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Genomic Structure and Expression of the Medaka Fish Homolog of the Mammalian Guanylyl Cyclase B[1]

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We have isolated a 3.9-kbp-long cDNA and approximately 93 kbp long genomic DNA encoding a membrane guanylyl cyclase (designated *OlGC1*) from the medaka fish *Oryzias latipes*, and determined their nucleotide sequences. The open reading frame for the *OlGC1* cDNA predicts a protein of 1,055 amino acids. Phylogenetic analysis indicates *OlGC1* to be a medaka fish homolog of mammalian guanylyl cyclase B (GC-B), a member of the natriuretic peptide receptor family. Northern blot analysis demonstrated 3.9 kb *OlGC1* transcripts in the eye and brain, while reverse transcription-polymerase chain reaction (RT-PCR) analysis showed *OlGC1* transcripts in a number of adult peripheral organs as well as embryos during early stages of development. The *OlGC1* gene consists of 22 exons with an exon/intron organization similar to that of the human and rat GC-A genes, except that *OlGC1* has several very large introns. The *OlGC1* gene has no apparent TATA box or CAAT box in the region 1.2 kbp upstream of the putative transcription initiation point, but several consensus sequences for *cis*-regulatory elements, including an NF-IL6-binding element, a shear stress responsive element, and multiple GM-CSF-binding elements, are present in that region.

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

Natriuretic peptide receptors comprise three members, guanylyl cyclase-A (GC-A), guanylyl cyclase-B (GC-B), and natriuretic peptide receptor-C (NPR-C). GC-A is a membrane-bound guanylyl cyclase (membrane GC) coupled with receptors for both artrial natriuretc peptides (ANP) and brain natriuretic peptides (BNP); it is considered to mediate the effects of these natriuretic peptides in controlling systemic blood pressure and body fluid homeostasis (*1, 2*). GC-B appears to bind C-type natriuretic peptides (CNP) with the highest affinity and to regulate cell growth and blood pressure while its *in vivo* functions remain unclear (*3, 4*). NPR-C consists of an extracellular domain for ligand-binding, a transmembrane domain, and very small intracellular domains with no guanylyl cyclase activity (*5*). NPR-C is thought to play a role in the elimination of natriuretic peptides from blood circulation and to diminish the biological effects of natriuretic peptides (*6*).

It has been reported that a shift in gene expression from predominantly GC-B to NPR-C occurs in chondrocytes during culture (*7*), and that in vascular endothelial cells, the expression of the NPR-C gene is downregulated by cGMP produced by activated GC-B (*8*). In addition, a recent study has demonstrated that the expression of natriuretic peptide receptor genes changes from NPR-C to GC-A and GC-B during the culture of rat proximal tubular cells (*9*). The above results suggest that switches in the gene expression of natriuretic peptide receptors may be under the control of endogenously released ANP/BNP. In this regard, knowledge of the genomic structure of natriuretic peptide receptors seems to be essential for understanding the transcriptional and translational regulatory mechanism of these receptors. There have been several papers reporting the exon-intron organization of human GC-A (*10*), rat GC-A (*11*), and bovine NPR-C (*12*). However, to our knowledge there has been no paper reporting the genomic structure of GC-B. In this paper, we describe the entire genomic structure of the medaka fish homolog of the mammalian GC-B gene and its expression patterns in adult fish organs and during embryogenesis.

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 kept in indoor tanks under artificial reproductive conditions (10 h dark and 14 h light

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Abbreviations: GC, guanylyl cyclase; cGMP, guanosine 3′,5′-cyclic monophosphate; *OlGC*, gene encoding *Oryzias latipes* membrane guanylyl cyclase; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; UTR, untranslated region.

at 27°C) and fed on TetraMin flakes (TetraWerke, 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 at Day 10.

Preparation of RNA, cDNA Library Construction, and Screening—Total RNA was prepared from the brains of adult medaka fish by the LiCl method (*13*). Poly(A)$^+$ RNA was purified by two passages of the total RNA over a column of oligo(dT)-cellulose (Pharmacia). cDNA was synthesized from 5 μg of the poly(A)$^+$ RNA using a ZAP-cDNA Synthesis Kit (Stratagene) and packaged using a GigapackII Gold *in vitro* packaging Kit (Stratagene). A cDNA library of approximately 2.3×10^6 pfu was used to screen the medaka fish membrane GC throughout this study. When we started cloning the medaka fish membrane GC seven years ago, no adequate probe, other than sperm membrane GC from sea urchin *Hemicentrotus pulcherrimus* (*14*), was available to us. Therefore, we first used a 1.9-kbp *Eco*RI-digested cDNA fragment of sperm membrane GC as a probe for screening, but the results were negative. We then screened the medaka fish membrane GC from a medaka fish (white variety) genomic DNA library and obtained a positive clone (λFIX10-1-1) containing several putative exons for a membrane GC (designated as *OlGC1*) (see detailed description below).

A cDNA fragment corresponding to the putative *OlGC1* exons was obtained by polymerase chain reaction (PCR) using a combination of two different 5′- and 3′-primers and cDNA reverse-transcribed from the total RNA of adult medaka fish brain as described previously (*15*). The oligonucleotide primers used for PCR were as follows: primer I-1 (5′-GGAGGGCAGCACCAGTA-3′) and primer I-2 (5′- CTGGTGGAGGAGCGCAC-3′) as 5′-primers, and primer II-1 (5′-AGCTCCCTCAGCTTTTG-3′) and primer II-2 (5′-CTCCCCAAGAAGCCAAT-3′) as 3′-primers. The first PCR product was 895 bp, and the second was 685 bp. The second PCR product was labeled with [α-^{32}P]dCTP using the Random Primer DNA labeling Kit, Version 2 (Takara Shuzo) and used as a probe for screening *OlGC1* cDNA clones from approximately 9×10^4 recombinant phages. The insert DNA from positive clones was rescued by pBluescript SK(−) using the ExAssist helper phage and characterized by digestion with various restriction enzymes and at the 5′- and 3′-end terminal portions of the nucleotide sequences.

5′-Rapid Amplification of cDNA Ends (5′-RACE)—To obtain a full length *OlGC1* cDNA, the 5′-portion was obtained by the 5′-RACE method using the 5′-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Life Technologies). One microgram of poly(A)$^+$ RNA was reverse-transcribed with a gene-specific antisense oligonucleotide primer (GSP1). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase, and amplified by PCR with the Abridged Anchor Primer (Life Technologies) and another gene-specific antisense oligonucleotide primer (GSP2). Amplification was performed as follows: denaturation at 94°C for 1 min 30 s followed by 30 cycles of denaturation for 30 s, annealing for 1 min at 56°C, and extension for 2 min at 72°C. To enrich the 5′-RACE products, the primary 5′-RACE products were reamplified by 35 additional cycles using the Abridged Universal Amplification Primer (AUAP; Life Technologies, Inc.) and nested GSP2 (annealing temperature 64°C). The gene-specific primers used were as follows: nucleotides 2370-2390 (GSP1), 2335-2357 (GSP2), and 2072-2091 (nested GSP2), 1983-2002 (GSP1), 1883-1901 (GSP2), and 1688-1710 (nested GSP2), 1180-1200 (GSP1) and 1140-1162 (GSP2). Each 5′-RACE product overlapped by 54-249 bp with the 5′-end. To confirm the sequence of *OlGC1* obtained by screening and 5′-RACE, a cDNA fragment containing the overlapping region was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with a pair of primers corresponding to nucleotides 1-26 and 3586-3613, and then subcloned into pBluescript II (KS−) and sequenced.

