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Genomic Structure and Expression of the Soluble Guanylyl Cyclase α_2 Subunit Gene in the Medaka Fish *Oryzias latipes*

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ABSTRACT—A cDNA clone encoding the soluble guanylyl cyclase α_2 subunit was isolated from medaka fish (*Oryzias latipes*) and designated as *OlGCS-α_2*. The *OlGCS-α_2* cDNA was 3192 bp in length and the open reading frame (ORF) encodes a protein of 805 amino acids. The deduced amino acid sequence has high similarity to that of the mammalian α_2 subunit gene except for the N-terminal regulatory domain. The C-terminal 5 amino acids, "RETSL", which have been reported to interact with the post synaptic density protein (PSD)-95 were conserved. An RNase protection assay with adult fish organs showed that *OlGCS-α_2* was expressed mainly in the brain and testis. The complete nucleotide sequence (about 41 kbp) of the *OlGCS-α_2* genomic DNA clone isolated from a medaka fish BAC library indicated that the *OlGCS-α_2* gene consisted of 9 exons and 8 introns. The 5'-flanking region and larger introns, such as introns 1, 4, and 7, contained the several fragments conserved in the nucleotide sequences of *Rex6* (non-long terminal repeat retrotransposon), MHC class I genomic region, and *OlGC1*, the medaka fish homolog of the mammalian guanylyl cyclase B gene. Linkage analysis on the medaka fish chromosome demonstrated that the *OlGCS-α_2* gene was mapped to LG13; this mapping position was different from those for the *OlGCS-α_1* and *OlGCS-β_1* genes (LG1).

Key words: soluble guanylyl cyclase, cGMP, medaka fish, gene expression, exon/intron organization

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INTRODUCTION

Soluble GC, a nitric oxide (NO) and carbon monoxide (CO)-sensitive guanylyl cyclase (GC), is an enzyme that catalyzes the conversion of GTP to cGMP through binding of these gaseous ligands to the heme in the enzyme (Gerzer *et al*., 1981). cGMP thus synthesized by the soluble GC activates cGMP-dependent protein kinases (cGKs), phosphodiesterases (PDEs), and cyclic-nucleotide gated (CNG) cation channels. Through the activation of these downstream effectors, the NO/cGMP signaling pathway plays important roles in various physiological phenomena, e.g., smooth muscle relaxation, platelet aggregation, and neural development (Schmidt and Walker, 1994; Kusakabe and Suzuki, 2000; Lucas *et al*., 2000).

Soluble GC is a heterodimeric enzyme consisting of α and β subunits (Garbers *et al*., 1994). In mammals, two isoforms of each subunit have been identified (α_1 and α_2, β_1 and β_2). The α_1/β_1 heterodimer was purified first from the mammalian lung (Gerzer *et al*., 1981), and subsequently,

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the cDNA clone for each subunit was isolated and sequenced (Koesling *et al*., 1988). The α_2 and β_2 subunit cDNA clone were isolated by homology screening from a human fetal brain and rat kidney cDNA library, respectively (Yuen *et al*., 1990; Harteneck *et al*., 1991). The α_1 or α_2 subunit was demonstrated to form an active heterodimeric enzyme (α_1/β_1 or α_2/β_1) with the β_1 subunit when both the subunit cDNAs were co-expressed in Sf9 cells (Russwurm *et al*., 1998), while the β_2 subunit has been reported to be able to form an active enzyme in the absence of a second subunit (α_1 or α_2) (Koglin *et al*., 2001).

It has been demonstrated that the α_2 subunit mRNA is expressed in the human brain, uterus, and placenta (Budworth *et al*., 1999), and the α_2 subunit protein was detected in the placenta, where the β_1 subunit protein was also detected (Russwurm *et al*., 1998). Moreover, it has been demonstrated the interaction of the C-terminal peptide of soluble GC α_2 subunit with the post synaptic density-95 protein (PSD-95), which is a synaptic scaffold protein, suggesting that the α_2/β_1 heterodimer can be recruited to the membrane (Russwurm *et al*., 2001). Several studies have reported that both the α_1/β_1 heterodimeric soluble GC and the endothelial NO synthase (eNOS) are translocated in a caveolae of the lung endothelial cells, and Hsp 90 acts as a scaffold protein between these proteins (Zabel *et al*., 2002; Nedvetsky *et al*., 2002). Together, these findings indicate the potential importance of the α_2/β_1 heterodimeric soluble GC, which shows enzyme kinetics and a tissue distribution similar to those of the α_1/β_1 heterodimeric soluble GC. Recently, we demonstrated that the α_2 subunit mRNA was expressed during embryogenesis of the medaka fish *Oryzias latipes* and suggested that the α_2/β_1 heterodimeric enzyme plays an important role in the early development of the eye (Harumi *et al*., 2003; Yamamoto *et al*., 2003). In this study, we report the structures of the cDNA and genomic DNA clones encoding the medaka fish soluble GC α_2 subunit gene (*OlGCS-α_2*) and assess their tissue distribution by means of an RNase protection assay using various adult medaka fish organs.

MATERIALS AND METHODS

Animals

Mature adults of the orange-red variety of the medaka fish *O. latipes* were maintained as described previously (Yamagami *et al*., 2001). Mature male individuals of the *O. latipes* Hd-rR inbred strain (Hyodo-Taguchi and Sakaizumi, 1993) were fixed in ethanol and used for isolation of the genomic DNA.

Preparation of RNA and isolation of an *OlGCS-α_2* cDNA fragment by RT-PCR

Total RNA was prepared from the adult brain and kidney of the orange-red variety of the medaka fish *O. latipes* using TRIZOL™ reagent (Invitrogen, Carlsbad, CA, USA). Poly (A)$^+$ RNA was isolated using Oligotex-dT30<Super> (Roche, Mannheim, Germany), according to the manufacturer’s protocol. Three µg of the poly (A)$^+$ RNA was reverse-transcribed with Superscript II (Invitrogen) in 50 µl scale. The degenerate oligonucleotide primers were designed and synthesized based on the amino acid sequences conserved in all reported soluble GC subunit proteins (sense-KGQMI: 5’-CCCGCGGAATTCAGCTTMRIGGICARATGRTI-3’; antisense-MPRYCLF: 5’-GAATTCTCGAGGATCCRAAIARRCARTAICIIGGCAT-3’). The first PCR amplification was carried out with the first strand cDNA as a template and performed for 30 cycles under the following reaction conditions: 94°C for 1 min (denaturation), 50°C for 1 min (annealing), 72°C for 45 sec (elongation), and an additional elongation reaction for 5 min at 72°C. Then, the second PCR was performed using the nested primers synthesized based on the amino acid sequences conserved in mammalian soluble GC α_2 (a2-5’-1: 5’-GCNAARGCNCARGAYGG-3’ for the amino acid sequence AKAQDG) and the antisense-MPRYCLF primer that was used in the first PCR. The PCR products were separated by electrophoresis with a 1.5% SeaKem GTG agarose gel (BMA, Rockland, ME, USA) and purified using MinElute Gel Extraction Kit (QIAGEN, Hilden, Germany). The cDNA fragments were subcloned into the plasmid vector pBluescript II KS (Stratagene, La Jolla, CA, USA) and sequenced.

