J. Biochem. 130, 39–50 (2001)

Expression and Exon/Intron Organization of Two Medaka Fish Homologs of the Mammalian Guanylyl Cyclase A[1]

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Received March 21, 2001; accepted April 11, 2001

Two cDNA clones (*OlGC2* and *OlGC7*) and their genomic DNA clones encoding medaka fish homologs of mammalian natriuretic peptide receptor/membrane guanylyl cyclase A (GC-A) were isolated, and their complete nucleotide sequences were determined. The open reading frame predicts a protein of 1,063 amino acids for *OlGC2* cDNA (4,283 bp), and one of 1,055 amino acids for *OlGC7* cDNA (3,721 bp), respectively. Northern blot analyses demonstrated 4.7 kb *OlGC2* transcripts in the kidney and gill, and 4.0 kb *OlGC7* transcripts in the kidney, brain, and ovary, while RNase protection analyses revealed that both genes are expressed in various adult organs. Both the *OlGC2* (about 33.0 kbp) and *OlGC7* (about 44.3 kbp) genes consist of 22 exons with an exon/intron organization similar to those of the human GC-A gene (about 16.6 kbp) and medaka fish GC-B homolog gene (*OlGC1*, about 93 kbp). Intron 4 of *OlGC2* contains two repeated sequence (RS) clusters, designated as RS1 (about 1 kbp) and RS2 (about 5 kbp), consisting of nucleotide 5′-AGCCTCTGCTCCTCCTTC-3′. In addition, many identical but variably sized nucleotide sequences were found in introns in *OlGC1*, *OlGC2*, *OlGC6*, and *OlGC7*. The *OlGC2* and *OlGC7* genes both have no apparent TATA box in the 5′ flanking region upstream of the putative transcription initiation point, but several consensus sequences for *cis*-regulatory elements, including C/EBP, CREB, NF-IL6, and Sp1 and AP-2, NF-IL6, c-Myb, and Sp1 are present in the 5′-flanking region of *OlGC2* and *OlGC7*, respectively.

Key words: exon-intron organization, gene expression, receptor, medaka fish, membrane guanylyl cyclase, repeated sequence.

Cyclic GMP (cGMP), which is formed from GTP by guanylyl cyclase (GC), is a ubiquitous second messenger in intracellular signaling cascades and responsible for a wide variety of physiological responses. GC is classified into two groups, those found in the plasma membrane (membrane GC) and those in the cytoplasm (soluble GC) (*1*). Soluble GC is a heme-containing heterodimeric protein that is activated by nitric oxide and related vasodilator agents such as nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-L-penicillamine (SNAP) (*2*). One of the richest sources of membrane GC is sea urchin spermatozoa, in which the enzyme appears to serve as a cell surface receptor for a sperm-activating peptide, IIA (SAP-IIA), which possesses both chemokinetic and chemoattractant properties (*3*), and causes marked but transient elevation of the intracellular cGMP concentration. A cDNA encoding the membrane GC was first isolated from a sea urchin testis cDNA library (*4*). Since then, a number of membrane GCs has been isolated from a number of animal tissues, including a natriuretic peptide receptor/GC, an enterotoxin/guanylin receptor/GC, and a sensory-organ-specific GC (*5*).

Membrane GC consists of a single polypeptide of about 120–150 kDa. In mammals, the natriuretic peptide/GC signaling pathway is thought to be involved in the regulation of blood pressure, kidney function, and bone formation (*6*). Two membrane GCs, GC-A and GC-B, are known to be the receptors for the natriuretic peptides. They consist of four domains, an extracellular ligand-binding domain, a transmembrane domain, an intracellular kinase-like domain, and an intracellular cyclase catalytic domain (*2*). GC-A is activated by low concentrations of both atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and GC-B is activated by ones of C-type natriuretic peptide, although GC-A and GC-B are localized in the vasculature, kidney, adrenal gland, and brain (*7*). In the euryhaline eel, on the other hand, the natriuretic peptide/GC signaling system is believed to play an important role in seawater adaptation (*8*, *9*). Two natriuretic peptide receptor/GCs (NPR-A/GC-A and NPR-B/GC-B) have been identified in the osmoregulatory organs, and cDNAs for both receptor/

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (No. 11236202). The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases, and are available under accession numbers AB030274 for cDNA of *OlGC2*, AB054294, AB054295, and AB054296 for genomic DNA of *OlGC2*, AB023489 for cDNA of *OlGC7*, and AB054293 for genomic DNA of *OlGC7*.

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Abbreviations: ANP, atrial natriuretic peptide; BAC, bacterial artificial chromosome; BNP, brain or B-type natriuretic peptide; CNP, C-type natriuretic peptide; GC, guanylyl cyclase; cGMP, guanosine 3′,5′-cyclic monophosphate; HDR, high-density replica; LA-PCR, long and accurate-polymerase chain reaction; NPR, natriuretic peptide receptor; *OlGC*, gene encoding *Oryzias latipes* membrane guanylyl cyclase; RACE, rapid amplification of cDNA ends; ORF, open reading frame; RT-PCR, reverse transcription–polymerase chain reaction; SINE, short interspersed repetitive element; UTR, untranslated region; VNP, ventricular natriuretic peptide.

GCs have been isolated.

Information on the gene organization of natriuretic peptide receptor/GCs in different organisms is important for understanding the transcriptional and translational regulatory mechanism. There have been several papers describing the exon/intron organization of mammalian natriuretic peptide receptor/GC (*10–12*). In previous papers, we reported that the medaka fish possesses one homolog each of mammalian GC-A and GC-B, respectively (*13*, *14*). The genomic DNA of the GC-B homolog, *OlGC1*, is extremely large compared with that of mammalian natriuretic peptide receptor/GC (*14*). However, the structures of cDNA and genomic DNA of *OlGC2*, a homolog of mammalian GC-A, were not completed. In this paper, we report the complete structures of cDNA and genomic DNA of *OlGC2*. We also report the complete nucleotide sequences of cDNA and genomic DNA of *OlGC7*, another natriuretic peptide receptor/GC homolog, and describe the expression pattern of the two genes.

MATERIALS AND METHODS

Animals and Embryos—Mature adults of the orange-red variety of the medaka fish *Oryzias latipes* were purchased from a dealer. They were fed on TetraMin flakes (Tetra-Werke, Germany). Naturally spawned and fertilized eggs were collected, and the embryos were cultured in distilled water containing 0.6 ppm methylene blue at 27°C. The developmental stage was expressed in days and the day of fertilization was referred to as Day 0. Hatching usually occurs on Day 10. Mature male individuals of *O. latipes* HdrR inbred strain (*15*) fixed in ethanol were provided by Professor Hiroshi Hori (Nagoya University).

Preparation of RNA and Amplification of cDNA Fragments by Reverse Transcription–Polymerase Chain Reaction (RT-PCR)—Total RNA was prepared from various adult organs of the orange-red variety of the medaka fish *O. latipes* according to the acid guanidinium thiocyanate/phenol/chloroform extraction method (*16*). Poly(A)$^+$ RNA was isolated using Oligotex-dT30<Super> (Roche), according to the manufacturer's protocol.

Three degenerate oligonucleotide primers (P2: 5′-GAYAT-HGTNGGNTTYAC-3′; P6: 5′-GTRTTNACNGTRTCNCC-3′; and P7: 5′-ARRCARTANCKNGGCAT-3′) were synthesized based on the amino acid sequences of three conserved regions (DIVGFT, GDTVNT, and MPRYCL) in known membrane GCs. These primers were used to amplify cDNA fragments encoding membrane GCs from cDNA reverse-transcribed from total RNA of the medaka fish gonad, as described previously (*17*). The PCR products were purified and subcloned into the plasmid vector pBluescript II KS(−) (Stratagene).

5′- and 3′-Rapid Amplification of cDNA Ends (5′- and 3′-RACE)—To obtain the full-length sequence of *OlGC2* or *OlGC7* cDNA, the 5′-portion of the cDNA was amplified by the 5′-RACE method (*18*) using the 5′-RACE System for Rapid Amplification of cDNA Ends, ver 2.0 (Life Technologies). During 5′-RACE to obtain a full-length cDNA sequence of *OlGC2* with cDNA reverse transcribed from the total RNA of Day 9 embryos as a template, we obtained a partial length of cDNA, which is a new membrane GC in the medaka fish, and designated it as *OlGC7*.