Northern Blot Hybridization and RT-PCR—Total RNA (30 μg) was separated in 1% agarose gels containing 6.7% formaldehyde. The RNA was transferred onto nylon membranes (Hybond-N$^+$, Amersham) using 20×SSC as the transferring solution. The blot was prehybridized in 30% formamide, 5×Denhardt's solution, 5×SSPE, 100 μg/ml sheared and denatured salmon sperm DNA, and 0.5% SDS at 42°C for 2 h. A PCR product (identical to nucleotides 3368-3613) was ^{32}P-labeled and added to the membrane in prehybridization solution and incubated for 20 h at 42°C. The membrane was washed with 2×SSC containing 0.1% SDS at 50°C for 30 min, then with 1×SSC containing 0.1% SDS at 50°C for 30 min, and finally with 0.1×SSC containing 0.1% SDS at 50°C for 30 min. The radioactive signals were visualized using a FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film).

For RT-PCR, 1 μg of total RNA was used as the template to synthesize the first-strand cDNA using an oligo(dT) primer according to the manufacturer's protocol (Super Script Preamplification System for First strand cDNA Synthesis, Life Technologies). The cDNA fragment containing the 3′-noncoding region of *OlGC1* was amplified by PCR from the first strand cDNA. A pair of primers specific to the medaka fish cytoplasmic actin gene *OlCA1* was used to amplify the *OlCA1* cDNA fragment as an internal control, as described previously (*15*). The primer pairs used were as follows: for *OlGC1*, 5′-GGCCGAGCCGTGACTTTATTTGTTCTAG-3′ (identical to nucleotides 3368-3392) and 5′-GAGGGTGCTTTGCTCCACAGTTACACAA-3′ (complementary to nucleotides 3586-3613); for *OlCA1*, 5′-CAGACACGTATTTGCCTCTG-3′ (identical to nucleotides 883-902) and 5′-CAAGTCGGAACACATGTGCA-3′ (complementary to nucleotides 1175-1194). The PCR products were transferred onto a nylon membrane (Hybond-N$^+$, Amersham) and hybridized with a nucleotide probe corresponding to nucleotides 3368-3613 of *OlGC1* cDNA, and then DIG-labeled using a DIG High Prime Labeling and Detection Starter Kit I (Boehringer Mannheim).

Cloning of Genomic DNA for OlGC1 from a Medaka Fish Genomic Library—A genomic library constructed in λFIXII using a *Sau*3A1-partially digested genomic DNA from medaka fish (white variety) was screened using a ^{32}P-labeled 1.9-kbp *Eco*RI-digested cDNA fragment corresponding to nucleotides 2246 to 4113 of sea urchin sperm membrane GC as a probe. Approximately 1.4×10^5 phages were plated at a density of 15,000 plaques/15-cm plate. Duplicate filter (Hybond N$^+$, Amersham) lifts were made

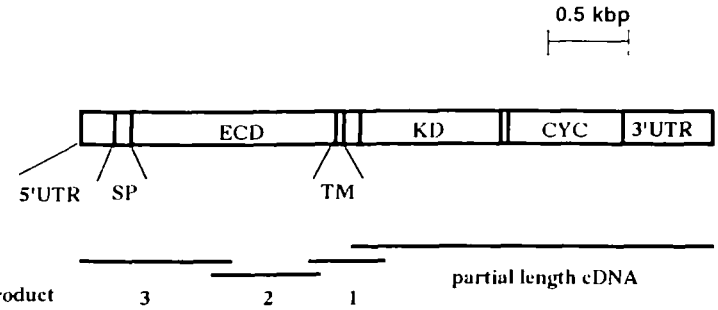
from each plate and hybridized with the ^{32}P-labeled cDNA probe in 30% formamide plus 6×SSC, 5×Denhardt's solution, 1% SDS, and 100 μg/ml sonicated herring sperm DNA at 37°C for 15 h. The filters were washed at 37°C in 4×SSC containing 0.1% SDS for 30 min. Autoradiography was carried out for 12 h at −70°C with Kodak X-Omat AR X-ray film using intensifying screens. Eight positive clones obtained by the first screening were purified by repeated screening. The phage DNA isolated from seven positive clones was characterized separately by digestion with various restriction enzymes. The clone containing the longest 5′-sequence of the *OlGC1* gene, designated λFIX10-1-1, was digested with *Xba*I, and the resulting DNA fragments were subcloned into pBluescript II (KS−). The sequencing of λFIX10-1-1 indicated that only about 10.5 kbp of the clone nucleotides corresponded only to the 3′-end portion of the *OlGC1* gene. Therefore, a second screening was carried out for about 2.4×10^5 phage plaques using the 2.2-kbp *Xho*I-*Eco*RI cDNA fragment (nucleotides 1842 to 3894) of the *OlGC1* cDNA as a probe. Two positive clones were isolated and named λG2-1 and λG3-1, respectively. The insert DNA of λG2-1 and λG3-1 were excised by digestion with *Sac*I and *Xba*I, subcloned into pBluescript II (KS−), and sequenced. The results indicated that λG2-1 and λG3-1 still did not cover the entire nucleotide sequence of the *OlGC1* gene. To isolate the remainder of the *OlGC1* gene, a third screening was carried out under the same conditions as above using a 5′-terminal 4.5-kbp *Sac*I genomic DNA fragment obtained from λG2-1 as a probe. Three positive clones were obtained from 2.4×10^5 plaques and a clone designated λGR4-1-1 was sequenced, the results indicating that the clone still did not contain all the remaining exons. A fourth screening was performed using a probe made by PCR using *OlGC1* cDNA as a template and the following primers: the 5′-GSP primer (5′-CATCACTCTCCGAGGGGCCACCACCTCTG-3′) and the 3′-GSP primer (5′-TGAGACAGTGCTACGTTTCA-3′), corresponding to nucleotides 1-26 and 1983-2002 of the *OlGC1* cDNA, respectively. Four positive clones, λG1, λG2, λG3, and λG4-1, were isolated from about 2.0×10^5 plaques and characterized as described above, indicating no overlap and none containing the entire nucleotide sequence of the *OlGC1* cDNA. A fifth screening was then carried out with the same library using a different probe made by PCR using *OlGC1* cDNA as a template and the following primers: EX9 primer (identical to nucleotides 1688-1710, 5′-TGAAGGTCTTCCCACTGAACTCT-3′), EX2 primer (identical to nucleotides 923-940, 5′-CTGGAAACGTTCC-TGAGC-3′). From this screening, two positive clones were isolated and named λGF1 and λGF3. The insert DNAs of λGF1 and λGF3 were excised, respectively, by digestion with *Sac*I and *Xba*I. The fragments obtained from the respective clones were subcloned into pBluescript II (KS−). However, there were still sequence gaps, one between clones λG2 and λGF3 and another between λG1 and λG4-1. In order to obtain a genomic DNA fragment with a sequence would cover the gap between λG2 and λGF3, we performed PCR using *O. latipes* genomic DNA as a template and two sets of gene-specific primers. The sequences for the first set of primers were 5′-CTGTGCTG-GAAGGTTAAGATGAAGAA-3′ and 5′-GCTAGAAGC-AGCCCCAGGCTCT-3′, which correspond to nucleotides 2979-3004 and 8259-8280 of the *OlGC1* genomic DNA, respectively; the sequences for the second set of primers were 5′-TAGAGCGGCCGCCTGTGCTGGAAGGTTA-3′ and 5′-TAGAGCGGCCGCGCTAGAAGCAGCCCCA-3′, corresponding to nucleotides 2979-2994 and 8249-8264 of the *OlGC1* genomic DNA, respectively. These primers were designed to contain the *Not*I site in the 5′-region to enable easy subcloning into the sequencing vector. Thirty cycles of PCR were performed with denaturation at 95°C for 30 s, annealing at 68°C for 5 min, and the polymerase reaction at 68°C for 5 min. The amplified 5.5-kbp DNA fragment was purified, digested with *Not*I, and subcloned into the corresponding site of Bluescript II (KS−). In order to obtain a genomic DNA fragment with a nucleotide sequence covering the gap between λG1 and λG4-1, we also carried out the PCR described above using *O. latipes* genomic DNA as a template and two different sets of gene-specific primers. The sequences for the first set of primers were 5′-GAGGGGAAGTGGGTTACTCTGCA-3′ (identical to nucleotides 43244-43266) and 5′-CCCGTAA-CAGGAAGCAATGGTGA-3′ (identical to nucleotides 50389-50411), and the sequences for the second set of primers were 5′-TAGAGCGGCCGCGAGGGGAAGTGGG-TTA-3′ (identical to nucleotides 43244-43259) and 5′-TA-GAGCGGCCGCCCCGTAACAGGAAGCA-3′ (identical to nucleotides 50396-50411). These primers were also designed to contain the *Not*I site in the 5′-region. By this PCR, a 7.5-kbp DNA fragment was amplified. The DNA fragment was purified, digested with *Not*I, and subcloned into the corresponding site of pBluescript II (KS−).