5’- and 3’- Rapid Amplification of cDNA Ends (5’- and 3’-RACE)

To obtain the full-length sequence of the *OlGCS-α_2* cDNA, the 5’-portion of the cDNA was amplified by the 5’-RACE method (Frohman *et al*., 1988) with a 5’-RACE System for Rapid Amplification of cDNA Ends, ver. 2.0 (Invitrogen). Briefly, 1 µg of total RNA isolated from the adult brain was reverse-transcribed with several gene-specific antisense primers (5’A: 5’-ACTTGCGAGCAGGCACTGGC-3’ [cDNA nucleotide no. 2023-2004]; 5’D: 5’-ATCAGCGAGCAGGATCCGGC-3’ [1733–1713]; 5’G: 5’-AAGATCCGCCTGGCAACAGC-3’[1130-1111]; 5’J: 5’-CACGGCTCGAAGAACTCGC-3’ [876–858]). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase and then amplified with the Abridged Anchor Primer and gene-specific primers (5’B: 5’-TTCTGTGCCACATCACCCGG-3’ [1988-1969]; 5’E: 5’-TTCTTTGGTGCCGGCCTGCG-3’ [1677-1658]; 5’H: 5’-ACAGCTCTGATCAAGCCTGG-3’ [1115–1096]; 5’K: 5’-CAGAGACCAAAGAACTCTTCGC-3’ [842-821]). Nested PCR was performed with the Abridged Anchor Primer and nested gene-specific primers (5’C: 5’-GGTGGGTCCGCTCTAATGTG-3’ [1915-1896]; 5’F: 5’-GGTCCGGATGGTAAAGGGGG-3’ [1645–1625]; 5’I: 5’-GCAGGGTTGAATCAGTGCAG-3’ [1073–1054]; 5’L: 5’-GTTCTCGCAGTTCACAAAACGG-3’ [814–792]). The 3’-portion of the cDNA was amplified by the 3’-RACE method (Frohman *et al*., 1988) using the 3’-Full RACE Core Set (TaKaRa, Otsu, Japan). Total RNA (1 µg) of the medaka fish brain was reverse-transcribed with an Oligo dT-3’ sites Adaptor Primer and gene-specific 3’Z primer: 5’-ATACTGTGTGGCTGGAGGAC-3’ [2187–2206]. The second PCR was carried out with the 3’ site Adaptor Primer and nested gene-specific 3’Y primer: 5’-AATTCACACAGGCTCGGTGC-3’ [2322–2341]. The RACE products were subcloned into pBluescript II KS vector and sequenced.

Determination of the transcription start site of the *OlGCS-α_2* gene by the oligo-capping 5’-RACE method

The transcription start site of the *OlGCS-α_2* gene was determined by the oligo-capping 5’-RACE method (Maruyama and Sugano, 1994) using a First Choice™ RLM-RACE kit (Ambion, Austin, TX, USA). All steps were carried out according to the manufacturer’s protocol. Adaptor-ligated RNA from the total RNA (10 µg) of the medaka fish brain was reverse-transcribed with the gene-specific 5’N primer: 5’-AGCTGCGTCCGTTCCAGAGG-3’ [454–435]. The first PCR was carried out with the 5’-RACE Outer Primer and the gene-specific 5’O primer: 5’-GAGGAGCGCTCTTTGGGAGG-3’ [438–419]. The conditions were 30 sec at 96°C, 30 sec at 60°C, and 30 sec at 72°C for 35 cycles, followed by elongation at 5 min at 72°C. The nested PCR was performed with the 5’-RACE Inner

Primer and nested gene-specific 5'P primer: 5'-CCGAGCTACTGAATGACTCG-3' [316–297], and the PCR program was the same as that for the first PCR. The RACE products were subcloned into pBluescript II KS vector and sequenced.

Molecular phylogenetic analysis

The partial amino acid sequence (residues 528 to 766) of *OlGCS*-α_2 was compared with those of the corresponding part of known fish and mammalian soluble GC subunit isoforms using the Clustal W program (Thompson *et al*., 1994) and the sequence editor SeqPub (Gilbert, Indiana University). An unrooted phylogenetic tree was constructed using the aligned sequences by means of the neighbor-joining algorithms (Saitou and Nei, 1987) in the PROTRAS program of PHYLIP version 3.572 (Felsenstein, 1989) and Clustal W program (Thompson *et al*., 1994). For neighbor joining analysis, the evolutionary distance was estimated using Kimura's empirical method for protein distances (Kimura, 1983).

GenBank/EMBL/DDBJ accession numbers for the sequences used for comparison are as follows: human GCS-α_1 (Y15723), rat GCS-α_1 (M57405), FrGCS-α_1 (AB062171), OlGCS-α_1 (AB000849), human GCS-α_2 (X63282), rat GCS-α_2 (AF109963), human GCS-β_1 (X66533), rat GCS-β_1 (M22562), FrGCS-β_1 (AB062172), OlGCS-β_1 (AB000850), human GCS-β_2 (NM_004129), rat GCS-β_2 (AB058888), and OlGC1 (AB004921).

RNase protection assay

The cDNA fragment of 333 bp, 412 bp, or 216 bp containing the 3'-UTR region of *OlGCS*-α_1 (2200–2532), *OlGCS*-β_1 (1897–2308), or *OlGCS*-α_2 (2694–2909) was subcloned into pBluescript II KS vector for preparation of the probe. After digestion with *Eco*RI, a cRNA probe was synthesized using T3 RNA polymerase with ATP, CTP, GTP, and [α-^{32}P] UTP and a DIG RNA Labeling kit (Roche) according to the manufacturer's protocol. The synthesized probe (1×10^5 cpm) was treated with RNase-free DNase I (Roche), then extracted by phenol and purified using CHROMA SPIN-30 columns (CLONTECH, Palo Alto, CA, USA). The purified cRNA probe was applied to the pool of total RNA (10 µg) extracted from various adult medaka fish organs (brain, eye, gill, heart, gall bladder, spleen, kidney, testis, ovary, liver, and intestine). The mixture was ethanol-precipitated and dissolved in a hybridization buffer containing 80% formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, and 1 mM EDTA, followed by incubation to anneal each other overnight at 50°C. Single stranded RNA was treated with RNase A for 30 min at 30°C in the solution containing 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 5 mM EDTA. The protected fragment was treated with 125 µg/ml Proteinase K and 0.5% SDS, and then phenol/chloroform-extracted, ethanol-precipitated, and electrophoresed on a 6% polyacrylamide gel containing 7 M urea. The gel was dried and analyzed using a FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

Northern hybridization

Poly (A)$^+$ RNA (7.5 µg) from various organs of medaka fish was separated on 1% agarose gel containing 6.7% formaldehyde. The RNA was transferred to a nylon membrane, Hybond-H$^+$ (Amersham Pharmacia Biotech, Little Chalfont Bucks, UK) with 10xSSPE as a transferring solution. A cDNA fragment (nucleotides 226–1645) of *OlGCS*-α_2 was labeled with [α-^{32}P] dCTP using the Random Primer DNA Labeling kit Version 2 (TaKaRa) and used as a probe. The blot was pre-hybridized in 50% formamide, 5xSSPE, 5xDenhardt's solution, 0.5% SDS, and 100 µg/ml denatured herring sperm DNA at 42°C for 1 hr. The radioactive probe was added to the pre-hybridization buffer and incubated overnight at 42°C. The radioactive signals were visualized using a FUJIX Bio-Imaging Analyzer BAS2000.