To obtain the full-length sequences of *OlGC7*, total RNA (3 μg) isolated from Day 9 embryos was reverse-transcribed with gene-specific antisense oligonucleotide primers (GSP1, nucleotides 2922–2941; GSP3, 2476–2495; GSP5, 1406–1421; and GSP7, 937–957). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase and then amplified by PCR with the Abridged Anchor Primer (Life Technologies) and other gene-specific antisense oligonucleotide primers (GSP2, GSP4, GSP6, and GSP8). The PCR conditions are given in Table I. The PCR products were cloned into pBluescript II KS(−) and sequenced. The 5′-RACE products overlapped by 46–240 bp with the 5′ end of the clone that had been isolated. Otherwise, a 3′-portion of the cDNA was amplified by the 3′-RACE method (*18*) using the 3′-Full RACE Core Set (Takara). Three micrograms of total RNA was reverse-transcribed with an Oligo dT-3′sites Adaptor Primer (Takara). The cDNA was amplified by PCR with the 3′sites Adaptor Primer (Takara) and another gene-specific oligonucleotide primer (GSP9). A one-tenth volume of the PCR product was amplified by PCR with the 3′sites Adaptor Primer (Takara) and another gene-specific oligonucleotide primer (GSP10) (see Table I). The 3′-RACE product overlapped by 240 bp with the 3′ end of the previously isolated clone.

To obtain the full-length sequence of *OlGC2* cDNA using the 5′-RACE method (*18*), total RNA (2 μg) isolated from the adult medaka fish ovary was reverse-transcribed with gene-specific antisense oligonucleotide primers (GSP11, nucleotides 2942–2961; GSP13, 2213–2234; GSP15, 1340–1358; GSP17, 941–959; and GSP19, 519–540). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase, and then amplified by PCR with the Abridged Anchor Primer (Life Technologies) and other gene-specific antisense oligonucleotide primers (GSP12, GSP14, GSP16, GSP18, and GSP20). To enrich the 5′-RACE products, a one-tenth volume of the primary 5′-RACE products was re-amplified using the Abridged Universal Amplification Primer (AUAP; Life Technologies) and nested primers (GSP19, GSP20, GSP21, GSP22, and GSP23). The PCR conditions are given in Table I. Each 5′-RACE product overlapped by 55–84 bp with the 5′-end of the clone that had been isolated in the previous PCR.

TABLE I. **5′- and 3′-rapid amplification of cDNA ends (5′- and 3′-RACE) PCR conditions.**[a]

	primer	cycles	°C/min	position in cDNA
OlGC7 5′-RACE	GSP2	40	94/1, 59/1, 72/2	2885-2906
	GSP4	40	94/1, 57/1, 72/2	2445-2464
	GSP6	40	94/1, 55/1, 72/2	1375-1391
	GSP8	40	94/1, 53/1, 72/2	912-931
OlGC7 3′-RACE	GSP9	30	94/1, 61/1, 72/2	2575-2594
	GSP10	30	94/1, 57/1, 72/2	2667-2689
OlGC2 5′-RACE	GSP12	40	94/0.5, 59/1, 72/1.5	2905-2926
	GSP19	35	94/0.5, 59/1, 72/1.5	2842-2844
	GSP14	40	94/0.5, 58/1, 72/1.5	2189-2210
	GSP20	40	94/0 5, 61/1, 72/1.5	2128-2147
	GSP16	40	94/0.5, 59/1, 72/1.5	1297-1319
	GSP21	40	94/0.5, 63/1, 72/1.5	1262-1279
	GSP18	40	94/0.5, 60/1, 72/1.5	903-922
	GSP22	40	94/0.5, 62/1, 72/1.5	880-899
	GSP20	40	94/0.5, 60/1, 72/1.5	474-497
	GSP23	40	94/0.5, 60/1, 72/1.5	419-439

[a]The first denaturation and final extension reactions were carried out at 94°C for 5 min and 72°C for 7 min, respectively.

Molecular Phylogenetic Analysis—The deduced amino acid sequences of *OlGC2* and/or *OlGC7* were compared with those of known fish and mammalian natriuretic peptide receptor/GC isoforms using the Clustal W program (*19*) 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 in the PROTRAS program of PHYLIP (version 3.572) (*20*) and the Clustal W program (*21*). For neighbor-joining analysis, the evolutionary distance was estimated using Kimura's empirical method for protein distances (*22*). The GenBank/EMBL/DDBJ accession numbers for the sequences used are as follows: human GC-A (X15357) (*23*); human GC-B (P20594) (*24*); rat GC-A (X14773) (*25*); rat GC-B (M26896) (*26*); eel GC-A (AB012869) (*8*); eel GC-B (D25417) (*9*); *Squalus* GC-B (AF054285) (*27*); OlGC1 (AB 004921) (*14*); and sea urchin (*Hemicentrotus pulcherrimus*) sperm GC (HpGC) (D21101) (*28*).

Northern Blot Hybridization—Total RNA (30 μg) or poly(A)$^+$ RNA (5 μg) was separated on a 1% agarose gel containing 6.7% formaldehyde. The RNA was transferred to a nylon membrane (Hybond-N$^+$, Amersham) with 10× SSPE as the transferring solution. A cDNA fragment (nucleotides 35–439 for *OlGC2* or nucleotides 23–905 for *OlGC7*) was labeled with [α-^{32}P] dCTP using the Random Primer DNA Labeling kit Version 2 (Takara) and then used as the probe. The blot was prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured herring sperm DNA at 42°C for 1 h. The radioactive probe was added to the prehybridization buffer and incubated overnight at 42°C. The membrane was washed twice with 2× SSC containing 0.1% SDS at 42°C for 30 min and with 0.1× SSC containing 0.1% SDS for 10 min at 42°C. The radioactive signals were visualized using a FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film).

RNase Protection Analyses—A plasmid containing a 228-nucleotide fragment corresponding to the 3′-noncoding region (3639–3866) of *OlGC2* cDNA or the 226-nucleotide fragment corresponding to the 3′-noncoding region (3377–3602) of *OlGC7* cDNA was used as a template for the synthesis of a cRNA probe. A plasmid containing a cDNA fragment of the 3′-noncoding region (1741–1840) of medaka fish cytoplasmic actin gene *OlCA1* (*29*) was used as an internal control. After the template cDNA in the vector had been treated with *Eco*RI to linearize it, an antisense cRNA probe was synthesized using T7 RNA polymerase, [α-^{32}P]UTP and a DIG RNA Labeling kit (Boehringer Mannheim) according to the manufacturer's protocol. The cRNA probe was treated with RNase-free DNase I, phenol extracted, passed through a CHROMA SPIN-30 column (CLONTECH Laboratories), and then ethanol precipitated. The cRNA probe (1×10^6 cpm) was annealed with 10 μg of total RNA prepared from various organs of medaka fish at 50°C for 16 h in hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, and 1 mM EDTA). Non-annealing nucleic acid was then digested with ribonuclease A in 300 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 5 mM EDTA at 30°C for 30 min. Proteinase K (final, 125 μg/ml) and SDS (final, 0.5%) were then added and the incubation was continued for another 15 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. The protected fragment was analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea and detected using a FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film).

Southern Blot Hybridization—One *O. latipes* Hd-rR inbred strain individual was homogenized in a solution containing 10 mM Tris/HCl, pH 8.0, 0.1 M EDTA, and 0.5% SDS, treated with 1.2 μg/ml RNase A for 1 h at 37°C, and then digested overnight with 130 μg/ml proteinase K. After repeated extraction with phenol, swirling isolated genomic DNA was precipitated by ethanol. The genomic DNA (10 μg) was digested with *Bam*HI, *Eco*RI, or *Hin*dIII (Takara), and then separated by 0.7% agarose gel electrophoresis. The DNA in the gel was transferred to a nylon membrane (Hybond-N$^+$, Amersham) with 20× SSC as the transferring solution. The blot was prehybridized for at least 1 h at 42°C in a buffer containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured herring sperm DNA.

A cDNA fragment encoding a part of the extracellular domain of *OlGC1* (nucleotides 1–852) (*14*), *OlGC2* (nucleotides 237–439), or *OlGC7* (nucleotides 264–464) was labeled with [α-^{32}P]dCTP using the Random Primer DNA Labeling kit Version 2 (Takara) and then used as the probe. The radioactive probe was added to the prehybridization buffer, followed by incubation overnight at 42°C. The membrane was washed three times with 2× SSC containing 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).

Isolation of Genomic DNA Clones for OlGC2 and OlGC7 from a Medaka Fish Bacterial Artificial Chromosome (BAC) Library—High-density replica (HDR) membranes of an *O. latipes* Hd-rR inbred strain genomic BAC library kindly provided by Professor Hiroshi Hori (Nagoya University) were treated with alkaline lysis buffer (1.5 M NaCl and 0.5 M NaOH) for 10 min at room temperature. After exposing the side of the membrane carrying the BAC DNA to an ultraviolet irradiation source, it was treated with the colony lysis buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl, and 1% *N*-lauroylsarcosine sodium salt at 37°C for 1 h, and then the membrane was baked for 2 h at 80°C. Colony hybridization was performed overnight at 55°C with an AlkPhos DIRECT kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. To isolate the *OlGC2* gene, the hybridization was carried out using a probe made by PCR with *OlGC2* cDNA as a template and the following primers: the gs2-5′ primer (5′-TCACCTGCTGGAAGTGGACC-3′) and the SR41 primer (5′-CATAGGTAAGGTGGTGGTTGGG-3′). For isolation of the *OlGC7* gene, the hybridization was performed with a probe made by PCR using genomic DNA of medaka fish (orange-red variety) as a template and the following two primers: the GS5′ primer (5′-CCACGGTCTGAGGAGCCG-3′) and the gs3′ primer (5′-CAGAGTTCACACTGTCCACGG-3′).

