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The Spotted Flounder (*Verasper variegatus*) Growth Hormone cDNA and Its Evolutionary Implications

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The full-length cDNA encoding the pre-protein growth hormone (sfGH) from spotted flounder (*Verasper variegatus*) was amplified by the rapid amplification of cDNA ends (RACE) using degenerated oligonucleotide primers derived from conserved growth hormone sequences. It consists of 901 nucleotides in length, including the coding region of 609 nucleotides, 111 nucleotides of a 5' untranslated region, and 181 nucleotides of a 3' untranslated region. The conserved polyadenylation signal (AATAAA) lies 12 bases upstream from the poly (A) tail. The deduced amino acid sequence shows an open reading frame encoding a pre-protein of 203 amino acids and a putative signal peptide of 17 amino acids, suggesting that the mature hormone consists of 186 amino acids. The analyses of sfGH reveal some unique structural features. The repetitive sequences are located in the 5' untranslated region of sfGH cDNA and consist of tandem arrays of imperfect direct repeat monomers. Moreover, sfGH contains six Cys residues, as opposed to four or five in other GHs, and it is clearly distinguishable from olive flounder (*Paralichthys olivaceus*) GH, which lacks a region corresponding to residues 175-188 in alignment positions. It has important implications from an evolutionary standpoint, suggesting possible divergence among flatfishes.

Key words: Spotted flounder, *Verasper variegatus*, Growth hormone, RACE, Evolution

Introduction

Growth hormone (GH), a single-chain polypeptide secreted by pituitary gland, plays an essential role in the development and growth of vertebrates. Together with prolactin (PRL) and placental lactogen (PL), GH forms a set of proteins that are structually related and have partially overlapping biological activities. Primary structure analysis of the peptides and of the genes suggests that these genes may have evolved from a common ancestral origin (Niall et al., 1971; Seeburg et al., 1977; Shine et al., 1977; Cooke et al., 1980). Therefore, these genes would provide an excellent model system for understanding of the interrelationship between the structure, function and evolution of fish hormones. To obtain information about the evolution and mechanisms of organization of this set of genes, it is essential to compare the structures of these genes isolated from many organisms belonging to various evolutionary stages.

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GH cDNAs have been cloned from numerous fish species including salmon (Sekine et al., 1985), rainbow trout (Agellon and Chen, 1986), tuna (Sato et al., 1988), eel (Saito et al., 1988), yellow tail (Watahiki et al., 1988), red seabream (Momota et al., 1988), carp (Koren et al., 1989), tilapia (Rentier-Delrue et al., 1989), olive flounder (Watahiki et al., 1989) and greenling (Nam and Kim, 2002). However, there has been still limited information on the GH genes of flatfishes, which may give the more complete comparative analysis of picine GHs. In this report we describe the cloning and characterization of cDNA encoding spotted flounder (*Verasper variegatus*) GH and compare the amino acid sequence with those of other vertebrate GHs.

Materials and Methods

RNA isolation and first-strand cDNA synthesis

Total RNA was extracted from the pituitaries of spotted flounder using TRIZOL Reagent (Life

Technologies, Rockville, Maryland). Complementary DNA (cDNA) was synthesized with 4 μg of total RNA using SuperScript II reverse transcriptase (GIBCO BRL).

3' RACE (the rapid amplification of 3' cDNA end)

To obtain the 3' end of the cDNA, 3' RACE reaction was performed with a degenerated primer (Table 1), UGH2F from the sequences conserved in the GH cDNAs for olive flounder (Watahiki et al., 1989), tuna (Sato et al., 1988), yellow tail (Watahiki et al., 1988), red sebream (Momota et al., 1988), salmon (Sekine et al., 1985), rainbow trout (Agellon and Chen, 1985), eel (Saito et al., 1988), bovine (Miller et al., 1980), rat (Seeburg et al., 1977) and human (Martial et al., 1979). Thirty cycles of PCR amplification were run with denaturation for 1 min at 95℃, annealing for 1 min at 50℃, extension for 1.5 min at 72℃, and final extension for 10 min at 72℃ after the last cycle. Amplification products were separated onto an agarose gel, and the band of desired size was excised and purified. The purified DNA fragment (about 700 bp) was subcloned into pGEM-T-Easy vector (Promega, Madison, WI). Plasmid DNAs from 25 clones were sequenced with T7 and SP6 primer in both directions.