Isolation of genomic DNA clones for *OlGCS*-α_2 from a medaka fish bacterial artificial chromosome (BAC) library

A high-density replica (HDR) membrane of an *O. latipes* Hd-rR inbred strain genomic BAC library (Asakawa *et al*., 1997; Matsuda *et al*., 2001) was used for screening of the *OlGCS*-α_2 gene. The treatments of the pre-hybridization membrane were performed as described previously (Yamagami *et al*., 2001). To isolate the *OlGCS*-α_2 gene, hybridization was carried out using a probe constructed by PCR with the *OlGCS*-α_2 cDNA as a template and the following primers: precap: 5'-TGCATCCCCCTTTACCATCC-3'; a2tail: 5'- AAATCCAAAGCTCAGCACCC -3'. Positive BAC clones were detected using CDP-star detection reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions. A QIAGEN plasmid maxi kit (QIAGEN) was used for BAC DNA isolation from the bacterial culture. BAC DNA was digested with *Eco*RI and *Hin*dIII, and then subjected to Southern hybridization with the same probe described above to confirm the isolation of positive clones. Following the Southern hybridization, to check whether or not the clones contained the full-length of the *OlGCS*-α_2 gene, PCR was performed with the following primer pairs: a2test: 5'-CTGCACTGATTCAACCCTGC-3' and 5'G: 5'-AAGATCCGCCTGGCAACAGC-3'; UTRUP: 5'-TCAGACCGTGTTACAAAGGC-3'; and a2tail: 5'-AAATCCAAAGCTCAGCACCC-3'. The conditions were as follows: 30 cycles at 96°C for 30 sec, 61°C for 30 sec, and 72°C for 1 min, and an additional incubation at 72°C for 5 min.

Genomic Southern hybridization

A membrane being blotted with the restriction enzyme-treated genomic DNA of an individual of the *O. latipes* Hd-rR inbred strain was prepared as described previously (Yamagami *et al*., 2001). The membrane was pre-hybridized for at least 1 hr at 42°C in a solution containing 50% formamide, 5xSSPE, 5xDenhardt's solution, 0.5% SDS, and 100 µg/ml denatured herring sperm DNA. A 592 bp cDNA fragment of *OlGCS*-α_2 (1054–1645) was labeled with [α-^{32}P] dCTP using the Random Primer DNA Labeling kit version 2 (TaKaRa) and was used as a probe. The radioactive probe was added to the pre-hybridization solution, followed by incubation overnight at 42°C. The membrane was washed three times with 2xSSC/0.1% SDS at 50°C for 15 min. Imaging of the radioactive signals was performed with a FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

Linkage analysis of the *OlGCS*-α_1, -α_2, and -β_1 subunit genes

Assignment of the loci encoding *OlGCS*-α_1, *OlGCS*-α_2, *OlGCS*-β_1 to each linkage group was carried out by the method described previously (Naruse *et al*., 2000). The primers and restriction enzymes used were as follows: LR-RT for *OlGCS*-α_1, 5'-GTAAAAGAAATGTGGGGA-3'; LF-2 for *OlGCS*-α_1, 5'-TTATTGATGTCTGACAGCCTA-3'; *Mse* I for *OlGCS*-α_1; 5'd for *OlGCS*-α_2, 5'-TAGGAACATGGTTCCAATGCTG-3'; 3'-Y for *OlGCS*-α_2, 5'-AATTCACACAGGCTCGGTGC-3'; *Hae* III for *OlGCS*-α_2; s-b1 for *OlGCS*-β_1, 5'-AGTACAAGCTGACCCAAG-3'; s-b5 for *OlGCS*-β_1, 5'-TCTGTCAGGATGTCAAAG-3'; *Hae* III for *OlGCS*-β_1.

Other methods

The nucleotide sequence of cDNA and genomic DNA fragments was determined by the dideoxy chain termination method (Sanger *et al*., 1977) with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). Sequence data was analyzed with GENETYX-MAC/version 7.2.0 (Software Development, Tokyo, Japan). The homology search was performed at the Web site NCBI BLAST (http://www.ncbi.nlm.hih.gov/BLAST/).

RESULTS

Characterization of *OlGCS-α_2* cDNA

To obtain the cDNA fragment of the medaka fish soluble GC α_2 subunit gene, RT-PCR was performed with three degenerate primers (antisense-MPRYCLF, sense-KGQMI, and AKAQDG) synthesized based on the conserved amino acid sequences among mammalian soluble GCs. A homology search of a 526 bp cDNA fragment obtained from the medaka fish brain and kidney samples revealed that it was a partial nucleotide sequence of the cDNA of a medaka homolog of the mammalian soluble GC α_2 subunit gene and

