-624.
35. Tyler, C. R., J. P. Sumpter, H. Kawauchi, and P. Swanson. 1991. Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **84:** 291-299.
36. Yoshiura, Y., H. Suetake, and K. Aida. 1999. Duality of gonadotropin in a primitive teleost, Japanese eel (Anguilla japonica). *Gen. Comp. Endocrinol.* **114:** 121-131.