5' RACE (the rapid amplification of 5' cDNA end)

From the above sequence information obtained, SFGH-1 was synthesized for the rapid amplification of 5' cDNA end (5' RACE) and used as a primer for the first strand cDNA synthesis. After cDNA purification, the other steps of the procedure were performed following the instruction of the manufacturer. The final product was synthesized by PCR amplification using a second primer SFGH-2 as nested oligonucleotide and supplied complementary anchor primer. The desired PCR bands about 350 bp were purified and subcloned into pGEM-T-Easy vector. The sequence of 5' clone was aligned with that of 3' clone in order to generate a contig sequence.

RT-PCR for full-length spotted flounder GH cDNA

To isolate the full-length growth hormone cDNA, SFGH-3 and SFGH-4 were designed based on the sequence information from 5' and 3' RACE. Reverse transciption-PCR (RT-PCR) using these primers produced the amplified fragment of about 900 bp. The RT-PCR product was purified and subcloned into the pGEM-T-Easy vector for sequencing.

Tissue distribution of spotted flounder GH mRNA

Total RNA was extracted from pituitary gland, brain, liver and fin using TRIZOL Reagent (Life Technologies, Rockville, Maryland). First strand cDNA was prepared from total RNA using SuperScript II reverse transcriptase (GIBCO BRL) and used as templates for PCR reaction. Two oligonucleotide primers, SFGH-5 and SFGH-6 specific to sfGH cDNA were used. PCR were carried out for 30 cycles of 95℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min following a final extension of 10 min at 72℃. Expected size of the product is 630 bp.

Phylogenetic analysis

Deduced amino-acid sequences of sfGH were aligned with other related GHs using the software program CLUSTAL X. The phylogeny tree was built by the neighbor-joining method based on a sequence alignment generated using CLUSTAL X (Jeanmougin et al., 1998).

Results

Analysis of the full-length cDNA encoding spotted flounder growth hormone

The complete nucleotide sequence of sfGH cDNA

Table 1. Oligonucleotide primers used for RACE and RT-PCR

Name	Nucleotide sequence[a]	Usage
SFGH2F	5'-AGARACTCTTCWSYR ACTTYGAG-3'	for 3'-RACE
SFGH-1	5'-TGCAGAACCAACAGAGGCTAAC-3'	for 5'-RACE
SFGH-2	5'-CGTTGTGTCTCGTGTTTGTCATCG-3'	for nested 5'-RACE
SFGH-3	5'-ACACTGAAGAACTGAACCTGACC-3'	for full-length cloning
SFGH-4	5'-CTGAATGAACCCTTTATTTGCTGACAGTATGAC-3'	for full-length cloning
SFGH-5	5'-CGTCTGTTCTCGATCGCAGTTGGTC-3'	for RTPCR
SFGH-6	5'-ATGCTAGTTAGCTTGGCAACCATGG -3'	for RTPCR