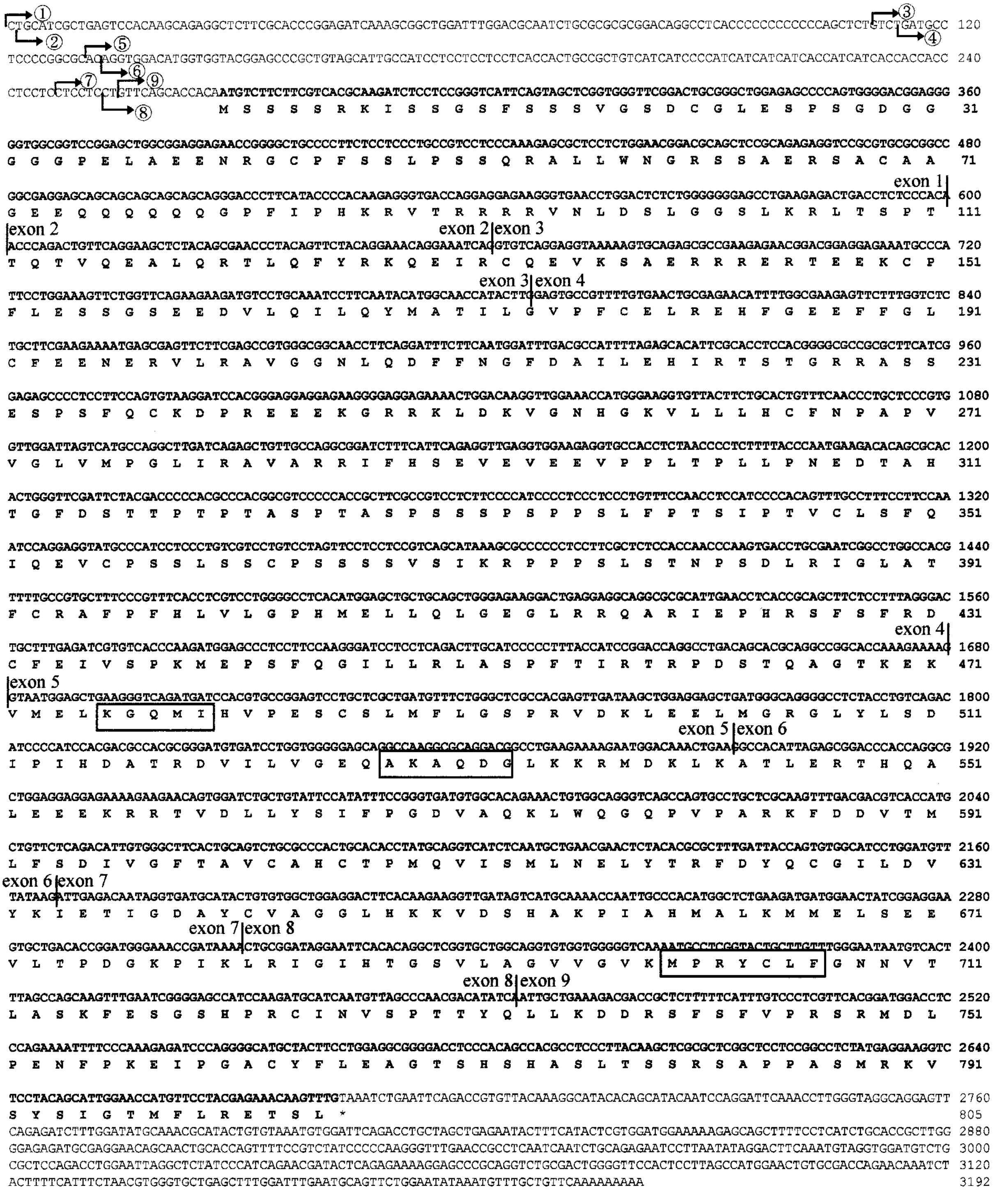


Fig. 1. Complete nucleotide sequence of the *OlGCS-α_2* cDNA. Arrows indicate the transcriptional start sites. Vertical lines indicate exon-intron boundaries. The numbers at the end of each line indicate the number of nucleotides or amino acids. The degenerate primers used in RT-PCR were synthesized based on the boxed amino acids.

(A)

(B) **N-terminal domain**

α_1 medaka	100							
α_1 rat	41.9	100						
α_2 medaka	**19.7**	**21.7**	**100**					
α_2 rat	23.8	30.0	**27.1**	100				
α_2 human	23.3	29.6	**38.6**	84.8	100			
β_1 medaka	13.1	12.1	**10.5**	15.9	14.9	100		
β_1 rat	14.5	13.2	**10.7**	14.7	15.8	88.2	100	
β_2 rat	10.7	14.6	**11.8**	12.8	12.4	29.2	28.8	100
	α_1 medaka	α_1 rat	**α_2 medaka**	α_2 rat	α_2 human	β_1 medaka	β_1 rat	β_2 rat

Central domain

α_1 medaka	100							
α_1 rat	61.3	100						
α_2 medaka	**56.7**	**51.0**	**100**					
α_2 rat	60.0	53.7	**69.6**	100				
α_2 human	57.3	52.3	**71.6**	92.6	100			
β_1 medaka	43.0	43.7	**37.3**	39.2	39.9	100		
β_1 rat	44.3	43.7	**37.3**	39.2	39.2	91.1	100	
β_2 rat	30.6	31.5	**30.2**	30.2	34.1	34.1	35.4	100
	α_1 medaka	α_1 rat	**α_2 medaka**	α_2 rat	α_2 human	β_1 medaka	β_1 rat	β_2 rat

Catalytic domain

α_1 medaka	100							
α_1 rat	71.1	100						
α_2 medaka	**59.4**	**65.4**	**100**					
α_2 rat	69.2	68.4	**79.5**	100				
α_2 human	85.2	69.6	**80.7**	97.6	100			
β_1 medaka	38.8	36.5	**37.4**	37.6	38.0	100		
β_1 rat	38.8	37.8	**38.2**	40.4	40.8	85.7	100	
β_2 rat	39.2	40.8	**39.7**	38.2	38.6	39.7	38.2	100
	α_1 medaka	α_1 rat	**α_2 medaka**	α_2 rat	α_2 human	β_1 medaka	β_1 rat	β_2 rat