Positive BAC clones were detected using CDP-Star detection reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol. QIAGEN plasmid midi and maxi kits (QIAGEN) were used for BAC DNA isolation from the bacterial culture, which was kindly provided by Professor Hiroshi Hori (Nagoya University). BAC DNA was digested with *Eco*RI and *Hin*dIII (Takara) and then subjected to Southern blot hybridization analysis with the same hybridization probes as used in the above experi-

ments to confirm the isolation of positive clones.

Based on the nucleotide sequence of *OlGC2* or *OlGC7* cDNA, we designed various specific oligonucleotide primers to amplify the intron regions of positive BAC clones and to carry out sequencing effectively (Table II). Long and accurate polymerase chain reaction (LA-PCR) was performed using a combination of these primers under the following conditions: After an initial denaturing step of 1 min at 94°C, the PCR conditions were 30 cycles of denaturation for 20 s at 98°C, annealing and extension for 20 min at 59–68°C (depending on primers), and final extension at 72°C for 10 min. Primers positioned in the exon were used to determine the intron size by PCR from a positive clone of the BAC DNA template, and the PCR product was purified and subcloned into the plasmid vector pBluescript II KS(−) or KS(+) (Stratagene). The subcloned PCR fragment was used as a template to determine the nucleotide sequence.

To determine the sequence of intron 4 of *OlGC2*, a region containing intron 4 was amplified using a specific primer set (LA3 and LA7), and the PCR fragment (4 kbp) was subcloned into the corresponding site of pBluescript II KS(−). While sequencing the PCR fragment of intron 4 of *OlGC2*, we found that the intron contains a nucleotide fragment of considerable size consisting of repeated 5′-AGCCTCTGCTCCTCCTTC-3′. The size of the nucleotide fragment designated as RS1 was more than 1 kbp and it was difficult to determine the correct nucleotide sequence by the regular dideoxy chain termination methods. The nucleotide fragment containing RS1 was isolated as follows: the BAC clone containing the *OlGC2* gene was digested with *Eco*RI and then subjected to 0.7% agarose gel electrophoresis (5 μg BAC DNA/lane). BAC DNA fragments in the gel were transferred to a filter and then hybridized with the ^{32}P-labeled probe (complementary to 294–1,342 bp downstream of exon 5). The filter was washed twice with 2× SSC containing 0.1% SDS at 42°C for 15 min and with 0.1× SSC containing 0.1% SDS at 42°C for 5 min. Imaging of the radioactive signals was performed with a FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film). A DNA band containing RS1 was cut out of the gel and purified, subcloned into pBluescript II KS(−) using the *Eco*RI site, and then sequenced using the vector-specific primers (M13-20 primer, 5′-TGACCGGCAGCAAAATG-3′; reverse primer, 5′-GGAAACAGCTATGACCATG-3′). Although the 5′ part of the subcloned fragment had the same sequences as the probe, the 3′ part was different from the expected sequence (exon 5). Therefore, we synthesized various specific oligonucleotide primers to determine the sequence of downstream of the RS1 region directly using BAC DNA as a template. We determined the nucleotide sequence of a 5,010-bp fragment downstream of RS1, and during the sequencing we found another nucleotide fragment consisting of repeated 5′-AGCCTCTGCTCCTCCTTC-3′ designated as RS2, downstream of the 5,010-bp fragment. Determination of the sequence of the fragment containing RS2 was carried out in a manner similar to in the case of RS1. The BAC clone containing the *OlGC2* gene was digested with *Bam*HI and *Sac*I, and the DNA fragments in the digest were separated by 0.7% agarose gel electrophoresis (5 μg BAC DNA/lane), then transferred to a filter and then hybridized with a ^{32}P-labeled 289-bp probe. The 289-bp probe was prepared by PCR using the BAC DNA containing *OlGC2* as a template with the following primers: 5′-11, 5′-GAATTGCTCCTCCTTCAGCC-3′ (identical to 16–35 bp upstream of exon 6) and LA3-2, 5′-ATCGAGAGGACGAGCTCCG-3′ (corresponding to nucleotides 1634–1616 of *OlGC2* cDNA). The filter was washed two times with 2× SSC containing 0.1% SDS at 42°C for 15 min and one time with 0.1× SSC containing 0.1% SDS at 42°C for 5 min. Imaging of the radioactive signals was performed as described above. A DNA band-containing RS2 was cut out of the gel and purified, subcloned into pBluescript II KS(−) at the *Bam*HI–*Sac*I site, and then sequenced.

Primer Extension Analysis—In order to identify the transcription start site, a primer extension experiment was carried out using poly(A)$^+$ RNA prepared from the adult

TABLE II. **Primers used for LA-PCR analysis of the *OlGC2* and *OlGC7* genes.**

primers	sense primers sequence	position in cDNA	primers	antisense primers sequence	position in cDNA	exon No.	annealing temp. (°C)
OlGC2							
gc2-5′	5′-GAGCTTTTGGCGCTCGTGCGCGG-3′	35-57	SR43	5′-GCTGTGTTGGTCTCTGGTAGG-3′	419-439	0-1	59.5
LA8	5′-GAGCGGCCACGGCAGAACATCACGC-	384-408	LA6	5′-GGCCCAGGGCTGCTTGTTGCTGAGG-3′	1133-1157	1-2	69
51	5′-GACAACAAGGAGGACGACCG-3′	858-877	21	5′-GTAGACCATGAGGCCGTCG-3′	1340-1358	1-4	61
LA7	5′-GCGTAAAGATCCTGTCGT- ACCGTGAACCTCAG-3′	1201-1232	LA3	5′-GGTTGTCGTTCTTGAA- GCCGCACTTGGG-3′	1638-1665	3-6	68
62	5′-GACGGACTTTGCTCTGTGGG-3′	1493-1512	91	5′-TCCCGCTCCGCAGAACC-3′	1847-1863	5-8	61
61	5′-GCTGTGGAGGATTTCCTGGG-3′	1799-1818	PR03	5′-GTAGGATCTGGTACAGCAGGG-3′	2824-2844	8-17	61
81	5′-GAAGATCACAGACTACGGGC-3′	2300-2319	gc2-3′	5′-GGAGCAGATGCTGGGTCTCTGAGGAC-3	4185-4210	13-21	63
OlGC7							
3RA1	5′-GCCGGGAAGCTGGAAGATCAC-3′	23-43	RP32	5′-GGTTGATGTTGCTCTCTACGA-3′	911-931	0-1	60
7-5	5′-TGCTACTTCGCCATCGAGGGG-3′	844-864	7-7	5′-GGGCTGCGTGGTCGTCC-3′	1131-1147	1-2	61
7-6	5′-CGAGAAGCCCGGCATGCC-3′	1095-1112	RP22	5′-TCCCCTTTGGGTCTCTG-3′	1375-1391	2-4	60
7-82	5′-TGAGCAAACGGAAACCAGGGC-3′	1343-1363	7-92	5′-GCCTTATGGGACATGACAGACATC-3′	1483-1506	4-5	62
7-83	5′-GAGAGATAGACTTTGCCTTATGGG-3	1469-1492	7-10′	5′-CGAAGCAGATCACCATGGC-3′	1687-1705	5-7	60
7.1.2	5′-CTCCATAAAGGAGCTGGTCCTGC-3′	1545-1567	7.2.2	5′-CCTGGTCAGTTCAATGCGTTTCTTG-3′	1971-1995	6-10	61
7-10	5′-GCCATGGTGATCTGCTTCG-3′	1687-1705	7-123	5′-GGGGCAGTATTCTGTGATGATGC-3′	2087-2109	7-11	60
7-122	5′-CGCCTGCGTTGACCCTCC-3′	2061-2078	7-132	5′-GGCGTGAGTGTCGTCGGG-3′	2329-2346	11-13	62
36-2	5′-GCGAAAGCATGACGTTGGAC-3′	2138-2157	RP03	5′-GTAGAATCTGGTACAGCAGGG-3′	2804-2824	12-16	59
3R1	5′-AGTCACAGCGAGGAGCTCGG-3′	2575-2594	AR2	5′-GTTCCCCACCATATTAGACAG-3′	3528-3548	15-21	56

medaka fish ovary and two oligonucleotide primers, PE4 for *OlGC2*, 5′-CACGAGCGCCAAAAGCTC-3′ (complementary to nucleotides 35–52 of *OlGC2* cDNA) and PE5 for *OlGC7*, 5′-AAGGCAAGAACCCCACGC-3′ (complementary to nucleotides 857–874 of the *OlGC7* gene). Five μg of the medaka fish ovary poly(A)$^+$ RNA was hybridized at 65°C for 90 min in a total 20 μl of 10 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 0.25 M KCl with the oligonucleotide primer (5×10^4 cpm), which had been endo-labeled with T4 polynucleotide kinase (Takara) and [γ-^{32}P]ATP at 37°C for 30 min. The extension reaction was carried out in 45 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, and 200 U of Superscript II reverse transcriptase (Life Technologies) at 42°C for 1 h. The primer-extended products were treated with RNase A and then separated on a 3.5% (for *OlGC2*) or 6% (for *OlGC7*) polyacrylamide gel containing 7 M urea, along with a sequence ladder generated with the corresponding unlabeled primers using [α-^{32}P]dCTP and a Sequenase 7-deaza dGTP DNA Sequencing Kit (USB). The radioactive signal was analyzed with a FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film).