Determination of the Transcription Initiation Site—For determination of the transcription initiation site within the *OlGC1* promoter, primer extention experiments were performed using poly(A)$^+$ RNA isolated from adult medaka fish brain as a template. The ^{32}P-labeled synthetic primer (5′-TTTATCCAGGCGATGTCTTC-3′), complementary to the upstream region of the ATG codon, was hybridized with 5 μg poly(A)$^+$ RNA in a solution containing 250 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1 mM EDTA at 65°C for 90 min and then at room temperature for 1.5 h. The extension reaction was carried out in 45 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM DTT, and 0.5 mM each of dNTP and 200 U reverse-transcriptase (Life Technologies). The reaction products were separated by polyacrylamide gel electrophoresis in 4.5% gels in 7 M urea. Dideoxy sequencing products primed by the corresponding primers were run in parallel for size comparison. Imaging of the radioactive signals was performed as described above.

Genomic Southern Blot Analysis—Medaka fish genomic DNA was isolated from adult medaka fish brain as described previously (*16*). An 852-bp *OlGC1* cDNA fragment

Fig. 1. **Schematic diagram of cDNA clones for *OlGC1* and the complete nucleotide sequence.** (A) The structure predicted for the medaka fish *OlGC1* cDNA is presented at the top. SP, signal peptide; ECD, extracellular domain; TM, transmembrane domain; KD, kinase-like domain; CYC, cyclase catalytic domain. The lower lines represent partial length cDNA clones obtained through library screening and the 5′-RACE method (see "MATERIALS AND METHODS"). (B) The numbers on the right side indicate the nucleotide sequence numbers. Nucleotides for the transmembrane domain are underlined. The bent arrow indicates a putative translation initiation site and the open box denotes a termination codon. The locations of introns are indicated by arrowheads with numbers.

(A)



(B)

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CATCACTCTCCGAGGGGCCACCTCTGCGGAAACCACGCTGCGCTTCGGAACTTCATTCGCGTGGACTTACACAGCGGAGACTCACGCGCGCCCTGGCGTGACAGCATGACGCATTTGTGC  120
GACTTTTGAAGCAGAAAACCGTCCAGTCAGCCGCAGAGCTTCAAGACGGTCGAGTGTCCTCTCC ATGCCGCTCCCTGGACAGAGAATGGGATGCAGAGGCGCGTTGGGATGCTGTCTGTT  240
CGCGGCGCTGTGCTTTTGCCTCCTGCCCGGCTGCAGAAGTAACATCACCGCGGCGGTCATGCTTCCGGACAACTATCACAAGTACCCGTGGGCTCTGCCGCGCGTGTTCCCGGCCCTGCT  360
CATGGCGCAGGAGGATCTGCACACCAAACACAAGCTGCTGCTCGGGCACACCATCACCATTCTCAACTACAGCACGGAGAACCCCGCCGCGCCAGGCTCGTGCGCAGAGAGCCGAGCGCA  480
GGTGGTGGTCGTGGACGCCAAACTTTACAGCCGCCCAGATGTTTTCTTCGGACCCGGGTGCGTGTACCCGCTGGCCTCCGTGGGGCGCTTCGTGTCTCACTGGAAGCTCCCGCTGATCAC  600
AGCCGGAGGACCCGCTTACGGCTTCGAGAAGCTGGATGAGTACAGAACAATAGTGCGCATCGGGCCGTCCACCACTAAGCTGGGGGCCTTCGTCAACGTGCTGCACACGCAGTTCAACTG  720
GACGTCCCGGGCCGTAGTCATCTTCTACGACCTGAAACAGGACGACCGGCCGCACTACTTTCTCTCTGAGGGAATTTTCATGAACCTGAAGGACGAAATGAACATGACCGTGTCGGCGCG  840
GCCGTACACGAACGAACAGGACTACAAGGAGCTGGTTTCTTTCATTAAGGAGAACGGAAGAATCATATACATCTGTGGGCCTCTGGAAACGTTCCTGAGCATCATGAAGCTGTTCCAAAG  960
TGAGATCCAGGACCCAGAGAGCTACGCCATCTTCTATCTGGATGTGTTTGCGGAGAGCTTGACTCACCGTAAGCCCTGGCAGAACGCTAAATTTGACTGGACCAATCCCATCCAGGTCTT 1080
CAAGTCTGTTTTCGTCATAACGTACCATCCTCCGGATAATCCGGAATACAAGGACTTCCAAAGGAAGCTCCATGCCAGAGCTCAGAGGGATTTCGGAGTTAATTTGGAGCCATCGCTGAT 1200
GGACTACATCGCTGGCAGCTTTTATGATGGCTTTGTGCTGTATGCAATGGCTTTGGATGAGACTCTTGCAGAAGGCGGAGCCCAGAACAACGGCATCAACATAACAAGGAGGACACAGAA 1320
CCGCAGCTTCTGGGGAGTGACAGGACTTGTTTCCATTGATAAAAGGAATGCCAGAAATATAGATGTCGACCTGTGGGCCATGACCAACCAGGAGACAGGAGAGTACGGGGTTGTTTCATA 1440
CTACAATGGCAGCACCAAAGAGATCGTTTGGTCCCAGACAGAGAAGATCCACTGGCCCAGTTGTGGACCGCCACTGGATAACCCTCCTTGTGTCTTCTCCACGGATGACCCCTCCTGTAA 1560
CGATGGTCTCCCAGTTCTGGGAATTGTTGCAGTGGGTTCTGGGCTGGCGCTCATCATTTTTGGAATCTCCAGCTTCCTTATCTACAGGAAACTGAAGCTGGAGAAGGAGCTGGCTGGAAT 1680
GCTGTGGAGAGTTCAGTGGGAAGACCTTCAGTTCGAGAGCCCCAACAAATACCACAAACGAGCAGGCAGCCGCCTGACTCTCTCACAGAGAGGCTCCAGCTACGGCTCCTTGATAACTGC 1800
CCAGGGAAAATATCAACTGTTTGCAAAAACCGGCTATTTTAAGGGAAACCTGGTGGCCATCAAGCACGTCAACAAGAAGAGGATAGAGCTGACGAGACAGGTCCTGATGGAGCTGAAGCA 1920
TATGAGGGACGTTCAGTTCAACCATCTAACCAGGTTCATCGGGGCCTGCATCGACCCCCCCAACATTTGCATCGTGACAGAGTACTGTCCCAGAGGCAGCCTGCAGGACATTCTGGAGAA 2040
CGAGAGCATCAACCTGGACTGGATGTTCCGCTACTCGCTGATCAACGACATCGTGAAGGGCATGAACTTTCTCCACAACAGCTACTTTGGCTGCCATGGAAACCTGAAGTCCTCCAACTG 2160
CGTGGTGGACAGTCGCTTCGTTTTGAAGATCACAGATTACGGCTTGGCAAGCTTCCGCTCGTCCTGCAAGAACGATGACTCCCACGCGCTCTACGCCAAGAAACTGTGGACGGCTCCGGA 2280
GCTGCTCATTTATGACCACCATCCTCCCCAGGGCACTCAGAAGGGCGATGTGTACAGTTTCGGCATCATCCTGCAGGAGATAGCTCTGAGAAATGGACCGTTCTATGTGGAAAGCATGGA 2400
TCTCAGCCCCAAAGAGATCGTGCAGAAAGTGAGGAACGGGCAGCGGCCGTACTTTCGCCCGACCACGGACAGCAGGTTTCACTCGGAGGAGCTGACCATTTTGATGGAGGGCTGCTGGGC 2520
TGAAGACCCTGCAGAGAGGCCGGACTTTGGCCACATCAAGATCTACATGGCCAAACTCAACAAGGAGGGCAGCACCAGTATCCTCAACAACCTGCTGTCCAGGATGGAGCAGTATGCCAA 2640
CAACCTGGAGAACCTGGTGGAGGAGCGCACGCAGGCGTACCTGGAGGAGAAGCGGAAGGCAGAAAACCTGCTCTATCAGATTCTGCCTCACTCCGTAGCAGAGCAGCTGAAAAGAGGGGA 2760
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CGCCAGTATGTCGCTGGCTTTGTTGGAGCAGGTCAAAACCTTCAAGATCCGCCACAGACCCAACGACCAGCTGAGGCTCAGGATTGGTATCCACACAGGTCCAGTGTGCGCCGGTGTGGT 3120
TGGTCTAAAAATGCCCAGATATTGTCTGTTCGGAGATACTGTCAACACCGCCTCCCGCATGGAGTCAAACGGAGAAGCCTTAAAGAACCACGTATCTTCAGCCACCAAAGAAGTGCTGGA 3240
TGAGTTTTGTTACTTTAACCTTGAGTTACGAGGGGACGTAGAAATGAAGGGCAAAGGTAAAATGAGAACATATTGGCTTCTTGGGGAGAAGACCGATGTGTACGTTATCTGAGTCGCCCG 3360
CTCTTACGGCCGAGCCGTGACTTTATTGTCTAGAATAAGTCCATAAACAGTGACTTCCCCGCAAAGGCCAATAGGAGCCTTCGACAAAAGCTGAGGGAGCTCCAGGTATATTTATTACCT 3480
GCTGGCTGTCACTGAAAGACATGACTATTAAGGATGAGTGCTTTTATATGAAGATTTTGATGTGGTAAAAGTGTCTGTTTTTCATGGAGAAAATCCTATTTTTGTGCTTTCTACGTTAAA 3600
ATTTGTATAATGTGTTTGAAAGTTGATGATTTAGTAGTATTTCCTTGAAAGATCCGTTAAAGATTGATGGCATAGAAATGAAATCACTGTCACTGCAGCGGTTCATTGTGTAACTGTGGA 3720
GCAAAGCACCCTCTATATATACAATACACACACACACACACGAATATATATTTATATATACATGTATATGTATATCCCACTGTAAAATATTTTGTTGAATAAACGGAGTTAAACACTCCT 3840
TTTACATTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                                                  3894
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in the 5′-flanking region of the *OlGC1* cDNA was amplified by PCR using the *OlGC1* cDNA as a template with the following primers: 5′-CATCACTCTCCGAGGGGCCACC-TCTG-3′ (identical to nucleotides 1-26) and 5′- TTCGTG-TACGGCCGCGCCGACAC-3′ (identical to nucleotides 830-852). The 852-bp PCR product was ^{32}P-labeled and used as a probe. The genomic DNA was digested completely with restriction enzymes including *Bam*HI, *Eco*RV, and *Hin*dIII, and then separated by 0.7% agarose gel electrophoresis (10 μg DNA/lane). The DNAs in the gel were transferred onto a filter and then hybridized with the ^{32}P-labeled probe as described above. The filter was washed once in 1×SSC, 0.1% SDS at 60°C for 30 min and then in 0.1×SSC, 0.1% SDS at 60°C for 30 min. The imaging of the radioactive signals was performed as described above.