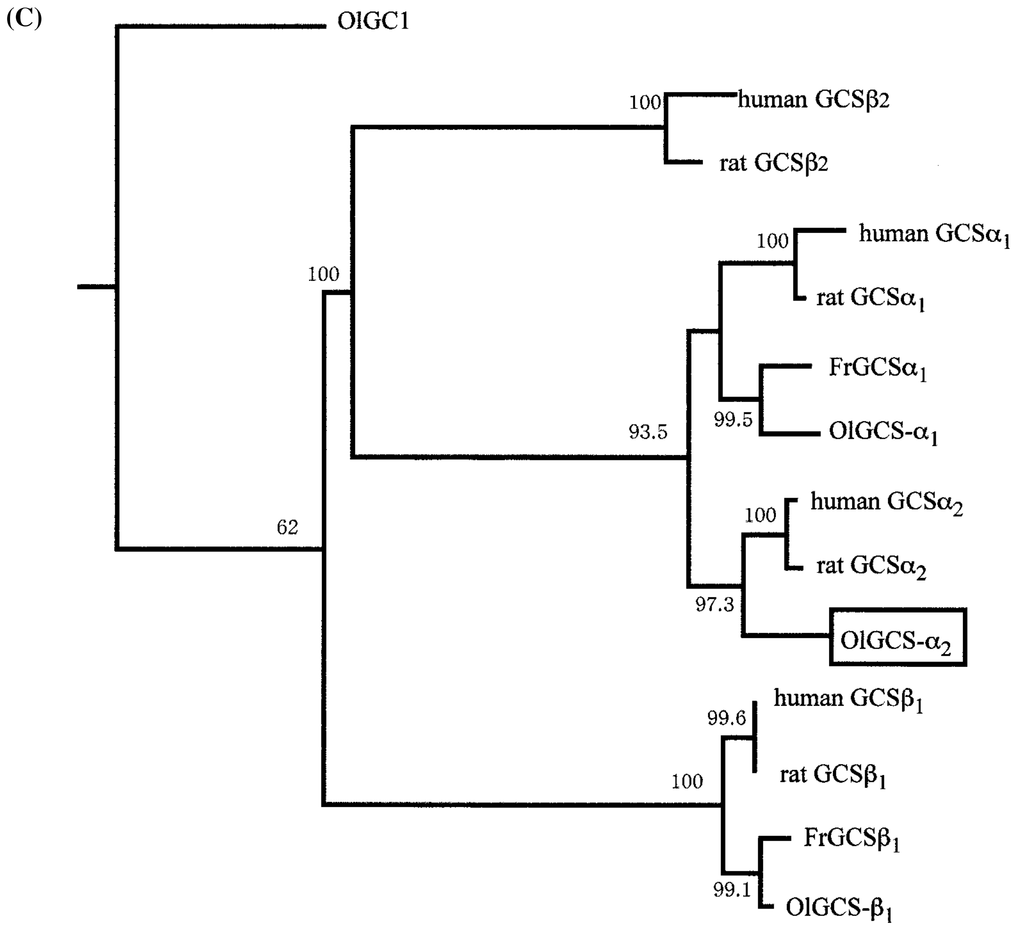


Fig. 2. (A) Alignment of amino acid sequence of OlGCS-α_2 with those of mammalian soluble GC α_2 subunits. The degenerate primers used in RT-PCR were synthesized based on the amino acids designated by thick arrows at the top of the sequence. Boxed amino acids indicate sites that may interact with PSD-95 (Russwurm *et al.*, 2001). An asterisk indicates an amino acid that is identical among the three proteins, and a pair of dots indicates two amino acids that are identical and one amino acid that has a similar nature among the three proteins. A single dot indicates that the two proteins have an identical amino acid and one amino acid with a different nature. Gaps in the sequence are indicated by dashes. Domains are designated as suggested by Stone and Marletta (1995). Exon-intron boundaries of the *OlGCS-α_2* gene are designated by a V-shaped symbol with the number of exons given beside it. (B) Sequence identity and similarity among the predicted amino acid sequences of three domains (N-terminal, central, and catalytic domain) of known soluble GC subunits. (C) Molecular phylogenetic tree of OlGCS-α_2 and the other soluble GC subunits. The amino acid sequences of various soluble GC subunits were subjected to phylogenetic analysis and the amino acid sequence of OlGC1 was used as an outgroup. The numbers indicate the bootstrapping value. Sources and their accession numbers are described in the MATERIALS AND METHODS. Abbreviations: Ol, medaka fish *Oryzias latipes*; Fr, Fugu fish *Fugu rubripes*.

was designated as *OlGCS-α_2*. By performing repeated 5'- and 3'- RACE, the full-length cDNA of *OlGCS-α_2* was obtained. It was 3192 bp in length, which size was in agreement with the result of Northern blot analysis using the adult brain RNA (data not shown). The *OlGCS-α_2* cDNA consisted of the 2418 bp-open reading frame (ORF) and the 507 bp-3'-untranslated region (UTR).

As shown in Fig. 1, the oligo-capping 5'-RACE with the adult brain RNA demonstrated that there were nine distinct transcription start sites at the 13 to 267 nucleotides upstream of the translation start site “ATG”, and the nucleotide “C” at 267 bp upstream of the first methionine was designated as “+1”. There were GC-rich nucleotides and no typical TATA box around these transcription start sites, which findings were in good agreement with those for the gene having the TATA-less promoter (Smale, 1997).

Comparison and phylogenetic analysis of the amino acid sequences of OlGCS-α_2 with those of other soluble GC subunits

The deduced amino acid sequence (805 residues) of the *OlGCS-α_2* cDNA was aligned with those of the mammalian soluble GC α_2 cDNAs (Fig. 2A). The OlGCS-α_2 consisted of a regulatory domain (residues 1 to 401), a central domain (residues 402 to 539), and a catalytic domain (residues 540 to 766). The catalytic domain of OlGCS-α_2 was 79.5% identical to that of rat soluble GC α_2 and 59.4% identical to that of OlGCS-α_1 (Fig. 2B). The central domain was 69.6% and 56.7% identical to those of rat soluble GC α_2 and OlGCS-α_1, respectively (Fig. 2B). However, the N-terminal regulatory region of OlGCS-α_2 had several amino acid insertions (7 to 15 residues) compared with that of mammalian soluble GC α_2, and its similarity to those of mammalian soluble GC α_2 and OlGC-α_1 was low at 36.3% and 41.2%, respectively (Fig. 2A). Moreover, the C-terminal 5 amino acids "RETSL" of OlGCS-α_2 were almost the same as those of the motif that has been demonstrated to be the site for interaction with rat brain PSD-95 (Russwurm *et al.*, 2001).

As shown in Fig. 2C, the molecular phylogenetic analysis using the amino acid sequence of the catalytic domain of OlGCS-α_2 (residues 528 to 766) and those of the corresponding domain of other soluble GC subunits (α_1, α_2, β_1, and β_2) indicated that OlGCS-α_2 belonged to the α_2 group to which the rat and human soluble GC α_2 subunits belonged.

Expression of *OlGCS-*α_2 in various medaka fish adult organs assayed by the RNase protection method

The organ distribution of the three subunit mRNAs (*OlGCS-*α_1, *OlGCS-*α_2, and *OlGCS-*β_1) was examined by the RNase protection method using the total RNA from various medaka fish adult organs. The results indicated that all mRNAs were expressed mainly in the brain and testis, although a weak signal due to the *OlGCS-*α_2 mRNA was detected in the eye, gall bladder, spleen, ovary, and intestine, where *OlGCS-*β_1 was also expressed (Fig. 3).