Other Methods—The nucleotide sequences of cDNA or genomic DNA fragments in pBluescript vectors (Stratagene), the purified PCR products, and the purified BAC DNA were determined by the dideoxy chain termination method (*30*) with an ABI PRISM® 377 DNA Sequencer or an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems), and analyzed with DNASIS software (Hitachi Software Engineering) and GENETYX-MAC/version 7.2.0 (Software Development).

RESULTS

Isolation and Characterization of cDNA Clones Encoding OlGC2 and OlGC7—In the present study, we obtained a full-length cDNA clone for *OlGC2* by repeated 5′-RACE and determined its complete nucleotide sequence. The *OlGC2* cDNA was 4,383 bp in length, and consisted of a 269-bp 5′-untranslated region (UTR), a 3,189-bp open reading frame (ORF), and a 926-bp 3′-UTR with a poly(A)$^+$ tail. Termination codons occur in all three frames upstream of the putative initiation codon (ATG), and nucleotides around the putative initiation codon fit within the preferred sequence context for initiation of protein synthesis in eukaryotic mRNAs (*31*). The ORF of *OlGC2* cDNA predicts a protein of 1,063 amino acids which contains an amino-terminal signal sequence of 22 amino acids (*32*). Cleavage of the signal sequence would result in a mature protein of 1,041 amino acids. The eukaryotic polyadenylation signal AATAAA is present at 24 bp upstream from the beginning of the poly(A)$^+$ tail.

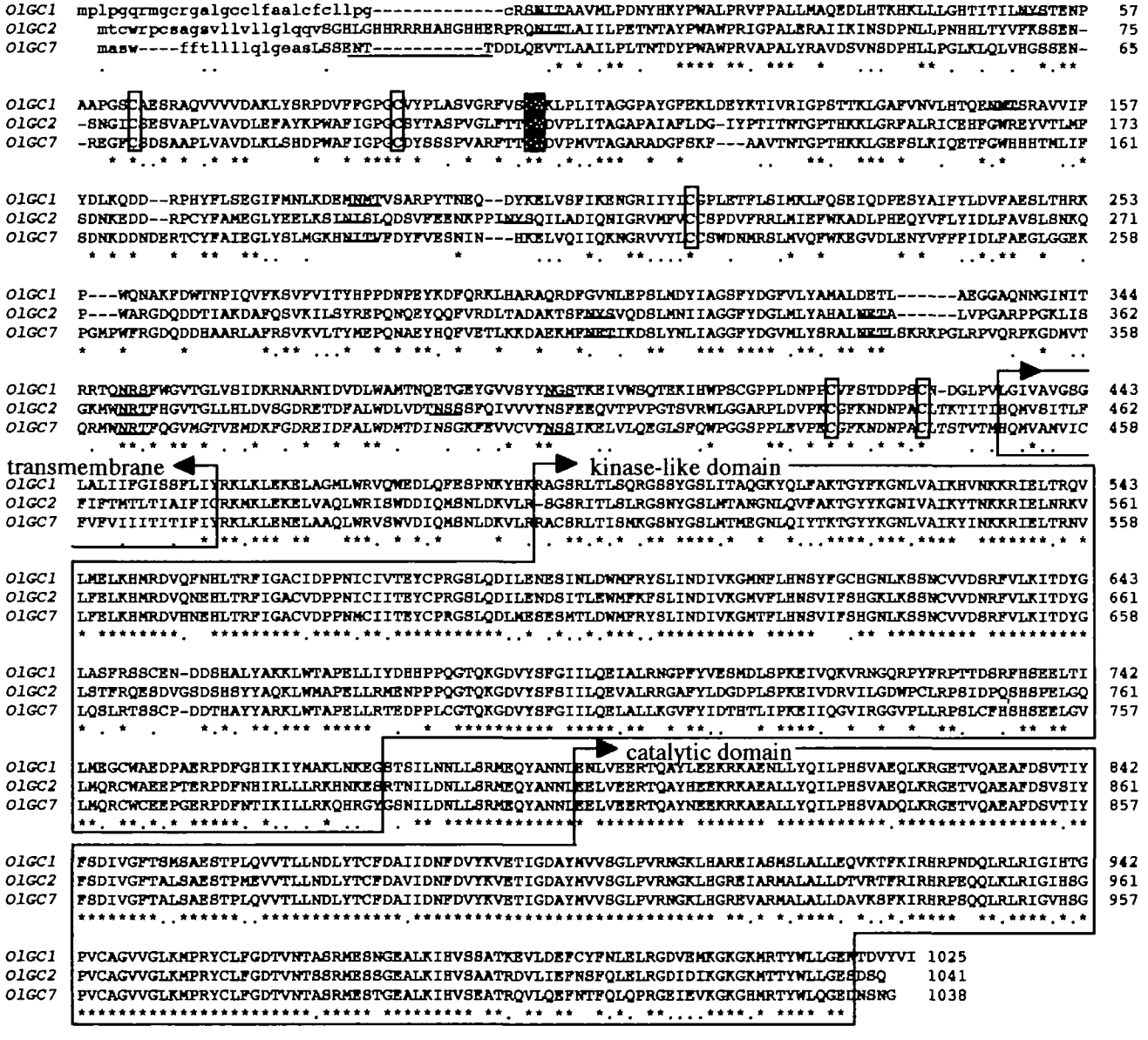
In a previous study, we obtained a new type of membrane GC using RT-PCR with the medaka fish gonad total RNA (*33*). In order to obtain a full-length cDNA sequence of the new membrane GC, the 5′- and 3′-portions of the cDNA were amplified by the 5′- and 3′-RACE method, respectively, using the gene-specific antisense oligonucleotide primers. The amplified cDNA clone named *OlGC7* is 3721 bp in length, and consists of a 274-bp 5′-untranslated region (UTR), a 3,165-bp open reading frame (ORF), and a 282-bp 3′-UTR with a poly(A)$^+$ tail. Termination codons occur in all three frames upstream of the putative initiation codon and nucleotides around the putative initiation codon of *OlGC7* fit within the preferred sequence context for initiation of protein synthesis in eukaryotic mRNAs (*31*). The ORF of *OlGC7* cDNA encodes a protein of 1,055 amino acids which contains an amino-terminal signal sequence of 17 amino acids (*32*), and cleavage of the signal sequence would result in a mature protein of 1,038 amino acids.

Comparison and Phylogenetic Analysis of the Amino Acid Sequences of Various Natriuretic Peptide Receptor/GCs—Hydropathic analysis (*34*) and comparison of the deduced amino acid sequence of *OlGC2* or *OlGC7* with those of other known membrane GCs suggest that the domain organization of OlGC2 and OlGC7 is similar to that of known natriuretic peptide receptor/GCs (data not shown). As shown in Fig. 1A, the mature OlGC2 protein is composed of a large extracellular domain (residues 1–453), a single membrane-spanning domain (residues 454–476), a protein kinase-like domain (residues 508–791), and a cyclase catalytic domain (residues 811–1038). The mature OlGC7 protein comprises a large extracellular domain (residues 1–449), a single membrane-spanning domain (residues 450–472), a protein kinase-like domain (residues 504–787), and a cyclase catalytic domain (residues 807–1034). The amino acid sequences of both the catalytic and kinase-like domains of OlGC2 and OlGC7 as well as OlGC1 are quite similar to each other. Five cysteine residues in the extracellular domain of known vertebrate natriuretic peptide receptor/GCs such as GC-A and GC-B are predicted to form two disulfide-linked loops, and are found at the corresponding positions of OlGC2 (residues 80, 106, 232, 437, and 446) and OlGC7 (residues 70, 96, 219, 433, and 442). Histidine-tryptophan residues in the extracellular domain of GC-A, which are believed to be the ligand-binding site (*35*), are conserved at the corresponding positions of OlGC2 (residues 119 and 120) and OlGC7 (residues 109 and 110), respectively.