[a]Abbreviations for degenerate nucleotides: R, A or G; W, A or T; S, G or C; Y, C or T.

```
                                                   SFGH-3
                                    5'··· ACACTGAAAAACTGAACCTGAACCTGAACTGAACCAAAAC

CCGAACCCGAACCCGAACCTGAACCAAAACCAAAACCAAAACTTGAACTAAAACCTGAACTAAAACCAGCC

112
ATG AAC AGA GTC ATC CTC CTG CTG TCA GTC ATG TGT GTG GGC GTG TCC TCT CAG
Met Asn Arg Val Ile Leu Leu Leu Ser Val Met Cys Val Gly Val Ser Ser Gln
-17                                                                 -1   1

166                                             SFGH-5
CCA ATC ACA GAG AAC CAG CGT CTG TTC TCG ATC GCA GTT GGT CGG GTT CAG TAT
Pro Ile Thr Glu Asn Gln Arg Leu Phe Ser Ile Ala Val Gly Arg Val Gln Tyr
                                                                 19

220                                         SFGH-1
CTG CAC CTG GTT GCT AAG AAA CTC TTC AGT GAA TTC GAG AAC TCT CAG TTG GAG
Leu His Leu Val Ala Lys Lys Leu Phe Ser Glu Phe Glu Asn Ser Gln Leu Glu
                                                                     37

274
GAT CAA CAT CCA CTC AAC AAA ATC TTT TTA CAA GAT TTT TGT CAT TCG GAT TAT
Asp Gln His Pro Leu Asn Lys Ile Phe Leu Gln Asp Phe Cys His Ser Asp Tyr
                                                                     55

328                                     SFGH-2
TTC TTG AGT CCG ATC GAC AAA CAC GAG ACA CAA CGC AGC TCA GTT CTG AAG CTG
Phe Leu Ser Pro Ile Asp Lys His Glu Thr Gln Arg Ser Ser Val Leu Lys Leu
                                                                     73

382
TTA TCG ATC TCT TAT CGA TTG ATT GAG TGC TGG GAG TTT TCC AGT CGC TTC CTG
Leu Ser Ile Ser Tyr Arg Leu Ile Glu Cys Trp Glu Phe Ser Ser Arg Phe Leu
                                                                     91

436
GTC GCA GGT TTC GCT GAG AGG GCC CAG GTT ACA TCC AAA CTG TCA GAG CTG AAG
Val Ala Gly Phe Ala Glu Arg Ala Gln Val Thr Ser Lys Leu Ser Glu Leu Lys
                                                                    119

490
ACG GGA CTC ATG AAG CTG ATA GAG GCC AAT CAG GAC GGA GCG GGT GGA TTC TCT
Thr Gly Leu Met Lys Leu Ile Glu Ala Asn Gln Asp Gly Ala Gly Gly Phe Ser
                                                                    137

544
GAG AGC TCG GTG ATC CAG CTC ACG CCG TAC GGA AAC TAC TAC CAG AGC GTC GGA
Glu Ser Ser Val Ile Gln Leu Thr Pro Tyr Gly Asn Tyr Tyr Gln Ser Val Gly
                                                                    155

598
GTC GAT GAG TCA TTC AGA CTA AAC TAC GAA CTG TTC GCC TGC TTT AAG AAG GAT
Val Asp Glu Ser Phe Arg Leu Asn Tyr Glu Leu Phe Ala Cys Phe Lys Lys Asp
                                                                    173

652
ATG CAC AAG GTG GAG ACG TAT CTG ACC GTG GCC AAA TGC CGA CTC TCT CCA GAA
Met His Lys Val Glu Thr Tyr Leu Thr Val Ala Lys Cys Arg Leu Ser Pro Glu
                                                                    191
706
GCT AAC TGC ACC CTG TAG CCCCGCCTCTCCACCGCGAAGCACCTCCCTGCAGATGACATCATATG
Ala Asn Cys Thr Leu

771                                     SFGH-6                       SFGH-1
CATTCTGTAGCCCCACCCCCATGGTTGCCAAGCTAACTAGCATAAATGTTAGCCTCTGTTGGTTCTGCATT

842                                                   ******         901
CCAAACTGATGATGTCATTGTGATATTGTCATACTGTCAGCAAATAAAGGGTTCATTCAGAAAAAAAAAAA
                                                        SFGH-4

AAAAAAAAAAAAAAAAAA ···3'
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Fig. 1. Nucleotide sequence of spotted flounder GH cDNA and predicted amino acid sequence. The amino-terminal ends of mature sfGH are indicated by 1. The sequences used for the PCR primers are indicated by underlines, and imperfect direct repeat sequences by italic, respectively. The asterisk marks the stop codon, polyadenylation signal (AATAAA), and potential N-adenylation site. Circles indicate conserved cysteines which are known to from disulfide bonds in other GHs, and boxes indicate two extra Cys residues. The sequences has been desposited in GenBank under the accession number AF086787.