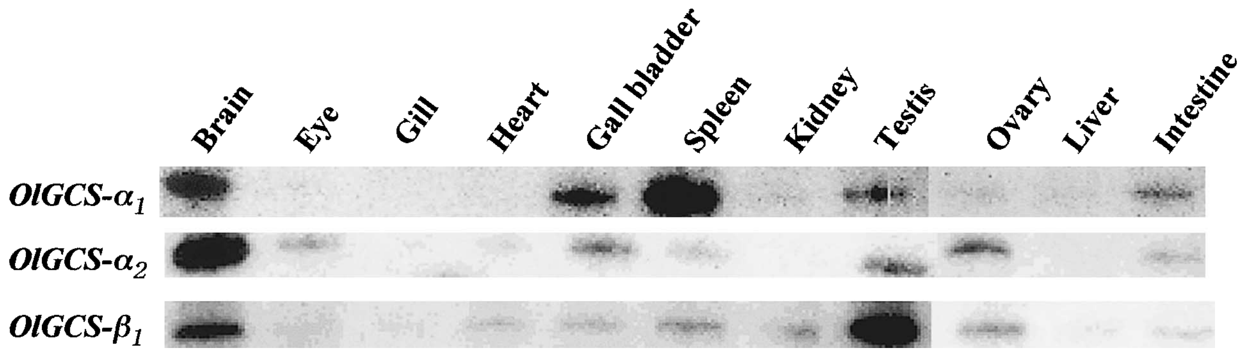
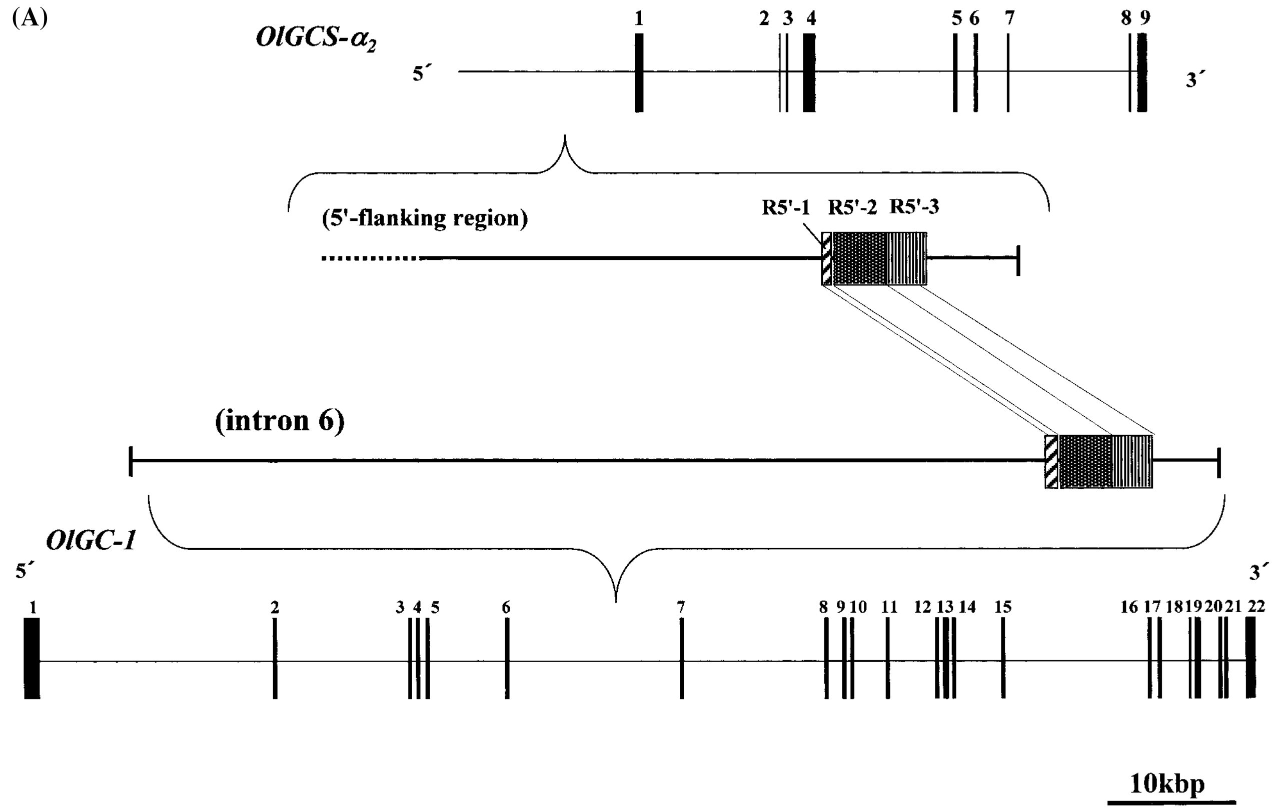
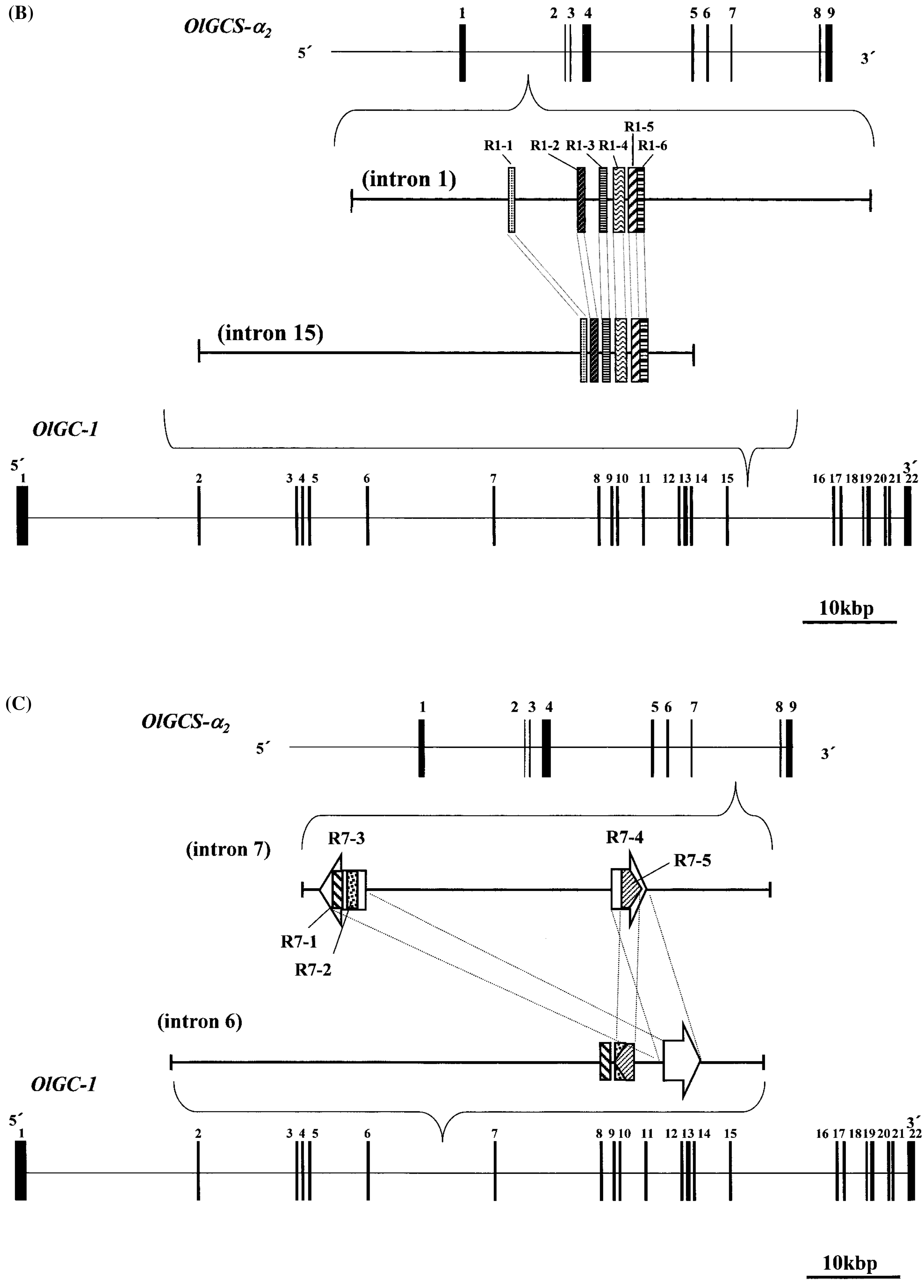


Fig. 3. RNase protection assay of *OlGCS-*α_1, *OlGCS-*β_1, and *OlGCS-*α_2 mRNAs. The expression of each gene was examined by an RNase protection assay using total RNA from various adult organs and an antisense cRNA probe for *OlGCS-*α_1, *OlGCS-*β_1, or *OlGCS-*α_2.