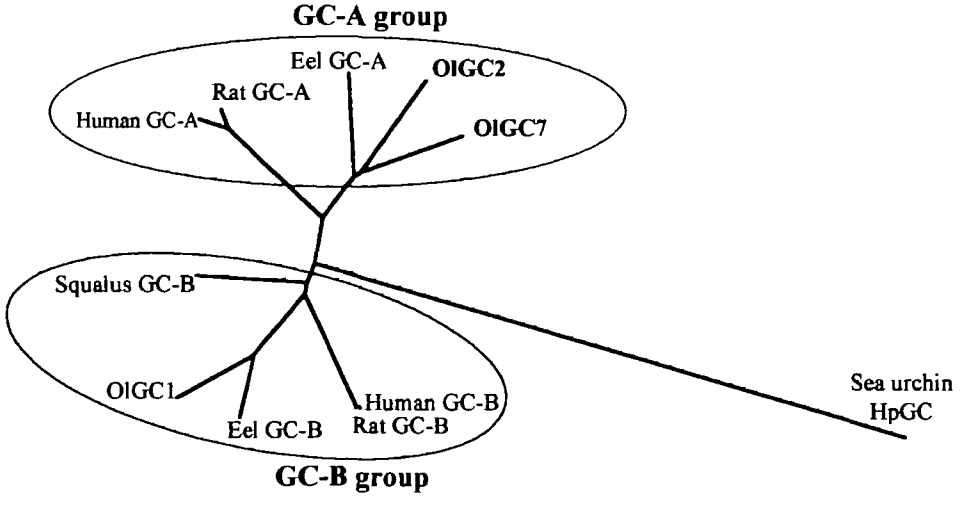
As shown in Fig. 1B, the unrooted phylogenetic tree constructed using the amino acid sequences of the extracellular domains of various isoforms of the natriuretic peptide receptor/GCs demonstrates that these isoforms can be divided into two groups, (I) GC-A and (II) GC-B, and that OlGC2 and OlGC7 belong to the GC-A group. Comparison of the amino acid sequence of OlGC2 or OlGC7 with that of eel GC-A indicates a high degree of similarity not only in the cytoplasmic domain, which contains the kinase-like and catalytic domains, but also in the extracellular domain. The amino acid sequence identity in the extracellular domain of OlGC2 or OlGC7 to OlGC1 is 36 or 38%, respectively, while that of OlGC2 or OlGC7 to eel GC-A is 53 or 51%, respectively, and that of OlGC2 to OlGC7 is 56%. On the other hand, the sequence identity in the kinase-like domain and catalytic domain of OlGC1, OlGC2, or OlGC7 to eel GC-A is 71–76 and 88–91%, respectively.

Northern Hybridization Analysis—To estimate the size of *OlGC2* or *OlGC7* mRNA, we carried out Northern blot analysis using total or poly(A)$^+$ RNA from various organs of mature adults of the orange-red variety of medaka fish and a cDNA fragment of 5′-UTR as a probe. A single hybridization signal was detected at the position corresponding to 4.7 kb (*OlGC2*) or 4.0 kb (*OlGC7*), respectively. The signals due to both the *OlGC2* and *OlGC7* transcripts were the strongest for the kidney sample, although the *OlGC2* transcript was detected in the gill, and the *OlGC7* transcript

(A)


transmembrane
kinase-like domain
catalytic domain

(B)


GC-A group
Eel GC-A
OlGC2
Rat GC-A
Human GC-A
OlGC7
Squalus GC-B
OlGC1
Human GC-B
Rat GC-B
Eel GC-B
GC-B group
Sea urchin
HpGC

was detected in the brain and ovary (data not shown).

Expression of OlGC2 and OlGC7 in Adult Organs—To determine the organ-specific expression of *OlGC2* and *OlGC7* transcripts, we performed RNase protection analysis using total RNA isolated from various adult medaka fish organs. As shown in Fig. 2, the analysis demonstrated that the *OlGC2* transcript is expressed at a higher level in the kidney, gall bladder, and gill, while the *OlGC7* transcript is significantly expressed in the kidney, gall bladder, brain, and gill. Among these organs, however, the strongest signal due to the *OlGC2* and *OlGC7* transcripts was in the kidney, which is in good agreement with the results of Northern blot analysis.

Genomic Southern Analysis—Southern blot hybridization of medaka fish (Hd-rR strain) genomic DNA using 5′-UTR or a part of exon 1 of *OlGC1, OlGC2*, or *OlGC7* as a probe demonstrated that the *OlGC7* probe produced only one positive signal in each of the three lanes, while the *OlGC1* or *OlGC2* probe produced one strong and one weak signal in the lane of genomic DNA fragments digested with *Eco*RI (Fig. 3). The size of the genomic DNA with the weak signal in the *Eco*RI lane was slightly different between *OlGC1* (16.6 kbp) and *OlGC2* (16 kbp). The size of the genomic DNA with the strong signal in each of the three lanes was consistent with that of the DNA fragment, which is expected to be obtained on digestion of the respective genomic DNA clone by the respective restriction enzyme.

Characterization of Genomic DNA Clones for OlGC2 and OlGC7—An *O. latipes* Hd-rR strain genomic BAC library was screened with a cDNA fragment of *OlGC2* (304-bp; a part of exon 1) or a genomic DNA fragment of *OlGC7* (4,149-bp; intron 0 and a part of exon 1) as a probe. Five and three positive clones were isolated for *OlGC2* and *OlGC7*, respectively, and a clone which contains a full sequence of *OlGC2* or *OlGC7* cDNA was sequenced.

While sequencing the fragment of intron 4 of *OlGC2*, we found that intron 4 contains two considerably sized nucleotide fragments consisting of repeated 5′-AGCCTCTGCTCCTCCTTC-3′. One fragment is about 1 kbp (designated as RS1) and the other one (designated as RS2) is predicted to be more than 5 kbp. Neither fragment could be sequenced by the regular dideoxy chain termination method. Therefore, we determined the sequences of both fragments by calculation using the accurate size of each fragment and the repeated sequence of 18 bp. To estimate the size of each fragment, we carried out Southern blot analysis and region-specific sequencing using several restriction enzyme fragments of RS1- or RS2-containing intron 4. *Eco*RI digestion of an RS1-containing intron 4 fragment produced a 3.8 kbp-repeated sequence-containing fragment, and *Bam*HI and *Sac*I digestion of an RS2-containing intron 4 fragment produced a 6.8 kbp-repeated sequence-containing fragment. RS1 is located 2,650 bp downstream of exon 4, and its size was estimated to be 1,130 bp (60 times repeat). RS2 is located close to the upstream of exon 5, and its size was estimated to be 6,300 bp long (350 times repeat). Since the nucleotides between RS1 and RS2 were determined to be 5,010 bp, the size of intron 4 was estimated to be 15,215 bp in length. In intron 0 of *OlGC2*, we found another repeated sequence-containing fragment consisting of seven repeated 5′-AGGAACCATTGGGGGTT-TTGCGGGGGGGTTGCTCCGCCCTG-3′ (1941–2227 in intron 0).

By combining all the sequence results, we estimated the size of the *OlGC2* gene to be 32,990 bp (GenBank accession Nos. AB054294, AB054295, and AB054296), and that of *OlGC7* to be 44,263 bp (GenBank accession No. AB054293) (Fig. 4). The *OlGC2* and *OlGC7* genes both consist of 22

Fig. 1. **(A) Alignment of the amino acid sequences of three medaka fish natriuretic peptide receptor/GC homologs.** The conserved cysteine residues in the extracellular domain are boxed, and the conserved histidine-tryptophan residues in the extracellular domain are indicated by visual inspection. The signal peptide sequence is indicated by lowercase letters. The potential N-linked carbohydrate binding sites are underlined. Identical amino acids in the three proteins are indicated by asterisks below the sequence. Gaps in the sequence are indicated by dashes (−). **(B) Molecular phylogenetic analysis of the amino acid sequences of fish and mammalian natriuretic peptide receptor/GCs.** The amino acid sequences of the extracellular domains of various membrane GCs were subjected to phylogenetic analysis (see "MATERIALS AND METHODS").