Molecular Phylogenetic Analysis—The nucleotide and deduced amino acid sequences of *OlGC1* were compared with those of known membrane GCs using the ClustalW program (*17*) and sequence editor SeqPup (Gilbert, Indiana University). The phylogenetic tree was constructed using the aligned sequences by the neighbor-joining algorithms in the PROTRAS program of PHYLIP (version 3.572) and the ClustalW program (*17*). For the neighbor-joining analysis, the evolutionary distance was estimated using Kimura's empirical method for protein distances (*18*). GenBank/EMBL/DDBJ accession numbers for the sequences used are as follows: rat GC-A (S03348) (*19*); rat GC-B (A33300) (*20*); rat GC-C (A36292) (*21*); rat GC-D (L37203) (*22*); rat GC-E (A55915) (*23*); rat GC-F (B55915) (*23*); rat GC-G (AF024622) (*24*); human GC-A (S4459) (*25*); human GC-B (S05514) (*26*); human GC-C (S40940) (*27*); human RetGC-1 (M92432) (*28*); human RetGC-2 (L37378) (*29*); *OlGC3* (AB000899) (*15*); *OlGC4* (AB000900) (*15*); *OlGC5* (AB000901) (*15*); *OlGC6* (AB007192) (*30*); eel GC-A (AB012869) (*31*); eel GC-B (D25417) (*32*); sea urchin (*H. pulcherrimus*) sperm GC (HpGC) (D21101) (*14*); sea urchin (*S. purpuratus*) sperm GC (SpGC) (M22444) (*33*).

Other Methods—The nucleotide sequences of the cDNA and genomic DNA fragments in the pBluescript vectors (Stratagene) were determined by the dideoxy chain termination method (*34*) with Applied Biosystems 373A and 377 DNA sequencers or a LI-COR sequencer model 4000L (LI-COR), and analyzed with DNASIS software (Hitachi Software Engineering) or GENETYX-MAC/version 9.01 (Software Development).

RESULTS

Isolation and Characterization of cDNA Clones Encoding OlGC1—Since there has previously been no useful cDNA probe available for screening membrane GC from a medaka fish cDNA library other than sea urchin sperm membrane GC (*14*), we used a 1.9-kbp sea urchin sperm cDNA fragment as a probe during the early stages of this study. However, despite all our efforts, this attempt was unsuccessful. We then switched from the cDNA library to a genomic library for screening. This switch was successful, yielding several positive clones. Characterization of these clones by digestion with various restriction enzymes and subsequent Southern blot analysis and nucleotide sequencing demonstrated that a clone, designated λFIX 10-1-1, contained the longest 5′-nucleotide sequence homologous to mammalian natriuretic peptide receptors/membrane GCs as well as to the sea urchin sperm membrane GC cDNA. RT-PCR using poly(A)$^+$ RNA from adult *O. latipes* brain and a combination of oligonucleotide primers synthesized based on the sequence of the mammalian membrane GC cDNA produced a 685-bp cDNA fragment highly homologous to part of the catalytic domain of rat GC-A and GC-B. Using this 685-bp cDNA fragment as a probe, we obtained two positive clones encoding a medaka fish membrane GC from an *O. latipes* brain cDNA library. The clone (*OlGC1*) with the longest cDNA insert (2.2 kbp) contained a nucleotide sequence corresponding to part of the catalytic domain and the 3′-untranslated region (3′-UTR) of mammalian membrane GCs including rat GC-A and GC-B. The remaining *OlGC1* nucleotides were obtained by the 5′-RACE method. The strategy for obtaining a full length *OlGC1* cDNA and the complete nucleotide sequence is shown in Fig. 1. The *OlGC1* cDNA consists of a 184-bp 5′-UTR, a 3,165-bp open reading frame (ORF), and a 545-bp 3′-UTR with a poly(A) tail. Termination codons occur in all three frames upstream of the putative initiation codon ATG. In addition, nucleotides around the initiation codon fit the preferred sequence context for the initiation of protein synthesis in eukaryotic mRNAs (*35*). The ORF of the *OlGC1* cDNA encodes a polypeptide of 1,055 amino acids (Fig. 2). Hydropathic analysis (data not shown) by the method of Kyte and Doolittle (*36*) predicted the OlGC1 polypeptide to contain a 30-residue amino-terminal signal sequence (*37*), and that cleavage of the signal sequence would result in a mature protein of 1,025 amino acids. A comparison of the amino acid sequence of OlGC1 with known membrane GCs suggests that OlGC1 is closely related to natriuretic peptide receptors including eel GC-B and mammalian GC-Bs (Fig. 3). The mature OlGC1 protein