was obtained with the untranslated region (Fig. 1). The sfGH cDNA consists of 901 nucleotides in length, including the coding region of 609 nucleotides, 111 nucleotides of 5' untranslated region and 181 nucleotides of 3' untranslated region. The conserved polyadenylation signal (AATAAA) lies 12 bases upstream from the poly (A) tail. The deduced amino acid sequence shows an open reading frame (ORF) encoding a preprotein of 203 amino acid and a putative signal peptide of 17 amino acids, suggesting that the mature hormone consists of 186 amino acids. The sfGH shows two possible sites for signal peptide cleavage similar to those of olive flounder GH. Although the amino-terminal structure of mature sfGH has not yet been determined, the cleavage sites of sfGH are similar to those of olive flounder GH, considering the homologous structure around the cleavage sites for both hormones. Thus, sfGH is considered to have cleavage site between serine and glutamine having 186 amino acid residues. A quite similar amino terminal structure has been reported for mature tuna GH (Sato et al., 1988) and mature yellow tail GH (Watahiki et al., 1988). One possible site for N-glycosylation site (Asn-X-Thr or Ser) was located in Asn-193 (Asn-193 - Cys-194 -Thr-195). The site is exactly located in the same position as that found in other fish GHs. Comparison of amino acid sequence between sfGH and other GHs reveals that sfGH shares 87%, 74%, 73%, 68%, 57%, 57%, 33%, 31%, 29% and 26% sequence similarity when compared to GHs of olive flounder, tuna, yellow tail, red sebream, salmon, rainbow trout, eel, bovine, rat and human, respectively (Fig. 2).

Molecular phylogenetic analysis

Molecular phylogenetic tree of several vertebrate GHs, including sfGH, was constructed using the methods of neighbor-joining for amino acid sequences (Fig. 3). Human GH used as an outgroup indicates the putative position of the root. Phylogenetic tree analysis indicated that the spotted flounder and olive flounder that belong to order pleuronectidae and paralichthyidae, respectively, are phylogenetically close species, and their GHs have evolved from a common ancestral gene.

Expression analysis of spotted founder GH mRNA

To detect the tissue distribution of sfGH mRNA, RT-PCR was performed with total RNAs from pituitary gland, brain, liver and fin tissues. A positive signal of 630 bp DNA fragment was detected in pituitary only (Fig. 4). The size of the PCR product fractioned in the agarose gel is identical to that calculated based on the sfGH cDNA sequence.

Discussion

In this report we describe the cloning and characterization of cDNA encoding spotted flounder GH (sfGH), allowing comparative analysis the amino acid sequence with other vertebrate GHs. The analyses of sfGH reveal some unique structural features. First, the repetitive sequences are present within the 5' untranslated region of sfGH cDNA consisting of tandem arrays of imperfect microsatellite-like repeat monomers (Tautz and Renz, 1984; Tautz, 1989). Analysis of the structures of rat GH, human GH and barramundi GH gene revealed the presence of imperfect direct repeat sequences flanking exons I, III, and V of these genes (Seeburg et al., 1977; DeNoto et al., 1981; Selby et al., 1984; Yowe and Epping, 1996). These results were taken as evidence that exons I and III of the ancestral GH gene arose by separate insertion events via a mechanism analogous to DNA transposition. Therefore, such repetitive sequences in 5' untranslated region may have been involved in the subsequence duplication and the divergence of the primordial GH