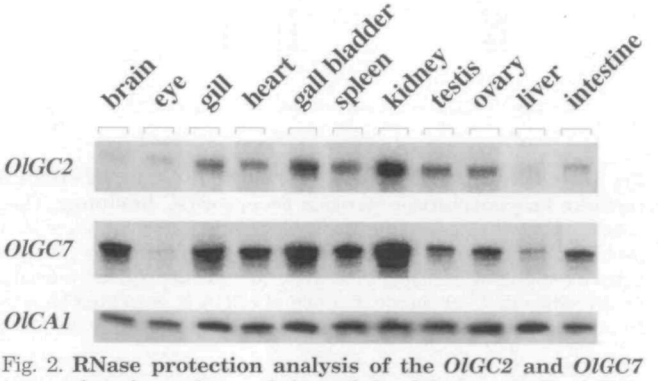


Fig. 2. **RNase protection analysis of the *OlGC2* and *OlGC7* transcripts in various adult medaka fish organs.** Total RNA (10 μg) obtained from various medaka fish organs was hybridized with an antisense cRNA probe for *OlGC2* (228 nucleotides) or for *OlGC7* (226 nucleotides). An antisense cRNA probe for *OlCA1* (100 nucleotides) was used as an internal control. After digestion with RNase, the protected fragments were separated by electrophoresis on a 6% polyacrylamide/7 M urea gel, followed by autoradiography.

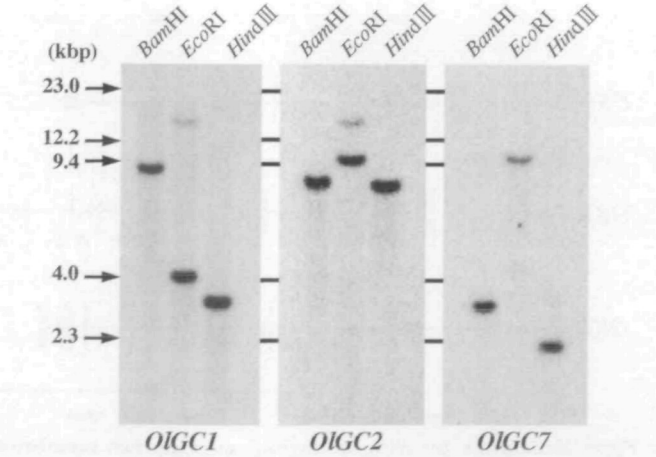


Fig. 3. **Genomic Southern hybridization analysis.** Genomic DNA (10 μg) from a single Hd-rR inbred strain individual was digested with *Bam*HI, *Eco*RI, or *Hind*III. The same blot was hybridized with a ^{32}P-labeled cDNA probe for *OlGC1, OlGC2*, or *OlGC7*. The positions and sizes of the markers are indicated.

exons, respectively, which are the same as those of *OlGC1* (*14*). In both *OlGC2* and *OlGC7* genomic DNAs, the GT-AG rule was conserved for all splice sites. The extracellular domain in the *OlGC2* or *OlGC7* gene is distributed in seven exons (exons 1–6), and the single transmembrane domain is coded by exon 7. The kinase-like and C-terminal cyclase catalytic domains in both genes are coded by eight (exons 8–15) and six (exons 16–21) exons, respectively. Introns in the extracellular domain are larger than those in the intracellular domain. In particular, introns 1 and 4 are quite

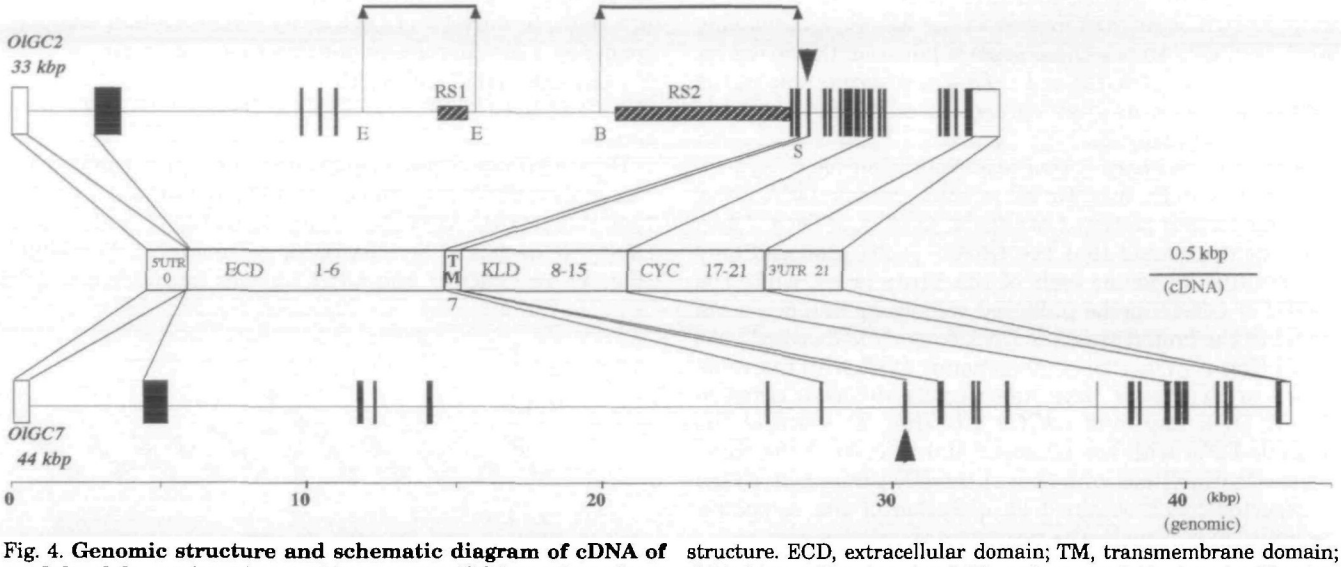


Fig. 4. **Genomic structure and schematic diagram of cDNA of medaka fish natriuretic peptide receptor/GC homologs.** Open boxes indicate 5′- and 3′-untranslated regions, and solid boxes show protein coding regions in the genomic structures. Introns are indicated by lines. The exons of the transmembrane domain are indicated by arrowheads. A schematic diagram of cDNA is presented between the two genomic structures. Exon numbers are shown in the cDNA structure. ECD, extracellular domain; TM, transmembrane domain; KLD, kinase-like domain; CYC, cyclase catalytic domain. The two bars above the *OlGC2* gene indicate the fragments containing repeated sequences (RS1 and RS2). The four arrows pointing to the end of the two bars indicate the active restriction enzyme sites, *i.e.* the *Bam*HI, *Eco*RI, and *Sac*I sites, which are denoted by B, E, and S, respectively.