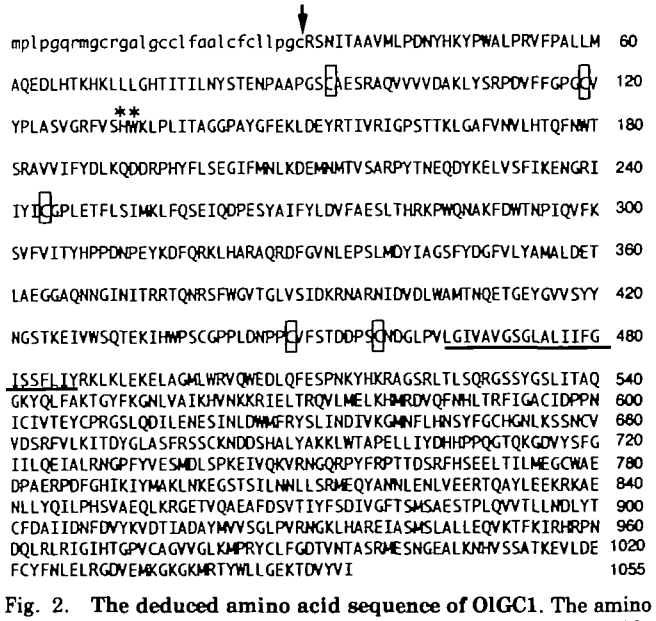
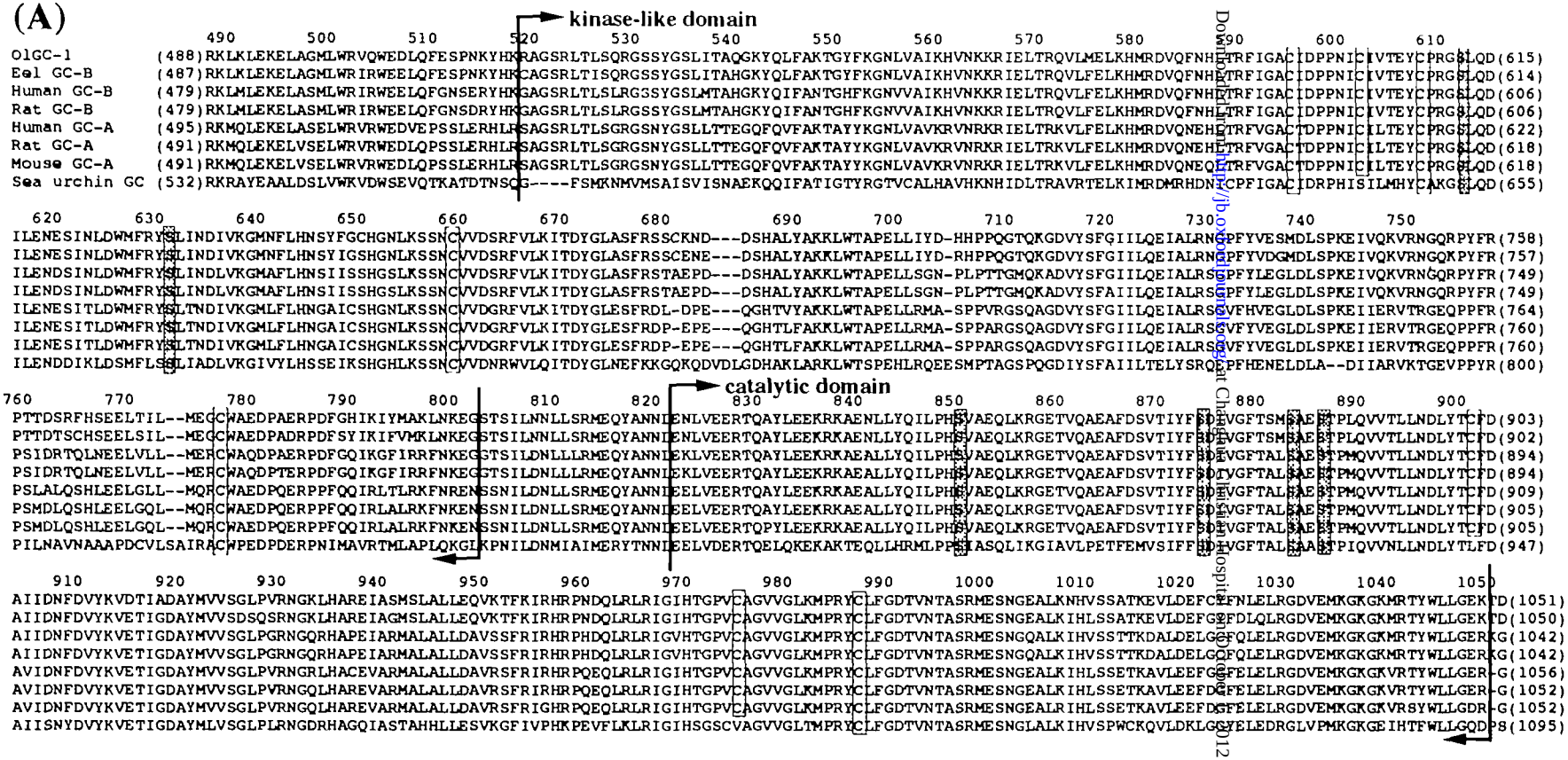


Fig. 2. **The deduced amino acid sequence of OlGC1.** The amino acid sequence is indicated in single letter code. The signal peptide sequence is indicated by lowercase letters and the signal peptide cleavage site is shown by the arrow. The putative transmembrane domain is underlined. Five cysteine residues at positions 93, 119, 243, 449, and 458 are boxed and histidine-tryptophan residues at positions 132 and 133 are indicated by asterisks.



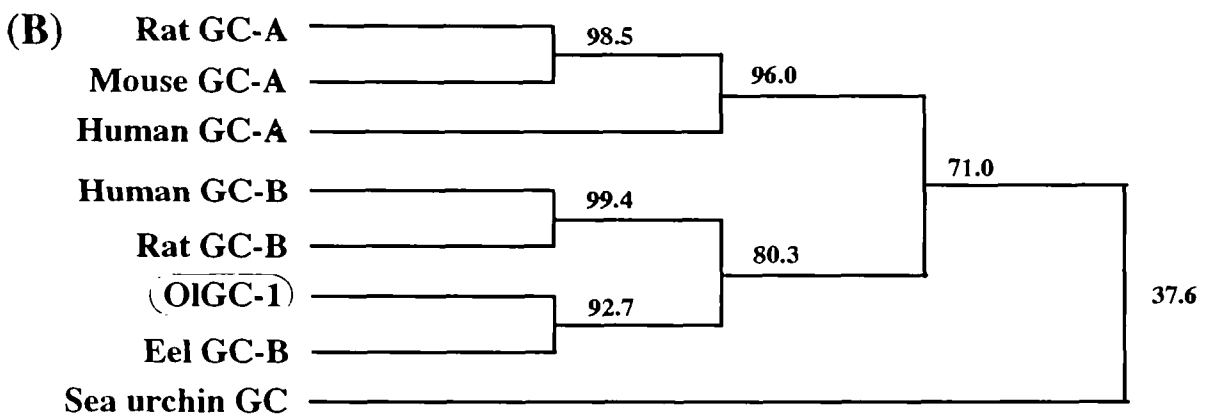


Fig. 3. **Alignment and phylogenetic tree of the amino acid sequences of membrane GCs.** (A) The amino acid sequence of the intracellular domain (562 residues) of OlGC1 was compared with sequences of various vertebrate membrane GCs and sea urchin sperm membrane GC. Eight conserved cysteine residues are open-boxed, and six conserved serine residues are indicated by shaded-boxes. (B) The amino acid sequences of the catalytic domain of various membrane GCs were subjected to phylogenetic analysis (see "MATERIALS AND METHODS").