gene. Second, sfGH contains six Cys residues rather than four or five found Cys residues in other GHs. One extra Cys located at position 83 cannot be aligned with Cys residues in other GHs, while the other extra Cys is located in the identical position within the signal peptide as that found in olive flounder GH. Rest of four Cys residues, however, are located at conserved positions and can be aligned with those of other GHs. It is widely accepted that these Cys residues mediate the formation of two intramolecular disulfide bridges. The current model implies that one disulfide bridge is formed between Cys-51 and Cys-169, while the other is formed between Cys-176 and Cys-194 (Vestling et al., 1991). Thus, Cys-83 may not be required for proper folding of sfGHs because the other extra Cys residue is located within the signal peptide.

During metamorphosis, all species of flatfish experience a 90 degree change in orientation between their vestibular and coordinate axes. Flatfishes, including spotted flounder (*Verasper variegatus*) and olive flounder (*Paralichthys olivaceus*), dramatically change their structures from symmetrical pelagic

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                              signal peptide                  mature GH
                                                             *   *    *    **
Spotted Flounder    1   MNRVILLLSV MCV------- ---GVSSQPI TENQRLFSIA VGRVQYLHLV
Olive Flounder      1   MNRVILLLSV MCV------- ---GVSSQPI TENQRLFSIA VGRVQYLHLV
Tuna                1   MDRVFLLLSV LSL------- ---GVSSQPI TDSQRLFSIA VSRVQHLHLL
Yellow Tail         1   MDRVVLLLSV LSL------- ---GVSSQPI TDSQHLFSIA VSRIQNLHLL
Red Seabream        1   MDRVVLMLSV LSL------- ---GVSSQPI TDGQRLFSIA VSRVQHLHLL
Salmon              1   MGQVFLLMPV LLVSCFL--- -----SQGAA IENQRLFNIA VSRVQHLHLL
Eel                 1   MASGFLLWPV LLV------- -SFSVNAVEP ISLYNLFTSA VNRAQHLHTL
Bovine              1   MMAAGPRTSL LLAFALLCLP WTQVVGAFPA MSLSGLFANA VLRAQHLHQL
Rat                 1   M-AADSQTPW LLTFSLLCLL WPQEAGALPA MPLSSLFANA VLRAQHLHQL
Human               1   M-ATGSRTSL LLAFGLLCLP WLQEGSAFPT IPLSRPFDNA MLRAHRLHQL

                        *    *                   *  *     *   *  *
Spotted Flounder   51   AKKLFSEFEN S-QLEDQH-P LNKIFLQDFC HSDYFLSPID KHETQRSSVL
Olive Flounder     51   AKKLFSDFEN SLQLEDQR-L LNKIASKEFC HSDNFLSPID KHETQGSSVQ
Tuna               51   AQRLFSDFES SLQTEEQR-Q LNKIFLQDFC NSDYIISPID KHETQRSSVL
Yellow Tail        51   AQRLFSNFES TLQTEDQR-Q LNKIFLQDFC NSDYIISPID KHETQRSSVL
Red Seabream       51   AQRLFSDFES SLQTEEQL-K LNKIF-PDFC NSDYIISPID KHETQRSSVL
Salmon             51   AQKMFNDFDG TLLPDERR-Q LNKIFLLDFC NSDSIVSPVD KHETQKSSVL
Eel                51   AAEIYLEFER SIPPEAHR-Q LSKTSPLAGC YSDSIPTPTG KDETQEKSDG
Bovine             51   AADTFKEFER TYIPEGQRYS -IQNTQVAFC FSETIPAPTG KNEAQQKSDL
Rat                51   AADTYKEFER AYIPEGQRYS -IQNAQAAFC FSETIPAPTG KEEAQQRTDM
Human              51   AFDTYQEFEE AYIPKEQKYS FLQNPQTSLC FSESIPTPSN REETQQKSNL

                        ** *  *    *    *                        *
Spotted Flounder  101   KLLSISYRLI ECWEFSSRFL VAGFAE--RA --QVTSKLSE LKTGLMKLIE
Olive Flounder    101   KLLSVSYRLI ESWEFFSRFL VASFAV--RT --QVTSKLSE LKMGLLKLIE
Tuna              101   KLLSISYRLV ESWEFPSRSL SGGSAP--RN --QISPKLSE LKTGIHLLIR
Yellow Tail       101   KLLSISYRLV ESWEFSSRFL SGGSAL--RN --QISPRLSE LKTGIQLLIT
Red Seabream      101   KLLSISYRLV ESWEFPSRSL SGGSAP--RN --QISPKLSE LKMGIHLLIR
Salmon            101   KLLHISFRLI ESWEYPSQTL IISNSLMVRN ANQISEKLSD LKVGINLLIT
Eel               101   YLLRISSALI QSWVYPLKTL SDAFSNSLMF GTSD-GIFDK LEDLNKGINE
Bovine            101   ELLRISLLLI QSWLGPLQFL SRVFTNSLVF GTSDR-VYEK LKDLEEGILA
Rat               101   ELLRFSLLLI QSWLGPVQFL SRIFTNSLMF GTSDR-VYEK LKDLEEGIQA