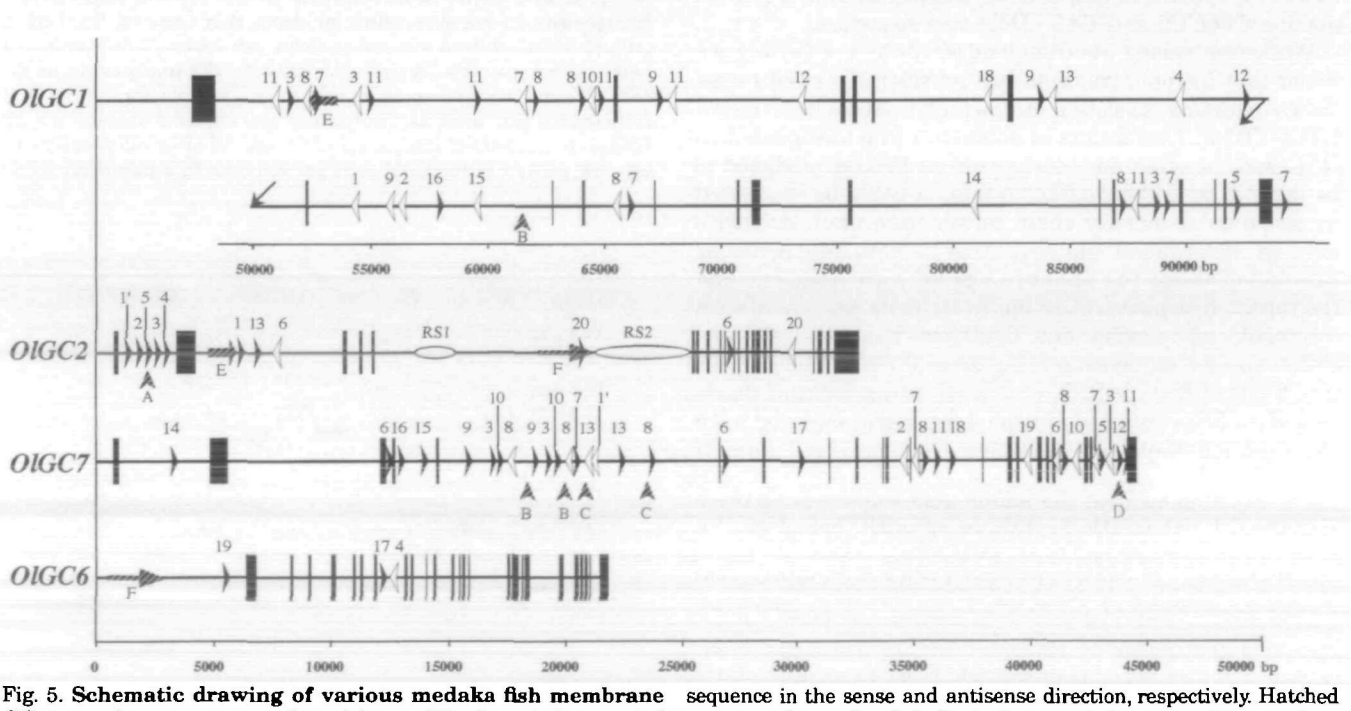


Fig. 5. **Schematic drawing of various medaka fish membrane GC genomic structures and positions of the inserted repeated sequences.** Exons are shown as shaded boxes. Introns and flanking sequences are indicated by lines. The two ellipses in the *OlGC2* gene denote the repeated sequences (RS1 and RS2). Open arrowheads pointing left and closed arrowheads pointing right indicate a repeated sequence in the sense and antisense direction, respectively. Hatched arrows and arrowheads indicate the repeated sequence. The numbers above the lines or the letters below the lines denote the positions of the identical sequences found in various medaka fish genomic DNA sequences including those of membrane GCs (see Table IV).

large. In *OlGC2*, the nucleotide sequences between exons (*O. latipes* strain Hd-rR) and cDNA (orange-red variety of *O. latipes*) were mismatched in nucleotides 56 (GGA→AGA) and 3788 (CTG→CTC), both of which are located in the UTR. Similarly, seven mismatches were found between the *OlGC7* gene and its cDNA in nucleotides 89, 90 (GCC→AGC), 132 (CAC→TAC), 972 (GTT→GTG), 1842 (GCA→GCT), 2820 (ATC→ATT), and 3381 (GAA→GAG).

We found a repeated sequence (nucleotides 3429–4991) between RS1 and RS2 in intron 4 of the *OlGC2* gene, which exhibits 95.2% identity with nucleotides 4511–6066 in the upstream of the putative initiation codon of the *OlGC6* gene, a medaka fish homolog of the mammalian GC-C (*13*). On the other hand, nucleotides 4625–4765 between RS1 and RS2 were found in the opposite strand in intron 17 of the *OlGC2* gene (nucleotides 285–425). Moreover, many fragments in introns of *OlGC2* or *OlGC7* are conserved in introns of other medaka fish membrane GCs (*OlGC1* or *OlGC6*) or of other medaka fish genes such as transferrin (*36*) and Tp53 (Fig. 5 and Table IV). These fragments (30 bp–1.5 kbp in length) are found in large-sized introns of respective genes and concentrated in a limited number of regions.

Primer Extension Analysis—Primer extension analysis demonstrated that 567(G) bp upstream of the putative initiation codon is the transcription initiation site for *OlGC2* and 631(C) bp upstream of the putative initiation codon is that for *OlGC7*. Therefore, a full-length *OlGC2* mRNA would be 4,682 bp in length and that for *OlGC7* would be 4,078 bp in length, which is consistent with the results of Northern blot analyses.

There is no typical TATA box in the 5′-flanking region upstream of the putative transcription initiation site of *OlGC2* or *OlGC7*, although TATA box consensus sequences, TATTTAT and TATAAA, are present at 75–69 bp and 186–180 bp upstream of the transcription initiation site of *OlGC7*, respectively. A consensus motif search indicated that several *cis*-regulatory elements, such as C/EBP, CREB, NF-IL6, and Sp1 binding sequences, are present in the 5′-upstream region (528 bp) of *OlGC2*, and that consensus sequences for binding to AP-2, NF-IL6, c-Myb, and Sp1 are present in the 5′-upstream region (801 bp) of *OlGC7*.

DISCUSSION

In this study, we isolated and characterized two medaka fish membrane GC clones, and designated them as *OlGC2* and *OlGC7*, respectively. Phylogenetic analysis indicated that both are homologs of mammalian GC-A. It is known that mammals each have two natriuretic peptide receptor/GCs, GC-A and GC-B (*1*). Based on the results of the present study and those of the previous study showing that the medaka fish possess one mammalian GC-B homolog (*14*), it is clear that the medaka fish contains three natriuretic peptide receptor/GCs. However, this is not surprising because (i) it has been reported that the medaka fish possesses four different sensory organ-specific membrane GCs (*17*, *37*), while mammals possess three of them (*38–40*), and (ii) it is also known that fish including the medaka fish, zebrafish, and Fugu contain more members of the same gene family than mammals do (*41*). For example, the number of genes in receptor tyrosine kinase subclass I (EGFR family) (*42*), neurotrophins (NGF family) (*43*), or orthodenticle-related genes (otx) in fish (*44*) is greater than in mammals. In a paper, Meyer and Schartl (*45*) suggested that fish have more genes than tetrapods because an additional (third) entire genome duplication took place during the evolution of actinopterygian fish that other vertebrates did not experience (*45*). Therefore, we presume that the existence of two GC-A subtypes in the medaka fish is due to the entire genome duplication. This hypothesis might be extended to other membrane GCs such as GC-B and GC-C, subtypes of which have not been found yet.

Alignment of the amino acid sequences of OlGC2 and OlGC7 with those of the other known GC-A subtypes indicated that in the extracellular domain of GC-A, two disulfide-linked loops and histidine-tryptophan residues considered an essential part of the ligand-binding site are completely conserved among OlGC2, OlGC7, and the other GC-A subtypes. Mammalian GC-A has been reported to be stimulated by both ANP and BNP. On the other hand, eel GC-A responds to eel ANP (*46*) and eel VNP (*47*), of which a mammalian counterpart is unknown. Therefore, we presume that in the medaka fish ligands similar to ANP and BNP or VNP, all of which have not yet been identified in the medaka fish, are responsible for the activation of OlGC2 and OlGC7.

RNase protection analysis demonstrated that the expression patterns of *OlGC2* and *OlGC7* are not identical but similar. Both gene transcripts are expressed in the kidney, gall bladder, gill, spleen, heart, ovary, and testis, although the *OlGC7* transcript is expressed in the brain and intestine at a considerable level, and the *OlGC2* transcript is expressed at a lower level in those organs. The expression patterns of *OlGC2* and *OlGC7* are markedly different from that of *OlGC1*, which is rather broad. Mammalian GC-A is expressed in the vasculature, kidney, adrenal gland, and

TABLE III. **Exon/intron organization of the *OlGC1*, *OlGC2*, and *OlGC7* genes.**

exon/intron	exon size (bp)			functional	intron size (bp)		
No.	*OlGC1*	*OlGC2*	*OlGC7*	domain*	*OlGC1*	*OlGC2*	*OlGC7*
0		523	612	5′UTR		2290	3918
1	995	792	712	EC	16989	5965	6490
2	182	182	191	EC	9696	507	282
3	114	114	114	EC	367	350	1727
4	136	136	154	EC	666	15215	11434
5	95	95	95	EC	5773	69	1789
6	136	136	136	EC	12506	310	2739
7	82	85	85	TM	10378	432	1102
8	121	118	121	KL	1173	79	1039
9	75	75	75	KL	519	276	75
10	78	78	78	KL	2432	99	933
11	105	105	105	KL	3477	73	2923
12	72	72	72	KL	521	68	1018
13	160	163	160	KL	85	74	183
14	156	156	156	KL	3834	71	773
15	169	169	169	KL	10505	135	205
16	147	147	147	CYC	472	70	136
17	124	124	124	CYC	2635	1722	985
18	69	69	69	CYC	76	111	96
19	175	175	175	CYC	1379	187	67
20	99	99	99	CYC	326	205	1521
21	92	1069	428	CYC	1436		
22	560			3′-UTR			
	3942	4682	4077				

*Exons coding for the extracellular (EC), transmembrane (TM), kinase-like (KL), and cyclase catalytic (CYC) domains.

brain, and it is thought to regulate the blood pressure and kidney function (*6*). GC-A of the euryhaline eel *Anguilla japonica* (*8*), which is the most closely related to *OlGC2* and *OlGC7*, is expressed at a higher level in osmoregulatory organs such as the kidney, brain, gill, and intestine. The expression patterns of *OlGC2* and *OlGC7* are similar to those of mammalian GC-A and eel GC-A, suggesting that their ligands and function in the respective organs are similar to those of mammalian and/or eel GC-A.