is composed of an extracellular domain (residues 31-464), a single transmembrane domain (residues 465-487), and intracellular protein kinase-like (residues 519-802) and cyclase catalytic (residues 822-1049) domains that are highly conserved among invertebrates and vertebrates. Six serine residues at positions 652, 894, 918, 927, 930, and 985 that have been shown to be phosphorylated in sea urchin sperm membrane GC (*38*) are found in the corresponding positions 612, 850, 874, 883, 886, and 941 in the intracellular domain of OlGC1 (Fig. 3). Furthermore, eight cysteine residues conserved in the intracellular domain of various vertebrate natriuretic peptide receptors are also conserved in the corresponding positions 595, 602, 608, 659, 777, 901, 975, and 987 of OlGC1 (Fig. 3). In the extracellular domain, in which the over-all amino acid sequence is less conserved among vertebrate natriuretic peptide receptors, five cysteine residues and histidine-tryptophan residues known to be conserved among vertebrate natriuretic peptide receptors including eel GC-B, rat GC-A, and rat GC-B and thought to play an important role in ligand-binding (*39*), are found in the corresponding positions 93, 119, 243, 449, and 458 for cysteine residues, and 132 and 133 for histidine and tryptophan in the extracellular domain of OlGC1 (Fig. 2).

Expression of OlGC1 in Adult Organs and during Embryogenesis—To estimate the size of the mRNA for *OlGC1* and to see whether the mRNA exists in adult organs other than brain, total RNA prepared from eye, brain, liver, and intestine was analyzed by Northern blot hybridization using a cDNA fragment of 3′-UTR as a probe. A signal was detected at a position corresponding to 3.9 kb in the total RNA from eye and brain, indicating that the size of the mRNA is in agreement with that of the *OlGC1* cDNA, and suggesting that the *OlGC1* transcript may be expressed

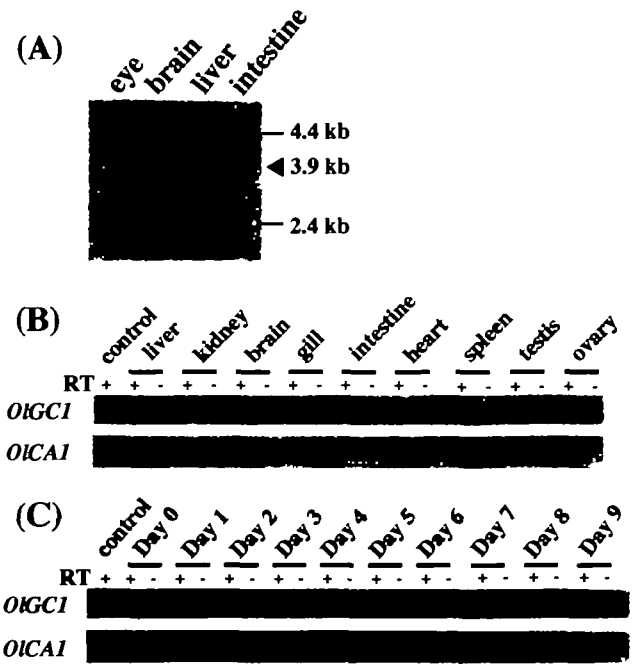


Fig. 4. **Northern blot and RT-PCR analyses of the *OlGC1* transcript in adult medaka fish and during embryogenesis.** (A) Northern blot analysis was carried out using total RNA (30 μg) from the brain, eye, liver, and intestine. A radioactive band is indicated by the arrowhead. The positions and sizes of the RNA markers are shown on the right. The *OlGC1* transcript in various adult organs (B) and embryos at various developmental stages (C) was detected by RT-PCR. The transcript of medaka fish cytoplasmic actin gene *OlCA1* was amplified as an internal control. RT+ and RT− represent amplification with and without reverse transcriptase, respectively.

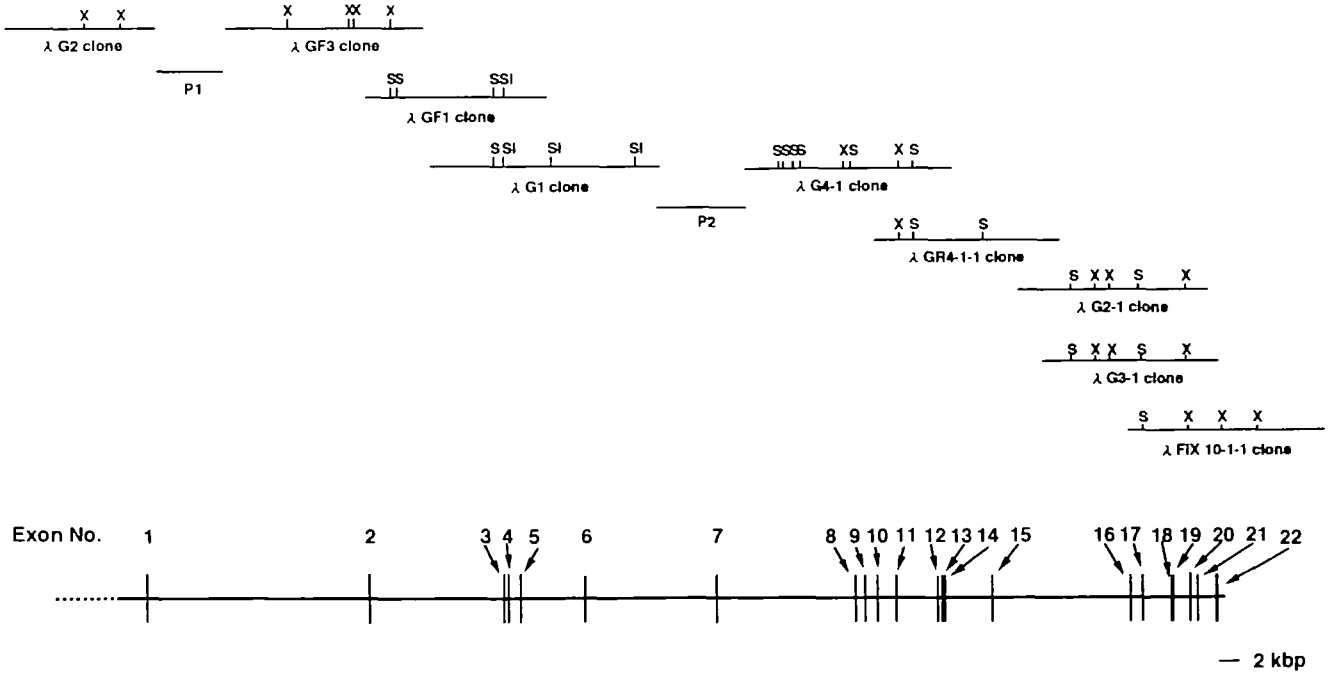


Fig. 5. **Restriction enzyme map and structural organization of the *OlGC1* gene.** The upper panel shows the location of genomic clones with restriction enzyme sites. X, S, and Sl denote *Xba*I, *Sac*I, and *Sal*I sites, respectively. The lower panel indicates the entire *OlGC1* gene structure. Exons are indicated by closed boxes with numbers.

in a limited number of organs (Fig. 4). However, RT-PCR with total RNA from various adult organs demonstrated the presence of the *OlGC1* transcript in organs including brain, kidney, spleen, liver, pancreas, gallbladder, ovary, testis, and intestine, although the signals for spleen, liver, pancreas, gallbladder, ovary, and intestine were rather weak (Fig. 4). RT-PCR using total RNA from embryos at various developmental stages demonstrated that the *OlGC1* transcript could first be detected in Day 1 embryos, with the signal becoming more distinct as development proceeds (Fig. 4).