(B)
OlGCS-α2
5´
1
2 3 4
5 6 7
8 9
3´
R1-5
R1-1
R1-2
R1-3
R1-4
R1-6
(intron 1)
(intron 15)
OlGC-1
5´
1
2
3 4 5
6
7
8 9 10
11
12 13 14
15
16 17 18 19 20 21 22
3´
10kbp
(C)
OlGCS-α2
5´
1
2 3 4
5 6 7
8 9
3´
(intron 7)
R7-3
R7-4
R7-5
R7-1
R7-2
(intron 6)
OlGC-1
5´
1
2
3 4 5
6
7
8 9 10
11
12 13 14
15
16 17 18 19 20 21 22
3´
10kbp

Characterization of genomic DNA clones for *OlGCS*-α_2

An *O. latipe*s Hd-rR strain genomic BAC library was screened to isolate the *OlGCS*-α_2 genomic clone with the partial cDNA fragment of *OlGCS*-α_2 (nucleotides 1543–2921), resulting in detection of 24 positive clones from 18432 clones of the medaka fish BAC library. To confirm the positivity of these clones, we carried out Southern hybridization and PCR amplification experiments using the probe described above. The results indicated that 15 out of 24 clones contained the *OlGCS*-α_2 gene and 12 out of the 15 clones possessed the 5'-UTR of *OlGCS*-α_2, and, subsequently, that 8 out of these 12 clones contained the 3'-UTR of *OlGCS*-α_2. We chose the clone 156J21 for use in later experiments. By sequencing this BAC clone, we finally determined the complete nucleotide sequence of 41 kbp for the *OlGCS*-α_2 gene (Fig. 4). Furthermore, we determined 6 kbp nucleotide sequences of the 5'-flanking region of the *OlGCS*-α_2 gene (data not shown). The sequence upstream of the transcription start sites contained GC-rich sequences and no canonical TATA box. As shown in Fig. 4 and Table 1, the *OlGCS*-α_2 gene consisted of 9 exons which was the

Table 1. Exon/Intron organization of *OlGCS*-α_2

Exon no.	Exon size (bp)	Intron size (bp)	5' splicing site	3' splicing site
1	600	11056	`ACA gtaagc`	`tttcag ACC`
2	62	502	`CAG gttctg`	`tgacag GTG`
3	125	1332	`TTG gtaagt`	`tgacag GTG`
4	893	11211	`AAG gtagag`	`tggcag GTA`
5	213	1449	`AAG gtaatg`	`tctcag GCC`
6	273	2420	`AAG gtatga`	`aaccag ATT`
7	144	9817	`AAA gtgagt`	`tttcag CTG`
8	155	527	`TCA gtaagt`	`ctacag ATT`
9	724			

Table 2. Characteristic regions of *OlGCS*-α_2 containing the fragments conserved with *OlGC-1*

OlGCS-α_2 5'-flanking region vs *OlGC-1* intron 6

region	nucleotide no. for *OlGCS*-α_2	size (bp)	nucleotide no. for *OlGC-1*	size (bp)	strand	identity (%)
R5'-1	–1941 to –1830	111	45670–45781	111	+/+	93
R5'-2	–1819 to –1215	604	45818–46422	604	+/+	96
R5'-3	–1211 to –761	450	46348–46887	449	+/+	97

OlGCS-α_2 intron 1 vs *OlGC-1* intron 15

region	nucleotide no. for *OlGCS*-α_2	size (bp)	nucleotide no. for *OlGC-1*	size (bp)	strand	identity (%)
R1-1	6359–6514	156	84623–84419	157	+/–	95
R1-2	10832–11023	192	84449–84647	199	+/+	85
R1-3	11275–11476	202	84698–84898	201	+/+	87
R1-4	11599–11877	279	85028–85309	282	+/+	88
R1-5	11908–12104	197	85336–85532	197	+/+	95
R1-6	12104–12274	171	85511–85677	157	+/+	87

OlGCS-α_2 intron 7 vs *OlGC-1* intron 6

region	nucleotide no. for *OlGCS*-α_2	size (bp)	nucleotide no. for *OlGC-1*	size (bp)	strand	identity (%)
R7-1	37031–37094	63	49809–49872	63	+/+	90
R7-2	37106–37201	95	49873–49977	104	+/+	94
R7-3	37126–37226	100	50942–50841	101	+/–	92
R7-4	43669–43761	92	50841–50942	101	+/+	90
R7-5	43693–43751	58	49970–49911	59	+/–	94

the number indicate the positions in genomic sequences.

Fig. 4. Characteristic regions found in the 5'-flanking region (A) and intron 1 (B), and intron 7 (C) of the *OlGCS*-α_2 gene. Diagrams at the top and bottom schematically show the genomic structure of *OlGCS*-α_2 and *OlGC1*; the scale is indicated below the genomic structure of *OlGC1*. Black boxes with numbers indicate exons and horizontal lines denote introns. The six regions conserved in the two introns are indicated by boxes with various patterns, and identical patterns are used to denote regions having almost identical nucleotide sequences. The nucleotide sequences in the fragment R5'-3 (A) and R7-1 (C) were highly conserved with that in *Rex 6* and *PSMB9/ABCB3*, respectively.

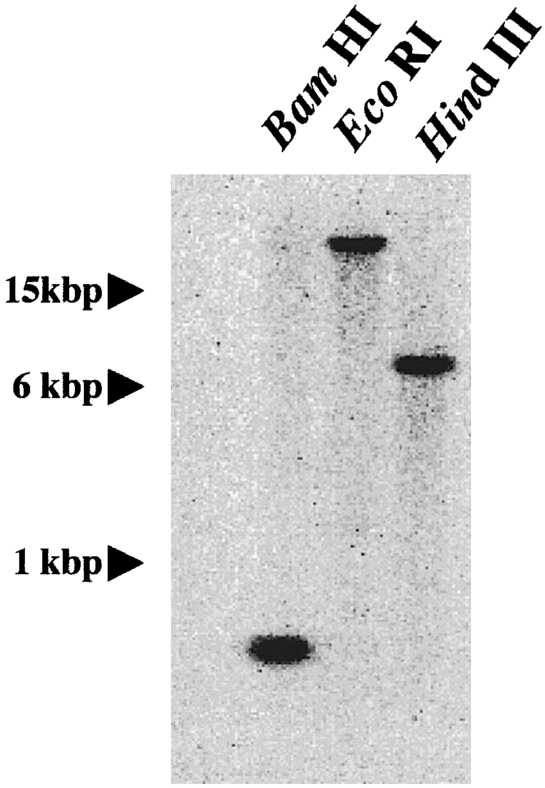


Fig. 5. Genomic Southern hybridization of *OlGCS-α_2*. Genomic DNA of an individual of the medaka fish *O. latipes* Hd-rR strain was digested with restriction enzymes and electrophoresed. The DNA was blotted to a membrane and hybridized with a probe against *OlGCS-α_2*.

same number of exons in the *OlGCS-α_1* gene (Mikami *et al.*, 1999), and the GT-AG rule was conserved for all splice sites (Table 1). Introns 1 (11,056 bp), 4 (11,211 bp), and 7 (9,817 bp) were especially larger than the others. In the 5'-flanking region (nucleotides from –1186 to –773) of the *OlGCS-α_2* gene, we found the nucleotide sequences that were conserved in *Rex 6*, a non-long terminal repeat (LTR) retrotransposon (Volff *et al.*, 2001). The nucleotide sequences in intron 1 (nucleotides 3737–3914) and intron 4 (nucleotides 21498–21658 and 21614–21658) contained the sequences conserved in the MHC class I genomic region (Matsuo *et al.*, 2002). Furthermore, in the nucleotide sequences in intron 7 (nucleotides 30279–30490 and 36894–37100), we found the sequences conserved in other MHC-related genes, immunoproteasome β subunit gene (*PSMB9*) and the transporter associated with antigen presentation gene (*ABCB3*) (accession no. AB073378). Most of these elements were also conserved in the nucleotide sequences of intron 6 and 15 of the *OlGC1* gene (a medaka fish homolog of atrial natriuretic peptide receptor type B) (Fig. 4 and Table 2). In particular, the 5'-flanking region of the *OlGCS-α_2* gene contained a 1.2 kbp-sequence found in the *OlGC-1* gene (Fig. 4A).