In this study, we performed genomic Southern hybridization experiments with genomic DNA from a single medaka fish inbred strain (Hd-rR) individual. As shown in

TABLE IV. **Positions of the elements of *OlGC2* or *OlGC7*, with other guanylyl cyclases or other genes of the medaka fish.**

Other guanylyl cyclases of medaka fish						Other medaka genes in the GenBank database		
No	gene	position in gene	No.	gene	position in gene	No.	gene	position in gene
1	*OlGC2*	6428-6468	9	*OlGC1*	57586-57557	A	*OlGC2*	1957-1995
	OlGC1	54361-54321		*OlGC1*	24785-24873		*Orla-DAB*	1627-1589
				OlGC7	15982-16085			
1'	*OlGC7*	20377-20424		*OlGC7*	19570-19687	B	*OlGC7*	19973-20002
	OlGC2	1535-1591		*OlGC1*	40370-40499		*ef*	1386-1415
2	*OlGC2*	1752-1785	10	*OlGC7*	17143-17192		*OlGC1*	62752-62709
	OlGC1	57839-57778		*OlGC7*	43348-43291		*OlGC7*	17338-17387
	OlGC7	34249-34193		*OlGC7*	19864-19937		*ef*	1646-1688
				OlGC1	21870-21787			
3	*OlGC1*	88924-88943				C	*OlGC7*	23237-23181
	OlGC7	43503-43469	11	*OlGC1*	8420-8375		*transferrin*	6369-6439
	OlGC7	19780-19825		*OlGC7*	43727-43767		*OlGC7*	20092-20139
	OlGC1	12492-12439		*OlGC1*	12603-12669			
	OlGC2	2582-2656		*OlGC1*	24659-24588	D	*OlGC7*	43511-43715
	OlGC1	8472-8542		*OlGC1*	16530-16617		*MRSQ20*	221-1
	OlGC7	16989-17074		*OlGC1*	21977-22065			
				OlGC1	88896-88808	E	*Tp53*	7276-7653
4	*OlGC1*	47154-47116		*OlGC7*	35186-35275		*OlGC1*	9910-8804
	OlGC2	2603-2653					*OlGC2*	5172-6242
	OlGC6	12962-12913	12	*OlGC1*	49712-49749			
				OlGC7	43681-43729		*Tp53*	5258-4732
5	*OlGC1*	92858-92893		*OlGC1*	31099-31051		*OlGC1*	9910-8804
	OlGC7	42857-42937					*OlGC2*	5172-6242
	OlGC2	1987-2068	13	*OlGC7*	20225-20205			
				OlGC2	6599-6630	F	*Fugu CR1*	3733-3832
6	*OlGC7*	40977-40949		*OlGC1*	40615-40584		*tnL3*	434-507
	OlGC7	26885-26927					*OlGC6*	826-2391
	*OlGC2**	7958-7904	14	*OlGC1*	80452-80407		*OlGC2**	3747-5299
	*OlGC2**	1956-2024		*OlGC7*	3829-3874			
7	*OlGC1*	66088-66105	15	*OlGC1*	55913-55849			
	OlGC1	94943-94966		*OlGC7*	14099-14163			
	OlGC7	12803-12825						
	OlGC7	42492-42458	16	*OlGC1*	53591-53620			
	OlGC1	18271-18227		*OlGC7*	13584-13613			
	OlGC1	89296-89394						
	OlGC7	20056-20139	17	*OlGC7*	30461-30486			
	OlGC7	35008-34886		*OlGC6*	12793-12818			
8	*OlGC7*	43192-43216	18	*OlGC1*	39894-39746			
	OlGC1	18368-18404		*OlGC7*	35347-35492			
	OlGC7	20023-19974						
	OlGC7	35061-35108	19	*OlGC6*	6196-6173			
	OlGC1	87881-87934		*OlGC7*	39478-39501			
	OlGC1	21693-21766						
	OlGC7	17277-17226	20	*OlGC2***	4939-5078			
	OlGC1	65980-65900		*OlGC2**	4151-4033			
	OlGC7	23341-23449						
	OlGC1	8788-8680						

OlGC1 (AB021490); *OlGC2* (AB054294); *OlGC2** (AB054295); *OlGC2*** (AB054296); *OlGC7* (AB054293); *Orla-DAB*, MHC class II antigen (AB033212); ef, polypeptide elongation factor 1 alpha (AB020734); *transferrin* (D64033); MRSQ20, *repetitive sequence (D88821); Tp53*, p53 tumor suppressor (AF212997); tnL3, Tilapia Nilotica clone L3 line-like repeat sequence (AF074486); Fugu CR1, Fugu Rubripes CR1-like LINE retrotransposon putative nucleic acid (AF086712). The relative positions of the elements described are shown in Fig. 5.

Fig. 3, using the *OlGC1* and *OlGC2* probes we detected two positive bands in the lane of genomic DNA fragments digested with *Eco*RI. The size of the genomic DNA with the strong signal is in good agreement with the expected size of the *OlGC1* or *OlGC2* DNA fragment, respectively. However, although we used the same blot membrane as in the above experiments, only a single positive band was detected with the *OlGC7* probe, suggesting the existence of an other natriuretic peptide receptor/GC gene or the existence of an related gene of *OlGC1* or *OlGC2*.

We determined the complete nucleotide sequences of two genomic DNA clones (*OlGC2* and *OlGC7*). The *OlGC2* gene is about 33 kbp and the *OlGC7* one is 44 kbp. The *OlGC1* gene has been reported to be about 93 kbp in length (*14*), and the mammalian natriuretic peptide receptor/GC gene has been reported to be much smaller than that of the medaka fish, that is, the human GC-A gene is 16 kbp (*17*), the human GC-B gene 16.5 kbp (*11*), and the rat GC-A gene 17.5 kbp (*12*). This is somewhat paradoxical, because the genome size of the medaka fish (*ca.* 800 Mbp) has been reported to be one-fifth that of mammals (*48*). In the present study, however, we demonstrated that the *OlGC2* and *OlGC7* genes both consist of 22 exons, respectively, which is the same number of exons in *OlGC1*, and mammalian GC-A and GC-B. Furthermore, each exon is almost the same size as that corresponding to *OlGC1*, and mammalian GC-A and GC-B (Table III). Therefore, the large size of genomic DNAs for *OlGC1, OlGC2*, and *OlGC7* is due to the extremely large size of the introns, particularly that of introns in the extracellular domain. However, the deduced amino acid sequences of exons in the extracellular domain are very variable, while those in the intracellular domain are highly conserved, suggesting that the exons in the extracellular domain are specialized for accepting different ligands. We presume that gene duplication and/or domain shuffling occurred in the process of molecular evolution of these genes.

In this regard, it should be mentioned that introns 1 and 4 in the *OlGC2* gene are of an especially large size and contain two fairly large repeated sequences consisting of 5′-AGCCTCTGCTCCTCCTTC-3′. A homology search showed that there is no such identical repeated sequence in invertebrates and vertebrates other than the medaka fish. Furthermore, many identical sequences, although the sizes varied, were found in the introns of *OlGC1, OlGC2, OlGC6*, and *OlGC7* (Fig. 5 and Table IV). Genetic linkage maps for the medaka fish demonstrated that the genes in the medaka fish membrane GC family including *OlGC1, OlGC2, OlGC3, OlGC4*, and *OlGC5* are not linked and are located on different chromosomes (*49*). Therefore, we presume that the repeated sequences might contribute to the genetic recombination, which would have involved gene duplication and/or domain shuffling.

On the other hand, several known repetitive sequences or conserved elements are found in the introns of *OlGC1, OlGC2*, and *OlGC7*. The nucleotide fragments (5′-TGTGTGGAGTTTGCATGTTCTCCCCGTGC-3′, nucleotides 34710–34738; and 5′-TGTGTGGAGTTTGCATGTTCT-3′, nucleotides 2512–2532) found in *OlGC1* are similar to those in the region of the zebra fish Mermaid element, which are highly identical to the sequences of the channel cat fish alpha-actin gene locus, a family of short interspersed repetitive elements (SINEs) (*50*). In intron 20 of *OlGC7*, there is a highly repetitive interspersed DNA sequence (MRE), which comprises 220 bp and is generally categorized as a SINE (*50*). The number of copies of MRE in the medaka fish genome has been calculated to be approximately 9800, *i.e.* 0.14% of the genome (*51*). Several nucleotides found in *OlGC1, OlGC2*, and *OlGC7* belong to SINEs, while the assignment of other nucleotides has not been reported. Although the functions of these sequences found in many genes are unknown, inserted elements found in the introns of medaka fish natriuretic peptide receptor/GC may contribute to the appearance of a greater number of membrane GC isoforms in the medaka fish than in mammals, and the larger size of the medaka fish membrane GC family genes.

The authors would like to thank the staff of the Research Center for Molecular Genetics and the Center for Animal Experiments, Hokkaido University, for allowing them to use the equipment for probe radiolabeling and culturing of the medaka fish.

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