Characterization of Genomic DNA Clones for OlGC1— The sequencing of λFIX10-1-1 indicated that the 5′-portion (about 10.5 kbp) of this clone contain only the 3′-end of the *OlGC1* gene. We then carried out many subsequent screenings of the genomic library using various probes (Fig. 5). By the second screening, two clones, λG2-1 (15.7 kbp) and λG3-1 (14.5 kbp), were obtained, and the third screening resulted in the isolation of λGR4-1-1 (15.7 kbp). Since none of these clones contained nucleotides covering all of the exons, fourth and fifth screenings were carried out with the same library using *OlGC1* cDNA fragments (nucleotides 1-2001 or 923-1710) as probes. Four positive clones, λG1 (19 kbp), λG2 (13.5 kbp), λG3 (17 kbp), and λG4-1 (17 kbp), were obtained by the fourth screening, and two positive clones, λGF1 (15 kbp) and λGF3 (16 kbp), were obtained by the fifth screening. However, sequence gaps remained, with one between clones λG2 and λGF3 and another between λG1 and λG4-1, indicating that the *OlGC1* gene is quite large. In order to cover the gaps, we carried out PCR using *O. latipes* genomic DNA as a template and two sets of gene-specific primers. PCR produced two genomic DNA fragments, 5.5-kbp P1 and 7.5-kbp P2. By sequencing these genomic DNA fragments, we finally obtained a complete nucleotide sequence of 93 kbp for the *OlGC1* gene (GenBank accession no. AB021490) containing 22 exons (Fig. 5). The nucleotide sequences of all the splice junctions are in good agreement with the GT/AG rule (Table I). Although phylogenetic analysis indicated that *OlGC1* is a medaka fish homolog of mammalian GC-B, the exon-intron organization and the exon sizes are similar to those of mammalian GC-A genes (Table I).

A homology search demonstrated that *OLR1*, a highly repetitive interspersed sequence found in *O. latipes* genomic DNAs (Genbank accession no. D89288), is located in intron 15 and that the *OlGC1* gene contains a retroelement, designated REX-3, that has been found by Jeam-Nicolas Volff *et al.* in *Poeciliidae xiphophorus* (personal communication), although the REX-3 element in the *OlGC1* gene is truncated, lacking the endonuclease domain and a part of the reverse-transcriptase domain. In addition to the above, we found a new repeat element in nucleotides 42580-43143 of the *OlGC1* gene, which shares 96.3% identity with the nucleotide sequence found in the 5′-flanking region of the *OlGC6* gene, a medaka fish homolog of mammalian GC-C (*30*).

Genomic Southern analysis using a 852-bp cDNA fragment encoding 5′-UTR and part of exon 1 of the *OlGC1* gene as a probe resulted in two positive bands, one of which was consistent with the size expected from the nucleotide sequence of the *OlGC1* gene (Fig. 6). Primer extension analysis demonstrated the presence of two different 5′-ends of the transcription initiation site at nucleotide positions 164 bp (C) and 277 bp (T) upstream of the putative initiation codon (Fig. 7). Since C, at 164 bp, is located in the 5′-UTR region of the *OlGC1* cDNA, T, at 277 bp, was assigned as the putative transcription initiation site and

TABLE I. **Exon-intron organization of *OlGC1*, rat GC-A, and human GC-A.**

Exon/Intron No.	*OlGC1*		Rat GC-A	Human GC-A	
	Intron size (bp)	Exon size (bp)	Exon size (bp)	Exon size (bp)	Functional domain
1	16,989	995	1,079	721	EC
2	9,696	182	200	200	EC
3	367	114	114	114	EC
4	666	136	136	136	EC
5	5,773	95	92	92	EC
6	12,506	136	136	136	EC
7	10,378	82	85	85	TM
8	1,173	121	122	121	KL
9	519	75	74	75	KL
10	2,432	78	78	78	KL
11	3,477	105	105	105	KL
12	521	72	72	72	KL
13	85	160	157	157	KL
14	3,834	156	156	156	KL
15	10,505	169	169	169	KL
16	472	147	147	147	CYC
17	2,635	124	124	124	CYC
18	76	69	69	69	CYC
19	1,379	175	175	175	CYC
20	326	99	99	99	CYC
21	1,436	92	92	92	CYC
22		560	>586	ND	CYC

Exons coding for the extracellular (EC), transmembrane (TM), kinase-like (KL), and cyclase catalytic (CYC) domains. ND, not determined.

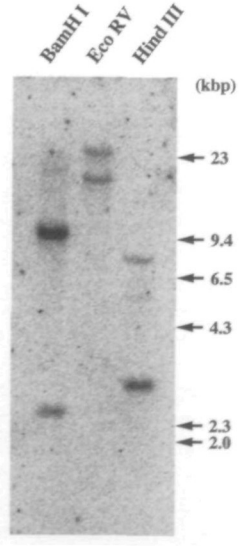


Fig. 6. **Genomic Southern blot analysis.** Ten micrograms of medaka fish genomic DNA was digested with several restriction endonucleases (*Bam*HI, *Eco*RI, and *Hind*III) and resolved by electrophoresis in 0.7% agarose gels, and then transferred onto a nylon membrane. The blot was hybridized with a ^{32}P-labeled probe. The restriction endonucleases used for digestion are indicated at the top of each lane. The positions and sizes of the DNA markers are shown on the right with horizontal arrows.

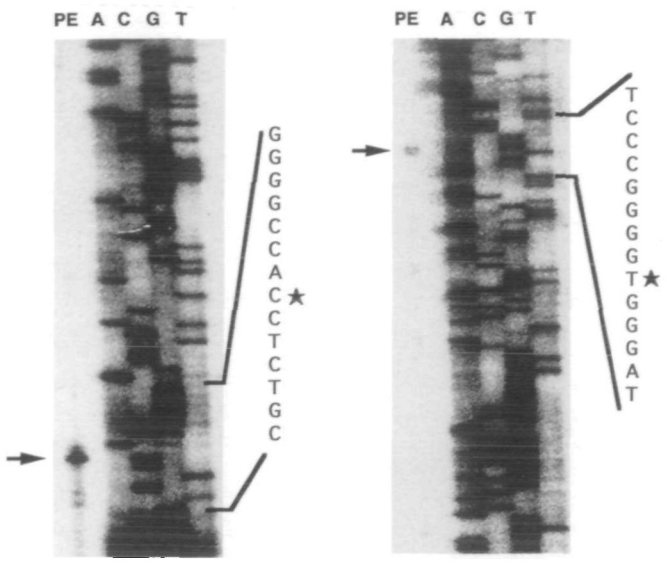


Fig. 7. **Determination of the transcription initiation site of the *OlGC1* gene.** A primer extension reaction was performed with 5 μg poly(A)$^+$ RNA isolated from adult brain using a 20-mer synthetic oligonucleotide primer, as described in the text. The left panel represents the upper part of the radioautogram, and the right panel shows the lower part of the radioautogram. The four right lanes, ACGT, are nucleotide sequencing using the *OlGC1* gene as a template. The left lane, PE, shows the extended products; the most likely end point of the extension reaction is indicated by an arrow on the left and by stars on the sense strand shown on the right.