Human             101   ELLRISLLLI QSWLEPVQFL RSVFANSLVY GASDSNVYDL LKDLEEGIQT

                                              *  <------------->  *   ** ** *
Spotted Flounder  151   ANQDGAGGFS ESSVIQLTPY GNYYQSVGVD ESFRLNYELF ACFKKDMHKV
Olive Flounder    151   ANQDGAGGFS ESSVLQLTPY GN-------- ------SELF ACFKKDMHKV
Tuna              151   ANQDGDEMFA DSSALQLAPY GNYYQSLGAD ESLRRSYELL ACFKKDMHKV
Yellow Tail       151   ANQDGAEMFS DVSALQLAPY GNFYQSLGGE ELLRRNYELL ACFKKDMHKV
Red Seabream      151   ANEDGAEIFP DSSALQLAPY GNYYQSVGAD ESLRRTYELL ACFKKDMHKV
Salmon            151   GSQDGVLSLD DNDSQQLPPY GNYYQNLGGD GNVRRNYELL ACFKKDMHKV
Eel               151   LMKVVGDGGI YIEDVRNLRY ENFDVHLRND AGLMKNYGLL ACFKKDMHKV
Bovine            151   LMRELEDGTP RAGQILKQTY DKFDTNMRSD DALLKNYGLL SCFRKDLHKT
Rat               151   LMQELEDGSP RIGQILKQTY DKFDANMRSD DALLKNYGLL SCFKKDLHKA
Human             151   LMGRLEDGSP RTGQIFKQTY SKFDTNSHND DALLKNYGLL YCFRKDMDKV

                        ** *    **    *  *
Spotted Flounder  201   ETYLTVAKCR LSPEANCTL
Olive Flounder    201   ETYLTVAKCR LFPEANCTL
Tuna              201   ETYLTVAKCR LSPEANCTL
Yellow Tail       201   ETYLTVAKCR LSPEANCTL
Red Seabream      201   ETYLTVAKCR LSPEANCTL
Salmon            201   ETYLTVAKCR KSLEANCTL
Eel               201   ETYLKVTKCR RFVESNCTL
Bovine            201   ETYLRVMKCR RFGEASCAF
Rat               201   ETYLRVMKCR RFAESSCAF
Human             201   ETFLRIVQCR -SVEGSCGF
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Fig. 2. Comparison of the spotted flounder growth hormone (sfGH; with the signal sequence) amino acid sequence with those of other species. The amino acid sequences are aligned to maximize homology, and residues conserved among the GHs are shaded. Asterisks indicate the invariant residues for all sequences, and boxes indicate the cysteine residues.

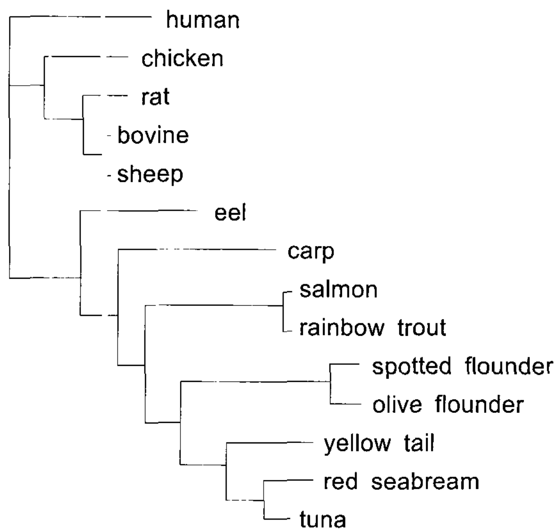

Fig. 3. Phylogenetic tree based on the alignment of amino acid sequences of the known full-length GHs in vertebrates. Alignment of amino acids sequences was performed with ClustalX using default values. N-J phylogenetic tree was generated using the option available in ClustalX program. Human GH was used as an outgroup. The scale bar beneath the tree corresponds to estimated evolutionary distance units.

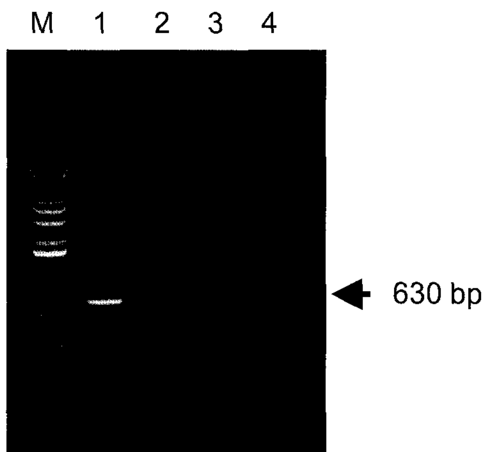

Fig. 4. Expression analysis of spotted founder GH mRNA. Total RNA was purified from the pituitary (lane 1), brain (lane 2), liver (lane 3) and fin (lane 4), reverse transcribed, and amplified by 30 cycles of PCR using SFGH-5 and SFGH-6. M, molecular weight size mark (1 kb DNA ladder, GIBCO BRL).

larvae to asymmetrical benthic juveniles in early life stages (Hotta et al., 2001). As a result, spotted flounder has both eyes on its embryonic right side in contrast to olive flounder. Phylogenetic tree analysis indicated that the spotted flounder and olive flounder are phylogenetically close species, and their GHs may have evolved from a common ancestral gene. Closer examination of the sequence data and phylogenetic analysis suggests that five Cys residues of the primordial flatfish GH may have been present prior to the divergence between the dextral and sinistral form of flatfish. In addition, it is interesting that sfGH, including other GHs, is clearly distinguishable from the olive flounder GH, in which a region correspoding to residues 175-188 in alignment positions is absent. The generation of an additional Cys residue within the signal peptide of the spotted flounder GH as well as the deletion event in olive flounder GH may have arisen relatively recently after its divergence from the primordial flatfish GH gene. Further investigation is necessary to understanding of the divergence of flatfish in the evolutionary process.

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