Genomic Southern analysis and Linkage analysis

Genomic Southern hybridization was performed using a 591 bp cDNA fragment as a probe and revealed a single positive band in each of three lanes (Fig. 5). The size of the positive bands was consistent with those of the DNA fragments obtained from the digestion of genomic clones by the restriction enzymes, suggesting that the medaka fish genome contains a single copy of the *OlGCS-α_2* gene. We also carried out linkage mapping of the *OlGCS-α_1*, *OlGCS-α_2*, and *OlGCS-β_1* genes on the medaka fish chromosome, and demonstrated that the *OlGCS-α_1* and *OlGCS-β_1* genes were mapped to LG 1, while the *OlGCS-α_2* gene was mappedto LG 13.

DISCUSSION

In the present study, we demonstrated that the medaka fish *O. latipes* possessed the soluble guanylyl cyclase α_2 subunit gene (designated as *OlGCS-α_2*), and that this gene was expressed in the organs where the *OlGC-β_1* gene—whose translation product was the essential counterpart of that of the *OlGCS-α_2* gene—was expressed, although its chromosomal localization was different. It has been reported that the soluble GC α_2 subunit gene is expressed in the human fetal brain (Harteneck *et al.*, 1991), and that the soluble GC α_2 subunit forms an active heterodimer (α_2/β_1) with the β_1 subunit, which shows enzymatic characteristics similar to those of the α_1/β_1 heterodimer (Russwurm *et al.*, 1998). To date, there has been no report showing the existence of the soluble GC α_2 subunit in non-mammalian animals. In this study, by RT-PCR using cDNA prepared from total RNAs of the adult medaka fish brain and kidney and primers synthesized based on the amino acid sequences conserved among all soluble GC subunits, we obtained a cDNA fragment having high similarity to that of mammalian soluble GC α_2 subunits (Fig. 2). The catalytic domain at the C-terminal of OlGCS-α_2 was highly conserved among various soluble GC subunits, while the regulatory domain at the N-terminus containing the insertion of 7–15 amino acids was much less similar than those of mammalian soluble GC α_2 subunits (Fig. 2A, 2B). In a previous study, we demonstrated that the regulatory region of OlGCS-α_1 had low similarity to those of the soluble GC α_1 subunits of other species (Mikami *et al.*, 1998). Therefore, we presume that these regions were not particularly important for the function of the enzyme, and thus many mutations which might have occurred during its molecular evolution were accumulated in the regulatory region of the α subunit genes. The C-terminal five amino acid residues, RETSL, of the soluble GC α_2 subunit were reported to interact with the PDZ domain of rat brain PSD-95, which has been proposed to form a signaling complex with other membrane proteins, including neuronal nitric oxide synthase (nNOS) and the *N*-methyl-*D*-aspartate (NMDA) receptor (Russwurm *et al.*, 2001). As described above, OlGCS-α_2 also possessed the same residues in the C-terminal region, suggesting that they play similar roles in medaka fish.

In the RNase protection assay of the *OlGCS-α_2* transcripts, the signals due to the *OlGCS-α_2* transcripts were mainly detected in the brain, eye, testis, and ovary (Fig. 3). Recently, we demonstrated using *in situ* hybridization that the *OlGCS-α_2* gene was expressed in the medaka fish embryonic brain and retina and that the expression in the embryonic retina became weaker with as the development proceeded (Harumi *et al*., 2003; Yamamoto *et al*., 2003). The expression of the *OlGCS-α_2* gene in the gonad is in good agreement with a recent report on the expression of the soluble GC subunit genes in mice (Mergia *et al*., 2003).

The *OlGCS-α_2* gene consisted of 9 exons and 8 introns (Fig. 4); some introns, such as introns 1, 4, and 7, were very large (Table 1) and contained many fragments conserved in introns 6 and 15 of a medaka fish homolog of the atrial natriuretic receptor gene (*OlGC-1*) (Takeda and Suzuki, 1999). The *OlGC-1* gene contained several repeated nucleotide sequences conserved in *Rex 6*, a non-LTR retrotransposon (Volff *et al*., 2001), and the MHC class I genomic region (Matsuo *et al*., 2002). These results suggest that several genetic recombinations via transposable elements between the *OlGCS-α_2* and *OlGC-1* genes occurred in the process of the *OlGCS-α_2* gene evolution, and this idea might be extensible to the mechanism of generation of the diverse numbers of membrane and soluble GC isoforms in vertebrates over a long period of time.

Linkage mapping of three soluble GC subunit genes (*OlGCS-α_1*, *OlGCS-α_2*, and *OlGCS-β_1*) demonstrated that the *OlGCS-α_2* gene was located in LG 13, which was different from the location (LG 1) of the *OlGCS-α_1* and *OlGCS-β_1* genes. In our previous report, the *OlGCS-α_1* and *OlGCS-β_1* genes were aligned tandemly in the medaka fish genome, separated by a 1 kbp-spacer sequence (Mikami *et al*., 1999). The α_1/β_1 and α_2/β_1 heterodimers are considered to be the only active soluble GCs in vertebrates. Therefore, it is rational to expect that the α_1 and α_2 subunits should be in competition for the association with the β_1 subunit to form an active enzyme. Moreover, the 5'-flanking regions of the *OlGCS-α_1* and *OlGCS-β_1* genes were shown to mutually influence each other's promoter activity in a study measuring the promoter activity in mammalian cultured cells and medaka fish embryonic cells (Yamamoto and Suzuki, 2002). On the other hand, we demonstrated that the *OlGCS-α_2* and *OlGCS-β_1* genes, but not the *OlGCS-α_1* gene, were coexpressed in the embryonic retina of the medaka fish *O. latipes* (Harumi *et al*., 2003). Taking these results together, we presume that in some organs the transcription and/or translation of the *OlGCS-α_2* and *OlGCS-β_1* genes are coordinated, and in the other organs the transcription and/or translation of the *OlGCS-α_1* and *OlGCS-β_1* genes are coordinated. In the former case, the transcription and/or translation of the *OlGCS-α_1* gene should be repressed and in the latter case the transcription and/or translation of the *OlGCS-α_2* gene should be repressed. In either case, the expression of the soluble GC subunit genes could be regulated at the transcriptional and/or translational level.

To date, the α_2 subunit of soluble GC has not attracted the attention of many investigators, probably due to the dearth of available information on its genomic structure, the organ distribution of the transcripts, and its function, relative to the many studies on the α_1 and β_1 subunits. However, our present and recent studies demonstrating that the wide distribution of the α_2 subunit mRNA and inhibition of translation of the *OlGCS-α_2* gene by means of an antisense oligonucleotide caused severe defects in medaka fish embryos should contribute to a deeper understanding of the unsolved but important biological roles of the α_2 subunit (Yamamoto *et al*., 2003). The differential functions between the α_1/β_1 and α_2/β_1 heterodimers in the NO/cGMP signaling pathway remained to be solved.

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