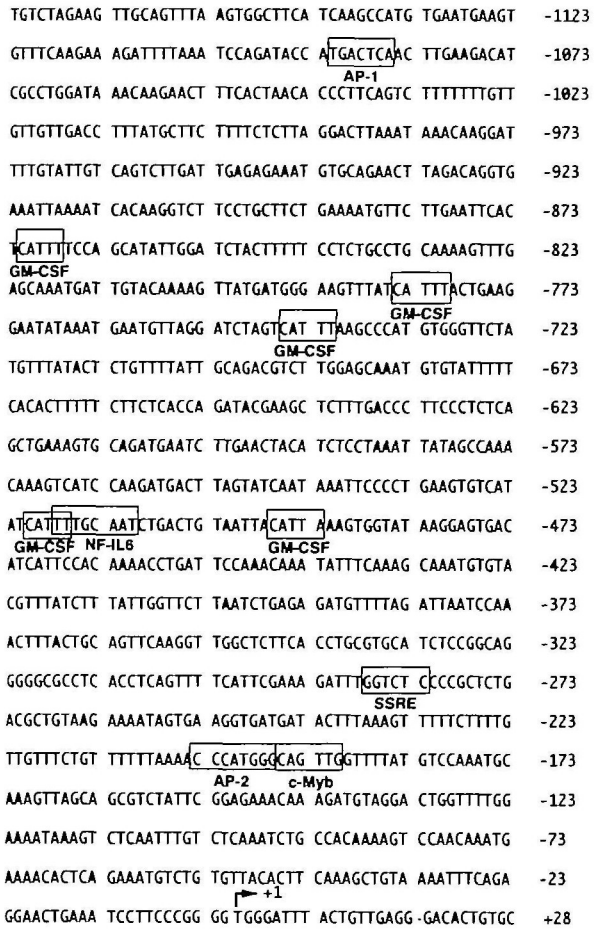


Fig. 8. **Nucleotide sequence of the 5′-flanking region of the *OlGC1* gene.** The nucleotide sequence from −933 to +148 is given with potential consensus binding sequences (boxed). The nucleotide, T, thought to be the transcription initiation site is indicated by a bent arrow.

designated as +1. There is no typical TATA or CAAT box in the 5′-flanking region upstream of the putative transcription initiation site (Fig. 8); however, a consensus motif search indicated that the 5′-flanking region contains a nucleotide sequence, GGTCTC, at nucleotides 886-891 identical to that of the shear stress responsive element (SSRE) that has been identified in the mouse ANP-CR gene (*40*) (Fig. 8). The search also indicated the presence of several other known nucleotide sequences for binding to activator protein-1 (AP-1), activator protein-2 (AP-2), and NF-IL6 and c-Myb. In addition, multiple binding sites for granulocyte-macrophage colony-stimulating factor (GM-CSF) are also present in the 5′-flanking region of the *OlGC1* gene.

DISCUSSION

In this study, we determined the entire genomic structure of the medaka fish homolog of mammalian GC-B. There have been several papers reporting the entire genomic structure of natriuretic peptide receptors, including rat GC-A (*11*), human GC-A (*10*), and bovine NPR-C (clearance receptor) (*12*). The exon-intron organization is similar in each of these genes, particularly in regions corresponding to the extracellular and transmembrane domains. However, despite many demonstrations of the cDNA structure of GC-B in various species and the importance of its biological functions, there has been no paper reporting the genomic structure of the GC-B gene. As a first step toward understanding the regulatory mechanism for GC-B gene expression, we characterized the cDNA and genomic DNA encoding the medaka fish homolog of mammalian GC-B, since the average genome size of the medaka fish is expected to be about one-fifth that of mammals and about half that of zebrafish.

OlGC1 cDNA is 3,894-bp long with the longest ORF predicting a protein of 1,055 amino acids. This protein contains a sequence, RGSSYGSL, that is conserved in all GC-B proteins including those of human, rat, cow, and eel (*20, 25, 32, 41*) (Fig. 3), and the overall identity of the amino acid sequence is the highest to that of eel GC-B (80%). Molecular phylogenetic analysis using several regions of the amino acid sequence of OlGC1 indicated that the protein is closely related to eel GC-B and mammalian GC-B, but not to eel GC-A or mammalian GC-A. However, as shown in Table I, the exon-intron organization of the *OlGC1* gene is similar to that of mammalian GC-A genes, although the *OlGC1* gene is much larger (about 93 kbp for the *OlGC1* gene *vs.* 17.5 kbp for the rat GC-A gene and 16 kbp for the human GC-A gene). Further, the exon sizes of the extracellular and intracellular domains of the *OlGC1* gene are very similar to those for the rat and human GC-A genes, and the nucleotide sequences of exons of the intracellular domain are also similar to those of the rat and human GC-A genes; in the *OlGC1* gene, the extracellular domain is distributed among six exons (exons 1-6), the

single transmembrane domain is coded by exon 7, and the kinase-like and C-terminal cyclase catalytic domains are coded by eight exons (exons 8-15) and seven exons (exons 16-22), respectively (Table I). Therefore, it is possible that the *OlGC1* gene arose from an ancestral gene common to the mammalian GC-A genes. In this regard, it may be worthwhile to note that our genomic Southern analysis suggested the presence of two copies of the *OlGC1* gene (Fig. 6). One of these copies might be an *OlGC1*-related gene with a different exon-intron organization. There have been numerous papers reporting the presence of many different membrane GCs in various organisms including mammals (human, bovine, rat, and mouse), insects, and nematoda *Caenorhabditis elegans* (*42*). In particular, it has been reported that *C. elegans* possesses many different membrane GCs (*42*). Therefore, in the future, such related gene(s) will likely be found in other organisms.

Northern blot analysis clearly demonstrated that a 3.9-kb *OlGC1* transcript is expressed in both adult fish brain and eye but not in the liver or intestine, although RT-PCR showed the presence of the *OlGC1* transcript in a number of peripheral organs and in embryos during early stages of development. CNP, a ligand for GC-B, has been reported to be coexpressed in various mammalian organs including kidney, heart, chondrocytes, and liver (*43-45*). Considering this and that endogenous CNP is thought to elicit paracrine and/or autocrine effects, we assume that it would be valuable to confirm the expression of CNP in the above-mentioned medaka fish organs.

The *OlGC1* gene lacks a typical TATA box in the region 500 bp upstream of the transcription initiation site, but contains several potential transcription factor binding sites including AP-1, AP-2, NF-IL6, and c-Myb. Further, the 5′-flanking region of the *OlGC1* gene possesses a consensus sequence to multiple binding sites for GM-CSF. It has been reported that NF-IL6 is expressed in macrophages and that its expression is inducible by lipopolysaccharide (LPS) (*46*). IL-6, TNF-α, and NO synthase, all of which contain a DNA recognition motif [T-G/T-N-N-G-C/T-A-A-G/T] in the functional regulatory region of their respective genes, have also been reported to be inducible in activated macrophages (*46-48*). In addition, ANP has been reported to inhibit the production of NO in LPS-activated primary macrophages and to stimulate phagocytosis and the production of reactive oxygen by macrophages in which mRNAs for all types of natriuretic peptide receptors have been detected (*49-52*). To acquire enhanced bactericidal and tumoricidal capacity, macrophages must be activated by microbial products including LPS, cytokines, and GM-CSF. Taking these findings regarding macrophages into consideration, it is possible to speculate that multiple binding sites for GM-CSF and a potential NF-IL6-binding site in the 5′-flanking region of the *OlGC1* gene may play a specific role in the regulation of gene transcription with relation to natriuretic peptide effects. Further, it has been reported that SSRE is present in the mouse endothelial CNP and ANP-CR genes (*40*), and that the levels of vasoactive peptides, including endothelin-1, adrenomedullin, and CNP, are affected by fluid shear stress. Considering this and that the *OlGC1* gene possesses a sequence similar to that of SSRE in the 5′-flanking region, the transcriptional regulation of the GC-B gene might be influenced by fluid shear stress.

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